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DETAILED REVIEW PAPER ON THE RETINOID SYSTEM

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NO. 343

DETAILED REVIEW PAPER ON THE RETINOID SYSTEM

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Paris 2021

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Foreword

1. The project to develop a Detailed Review Paper (DRP) on the Retinoid System was added to the Test Guidelines Programme work plan in 2015. The project was originally proposed by Sweden and the European Commission later joined the project as a co-lead. In 2019, the OECD Secretariat was added to coordinate input from expert consultants. The initial objectives of the project were to:

- draft a review of the biology of retinoid signalling pathway,
- describe retinoid-mediated effects on various organ systems,
- identify relevant retinoid *in vitro* and *ex vivo* assays that measure mechanistic effects of chemicals for development, and
- Identify *in vivo* endpoints that could be added to existing test guidelines to identify chemical effects on retinoid pathway signalling.

2. This DRP is intended to expand the recommendations for the retinoid pathway included in the OECD Detailed Review Paper on the State of the Science on Novel In vitro and In vivo Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors (DRP No 178). The retinoid signalling pathway was one of seven endocrine pathways considered to be susceptible to environmental endocrine disruption and for which relevant endpoints could be measured in new or existing OECD Test Guidelines for evaluating endocrine disruption. Due to the complexity of retinoid signalling across multiple organ systems, this effort was foreseen as a multi-step process. This DRP is intended, in part, to be an initial scoping effort to identify retinoid signalling pathway test methods, markers, and endpoints for consideration. Following the expert group discussion of the technical aspects and regulatory context, the initial scoping effort may lead to recommendations for identification of early screening assays that could be included as a multi-tiered approach, identification and development of biomarkers to be used in studies of humans and wildlife, and endpoints that could be added to enhance exiting in vivo test guidelines. This effort was intended to be modelled after the thyroid scoping effort (GD No 207).

3. The EU-Commission supported a first draft of DRP development through a contract with Brunel University and the sub-contractors Technical University of Denmark (DTU). Sections of the 2017 draft prepared for this project are included herein.

4. The Retinoid DRP project was discussed at two meetings of the Advisory Group on Endocrine Testing and Assessment (EDTA) in 2017 and a meeting in 2018. The EDTA recommended narrowing the scope of the original proposal to focus the DRP on specific organ systems for which some information is known regarding the role of retinoid signalling.

5. At the 2018 EDTA meeting, Sweden presented drafts of a section describing the role of retinoids on female reproduction (Appendix A). A section reviewing the overall biology of the retinoid pathway and additional information on the male reproductive system (Appendix A) were added in 2019.

6. The retinoid pathway signalling was also discussed at a 2017 European Commission workshop to identify gaps in current OECD Test Guidelines and prioritise new assays to bridge the gaps. The meeting attendees identified the development of retinoid pathway assays and endpoints for inclusion in

OECD Test Guidelines as a high priority. As a result, the European Commission supported the drafting of two additional sections reviewing retinoid effects craniofacial/skeletal system development (Annex B) and the central nervous system (CNS) (Annex C), also included in this DRP. These were coordinated by the OECD Secretariat and prepared by subject matter expert consultants noted below. In Q2 2019, OECD circulated a request for updated nominations to the Expert Group on Retinoid Pathway Signalling.

7. The draft DRP was circulated for a WNT commenting round in 2019 and comments were discussed by the OECD Expert Group on Retinoid Pathway Signalling in November 2019. The objectives of the meeting were to address any outstanding comments received during the first commenting round and to collate recommendation on possible next steps. The discussion of sections and recommendations are summarised in Annex D. Following the meeting, the sections were revised by the lead experts. The draft document was also sent to the OECD Advisory Group for Endocrine Disuptor Testing and Assessment (EDTA) in Q2 2020, comments reflecting a regulatory perspective were further discussed by the Expert Group on Retinoids, and a final WNT commenting round was initiated in Q4 2020. Resulting comments have been addressed in this draft document.

8. In addition, the overview of retinoid biology, along with the annex on the male and female reproduction, was published as a TemaNord Report (Retinoids in Mammalian Reproduction, with an Initial Scoping Effort to Identify Regulatory Methods, Nordic Council of Ministers, (Nordic Council of Ministers, 2020), is made available under the Creative Commons Attribution 4.0 International license (CC BY 4.0).This material has been reformatted herein, but the content has not been changed. The sections on skeletal development (Annex B; (Knudsen, et al., 2021)) and developmental neurotoxicity (Annex C; (Chen, et al., 2020)) were published the peer-reviewed journal, Reproductive Toxicology. They are reproduced herein with kind permissions from the publisher.

9. Sections of this DRP were drafted by different experts and the content of some sections was discussed extensively and revised several times prior to this draft, while other sections were prepared more recently and with less initial review. The following experts prepared the sections of this DRP indicated below: The body of the DRP and Appendix A which was adopted from the TemaNord report (see paragraph 7) was primarily authored by Charlotte Nilsson, Research Institutes of Sweden, in collaboration with the Swedish Chemicals Agency, with financial support from the Nordic Working Group for Chemicals and Health[1] and the Swedish Chemicals Agency[1]. The drafting of the body of the DRP and the section on male reproduction built on the work was performed by Brunel University and Technical University of Denmark 2016-2017, supported by the EU Commission. In addition, a number of experts outside the OECD retinoid expert group, made valuable contributions to the Nordic Report (please see the acknowledgement section “List of contributors” in (Nordic Council of Ministers, 2020) for details). Appendix B was drafted by Thomas B. Knudsen (Center for Computational Toxicology and Exposure (CCTE), Office of Research and Development, U.S. Environmental Protection Agency) and Nancy C. Baker (Leidos, Contractor to CCTE)[2]. Appendix C was drafted by Joshua F. Robinson (University of California, San Francisco), with financial support from the European Commission- DG Environment. Members of the OECD Expert Group made contributions via written comments and discussion at the November 2019 meeting.

[1] The contents of this working paper do not necessarily reflect the views, policies or recommendations of the Nordic Council of Ministers, or the Swedish Chemicals Agency

[2] The views expressed herein are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

10. The draft DRP was approved by the WNT in April 2021 and is published under the responsibility of the Chemicals and Biotechnology Committee.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 887268. Previous financial contributions from the European Union supported the development of publications referenced here published before 2020.

Abbreviations relevant to entire document

ADH	Alcohol dehydrogenase
AhR	Aryl hydrocarbon receptor
ALDH	Aldehyde dehydrogenase
AMH	Anti-müllerian hormone
AO	Adverse outcome
AOP	Adverse outcome pathway
ARAT	Acyl CoA:retinol acyltransferase
ATRA	All-trans retinoic acid
BMP	Bone morphogenic protein
BTB	Blood-testis barrier
CAR	Constitutive androstane receptor
CF	Conceptual framework
CRABP	Cellular retinoic acid-binding protein
CRBP	Cellular retinol-binding protein
CYP	Cytochrome P450
CYP17	Cytochrome P450 C17, 17,20-lyase, 17α-hydroxylase
Cyp2b10	Cytochrome P450, family 2, subfamily b, polypeptide 10
CYP26	Cytochrome P450, Family 26
Dazl	Deleted in azoospermia-like
DEHP	Diethyl hexyl phthalate
DHRS3	Retinaldehyde reductase 3
DMRT1	Doublesex and mab-3 related transcription factor 1
dpc	Days post coitum
DRP	Detailed Review Paper
Foxl2	Forkhead box protein L2
FSH	Follicle stimulating hormone
FXR	Farnesoid X receptor
GD	Gestational day
GR	Glucocorticoid receptor
GW	Gestational week
HSD	Hydroxysteroid dehydrogenase

KE	Key event
LH	Luteinizing hormone
LRAT	Lecithin:retinol acyltransferase
LXR	Liver X receptor
MEHP	Monoethyl hexyl phthalate
MIE	Molecular initiating event
MMP	Matrix metalloproteinases
Nanos2	Nanos C2HC-Type Zinc Finger 2
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
PCOS	Polycystic ovarian syndrome
PGC	Primordial germ cells
PND	Post-natal day
PPAR	Peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
(Q)SAR	(Quantitative) structure-activity relationship
RA	Retinoic acid
RAL	Retinaldehyde
RALDH	Retinaldehyde dehydrogenase
RAMBA	RA metabolism blocking agents
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBP	Retinol binding protein
RBP4	Retinol-binding protein 4
RDH10	Retinol dehydrogenase 10
RDH11	Retinol dehydrogenase 11
RE	Retinyl esters
Rec8	Meiotic recombination component gene Rec8
REH	Retinyl ester hydrolase
RIP140	Nuclear receptor interacting protein 1
ROH	Retinol
RXR	Retinoid X receptor
P450scc	Cytochrome P450 cholesterol side-chain cleavage enzyme
SDR	Short-chain dehydrogenase/reductase
Sox9	SRY -Box Transcription Factor 9
SRC-1; Ncoa1	Nuclear receptor coactivator 1
SREBP-1c	Sterol regulatory element binding protein-1c
Sry	Sex-determining region Y protein
Stra6	Stimulated by retinoic acid, gene 6
Stra8	Stimulated by retinoic acid, gene 8
TG	Test guideline
TTR	Transthyretin
VAD	Vitamin A deficiency

VDR	Vitamin D receptor
WEC	Whole embryo culture
Wnt4	Wnt Family Member 4

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Executive Summary

11. Retinoids are essential molecules that are needed for normal physiological functions, including neurodevelopment, growth, and cellular metabolism. The importance of retinoid signalling is reflected in the conservation of genes and pathways across many phyla, including vertebrates and invertebrates. It is therefore not surprising that dysmorphogenesis of various tissues associated with altered retinoid transport, metabolism and signalling is reported in wild populations of fish, birds, amphibians and mammals. Subtle increases or decreases in concentrations of retinoic acids (the main biologically active form of Vitamin A) or some of its metabolites can directly influence the expression of genes that regulate cell differentiation and maturation with direct consequences for fundamental life processes in virtually every organ and species. Examples include sex determination, neural tube formation and formation of craniofacial structures. Such biological consequences are mediated by the retinoic acid receptor (RAR) which acts in concert with the retinoid X receptor (RXR) as a functional heterodimer. Tissue remodelling by retinoids, including tissue sculpturing through programmed cell death, can be equally profound when alterations occur during very specific windows of development.

12. The precise balance of retinoids in animals is regulated by a complex interplay between enzymes that can convert dietary forms of retinoids into retinol, and then into retinyl esters for transportation in the blood and storage in tissues, and finally removal and clearance. In addition, retinoid signalling is highly intricate with multiple nuclear receptor isoforms (RARα, β and γ; RXRα, β and γ) and possible combinations of heterodimer pairs, and different co-activators and repressors - all with specific tissue distributions.

13. There is increasing evidence that certain environmental chemicals (including organochlorine pesticides, alkylphenols and styrene dimers) can bind to, and transactivate, the RAR. Considering the critical role of retinoids in key physiological processes, it is important to develop a thorough understanding of the extent of retinoid disruption in humans and wildlife, the most important mechanisms for disruption, and to initiate a systematic process to identify and develop a suite of assays to accurately test for potential retinoid system modulators.

14. The RXR functions as a central node in regulating various facets of reproduction, development, and lipid homeostasis through its heterodimerization with other nuclear receptors. In addition to the RAR, RXR agonists have the potential to disrupt signalling mediated by the Peroxisome Proliferator-Activated Receptor (PPAR), farnesoid X receptor (FXR), liver X receptor (LXR), thyroid hormone receptor (THR), and Vitamin D receptor (VDR). The RXR has been shown to be highly susceptible to activation by some xenobiotics, such as tributyltin, resulting in alterations in lipid homeostasis and intersex conditions in some invertebrates.

15. Despite the importance of retinoid signalling in many life processes, and the potentially broad adverse effects of disrupting this signalling system, there are currently no OECD test guidelines that specifically cover retinoid system modulation. The OECD DRP No. 178 highlighted the retinoid signalling pathway for inclusion in a chemical testing battery. The aim of this document is to provide a more detailed overview of the understanding of the role of retinoid signalling in several organ systems for which more information may be known, as well as evaluate potential gaps in knowledge, and identify

potential markers and endpoints that could be added to existing OECD test guidelines, in addition to *in vitro* and *ex vivo* test systems that measure retinoid pathway targets.

16. The overview of retinoid biology in the main text of this DRP highlights the complexity of the signalling pathway and potential cross talk with other pathways. Though more information is available for retinoid effects on the reproductive, craniofacial/skeletal, and central nervous systems, each detailed in the respective Annexes (A-C), considerable gaps in knowledge remain. The species, sex, window of susceptibility, organ, tissue, and specific tissue regions are all important variables to consider when identifying potential endpoints to add to existing *in vivo* OECD test guidelines.

17. The OECD Retinoid Expert Group was asked to make recommendations for potential *in vitro* and *in vivo* assays to measure the effects of environmental chemicals on retinoid signalling (Annex D). With respect to potential *in vitro* assays for development, the group suggested that it may be useful to focus on effects unique to retinoids and not covered by other MoAs considered in regulatory toxicology. While there are some relevant *in vitro* assays that are currently available, much of the current work is emerging science and the available tools would benefit from a critical evaluation of the strengths and weaknesses. Existing cell-based assays may be more easily interpreted, and for some endpoints that may be affected by multiple signalling pathways (*e.g.* cell migration), adding toxicogenomics may increase the specificity, though at this point, it may be difficult to know what genes to target. The Expert Group noted that some existing *in vivo* endpoints under consideration are at a high level technical relevance (*e.g*. mammalian pup gonadal histopathology), however, histopathology is a non-specific endpoint. Furthermore, the number of animals examined needs to be taken into account and if there is no observed effect on histopathology measures, one cannot be sure there is no effect of the chemical.

18. The Expert Group emphasized the need for using an AOP framework to help understand the link between specific *in vitro* and -omics targets with non-specific downstream effects. AOPs can also help to unravel the complexity of crosstalk between pathways and understand the relationships between key events in an AOP, as well as identify gaps in biological understanding. In the interim, while knowledge is being gained, and despite challenges posed due to the interplay of retinoid signalling with other pathways/bioregulators and spatial/temporal signalling complexities, a retinoid AOP approach may (or will) aid integrating useful AOPs and moving forward towards the goal of chemical screen development.

Introduction

1.1. History and general background

19. Retinoids[3], a chemically related group of compounds which includes vitamin A, can exist in several different forms (see Figure 1.1). As the name "vitamin" implies, they are essential micronutrients that must be supplied from the diet, either in the form of carotenoids (orange, red and yellow fat-soluble pigments) from vegetable sources, or retinol (ROH) and retinyl esters (RE) from animal sources (reviewed by Harrison 2012, Al Tanoury *et al.* 2013). Retinoids from animal sources are originally derived from carotenoids (reviewed in O'Byrne and Blaner 2013). The liver, an organ found in all vertebrate organisms, can be a rich source of retinoids and may have been used by the ancient Egyptians as a cure for night blindness, a typical symptom of retinoid deficiency (Wolf 1996). Conversely, Arctic cultures have long known to avoid eating polar bear liver, which can contain extremely high levels of retinoids, in order to avoid adverse effects such as blurred vision, nausea, skin loss, coma and even death (Rodahl and Moore 1943). Thus, both hypovitaminosis A and hypervitaminosis A can be detrimental.

20. In addition to being a nutrient-derived vitamin, retinoids can also be considered as hormones, based on their nuclear receptor signaling (Giguère *et al.* 1987, Petkovich *et al.* 1987). In contrast to classical hormones, there is no endocrine gland that synthesizes retinoids, controlled *via* feedback by the hypothalamus and pituitary gland. Instead, the levels of the active form of vitamin A (all-*trans* retinoic acid; RA) are tightly regulated via local cellular enzymatic mechanisms; a regulation that is critical for correct signaling *via* the nuclear receptors (reviewed in Ghyselinck and Duester 2019).

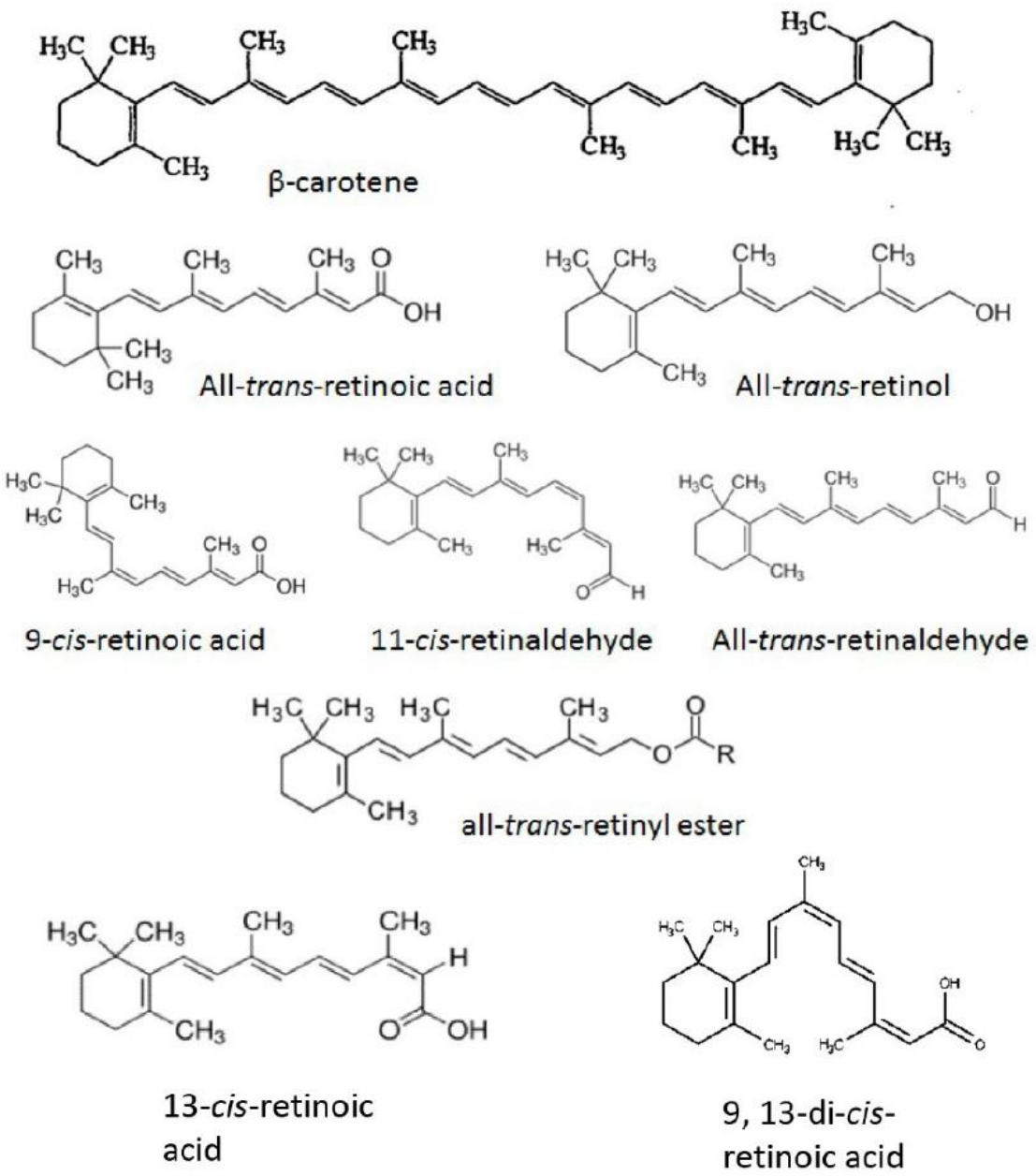
21. The requirement for retinoids in normal physiological functions has been studied for more than a century, by examining the effects of retinoid deficiency or excess in different species, and, more recently, in genetically modified mice (reviewed in Clagett-Dame and Knutson 2011). The importance of retinoids and the retinoid signaling pathways is reflected in both the ancestry and the conservation of genes and pathways among many phyla, including vertebrates and invertebrates (André *et al.* 2014). Dysmorphogenesis of various tissues associated with altered retinoid transport, metabolism and signalling is reported in wild populations of fish, birds, amphibians and mammals. Indeed, subtle increases or decreases in concentrations of RA or its metabolites can directly influence genes that regulate cell differentiation and maturation with direct consequences for fundamental life processes in virtually every organ and species (Novak *et al.* 2008). Examples include sex determination, heart looping, neural tube formation and formation of craniofacial structures. Such biological consequences are mediated by the RAR which acts in concert with the RXR as a functional heterodimer. The importance of the RAR-RXR heterodimer in mediating the biological functions of retinoids has been nicely illustrated in gastropods, where 9-cis-retinoic acid (9cRA) exposure induces imposex (the

[3] The term "retinoids" originally had a structural basis, referring to isoprene-derived compounds (IUPAC 1982), but currently the term refers to compounds (natural or synthetic) capable of activating a set of receptors (Sporn and Roberts 1985).

imposition of the male penis upon the female mollusc) in the same way as the organotin TBT (RXR agonist) by binding to the RAR and/or RXR (Nishikawa *et al.* 2004). Such types of tissue remodelling by retinoids, including tissue sculpturing through programmed cell death, can be equally profound when alterations occur during very specific windows of development. Retinoids are essential for vision, reproduction, embryo-fetal development, adult growth and development, and maintenance of immunity and epithelial barriers (reviewed in O'Byrne and Blaner 2013). Most diets contain sufficient amounts of retinoids, and the most fat-soluble forms, retinyl esters, can be stored within the body at relatively high levels, thereby counteracting periods of low dietary retinoid intake (reviewed in O'Byrne and Blaner 2013). In spite of this, vitamin A deficiency (VAD) is common in some parts of the world (WHO 2009), and VAD is the main cause of preventable blindness in the world (West 2003, Bastos Maia *et al.* 2019). It has also been hypothesized that one reason for hearing loss among humans in the developing world is gestational VAD (Emmett and West 2014). In addition, new metabolic functions for retinoids have been reported, in *e.g.* lipid metabolism and insulin response (reviewed in Napoli 2017, Cione *et al.* 2016).

22. For several decades, retinoids have been used for pharmaceutical purposes (reviewed in Theodosiou *et al.* 2010). For example, retinoids can be prescribed for treatment of cystic acne, where they have favorable effects on epithelial cell differentiation. In acute promyelocytic leukemia, retinoids can induce terminal differentiation in cancerous cells, and thereby proliferation ceases.

Figure 1.1 Chemical structures of various forms of naturally occurring retinoids and retinoid precursors. R (in the retinyl ester) represents an acyl chain of variable length (reviewed in Goodman 1984)



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1.2. Retinoid storage, uptake, transport and metabolism

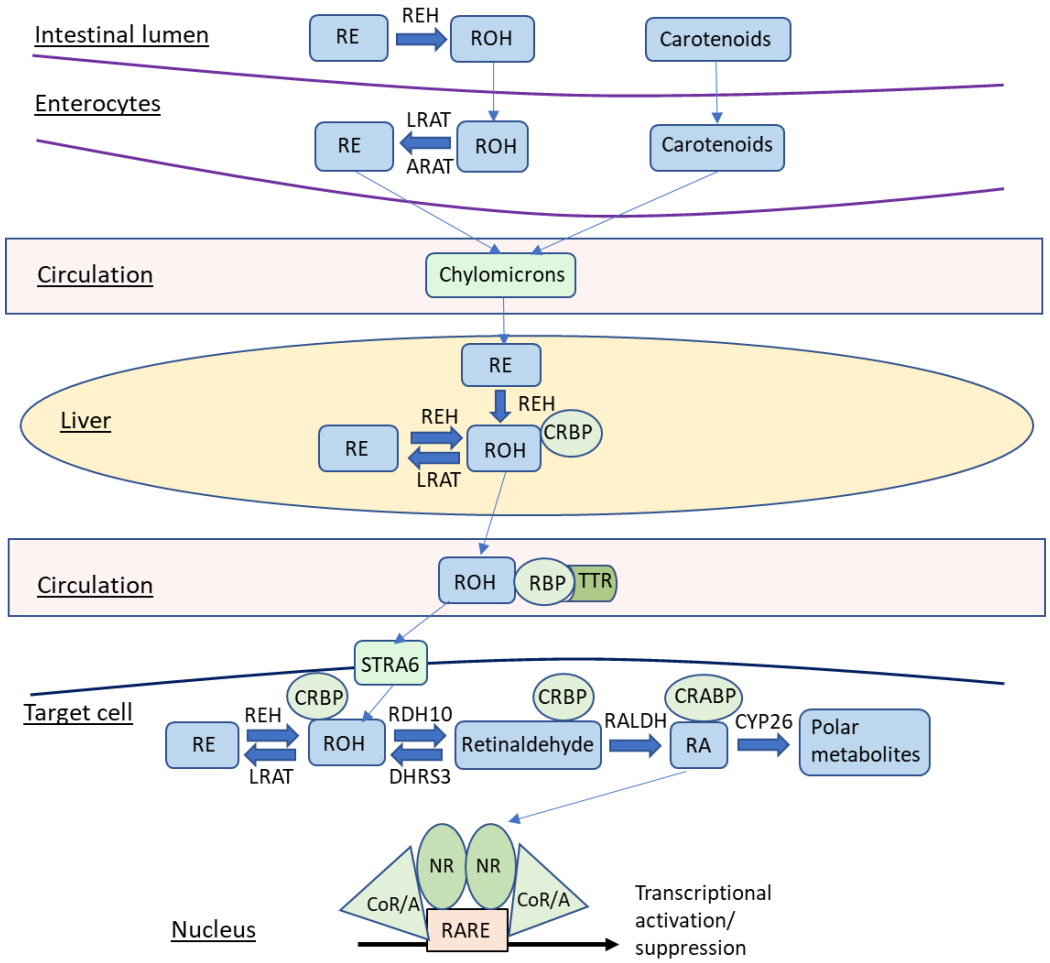
1.2.1. Storage

23. The vast majority, approximately 80-85%, of retinoids in the body are stored in the liver (reviewed in O'Byrne and Blaner 2013). Two main cell types, hepatocytes and hepatic stellate cells, have been identified as being central to metabolism and storage of retinoids. Hepatocytes contain 10-20% of hepatic retinoids, and they are also involved in the initial uptake of retinoids into the liver (Blomhoff *et al.* 1982). Hepatocytes are also the site of synthesis and secretion of the serum transport protein of ROH; retinol-binding protein type 4 (RBP4; Sauvant *et al.* 2001). Thus, hepatocytes are important both in the uptake and the mobilization of vitamin A into, and out of, the liver. Hepatic stellate cells have been found to contain 80-90% of hepatic retinoids (reviewed in Blaner and Li 2015). Studies in mice using radiolabeled RE have shown dietary retinoids to be initially taken up by the hepatocytes and then rapidly transferred to hepatic stellate cells for storage (Blomhoff *et al.* 1982). Nevertheless, the adipose tissue also stores significant amounts of retinoids as ROH and RE which are used to maintain its homeostasis by activation of nuclear receptors (section 1.3. Retinoid receptors and gene regulation). Additionally, retinoids are mobilised at demand of the organism from the adipose tissue which also synthesises a significant amount of RBP4 (Blaner *et al* 2019). Intersetingly, there is a correlation between retinoid levels in adipose tissue and those in the liver) (Sheftel *et al*., 2019). Smaller RE stores are also found in a number of other organs or tissues, including the lung, brain, skin, muscle, kidney, and testis (reviewed in Blaner and Li 2015).

24. Hepatic reserves of retinoids, i.e. total hepatic retinoid concentrations, are considered the gold-standard biomarker for vitamin A status (Tanumihardjo, 2011; Tanumihardjo *et al.*, 2016). Hepatic retinoid concentrations were proposed to be used to classify the vitamin A status as deficient (< 0.1 µmol / g), adequate (0.1 – 0.7 µmol / g), high (0.7 – 1.0 µmol / g), hypervitaminotic (> 1.0 µmol / g) and toxic (~ 10 µmol / g) (Tanumihardjo *et al.*, 2016). The cutoff set at <0.1 mmol/g was supported by the kinetics of retinoid as determined in experimental animals (Tanumihardjo *et al.*, 2016).

25. Another retinoid storage organ is the eye (reviewed in Palczewski 2012). Retinaldehyde has long been known to play a vital role in vision and eye function. The retina and retinal pigmented epithelium contain 11-*cis* and all-*trans*-isomers of retinaldehyde, but also ROH and RE. The retinal pigmented epithelium is the main ocular site of RE storage, while the photoreceptors of the retina contain large amounts of retinaldehyde (reviewed in Palczewski 2012).

Figure 1.2. Overview of retinoid uptake, transport, metabolism, and nuclear signaling, as described in detail in the text below.



Note: In the intestinal lumen, RE are hydrolyzed into ROH, which after uptake into enterocytes is re-esterified to RE. RE together with carotenoids are incorporated into chylomicrons and exported to the circulation. Eventually, the chylomicron remnants are taken up by the liver. Here, RE are again hydrolysed into ROH. ROH is either re-esterified for hepatic storage or bound to RBP4 for release into the circulation. In the bloodstream, the ROH-RBP4 complex associates with transthyretin (TTR). After uptake (possibly aided by STRA6) into target cells, ROH is reversibly oxidized to retinaldehyde by RDH10. Retinaldehyde may then be irreversibly oxidized to RA by retinal dehydrogenases (RALDHs), or be reduced back to ROH by DHRS3. RA is degraded to non-active polar metabolites by CYP26 enzymes, but can also be shuttled to the nucleus by cellular RA-binding proteins (CRABP), where RA acts as a ligand to nuclear receptors (NR).Non-liganded receptors interact with co-repressors (CoR), repressing transcription, while RA liganded receptors- binds co-activators (CoA), subsequently activating transcription, thereby modulating expression of Retinoic Acid Response Element (RARE)-regulated target genes. Additional abbreviations: ARAT, acyl CoA:retinol acyltransferase); CRBP, cellular retinol-binding protein; CYP26; cytochrome P450 hyroxylase type 26; DHRS3, retinaldehyde reductase 3; LRAT, lecithin:retinol acyltransferase; RA, retinoic acid; RE, retinyl esters; REH, retinyl ester hydrolase; RBP, retinol-binding protein 4; ROH, retinol; SDR, short-chain dehydrogenase/reductase; Stra6, stimulated by retinoic acid, gene 6; RDH10, retinol dehydrogenase 10.

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25. A number of enzymes are required for the balance of storing and re-mobilizing of retinoids during times of abundance and insufficiency, respectively (see Figure 1.2). The most important enzymes are those synthesizing RE (the physiologically important enzyme is LRAT; lecithin:retinol acyltransferase) and hydrolyzing RE to ROH (REH; retinyl ester hydrolase). These enzymes, along with accumulation of RE in lipid droplets (chylomicrons) within cells and tissues, manage the natural fluctuations in dietary retinoid intake (O'Byrne and Blaner 2013). The process of remobilization of stored

RE into circulating ROH seems reliant on interactions with RBP4, based on observations in RBP4-deficient mice (Quadro *et al.* 1999).

1.2.2. Uptake and transport

26. Intestinal uptake of retinoids in the diet (reviewed in Harrison 2012) involves hydrolysis of RE (by REH) into ROH in the intestinal lumen, followed by re-esterification of ROH into RE after absorption of ROH into the enterocytes. In addition to LRAT, the enzyme acyl CoA:retinol acyltransferase (ARAT) can play an important role when the levels of ROH exceed normal physiological levels (as after a meal rich in retinoids). The resulting RE, together with carotenoids, are released into the circulation as part of chylomicron particles.

27. In the circulation, a range of retinoid forms are found in fasting and/or non-fasting states. ROH bound to RBP4 and chylomicron-RE are considered the most important transport forms. Circulating RE levels are highly dependent on the dietary content of RE and carotenoids. During fasting, ROH is the main circulating form of retinoids. Serum ROH levels are maintained within a narrow range, unless the RE levels in liver and other tissues are very low (Green and Green 1994, and reviewed in Tanumihardjo *et al.* 2016).

28. Both circulating and intracellular ROH is generally bound to chaperone proteins. Of these, RBP4 transports ROH in the blood, and cellular retinol-binding protein types 1 and 2 (CRBP1, CRBP2) and inter-photoreceptor retinoid-binding protein are believed to manage intracellular transport (reviewed in Blaner and Li 2015). In contrast, there is no specific binding protein for the transport of RA in the plasma, as it is synthesized locally in target cells. Intracellular retinoic acid-binding proteins (CRABPs) are involved in RA transport from the cytoplasm to the nuclear retinoic acid receptors (RAR), and also play a role in determining intracellular RA levels by controlling the amount of RA that is available for degradation (reviewed in Napoli 2012, and in Napoli 2017).

29. Retinoids are delivered to tissues in a similar manner as vitamin D and thyroid hormone (reviewed in Blaner and Li 2015), *i.e.*, by transport of large quantities of the biologically-inactive form (in this case ROH), and relatively low quantities of the biologically-active form (RA) in the blood (reviewed in Blaner and Li 2015). ROH, bound to RBP4, forms a larger complex with the T4 transporting protein transthyretin (TTR) in the blood (See Figure 1.2). Both TTR and RBP4 are mainly synthesized in the liver and choroid plexus, secreted into plasma by the liver, and into cerebrospinal fluid by the choroid plexus, respectively (Shirakami *et al.* 2012).

30. Transport of ROH (bound to RBP4) across membranes may be facilitated in both directions by the membrane protein, stimulated by retinoic acid, gene 6 (STRA6) (reviewed in Kelly and von Lintig 2015). ROH uptake by STRA6 seems to be enhanced by the presence of LRAT and CRPB1 on the intracellular side of the membrane, in a manner that appears to depend on the availability of ROH inside the cell. Alternatively, with the exception of the eye, the role of STRA6, even under VAD conditions, may not be mandatory for ROH availability to tissues (Berry *et al.* 2013).

1.2.3. Metabolism

31. The most abundant forms of retinoids in the body are ROH and RE. A number of conversion steps are required to transform relatively inactive forms of retinoids into the biologically active RA form within target cells (reviewed in Shannon *et al.* 2017). Once ROH-RBP4 has been bound to STRA6 and taken up into a target cell, ROH is converted to RA via a two-step oxidation process (See Figure 1.2). ROH is first converted into retinaldehyde; this conversion is carried out by a short-chain dehydrogenase/reductase (SDR; a complex consisting of retinol dehydrogenase 10 [RDH10], and retinaldehyde reductase 3 [DHRS3]), and possibly also by the less specific cytosolic alcohol dehydrogenases (ADH) (reviewed in Shannon *et al.* 2017). The predominant fraction of intracellular

ROH is bound to CRBP and directed towards SDR (reviewed in Napoli 2017). The formed retinaldehyde can subsequently either be reduced back to ROH by SDR, or, under VAD conditions, by retinol dehydrogenase 11 (RDH11) (Belyaeva *et al.* 2018). In a second RA-conversion step retinaldehyde can be irreversibly oxidized into RA by retinaldehyde dehydrogenases RALDH 1, 2 or 3 (also known as aldehyde dehydrogenases ALDH1A1-3, reviewed in Shannon *et al.* 2017). The ratio of holo-CRBP to apo-CRBP[4] appears to signal whether retinaldehyde should be reduced back to ROH or further oxidized to RA (reviewed in Napoli 2017). The fate of the retinaldehyde also depends on a negative feedback response to available RA (reviewed in Shannon *et al.* 2017).)

32. RA has a short half-life (approximately one hour), and RA tissue levels are governed in a spatially and temporally controlled manner, mainly by a balance of local synthesis and metabolic breakdown with RA feedback loops (reviewed in Kedishvili 2013, and Teletin *et al.* 2017). Some tissue-specific uptake of RA from serum appears to take place, *via* a still unestablished mechanism (Kurlandsky *et al.* 1995). RA is catabolized mainly by cytochrome P450 hydroxylases (CYP) 26A1, B1 and C1 (Topletz *et al.* 2015). A large number of other CYP enzymes have also been shown to degrade RA *in vitro*, although the relevance of these enzymes in normal retinoid homeostasis *in vivo* is unclear (reviewed in Laudet *et al.* 2015). In the adult human, CYP26A1 is mainly expressed in the liver, and is responsible for more than 90% of the hepatic clearance of RA (Thatcher *et al.* 2010). CYP26A1 is also expressed in *e.g.* testis, epididymis, uterus, endometrium and placenta, while CYP26B1-expression is more dispersed and found *e.g.* in the brain, testes, placenta, ovaries and endometrium. (Human Protein Atlas[5]). In the human fetus, CYP26A1 is the form predominantly expressed in the brain, whereas CYP26B1 is expressed in all tissues except the brain (reviewed in Kedishvili 2013). At least in humans, CYP26C1 is mainly expressed during embryonic development, but is also expressed at low levels in adult tissues, *e.g*. in testis (reviewed in Ross and Zolfaghari 2011).

33. The physiological role of RA in cell differentiation has motivated its use in cancer treatment, including reproductive organ malignancies (Siddikuzzaman *et al.* 2011). The treatment increases the normal serum nM levels of RA up to µM levels, which over time leads to autoinduction of RA clearance *via* upregulation of CYP26 enzymes (Jing *et al.* 2017). Attempts have been made to counteract therapy resistance by developing *e.g.* CYP26 inhibitors (Nelson *et al.* 2013). However, side effects and low potency has limited the use of these inhibitors (Jing *et al.* 2017). Similar efforts to maintain RA homeostasis has also been observed in models of teratogenicity, and it has been speculated that some of the teratogenic effects of RA may be due to a prolonged local RA deficiency, caused by differential induction of genes coding for enzymes synthesizing (*Raldh1-3*) or breaking down (*Cyp26a1* and *Cyp26b1*) RA (Lee *et al.* 2012).

1.3 Analytical chemistry of retinoids

34. There are a number of reviews on the analytical chemistry of retinoids to which the reader is referred (Kane 2012; Wyss 1995; Gundersen and Blomhoff 2001; Gundersen 2006; Barua and Furr 1998). Liquid chromatographic methods along with ultraviolet and mass spectrometric detections are considered to be the most appropriate methods for challenges of measuring endogenous retinoids in biological samples.

[4] Ratio: holo-CRBP (CRBP-ROH/retinaldehyde) to apo-CRBP (CRBP without ROH/retinaldehyde)

[5] https://www.proteinatlas.org/ accessed in May 2019

1.3.1. Laboratory precautions

35. The polyene chain of retinoids make them vulnerable to light, oxidation, acids and heat as described below and reviewed in Wyss *et al.* (1995), Barua and Furr (1998), Gundersen and Blomhoff (2001), and Gundersen (2006). Therefore, retinoids should be protected from natural as well as from other common in-door light sources and hence, they are often handled under light above 500 nm, i.e. dim yellow or red light to avoid isomerization. For storage, amber coloured containers are preferred and are kept in the dark. Additionally, biological samples should be protected from light, though photo-induced isomerization of retinoids in tissues and blood occurs to a lower extent compared with transparent retinoid solutions in organic solvents.

36. With regard of oxidation, it is recommended purging solutions with argon or, if not available, nitrogen and, in any case, fill containers to reduce as much as possible the presence of air. Additionally, butylated hydroxytoluene was often used as an antioxidant in stock solutions for long- term storage. However, such antioxidant can be deleterious in the analysis, for which it is avoided in most of the current methods. With regard of biological samples, in many cases, immediate analysis of retinoids is not possible, for which tubes of serum samples were also flushed with argon or nitrogen in some studies.

37. Retinoids dissolved in organic solvents are stable for hours at room temperature. For long-term storage of retinoid solutions, temperatures below -30 ^{o}C, and if available at -80 ^{o}C, are preferred. When collecting biological samples, it is recommended use liquid nitrogen to flash freeze tissue samples to avoid degradation and store them at -80 ^{o}C.. Critically, tissues should be immersed in ice bath for homogenization (or other sample preparation techniques) and must be extracted immediately as retinoids can be degraded by endogenous enzymes and thiols.

38. Additionally, acidic solutions must be avoided for storage of retinoids as isomerization and hydrolysis of glucuronides or other conjugates might occur. Although saponification has been a method to hydrolyse retinyl esters into retinol, alkaline solutions should also be avoided for long-term storage of retinoids.

39. Furthermore, glass, plastic or steel materials often used in laboratories may adsorb retinoids. Thus, glass was recommended to be pyrolysed above 450 oC for re-cross-linking of the silanol groups of glass surfaces and decontamination of organic compounds (Schmidt *et al.* 2003).

40. Taken together, besides the implementation of proper laboratory precautions, it is necessary to use retinoid standards to monitor sample preparation and retinoid analysis at the different step to control the potential degradation of retinoids.

1.4 Retinoid receptors and gene regulation

41. RA acts mainly by interacting with nuclear retinoic acid receptors, RARs, which form heterodimers with retinoid X receptors (RXRs) and ultimately regulate gene transcription, thereby influencing a variety of cellular processes (reviewed in Germain *et al.* 2006). The receptor dimers bind to retinoic acid response elements (RARE), and, in the absence of ligand, recruit a co-repressor complex that suppress transcription (Vilhais-Neto and Pourquié 2008). In the presence of ligand, the co-repressor complex dissociates and is replaced by a co-activator complex, leading to transcription of the target gene (Germain *et al.* 2002). While RAR can be activated both by RA and 9-*cis*-RA as well as synthetic ligands, RA is considered to be the only endogenous RAR ligand (Allenby *et al.* 1993, Mouchon *et al.* 1999). RARs in an unliganded state are also known to act as transcriptional repressors (reviewed in Weston *et al.* 2003). The endogenous ligand for RXR was initially suggested to be 9-*cis*-RA, and other forms such as 9-*cis*-13,14-dihydro-RA have been put forth more recently (de Lera *et al.* 2016). However, the physiological relevance of the suggested ligands is still unclear (reviewed in Krężel *et al.* 2019). Non-enzymatic isomerization between different forms is likely to be important (reviewed in Blaner 2001),

which impedes conclusions regarding endogenous ligands. In RAR-RXR heterodimers, RXR is a “silent partner”, meaning that the RAR ligand is both necessary and sufficient for dissociating the corepressor complex (Mangelsdorf and Evans 1995, le Maire *et al.* 2019).

42. RARs and RXRs belong to the same nuclear hormone receptor family as steroid hormones, thyroid hormone and vitamin D receptors, as well as various orphan receptors and receptors activated by intermediary metabolites (*e.g.* PPAR; peroxisome proliferator-activated receptor, LXR; liver X receptor, FXR; farnesoid X receptor and PXR; pregnane X receptor) (Szanto *et al.* 2004). RXR is also the essential heterodimerization partner to these receptors. Some of these nuclear receptors may also be involved in retinoid signaling responses. For example, PPARα, β/δ and γ heterodimerize with RXR and function as transcription factors (Mangelsdorf *et al.* 1995; see also section on cross-talk below). RA has been reported to serve as a ligand for PPAR β/δ, but with a much lower affinity than for RAR (Al Tanoury *et al.* 2013).

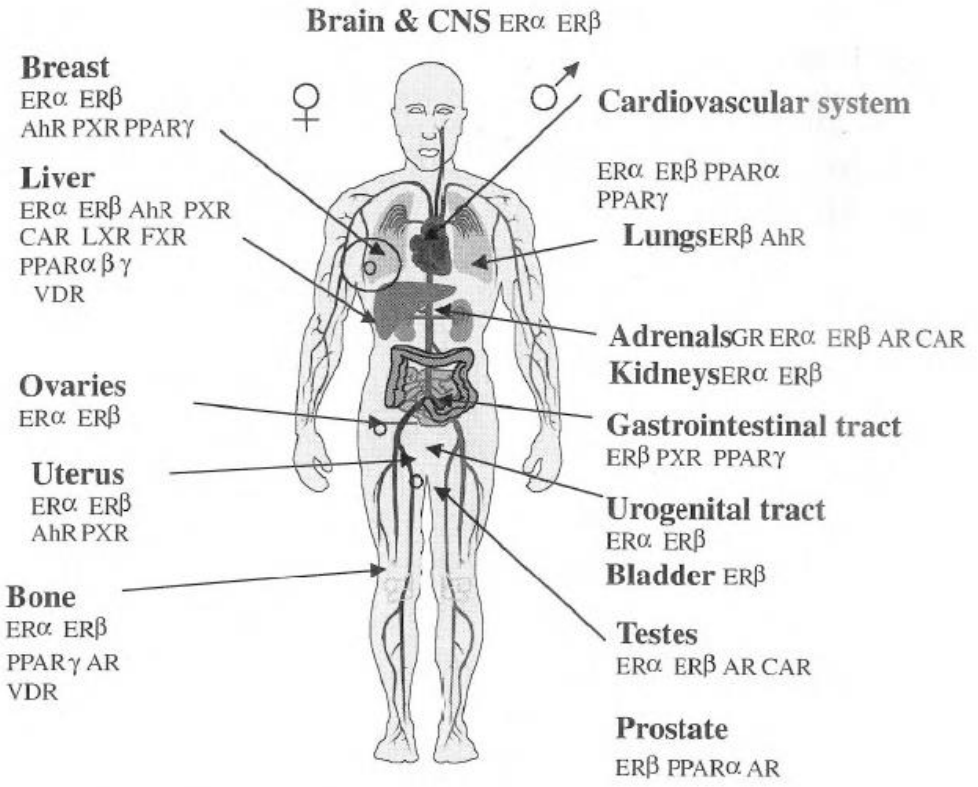
43. Three of the retinoid receptors (RARα, RXRα and RXRβ) have widespread expression patterns, whereas RARβ, RARγ and RXRγ show a more restricted, tissue-specific expression. Therefore, most tissues are potential targets of retinoid signalling, although different heterodimeric complexes can transduce the RA signal (reviewed in Rhinn and Dollé 2012). Many tissues will also be indirect targets *via* RXR heterodimerization with receptors (see Figure 1.3). Receptors mediate and interpret the information provided in the chemical structure and energy of a nuclear receptor ligand, in the context of the cell and its physiology, converting it into a sequence of receptor-protein and receptor DNA interactions. This can be *via* ligand binding, receptor phosphorylation, induction of allosteric changes in receptor docking surfaces including subunits of transcription, epigenetic machinery and enzyme induction. The next section explores these latter molecular aspects of retinoid mechanisms related to receptor cross-talk.

44. In addition to the classical genomic effects, RA has been found to have a number of non-genomic mechanisms such as kinase activation (reviewed in Rochette-Egly 2015, Park *et al.* 2019). More specifically, these effects may include activation by RA of phosphatidylinositol 3-kinase(PI3K)/Akt signalling during neural differentiation, rapid activation of p38 mitogen activated protein kinase (p38MAPK)/mitogen and stress-activated kinase 1 (MSK) pathways (reviewed in Laudet *et al.* 2015). Such effects are not further discussed in this report.

1.5 Heterodimerisation partners and receptor cross-talk

45. Members of the same nuclear receptor family share a common heterodimerisation partner, RXR. There is cross talk with other nuclear receptors and with a broad range of intracellular signaling pathways. Consequently, there may be competition for RXR for the dimerization stage of receptor activation of DNA. There may even be a cascade effect, in which metabolites produced through the activities of one receptor act as specific signaling molecules and ligands to modulate the next receptor, a link in the nuclear receptor intercommunication web of the body (See Figure 1.3).

Figure 1.3. Schematic diagram showing differences in tissue distribution of nuclear receptors, RXR; retinoid X receptor, and heterodimerisation partners.



Note: RXR; retinoid X receptor, is the heterodimerisation partner essential to the normal functioning of the main xenobiotic metabolizing receptors including CAR, PXR, PPARs, LXR, FXR Furthermore, cross-talk with ERα and ERβ is established. In addition, RXR is also the heterodimerization partner for VDR and the thyroid hormone receptor (THR) (not included in the Figure). *Abbreviations*: AhR; Aryl hydrocarbon receptor, AR; androgen receptor, CAR; constitutitive androstane receptor, ERα and ERβ; estrogen receptors α and β, FXR; farnesoid X receptor, GR; glucocorticoid receptor, LXR; liver X receptor, PPARs; peroxisome proliferator activated receptors, PXR; pregnane X receptor, VDR; vitamin D receptor.
Source: Jacobs 2005, reprinted with kind permission from the publisher: Taylor and Francis Inc.

46. The ubiquitous RXRα is the necessary heterodimerization partner for many receptors, and is essential for xenobiotic metabolism *in vivo*. The receptors include the THR, PXR, CAR, where retinoic acid has also been noted to repress CAR induction of the Cytochrome P450, family 2, subfamily b, polypeptide 10 (*Cyp2b10)* gene in mouse hepatocytes (Kakizaki *et al.* 2002), as well as LXR, FXR, GR, PPARα (Cai *et al.* 2002) and PPAR gamma (Dubuquoy *et al.* 2002) to bind to DNA. Crystal structure data of the PPARγ and RXRα heterodimer shows the asymmetric conservation heterodimerization interfaces between both receptors (Gampe *et al.* 2000). RXRα dimerizes through a 40-amino acid subregion within the ligand binding domain, known as the 'identity box'. Mutation of two important determinants (alanine 416 and arginine 421) within this box has been shown to impair the actions of receptor dimerization partners. RXRα is well established as a heterodimeric integrator of multiple physiological processes in the liver, and is a regulatory component of cholesterol, fatty acid, bile acid steroid and xenobiotic metabolism and homeostasis.

47. The retinoid ligands of RXR have distinct effects in different contexts and have been reported to significantly alter the response of the CAR-RXR heterodimer to CAR ligands (Tzameli *et al.* 2003) for example. Suppression of RXRα has a concomitant effect upon the heterodimerization partner. For example LXR is reported in this way to inhibit PPARα signaling in the nutritional regulation of fatty acid

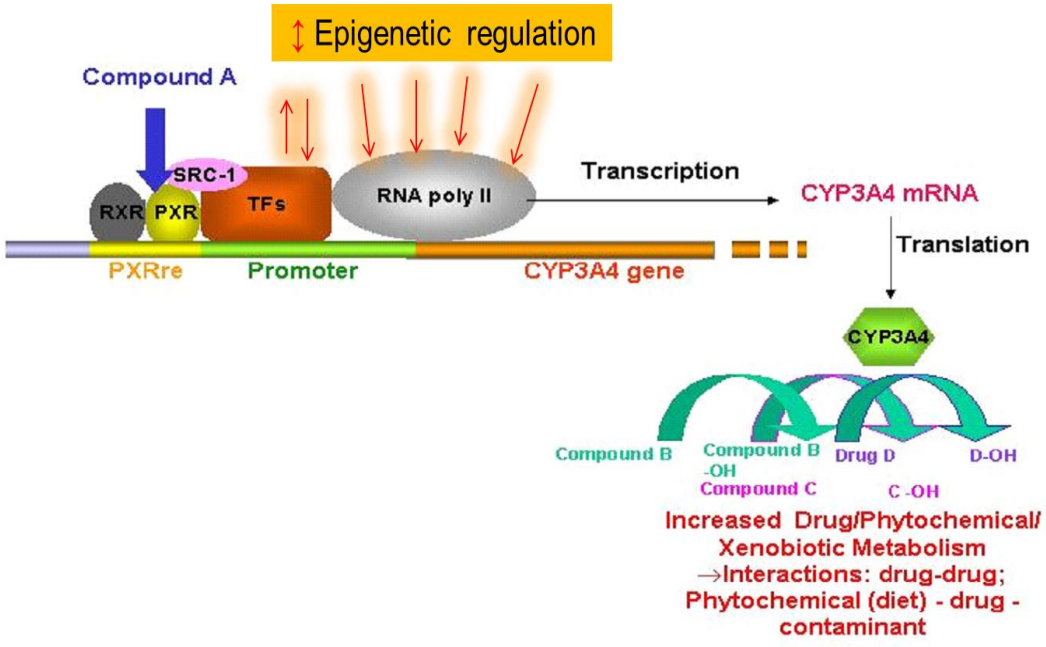
metabolism. PPARα has a counter-inhibitory action repressing LXR/RXR binding through the sterol regulatory element binding protein-1c (SREBP-1c) (Ide *et al.* 2003, Yoshikawa *et al.* 2003).

48. RXRα also has cofactors in common with other nuclear receptors, for instance, over 20 years ago Wiebel and co-authors described a competitive element between nuclear receptor interacting protein 1 (RIP140) and nuclear receptor coactivator 1 (SRC-1; Ncoa1) in binding with OR-1 with RXR to heterodimers of a novel orphan receptor (Wiebel *et al.* 1999). RIP140 (NRIP1) is also implicated in the potentiation of endocrine disrupting compounds *in vitro* (Sheeler *et al.* 2000). SRC-1 RXR phosphorylation can also be induced through stress pathway activation (Lee *et al.* 2000), which would reduce RXR availability for other receptor heterodimerization partners. Hua and co-authors have demonstrated competition between RAR/RXR heterodimers and ERα for binding sites in a breast cancer MCF-7 cell line. Indeed, the experimental work suggested that there may be antagonistic transcriptional regulation for up to 71% of the target genes that they evaluated (Hua *et al.* 2009).

49. As with many nuclear receptors, ERα- and RAR-binding sites appear to have co-evolved on a large scale throughout the human genome, often resulting in competitive binding activity at nearby or overlapping *cis*-regulatory elements. The intersection between these two critical nuclear hormone receptor signaling pathways is highly coordinated to give a unifying mechanism for balancing gene expression output via local regulatory interactions dispersed throughout the genome. This selection or competition of dimerization partners determines tissue/organ and biological system level outcomes. It can be affected by genetic, nutritional, and environmental factors, which for the latter can include both beneficial and adverse nutrient and chemical exposure.

50. An example for the RXR-PXR downstream activation of CYP3A4 is given in Figure 1.4.

Figure 1.4. Illustration of chemical-nuclear receptor-, and epigenetic, transcription factor regulation

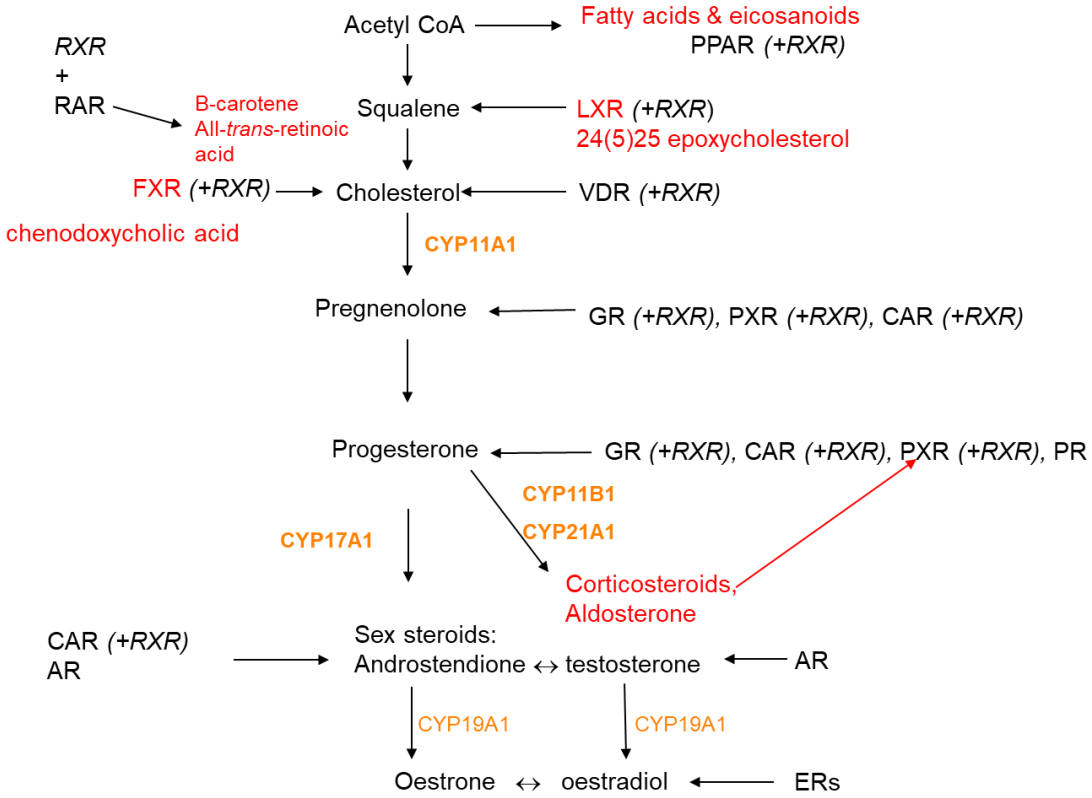


Abbreviations: PXRre; pregnane X receptor response element, RNA poly II; RNA polymerase II, TFs; transcription factors.
Source: Adapted from Jacobs *et al.* 2005.

51. Not only are the heterodimerization receptor cross-talk aspects of retinoid biology, via RXR, essential for many xenobiotic metabolic processes in the body, but they are also essential in the steroidogenic pathway, for the production of sex steroids that are prototype ligands for androgen and oestrogen receptor activity, and thus affect the pool of circulating sex steroid hormones (See Figure 1.5).

Figure 1.5 The steroidogenic pathway indicating RAR; retinoic acid receptor, RXR; retinoid X receptor, and heterodimerization partner interactions.

Regulation of steroid biosynthesis is principally mediated by the steroidogenic acute regulatory protein (StAR) delivery of cholesterol



Note: RXR and RAR play a pivotal role at the outset of the pathway that affects the entire subsequent cascade, whilst RXR also play a key role in the subsequent pathway steps as a heterodimerization partner for PPAR, LXR, FXR, GR, PXR and CAR. *Abbreviations*: AhR; Aryl hydrocarbon receptor, AR; androgen receptor, CAR; constitutive androstane receptor, ER; estrogen receptor, FXR; farnesoid X receptor, GR; glucocorticoid receptor, LXR; liver X receptor, PPARs; peroxisome proliferator activated receptors, PXR; pregnane X receptor, VDR; vitamin D receptor, PR; progesterone receptor.
Source: Adapted from Jacobs 2004.

52. Furthermore, these molecular initiating event level processes each have pathway outcomes that in the case of the PPARs is associated with lipid homeostasis, adiposity and obesity. For example, the delivery of retinoic acid to either RAR or PPARβ/δ/VDR determines its biological effects on adipose development (Wang B *et al.* 2016). In fibroblasts for example, binding with a ligand-activated VDR stimulates non-adipogenic gene transcription, whilst in adipocytes ligand activation of PPAR gamma together with heterodimerization with RXR stimulates adipogenic gene transcription.

53. Other retinoic acid regulation consequences include insulin stimulated glucose secretion, regulation of continuous asynchronous spermatogenesis (Chung *et al.* 2004, Chung and Wolgemuth

2004, Hogarth and Griswold 2013), and immunomodulatory roles in inflammation and cancer (Stevison *et al.* 2015), as well as RXR and RAR expression in tumours (Li *et al.* 2014).

54. In addition, cross-talk has also been reported with androgenic signaling (Long *et al.* 2019), as well as with xenobiotica-related receptor pathways, *via e.g.* the constitutive androstane receptor (CAR), PXR and AhR, has been demonstrated; possible responses include triggering or suppression of induction of xenobiotica-metabolizing enzymes (Murphy *et al.* 2007, Shmarakov *et al.* 2019).

55. Cross-talk has additionally been shown on the level of regulating expression of *e.g.* RA-catabolizing enzymes in the CYP26 family. It has been demonstrated that in human liver cells, PPARγ agonists rosiglitazone and pioglitazone induce CYP26A1 as well as the normally less abundant CYP26B1 (Tay *et al.* 2010). The non-endocrine sonic hedgehog (SHH) pathway is reported to regulate expression of the *Cyp26a1* and *Cyp26b1* genes, thereby preventing excessive RA levels during mouse embryonic development (El Shahawy *et al.* 2019). In the testicular Leydig cells, cross-talk between the retinoid signaling system and testosterone signaling has been shown to be crucial for steroidogenic cell function (Jauregui *et al.* 2018). Mechanistically this can be understood through the steroidogenic pathway, shown in Figure 1.5.

1.6 Epigenetics and its role in the retinoid system

56. Epigenetic changes are changes in gene expression that a) do not involve gene sequence alterations and b) may persist after the initial trigger is long gone (reviewed in Greally and Jacobs 2013, Villota-Salazar *et al.* 2016, and Jacobs *et al.* 2017). Epigenetic modifications are a feature of normal development, but can be modified by environmental chemicals. Epigenetic modifications include DNA methylation, histone modifications and microRNA (miRNA) signaling.

57. Histone deacetylation/methylation and DNA methylation are usually associated with repression of gene transcription, while histone acetylation/demethylation and lack of DNA methylation usually leads to transcriptional activation.

58. miRNAs are small noncoding RNAs that also act as endogenous regulators of gene expression. RA regulates the expression of many different miRNAs, with multiple fundamental biological roles. miRNAs have been extensively studied as targets and mediators of the biological activity of RA during embryonic development, as well as in normal and neoplastic cells. However, a recent review article reports that relatively few studies have experimentally explored the direct contribution of miRNA function to the RA signalling pathway. The tissue-specific roles of miRNAs modulated by RA include stem cell pluripotency, maintenance and regeneration, embryonic development, hematopoietic and neural differentiation, therefore playing a major general role in human disease pathogenesis (reviewed in Nervi and Grignani 2014).

59. Environmental factors, including nutrients and diet, can alter the epigenetic cellmechanisms, including the recruitment of transcription factors which regulate epigenetic modifications, and a good and topical example for this, is adipogenesis. Retinoic acid enhances adipogenic commitment in progenitor cells through altering epigenetic modifications in the promoters of key adipogenic genes, such as Zinc Finger Protein 423 (*Zfp423*), Extracellular signal-regulated kinase (*ERK*), Delta Like Non-Canonical Notch Ligand 1 (*Dlk1*)/Pre-adipocyte factor 1 (*Pref1*), SRY-Box Transcription Factor 9 (*Sox9*) and Kruppel Like Factor 2 (*Klf2*) in the development of preadipocytes. Epigenetic regulation of PPARγ and CCAAT Enhancer Binding Protein Alpha (*C/EBPα*) expression during adipogenesis has been reported (Ngo *et al.* 2014), and the PPARγ2 promoter for DNA demethylation has been detected in *in vitro* studies of a chemical flame retardant, BDE 47, using a 3T3-L1 model of adipogenesis (Kamstra *et al.* 2014).

60. As noted in the crosstalk section, retinoic acid can alter the partnership of RXRs with other nuclear receptors, and this has been mechanistically demonstrated for the regulation of adipogenesis in the current scientific literature.

61. RA is known to mediate cell differentiation also *via* epigenetic mechanisms (reviewed in Urvalek *et al.* 2014). Epigenetic changes are also involved in, *e.g.,* the process of transient induction of the *Cyp26a1* gene by RA (Yuan *et al.* 2012). RA itself is a significant regulator of miRNA expression, and there are several recent relevant studies using RA for the induction of proliferation or differentiation, or as a treatment, that also reveal which miRNAs RA can up- or down-regulate (*e.g.* Shen *et al.* 2016, Czaika *et al.* 2016, Wang JH *et al.* 2016, Ouimet *et al.* 2015). Identification of pivotal miRNA markers is presently being used in diagnostic clinical treatment, and such markers have potential for possible development of *in vitro* assay study designs and inclusion in *in vivo* test methods. However, the resulting downstream consequences do not necessarily then go through the retinoid pathway, but have multiple roles in various metabolic activities of the body such as ERK Mitogen-Activated Protein Kinase (MAPK) signaling (Shen *et al.* 2016), apoptosis (Wang B *et al.* 2016) and macrophage metabolism (Oiumet *et al.* 2015) in specific and highly varied disease outcomes. Additionally, it has been experimentally confirmed that miRNA-34-1 down regulates CYP3A4 by targeting RXRα (Pan *et al.* 2009), whilst miR-30c-1-3p (Vachirayonstien *et al.* 2016) and miR-27b (Oda *et al.* 2014) downregulate CYP3A4 *via* PXR and VDR, as both receptors need RXR as their heterodimer. In some cases, epigenetic actions of RARs appear to be independent of the ligand, *i.e.*, RA (Laursen *et al.* 2012).

1.7 Evolutionary Aspects of the Retinoid System

62. The RXR is an ancient member of the nuclear receptor family that has been identified in the majority of phylogenetic lineages (see Table 1.1, Table 1.2 and Figure 1.7). The RAR is thought to be more modern in comparison. However, RARs have now been identified in a number of invertebrate genomes (Table 1.1, Figure 1.6 although their function, in many cases, has yet to be fully elucidated.

Table 1.1 Invertebrate species comparison of nuclear retinoid receptors, transport

		RAR	RXR	RALDHs, ALDH1, ALDH8	SDHs/ SDRs	ADHs	CYP26 *	CRABP	CRBP	RBP	SRA6	TTR
Cephalochordates	Branchiostoma floridae	RAR	RXR	✓RALDHs ✓ALDH's-like	✓SDH/R-like	✓	✓	✓ CRABP-like	✓	?	✓ SRA6-like	?
Urochordate	Ciona intestinalis	RAR	RXR	✓ALDH1	✓	✓	✓	✓	X	X	X	?
Chordate; Ascidiacea	Polyandrocarpa misakiensis Halocynthia roretzi	RAR	RXR	✓RALDHs	✓	✓	✓	X	✓/X	X	X	?
Mollusc	**Gastropod**: Lottia gigantean, biomphalaria glabrata, *Osilinus lineatus*,	RAR	RXR	✓ALDH1, ALDH8	✓/?	✓ADH-like	✓	?	?	?	✓	?
	Bivalve: Crassostrea gigas	RAR	RXR	✓ALDH1	✓	✓	?	✓ CRABP-like	?	✓	✓ SRA6-like	?
Annelid	Capitella teleta	RAR	RXR	✓ALDH1, ALDH8	✓	✓	✓	?	?	X	X	?
Platyhelminthes	Schistosoma mansoni	X	RXR	✓RALDHs	?	?	X	?	?	?	?	?
Crustacean	**Daphnia pulex**		RXR	✓ALDH1, ALDH8	✓	✓	X	?	X	X	X	?
	Decapod: **Penaeus *monodon***	?	RXR	?	✓SDH/R-like	✓ADH-like	?	✓ CRABP-like	✓	✓RBP-like	?	?
Insect	Drosophila melanogaster	X	USP (RXR)	✓ALDH1	✓	✓/?	X	✓?	✓?	X	X	?
	Apis mellifera	X	USP(RXR)	✓RALDHs	✓	✓	X	?	?	✓RBP-like	?	?
nematode	Caenorhabditis elegans	ODR7	? RXR in other nematode	✓ALDH1, ALDH8	✓	✓	X	?	?	?	X	✓
cnidarian	Acropora digitifera	X	RXR	✓ALDH8	✓SDH/R-like	✓ADH-like	?	?	?	?	✓	?

Note: Species in bold text are currently used in OECD chemical toxicity testing guidelines. Question mark (?) means presence is not known. Retinoic acid receptor, RAR; retinoid X receptor, RXR; ALDH, aldehyde dehydrogenase; RALDH, Retinaldehyde Dehydrogenase; SDRs, Short-chain Dehydrogenase.Reductase, ; ADH, alcohol dehydrogenase; CYP26, cytochrome P450 subunit 26 protein; CRABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein; RBP, retinol binding protein; SRA6, stimulated by retinoic acid protein 6; TTR, transthyretin. Gene searches conducted NCBI website, additional gene information from (Laudet *et al.* 2015; Kaur *et al.* 2015; Albalat and Canestro 2009) CYP26* although a number of CYPs can be involved in RA breakdown, CYP26 is considered the major enzyme in normally functioning vertebrates *in vivo,* therefore it is used here for comparative analysis.

Table 1.2. Vertebrate species comparison of nuclear retinoid receptors, transport proteins and enzymes involved in production and breakdown of retinoids.

		RAR	RXR	RALDHs, ALDH1, ALDH8	SDHs/ SDRs	ADHs	CYP26*	CRABP	CRBP	RBP	SRA6	TTR
Human	*Homo sapiens*	RARα RARβ RARγ	RXRα RXRβ RXRγ	✓	✓	✓	✓ CYP26a1 CYP26b1	✓	✓	✓	✓	✓
Mouse	***Mus musculus***	RARα RARβ RARγ	RXRα RXRβ RXRγ	✓ ALDH1A1	✓	✓	✓ CYP26b1	✓	✓	✓	✓	✓
Rat	***Rattus novegicus***	RARα RARβ RARγ	RXRα RXRβ RXRγ	✓ ALDH1	✓	✓	✓ CYP26a1	✓	✓	✓	✓	✓
Fish	**Zebrafish** ***Danio rerio***	RARαA RARαB RARγA, RARγB	RXRαA, RXRαB RXRβA, RXRβB RXRγA, RXRγB	✓ ALDH1A2 ALDH1A3	✓	✓	✓ CYP26a1	✓	✓	✓	✓	✓
	Salmonid	RARαA RARαB RARγA	RXRαA RXRβA RXRγA, RXRγB	✓ ALDH1	✓	✓	?	✓	?	✓	?	✓
	Japanese Medaka ***Oryzias latipes***	RARα RARβ RARγ	RXRα RXRβ, RXRβA RXRγ, RXRγB	✓ ALDH1A2	✓		✓ cyp26a1 cyp26b1	✓ CRABP1 CRABP2-like		✓ RBP3	✓	?
Amphibian	***Xenopus laevis***	RARα RARβ RARγ	RXRα RXRβ RXRγ	✓ ALDH1A1 ALDH1A2	✓	✓	✓ CYP26a1	✓	?	✓	?	✓
	Xenopus tropicalis	RARα RARβ RARγ	RXRα RXRβ RXRγ	ALDH1A1 ALDH1A2 ALDH1A3	✓	✓	✓ CYP26a1	✓	?	✓	✓	?
Reptile	*Alligator mississippiensis*	RARα RARβ RARγ	RXRα RXRβ RXRγ	✓ ALDH1 ADH8	✓	✓	?	✓ CRABP1 CRABP2	?	✓	✓	✓
Avian	**Chicken** ***Gallus gallus***	RARα RARβ RARγ	RXRα ? RXRγ	✓ ALDH1	?	✓	✓ CYP26	✓	?	✓	✓	✓
	Japanese quail ***Coturnix japonica***	RARα RARβ RARγ	RXRα ?	✓ ALDH1A1	✓	✓	✓ CYP26a1	✓ CRABP1	?	✓	✓	✓

			RXRγ					CRABP2				
	Mallard duck *Anas platyrhynchos*	RARα RARβ ?	RXRα RXRβ RXRγ	✓ ALDHl1A	✓	✓	?	✓ CRABP1	?	✓	✓	✓

Note: Species in bold text are currently used in OECD chemical toxicity testing guidelines. Question mark (?) means presence is not known. Retinoic acid receptor, RAR; retinoid X receptor, RXR; ALDH, aldehyde dehydrogenase; RALDH, Retinaldehyde Dehydrogenase; short-chain dehydrogenase/reductase, SDRs; ADH, alcohol dehydrogenase; CYP26, cytochrome P450 subunit 26 protein; CRABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein; RBP, retinol binding protein; STRA6, stimulated by retinoic acid protein 6; TTR, transthyretin. Gene searches conducted NCBI website. CYP26* although a number of CYPs can be involved in RA breakdown, CYP26 is considered the major enzyme in normally functioning vertebrates *in vivo*, therefore it is used here for comparative analysis.

1.8 Vertebrate Cross-Species Comparison of Retinoid Source, Storage, Transport and Metabolism

63. Like mammals, other lower vertebrate groups obtain the vitamin A they need from dietary sources. Importantly, there appears to be a high conservation in the morphogenic role of retinoids particularly in early development. For example, there is a well-established need for adequate nutrition for proper fry development and subsequent growth and reproduction in fish; the subject of many aquaculture papers.

64. Analogues of Hepatic Stellate Cells (found in the liver of mammals and are important in RE storage) have also been identified in primitive vertebrates such as the lamprey, *Lampetra japonica* (Wake *et al.*, 1987). In lamprey the stellate cells are found in the hepatic parenchyma as well as the perivascular and capsular connective tissue of the liver and in the interstitium of the pancreatic tissue *(*Wake *et al.*, 1987*)*. Retinoid storage in fish has also been investigated in the arrowtooth halibut (*Atheresthes evermanni*) where the highest concentrations of stored retinoids (Rol and RE, 6199 nmol/g) were found in the pyloric cecum (teleost-specific organ projecting from the intestine), with lower amounts also found in the intestine (3355 nmol/g), liver (1891 nmol/g) and kidney (102 nmol/g) (Yoshikawa *et al.*, 2006). In comparison, Gesto *et al.* found a more 'mammal-like' storage profile in rainbow trout (*Oncorhynchus mykiss)* with hepatic levels of free and total (free + esterified) all-*trans*-ROL and all-*trans*-didehydro-ROL (a fish specific form) of at least one order of magnitude higher than in the other tissues (including spleen, intestine and kidney)(Gesto *et al.* 2012).

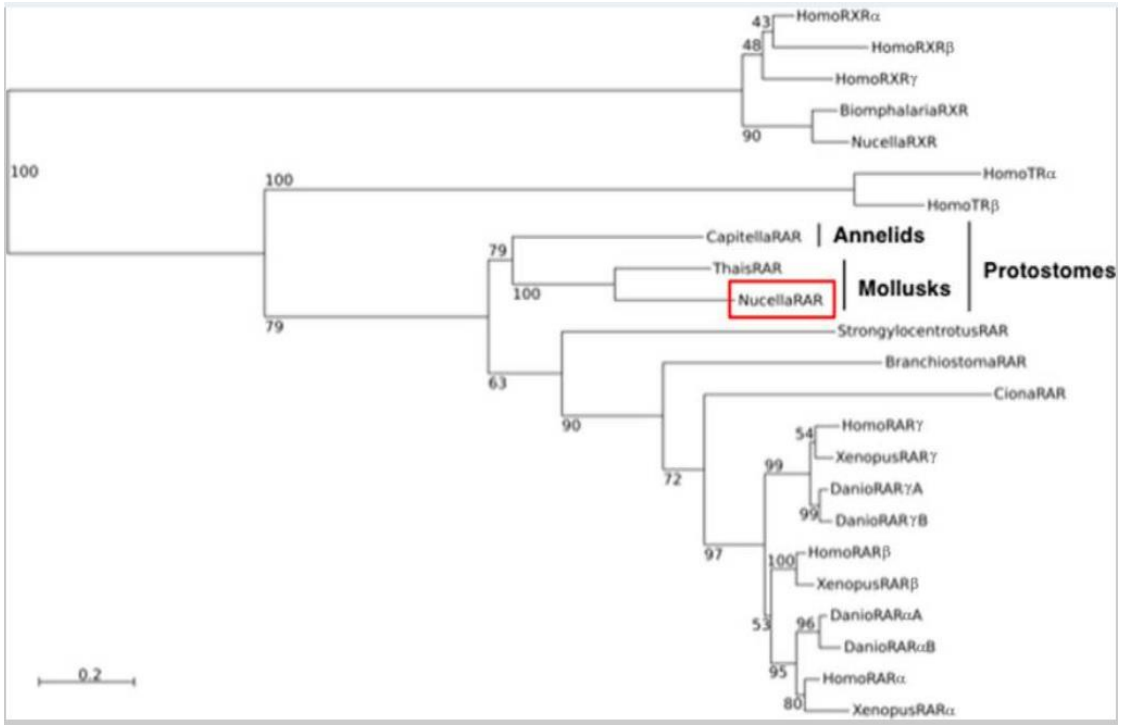
65. In vertebrates the enzymes involved in retinoid conversion and metabolism seem to be highly conserved and represented throughout mammals, birds, reptiles, amphibians and fish (Table 1.2).

66. RBP has been identified in the circulatory systems of all vertebrates studied (Shirakami *et al.*, 2012) (*e.g.* Table 1.2). Transthyretin (TTR) works with RBP to transport Rol in the blood stream. TTR is mainly produced in the liver and is also an important transport protein of thyroid hormone. It is found in the majority of vertebrates but often occurs at its highest levels during periods of known thyroid hormone action during early development and/or during important developmental stages (i.e. frog metamorphism, salmon smoltification) (Richardson 2002). In mammals and birds TTR also found at throughout adult life (Richardson *et al.* 2005), whereas in amphibians and fish much lower levels are seen in adulthood e.g. (Ishihara *et al.* 2012).

67. RXR forms heterodimers with a range of nuclear receptors other than RAR (Table 1.3) *e.g.* PPAR, THR, PXR. In vertebrates these additional RXR partners are very well conserved.

68. Compared to mammals, the fish retinoid system is more complicated (due to genome duplication events that have occurred throughout evolution) and complex (due to the possibility of greater redundancy resulting indifferent compensatory mechanisms compared to mammals). For example, fish have more retinoid receptor isotypes (see Table 1.2) and more active metabolites than mammals, and the transport of retinoids in fish is also different (Gesto *et al.* 2012).

Figure 1.6. Example of Phylogenetic tree of the RAR in mollusc, annelid and vertebrate species.



Note: Numbers on each branch are bootstrap support values in percentage of 1000 replicates. The tree was rooted with the RXR sequences. The species listed are the following: *Branchiostoma floridae*, *Biomphalaria glabrata*, *Capitella teleta*, *Ciona intestinalis*, *Danio rerio*, *Homo sapiens*, *Nucella lapillus, Strongylocentrotus purpuratus*, *Thais clavigera*, and *Xenopus tropicalis*.
Source: Figure taken from Gutierrez-Mazariegos *et al.* (2012) with kind permission from the publisher.

1.9 Non-vertebrate cross-species comparison of retinoid source, storage, transport and metabolism

69. As with other animals, invertebrates obtain retinoids from their diet. There is still debate as to whether invertebrates, such as molluscs, store retinoids in the form of RE. Comparative studies in some gastropod groups, have reported limpets (*Patella depressa*) to have the capacity to store retinoids in the form of inactive RE. In contrast, other gastropods groups, such as Whelks (*Nucella lapillus* and *Nassarius reticulatus*) lacked detectable amounts of Rol or RE (although they did have 13cRA, 9cRA and atRA) (Gesto *et al.*, 2013). It could be conceived that these differences relate to their differing diets, i.e. herbivorous limpet vs carnivorous whelks (Gesto *et al.*, 2013).

70. The recent identification of RAR in molluscs and annelids has lead researchers to investigate if these receptors are functionally similar to vertebrate RARs. Gutierrez-Mazariegos *et al.* identified RAR in the gastropod *Nucella lapillus* (Figure 1.6 Gutierrez-Mazariegos *et al.*, 2012) and demonstrated that it heterodimerises with RXR and is able to bind to a typical retinoic acid response element sequence (Gutierrez-Mazariegos *et al.,* 2012). However, in the same paper it was shown that the Nucella RAR (i) does not bind atRA or other retinoid compounds tested, and (ii) does not activate the transcription of reporter genes in response to exposure of retinoids *in vitro*. Three-dimensional modelling of the ligand-binding domain suggested that although Nucella RAR was similar to vertebrate RAR, the ligand-binding pocket (LBP) had alterations which might significantly reduce its interaction with the ligand (Gutierrez-Mazariegos *et al.,* 2012). The authors suggest that molluscs may have lost RAR ligand binding. In comparison it has been demonstrated that an RXR from another mollusc (*Biomphalaria glabrata*) can

bind 9cRA and activate transcription in response to 9cRA (Bouton *et al.*, 2005). In the Pacific oyster (*Crassostrea gigas*) RAR and RXR are both highly expressed in mollusc embryos during gastrulation, organogenesis and shell development (Vogeler *et al.*, 2016), suggesting that the RAR-RXR heterodimer may also play an important role in development in molluscs as in mammals. In addition, atRA, 13cRA, Rol, and RE have been measured in adult Nucella tissues (Gutierrez-Mazariegos *et al.*, 2012) , and RAR expression in adults has also been found, suggesting a role for retinoids in these animals.

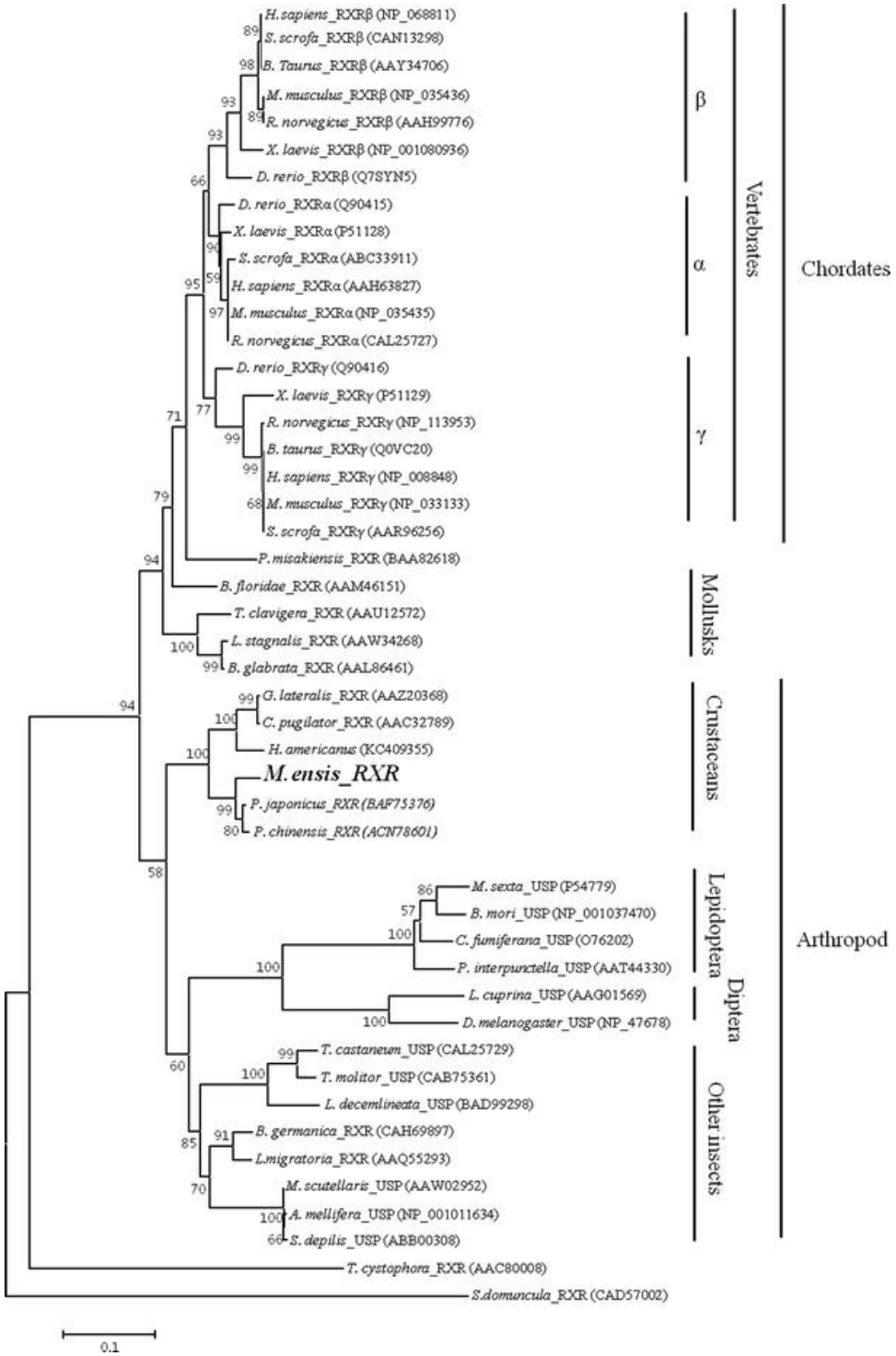
71. In primitive chordates, lophotrochozoa (molluscs and annelids) and Ambulacraria (e.g. echinoderms) homologs of vertebrate RA synthesising (RALDHs) and degrading (CYP26) enzymes have been identified (Table 1.1, Laudet *et al.*, 2015). For the lineages that only posses a RXR (not RAR) synthesising enzyme homologs (RALDHs and ALDH) have also been found (Table 1.1,Laudet *et al.*, 2015). CYP26 has only been identified in species/groups which also have a RAR.

72. To date, RBP has not been identified in invertebrates (Table 1.1).Transthyretin-like protein have been identified in bacteria, plants and invertebrates (e.g. *Escherichia coli*, *Arabidopsis thaliana* and *Caenorhabditis elegans* (Table 1.1) (Hennebry *et al.*, 2006). However, the function of these TTR-like proteins is considered to be distinct from vertebrate TTRs. For example, *C. elegans* ttr-52 gene is involved in cell corpse engulfment (phagocytosis) (Wang *et al.*, 2010).

73. Some, but not all (Table 1.3), of the additional nuclear receptor RXR heterodimer partners have been identified in non-vertebrate lineages. The primitive chordates and lophotrochozoa have PPAR, LXR and THR homologs.

74. More research is required to fully understand the role of retinoids, RAR and RXR in invertebrates. However, chemicals that interact with the RXR, such as organotins, are well known to cause developmental and reproductive dysfunction in molluscs (Nishikawa *et al.*, 2004; Gibbs *et al.,* 1987), and disruption to retinoid synthesizing/degrading enzymes are possible targets for chemical disruption in invertebrates despite possible differences in their functional roles. Therefore, invertebrates should not be ignored if retinoid regulatory tests are to be adapted or developed.

Figure 1.7. Phylogenetic (NJ) tree of RXR (USP) subfamily.



Note: Branch lengths are proportional to evolutionary change; the scale bar represents 0.05 substitutions per site. Number at each branch represents the percentage of bootstrap support from 1,000 pseudoreplicates *Source*: .Figure taken from Cui *et al.*, (2013).

Table 1.3. Cross-species comparison of RXR Heterodimer partners.

		RXR Possible Heterodimer partners						
		PPAR	FXR	LXR	PXR	CAR	VDR	THR
Human	*Homo sapiens*	PPARα, PPARδ/β, PPARγ	FXR	LXRα, LXRβ	PXR	CAR	VDR	THRα, THRβ
Mouse	***Mus musculus***	PPARα, PPARδ/β, PPARγ	FXR	LXR	PXR	CAR	VDR	THRα, THRβ
Rat	***Rattus novegicus***	PPARα, PPARδ/β, PPARγ	FXR	LXRα	PXR	CAR	VDR	THRα, THRβ
Fish	***Danio rerio*** (Zebrafish)	PPARαA, PPARαB PPARδ/βA, PPARδ/βB PPARγ	FXR	LXR	PXR	?	VDR	THRαA, THRαB THRβ
	Oncorhynchus mykiss (rainbow trout)	PPARα, PPARδ/β, PPARγ	FXR	LXR	PXR	?	VDR	THRβ
	Oryzias latipes (Japanese Medaka)	PPARα, PPARδ/β, PPARγ	FXR	LXR	PXR	?	VDR	THRα, THRβ
Amphibian	***Xenopus laevis***	PPARα, PPARδ/β, PPARγ	FXR	LXR	PXR	CAR	VDR	THRβ
	Xenopus tropicalis	PPARα, PPARδ/β, PPARγ	FXR	LXR	PXR	CAR	VDR	THRβ
Avian	***Gallus gallus***	PPARα, PPARδ/β, PPARγ	FXR	LXR	PXR	CAR	VDR	THRα, THRβ
	Coturnix japonica	PPARα, PPARδ/β, PPARγ	FXR	LXR	PXR	CAR	VDR	THRα, THRβ
	Anas platyrhynchos	PPARα, PPARδ/β, PPARγ	FXR	LXR	?	?	VDR	THRα, THRβ
Reptile	*Alligator mississippiensis*	PPARα, PPARδ/β, PPARγ	FXR	LXR	?	CAR	VDR	THRα, THRβ
Urochordate	*Ciona intestinalis*	PPAR	CiFXR	LXRβ	?	?	CiVDR	CiTHR
Mollusc	*Lottia gigantean*	LgPPAR1, LgPPAR2	-	? LgEcR	?	-	-	LgTHR
	Biomphalaria glabrata	BgPPAR1, BgPPAR2	-	? BgEcR	?	-	-	BgTHR
	Crassostrea gigas	CgPPAR	?	?	?	?	?	CgTHR
Crustacean	***Daphnia pulex***	?	?	?	?	?	?	?
Insect	*Drosophila melanogaster*	?	?	?	?	?	?	?
nematode	*Caenorhabditis elegans*	?	?	?	?	?	?	?

Note: Species in bold text used in OECD chemical toxicity testing guidelines. Question mark (?) indicates currently unknown if present or not. Bar (–) indicates not found in genome in specific search for nuclear hormone receptors (Kaur *et al.*, 2015). peroxisome proliferator-activated receptors (PPAR, PPARα; NR1C1, PPARδ/β; NR1C2, PPARγ; NR1C3), farnesoid X receptor (FXR, NR1H4), liver X receptor (LXR, NR1H3), pregnane X receptor (PXR, NR1I2), constitutive androstane receptor (CAR, NR1I3), vitamin D receptor (VDR, NR1I1), thyroid hormone receptor (THR, THRα; NR1A1, THRβ; NR1A2).
Source: Gene searches conducted NCBI website.

2.0 Additional information

75. Due to the inherent complexity of the retinoid signalling pathway, it is difficult to identify endpoints or candidate assays that clearly indicate altered pathway signalling. Endogenous retinoid synthesis, local tissue metabolic activation and inactivation, and gradient effects within tissues vary for species, organ, sex, and developmental stage. Receptor-ligand signalling is also considerably more complex than other endocrine signalling pathways (e.g. estrogen and androgen), for which current OECD test guidelines are available. Retinoids bind to the nuclear retinoic acid receptor (RAR) which may heterodimerise with the retinoid X receptor (RXR). Furthermore, the RXR does not require a ligand to be active, and it heterodimerises with a variety of other nuclear receptor partners.

76. As a first step towards identifying potential targets of altered retinoid signalling and candidate assays for evaluating the effects of environmental chemicals, annexes to this Detailed Review Paper provide additional information on the role of retinoids in specific organ systems including male and female reproduction (Annex A), skeletal and craniofacial development (Annex B), the central nervous system (Annex C). As more information becomes available, additional annexes may be added. Lastly, a summary of comments from an OECD Expert Group on Retinoid Signalling was provided to indicate

current gaps in knowledge and potential next steps for evaluating altered retinoid pathway signalling in a regulatory context (Annex D).

Annex A. Retinoid effects on the reproductive system

A.1. Annex Preface

77. Retinoids are essential for vision, embryonic development, adult growth and development, as well as for reproduction in both males and females. In addition to being a nutrient-derived vitamin (vitamin A), retinoids are also considered as hormones, based on the hormonal-like signaling of retinoid-specific nuclear receptors which affect gene transcription. In general, all-*trans* retinoic acid (RA) is viewed as the physiologically active form. Tissue levels of RA are maintained via tightly regulated enzymatic synthesis and catabolism, and are also dependent on proper uptake, transport and storage of different form of RA precursors.

78. RA acts mainly by interacting with nuclear retinoid acid receptors, RARs, which forms heterodimers with retinoid X receptors, RXR. These heterodimers bind to specific DNA response elements. The RXR receptor type also heterodimerizes with other nuclear receptors (*e.g.* PPARs, VDR, CAR, PXR, LXR and FXR). Thus, extensive cross-talk between nuclear receptor pathways depend on RXR. RA has also been implicated in epigenetic regulation.

79. During embryonic formation of reproductive organs and sex differentiation of germ cells, RA is initially available from several possible enzymatic sources in or near the gonad. RA is believed to have an important role in meiosis initiation, however, this is currently an area of active research. Meiosis initiation occurs *in utero* in the female, and postnatally in males. Investigations in rodents have shown that temporal and spatial expression of specific enzymes, involved in RA metabolism, is critical for avoiding premature meiosis in males.

80. RA has been reported to be of importance for ovarian somatic cell development and function, as well as for the implantation of the fertilized embryo into the endometrium. In addition, altered retinoid signaling has been associated with endometriosis, as well as polycystic ovarian syndrome in women.

81. In the adult male, RA signaling is important for proper spermatogenesis. More specifically, correct RA levels must be maintained inside the seminiferous tubule for proper spermatogonia differentiation, meiosis initiation and release of spermatozoa. In addition, RA has been suggested to play a role in the formation and maintenance of secondary male reproductive organs (seminal vesicles, epididymis, prostate). However, many of these processes depend also on other endocrine pathways, and extensive cross-talk between these pathways exist. Consequently, for many of the processes described above, more research is needed to elucidate the exact role of RA signaling, the mechanisms controlling spatial and temporal availability of RA in reproductive tissues, and the influence of RXR-cross-talk in retinoid homeostasis.

82. Some chemicals have been demonstrated to interfere with the retinoid pathway. Chemicals, for which there are at least some data, include pharmaceutical compounds, conazole fungicides and

organotins. However, there are a lack of studies investigating effects of chemicals, except a few pharmaceutical compounds, on reproduction, while simultaneously examining effects on retinoid related parameters in reproductive organs. This is a major data gap.

83. Four different visualizations of possible adverse outcome pathways, using available research, between effects on retinoid homeostasis and reproductive adversity, in males or females, is presented in this report. These visualization pathways can be used as starting points for future AOP development of retinoid disruption.

84. The initial scoping effort presented in this report identified the RA-catabolizing CYP26 enzymes and the RA-synthesizing RALDH enzymes that could be integrated at (CF) Level 1 and 2 in the OECD Conceptual Framework [6]. This could be possible with development of *in silico* methods, such as QSARs or molecular docking models for these enzymes, and/or *in vitro* assays. In current *in vivo* test guidelines (CF level 3-5), existing histopathological analyses include endpoints related to reproduction and that may be affected by altered retinoid signalling. However, as discussed in the present report, the regulation of reproduction involves many other endocrine modalities, and the interpretation of the observed effects is further impeded by extensive cross-talk between nuclear receptors. Consequently, there are no endpoints identified in this report that are specific for retinoid disruption to be added to the existing OECD test guidelines, although some non-specific endpoints may be affected by altered retinoid homeostasis and provide valuable information (e.g. ovarian histopathology, see section A.10.3).

85. In addition, no suitable reference chemicals, except for a few pharmaceutical compounds, known to affect fertility specifically *via* the retinoid pathway has been identified. Such chemicals will be needed in a future validation step.

86. In spite of these challenges, selected *in vitro* and/or *in silico* retinoid-related endpoints, presented in this report, could be part of a broader screening test battery aimed at developmental and reproductive toxicity, though the relevance to *in vivo* responses must be determined. For *in vivo* studies, histopathological readouts of, for example ovaries and testes, already included in the OECD test guidelines can provide information to support regulatory decision-making, without knowledge of exactly which pathway(s) have been disturbed. Hormone receptors and pathways are highly conserved across vertebrate species and cross-species extrapolations should be considered (OECD 2018), though potential species differences should be considered.

A.2. Formation of female and male reproductive organs, and the role of retinoids

87. The formation of the female and male reproductive organs is initiated early in fetal life. The genital ridges (the presumptive gonads) first appear around halfway through gestation in mice, or at 10.5 days post coitum (dpc) (Spiller *et al.* 2017), whereas they appear during the first trimester in humans (Johansson *et al.* 2017, Mamsen *et al.* 2017). The somatic cell progenitors of the genital ridges are initially bipotential, *i.e.*, they are capable of developing into either Sertoli or Leydig cells (in males) or granulosa or theca cells (in females) (reviewed in Svingen and Koopman 2013). The Sertoli and granulosa cells are the first somatic cells to differentiate in testes and ovaries, respectively, and they are important for supporting the germ cells and also for orchestrating the subsequent differentiation of somatic cell progenitors into the steroidogenic Leydig and theca cells (Rotgers *et al.* 2018).

[6] OECD (2018), Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption, OECD Series on Testing and Assessment, No. 150, OECD Publishing, Paris, https://doi.org/10.1787/9789264304741-en.

88. Soon after they are formed, the genital ridges begin to differentiate either as testes, in the presence of the Y-chromosomal gene, Sex-determining region Y protein (*Sry)*, or as ovaries, in the absence of *Sry* (reviewed in Svingen and Koopman 2013). The transcription factor SRY upregulates the expression of the gene SRY-Box Transcription Factor 9 (*Sox9)*, which in turn governs the expression of several male-specific genes, leading to differentiation of the bipotential somatic cell progenitors into Sertoli cells (Kashimada *et al.* 2011). In the absence of *Sry*, as in females, a different set of genes are expressed by default, such as Wnt Family Member 4 (*Wnt4)* and Forkhead box protein L2 (*Foxl2)*, leading to differentiation of somatic progenitor cells into granulosa cells and subsequently to ovarian development (Kashimada *et al.* 2011). During early testis differentiation, Sertoli cells encircle clusters of gonocytes and form testis cords; the future seminiferous tubules. Shortly thereafter, fetal Leydig cells emerge in the interstitial space and start producing testosterone, which is essential for masculinization of the male fetus (reviewed in Svingen and Koopman 2013).

89. At the time of gonad formation, RA appears to be available from several possible sources. In mice, the adjacent mesonephros has been suggested as an important source, where both dehydrogenase 10 (*Rdh10*) and retinaldehydrogenase 2 (*Raldh2*) are expressed, as well as *Raldh3* at a lower level, (Niederreither *et al.* 2002a, Niederreither *et al.* 2002b, Bowles *et al.* 2006, Spiller and Bowles 2015, Bowles *et al.* 2018). Using a RARE-controlled *LacZ* reporter gene system, RA was demonstrated to be localized in the anterior part (nearest the mesonephros) of the mouse fetal ovary (Bowles *et al.* 2006).

90. An alternative RA source is the coelomic epithelium, which covers the embryonic gonad, and where Raldh2 is also expressed (Niederreither *et al.* 1997, Teletin *et al.* 2017). Species differences appear to exist: RA-producing (retin)aldehyde dehydrogenases are present in both female and male fetal gonads in humans (Childs *et al.* 2011, Le Bouffant *et al.* 2010), suggesting a capacity for *de novo* RA synthesis in the gonad proper. Gonadal RA synthesis in the rabbit appears similar to the one in the human gonad (Diaz-Hernandez *et al.* 2019). Species differences are evident when it comes to gonadal architecture; the human and rabbit gonads both develop a well-defined cortex and medulla, where somatic and germ cells can interact differently than in the mouse, for example, which has a different gonadal architecture (Diaz-Hernandez *et al.* 2019).

91. In mice, Cyp26-dependent degradation of endogenous RA appears to be critical for somatic testis development, as evident from observations of mild ovotestes, impaired steroidogenesis and a feminized reproductive tract in *Cyp26b1*-null C57/BL6 dpc 13.5 dpc/ 14.5 dpc male mouse embryos (later time-point not examined as the embryos are not viable after ~15 dpc) (Bowles *et al.* 2018). However, in an earlier study using *Cyp26b1*-null mice on a different genetic background, testis formation and somatic cell differentiation in neonatal pups appeared normal, although some germ cells had prematurely entered meiosis while others appeared apoptotic. Germ cells were essentially absent in testes from neonatal *Cyp26b1*$^{-/-}$ pups (MacLean *et al.* 2007). In an *ex vivo* model using rat fetal testis, exogenous RA disrupted the proper formation of seminiferous cords, as well as the maintenance of the testicular cell fate of the somatic cells; following RA exposure, expression of ovarian-specific marker Foxl2 was observed (Spade *et al.* 2019a). In a human *ex vivo* testis model, exogenous RA appeared to disrupt the seminiferous cords and altered the expression of somatic cell markers (Jørgensen *et al.* 2015). Thus, there appears to be differences between rodents and humans with regard to where and how RA is synthesized and regulated in the developing gonads, although it is clear that RA influences both germ and somatic cell lineages in both species.

92. Even in adulthood, the sexual fate of the male gonads must be maintained, probably *via* the active presence of the transcription factor Doublesex and mab-3 related transcription factor 1 (DMRT1) (Minkina *et al.* 2014). The role of DMRT1 in the testes appears to be to prevent RA-initiated activation of specific potential feminizing genes (*Foxl2* and Estrogen receptor β; *Esr2*) in Sertoli cells; without DMRT1 present, male-to-female transdifferentiation of somatic cells was observed in the pre- and

postnatal mouse testes (Minkina *et al.* 2014), or even complete male-to-female sex reversal (Zhao *et al.* 2015).

93. In contrast, based on observations in mice with deletions/disruptions of either RARs or RA-synthesizing enzymes, it has been suggested that RA signaling is not critical for correct ovarian development in the female mouse embryo (Minkina *et al.* 2017), although earlier studies have suggested that RA might maintain ovarian differentiation and development (Minkina *et al.* 2014, Suzuki *et al.* 2015). Additional studies suggest that abnormal endogenous RA levels may be able to influence somatic cell differentiation and/or function in mice (Bowles *et al.* 2018). Further research is needed to clarify the role of RA in ovarian development and function.

A.3. Germ cells, meiosis and the role of retinoids

94. Most of our current understanding of meiosis initiation and early sex differentiation is derived from studies in mice. Data from humans are sparse (See Table A.1 and text below).

Table A.1. Sensitive windows in males and females, comparing rodents and humans.

Process	Time period in females	Time period in males
Early gonadal development	Mice: 11.5 dpc[a] Rats: 13.5 dpc Humans: just beyond GW[b] 7	Mice: just beyond 11 dpc Rats: just beyond 12 dpc Humans: just beyond GW 7
Meiosis initiation	Mice: 12.5 dpc Rats: 16.5 dpc Humans: GW 10 -12	Mice: end of first postnatal week at puberty Humans: at puberty
Follicular assembly	Rodents: post-natally (mice: primordial follicle formation initiated 2-3 days before birth, follicle assembly continues until PND 6) Humans: pre-natally (during mid-gestation stage)	-
Early follicle recruitment[c]	Rodents post-natally Humans: initiated pre-natally	-
Spermatogenesis	-	Mice: beginning at puberty Humans: beginning at puberty

Note: [a] dpc: days post coitum
[b] GW: gestational week
[c] Takes place immediately after follicular assembly

Source: This table from Retinoids in Mammalian Reproduction, with an Initial Scoping Effort to Identify Regulatory Methods, Nordic Council of Ministers, (Nordic Council of Ministers, 2020), is made available under the Creative Commons Attribution 4.0 International license (CC BY 4.0). Information compiled in the table is obtained from Le Bouffant *et al.* 2010, Grive and Freiman 2015, Johansson *et al.* 2017, Mamsen *et al.* 2017, Teletin *et al.* 2017.

95. In both male and female mouse embryos, a cluster of pluripotent primordial germ cell (PGC) precursors arise under the influence of bone morphogenic protein (BMP) at 6.25 dpc at the base of the allantois of the embryo (reviewed in Yadu and Kumar 2019). Subsequently, the PGCs migrate through the presumptive hindgut to the genital ridges (formed at 10.5 dpc), which will differentiate into testes or ovaries (see section 5). During the migration and colonization period, epigenetic reprogramming (genome-wide demethylation) occur in PGCs, which allows transcription of genes that are suppressed epigenetically in somatic cells. This may explain why only germ cells are capable of responding to the meiosis-inducing signal from RA (reviewed in Yadu and Kumar 2019).

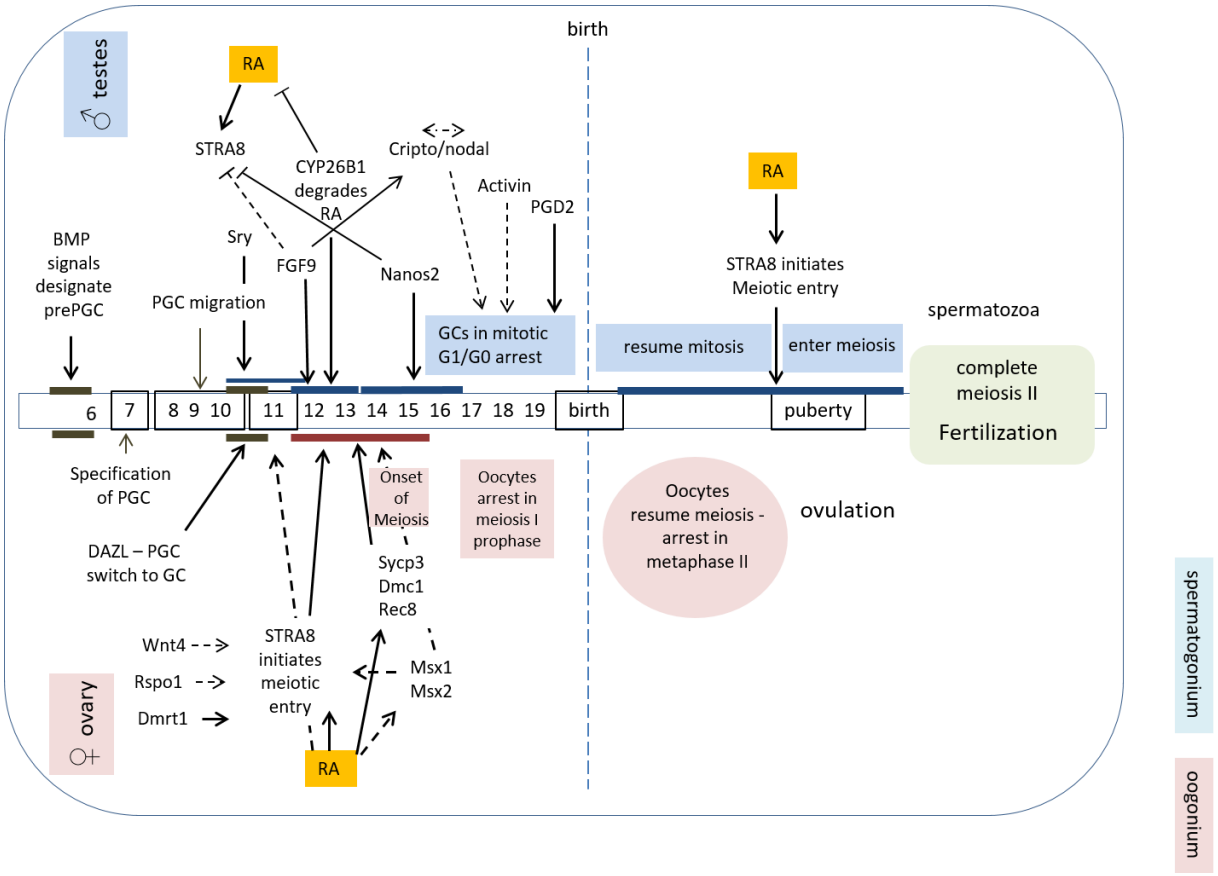
96. In both males and females, the haploid gametes are produced from primordial germ cells *via* meiosis in the gonads, following a similar process of events, although the timing differs. In the mouse ovaries, germ cells enter the prophase of the first meiotic division (at around 13 dpc), whereas in the testes, germ cells (now situated in the testis chords) instead enter quiescence by 12.5 dpc; they slow down their proliferation towards mitotic arrest as G_O/G_1-arrested gonocytes (Kashimada *et al.* 2011). Thus, in the fetal gonad the commitment of germ cells towards oogenesis involves entry into meiosis, whereas commitment to spermatogenesis involves the inhibition of meiotic initiation, suppression of pluripotency and mitotic arrest. This mitotic arrest is maintained until after birth (Spiller and Bowles 2015, Spiller *et al.* 2017) and consequently meiosis initiation occurs postnatally in males. Thus, all oocytes are produced before birth, while spermatocytes are produced continuously during post-pubertal life in males.

97. Meiotic entry during fetal development seems to be regulated by two master genes: Stimulated by retinoic acid, gene 8 (*Stra8*), required for the initiation of meiosis in female fetal germ cells, and Nanos C2HC-Type Zinc Finger 2 (*Nanos2),* expressed in male fetal germ cells (Rossitto *et al.* 2015). Nanos2 is required to prevent *Stra8* expression and meiosis initiation in male fetal germ cells (Rossitto *et al.* 2015). Other genes, such as the one coding for Fibroblast growth factor 9 (*Fgf9)*, have been implicated as being of importance for proper control of meiotic entry; Figure A.1 is an attempt to summarize this information. STRA8 appears to be critical for several meiotic cellular processes such as DNA replication, condensation of chromosomes and double-stranded DNA breaks (reviewed in Yadu and Kumar 2019). The role of *Stra8* in meiosis is also evident in *Stra8*$^{-/-}$ mice, as both females and males are infertile, while heterozygotes of both sexes are fertile (Baltus *et al.* 2006). *Stra8*$^{-/-}$ females display smaller ovaries lacking oocytes and follicles, and in *Stra8*$^{-/-}$ males, testes are smaller and testicular germ cell numbers are severely reduced (Baltus *et al.* 2006). In addition, spermatocytes in *Stra8*$^{-/-}$ males undergo apoptosis before reaching the leptotene and zygotene stages of meiotic prophase (Baltus *et al.* 2006). The *Stra8* gene contains putative retinoid acid response elements (RAREs) and its expression is activated by RA (Spiller *et al.* 2017). When two RAREs are mutated *in vivo*, *Stra8* expression is significantly diminishd, demonstrating direct regulation of *Stra8* by RA (Feng *et al*. 2021). RA-signaling is implied in Stra8 signaling *in vivo*, as evidenced by the lack of Stra8 induction in *ex vivo* cultured mouse fetal ovaries, exposed to the RAR antagonist BMS-204493. (Koubova *et al.* 2006). In line with these hypotheses, it has been demonstrated that exogenous RA added to *ex vivo* cultures of 12.5 dpc mouse testes induces expression of the meiois marker *Stra8* (Bowles *et al.* 2006, Koubova *et al.* 2006). Very low concentrations of RA (10 nM) has been shown to induce *Stra8* expression in 11.5 dpc germ cells *in vitro* (Bowles and Koopman 2007). The *Stra8* locus in non-germ cells appears to be epigenetically silenced, thus, only pre-meiotic germ cells respond to RA by *Stra8* induction (Wang and Tilly 2010, Spiller and Bowles 2015).

98. The sex-specific timing of *Stra8* expression is conserved between mice and humans (Childs *et al.* 2011). If STRA8 has other roles, they are still largely unknown (Griswold 2016). Interestingly, in humans, spermatogenic impairment, evident as azoospermia or oligozoospermia, was associated with a specific *STRA8* single nucleotide polymorphism (Lu *et al.* 2013).

99. Another gene essential for meiosis, *Rec8* meiotic recombination component gene *(Rec8)*, also contains a RARE, and is activated by RA independently of *Stra8* (Koubova *et al.* 2014). *Rec8* encodes a meiosis-specific component of the cohesion complex, and is required for several steps in meiotic chromosomal activities, *e.g.* chromatid cohesion and chiasmata formation (Koubova *et al.* 2014). Neither female nor male *Rec8*$^{-/-}$ mice that survive to reach sexual maturity are fertile (Xu *et al.* 2005). At 18.5 dpc, fetal ovaries of female *Rec8*$^{-/-}$ mice displayed apparently normal prophase I germ cells, but also abnormal germ cells with compacted chromosomes, while at PND5 and older (up to adult), no oocytes or ovarian follicles were present (Xu *et al.* 2005). Findings of involuted genital tracts in the same females were interpreted as being a consequence of ovarian hormone failure, due to the lack of (steroidogenic) follicles.

Figure A.1. Comparison of male and female germ cell development and meiotic progression in mice



Note: Blue indicates male-specific events, red/pink indicates female-specific events and grey, events shared by both sexes. Numbers indicate days post coitum (dpc). Arrows and bars indicate rough timing of gene expression, solid arrows indicate direct effect, and broken arrows indicate an incompletely characterized or likely indirect effect. Plus (+) or minus (-) indicate supportive/additive or inhibitory action on other genes.

Abbreviations: GC; germ cell, PGC; primordial germ cells, RA; retinoic acid, DAZL; deleted in azoospermia-like, BMP; bone morphogenic protein, mitotic pro; mitotic proliferation.

Source: The summary is adapted from Figure 3 in Clagett-Dame & Knutson, 2011; Figure 1 in Feng and co-authours, 2014; Rossitto and co-authours, 2015 and Figure 1 in Spiller & Bowles, 2015. Printed, with kind permission of the EU Commission.

100. RA has been implicated in controlling the onset of meiosis in both males and females (Bowles *et al.* 2006, Koubova *et al.* 2006). RA-treatment of cultured fetal mouse ovaries from 14.5 dpc, increases the number of meiotic cells (Livera *et al.* 2000b). Any response to RA in both male and female germ cells must be preceeded by expression of the gene Deleted in azoospermia-like (*Dazl*), which is present in the post-migratory germ cells after their arrival in the gonad (Lin *et al.* 2008). The RA necessary for meiosis initiation is synthesized locally in both fetal testes and ovaries. As mentioned in Section 5, several sources for RA in mouse fetal gonads have been suggested: the mesonephros (near the anterior part of the fetal gonads), the coelomic epithelium, and the fetal gonads themselves. In fact, an anterior-posterior pattern of *Stra8* expression has been demonstrated in mouse female fetal gonads (reviewed in Bowles *et al.* 2006), implying that the mesonephros may be the most important source of RA for the mouse gonad. Germ cell meiosis initiation in human gonads is asynchronous, as compared to the anterior-posterior wave observed in mice, which suggests a less important role for the mesonephros in humans (Le Bouffant *et al.* 2010, Childs *et al.* 2011). In the rabbit, contrary to the mouse, meiosis onset occurs after termination of the connection between the gonad and the mesonephros (Hayashi *et al.*

2000). It should also be noted that in a human fetal testis *ex vivo* model, RA was capable of inducing *Stra8* but not other meiosis-associated genes (Childs *et al.* 2011). Species differences are also evident from studies using rabbits, where both meiosis initiation and mitotic arrest occurs postnatally, and the gonadal expression profiles of *e.g. Raldh1*, *Raldh2*, *Stra8* and *Cyp26b1* suggest both similarities and differences when compared to humans and mice (Diaz-Hernandez *et al.* 2019).

101. An important difference between female and male mice is that the RA-catabolizing enzyme Cyp26b1, which is expressed in mouse fetal gonads of both sexes at 11.5 dpc, is no longer expressed in the female gonad from 12.5 dpc (Bowles *et al.* 2006). Consequently, RA is degraded in the fetal testis, and thereby meiotic entry is blocked (MacLean *et al.* 2007). In contrast, RA levels are maintained in the fetal ovary, *Stra8* and *Rec8* are expressed, and female gonocytes will subsequently enter meiosis I (Rossitto *et al.* 2015).

102. Anomalous meiosis initiation takes place in fetal mouse testes as a result of exogenous RA exposure (Bowles *et al.* 2006, Koubova *et al.* 2006). The expression of *Stra8* is upregulated in *Cyp26b1*$^{-/-}$ fetal testis (Bowles *et al.* 2006). Furthermore, increased *Stra8* expression has been observed in *ex vivo* cultured 12.5 dpc mouse fetal testes treated with either ketoconazole (a non-specific CYP inhibitor) or R115866 (a more CYP26-specific inhibitor) (Koubova *et al*. 2006). Ketoconazole has no effect in the presence of the RAR antagonist BMS-204493, which further proves the role of RA in Stra8 induction (Koubova *et al.* 2006). In concordance with these *ex vivo* studies, the absence of *Cyp26b1* in male mouse embryos would lead to sustained concentrations of RA in the testes, as well as subsequent induction of *Stra8* expression followed by meiosis. In line with this, suppressed induction of meiosis is observed when *Stra8* is knocked-out concomitantly with Cyp26b1 (Saba *et al.* 2014).

103. Species differences may exist for the expression of gonadal CYP26B1, as suggested by the higher than expected expression in human fetal ovaries at GW 14-16 (Childs *et al.* 2011). It has also been suggested that in the human fetal gonad, RA levels are regulated primarily *via* synthesis mediated by RALDH-subtypes rather than by CYP26B1 catabolism (Le Bouffant *et al.* 2010). This could explain the asynchronous initiation of meiosis in human ovaries. It has been noted that while the roles of RA and STRA8 in meiosis initiation appear to be conserved between humans and several animal species, the role for CYP26B1 differs between mice and other species such as humans and marsupials (Hickford *et al.* 2017). In mice, ovarian *Cyp26b1* expression is known to be downregulated prior to meiosis initiation, while such downregulation is not observed in the human ovaries (Hickford *et al.* 2017).

104. As discussed in the previous paragraph, RA levels are maintained in both male and female mouse gonads alike in the absence of Cyp26b1. In a *Cyp26b1*$^{-/-}$ mouse model, germ cell development was studied in ovaries and testes from embryos at 13.5 dpc and from neonatal pups (MacLean *et al.* 2007). In that study, ovarian germ cells at both stages appeared unaffected by the lack of Cyp26b1. In the embryonic *Cyp26b1*$^{-/-}$ testes, some germ cells had prematurely entered meiosis while others appeared apoptotic. Increased levels of RA were demonstrated in the embryonic *Cyp26b1*$^{-/-}$ testes. Germ cells were essentially absent in testes from neonatal *Cyp26b1*$^{-/-}$ pups. Premature germ cell meiosis could also be observed in male wildtype genital ridges cultured in the presence of a synthetic retinoid (Am580, which is resistant to Cyp26b1 metabolism), suggesting that excess RA was responsible for the effect (MacLean *et al.* 2007).

105. RA has been widely accepted to be important for the induction of meiosis (Bowles *et al.* 2016, Spiller *et al.* 2017, Teletin *et al.* 2017. However, it has been suggested that the role of RA in the induction of meiosis may be facilitating rather than critical (Kumar *et al.* 2011, Teletin *et al.* 2019, Bellutti *et al.* 2019). Kumar and coworkers reported that meiosis occurred normally in mouse fetal ovaries lacking RA due to ablation of *Aldh1a2* and *Aldh1a3* (Kumar *et al.* 2011). It was subsequently shown that in 11.5 dpc cultured mouse urogenital ridges with chemically inhibited Raldh2 and Raldh3, levels of the third RA-producing enzyme, Raldh1, were elevated (Bowles *et al.* 2016). Thus, at least in mouse ovarian germ

cells, RA appears indeed to be an inducer of meiosis (Yadu and Kumar 2019). This is currently a very active area of research and the exact role of RA in meiosis induction needs to be further clarified.

A.4 Cross-species comparison of retinoic acid and the reproductive system

A.4.1. Retinoic acid, Stra8 and meiotic regulation in other species

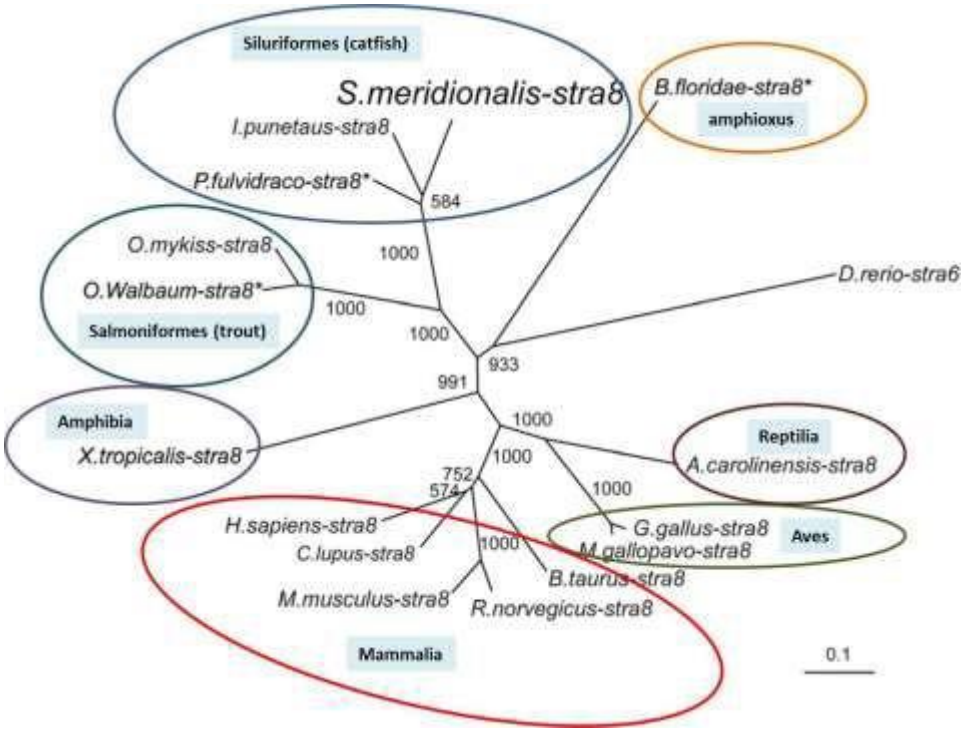
106. After the discovery of RA important role in germ cell fate and meiosis in mammals, researchers have very recently started to investigate if this function is shared by other 'lower vertebrate' models such as fish.

107. Rodríguez-Marí *et al.* (2013) found some shared underlying regulatory themes between zebrafish (*Danio rerio*) and mammals but also important genomic and developmental differences in the mechanisms of RA-regulated gonadogenesis and sex determination. Zebrafish genomic surveys of the RA-metabolic genetic machinery have shown that some aldh1a family genes (i.e. aldh1a1) have been lost in zebrafish and other teleosts, and that this gene loss has altered the functional evolution of the surviving aldh1a paralogs. In addition, in zebrafish, Cyp26a1 is the main Cyp26 paralog expressed in gonads during the critical period for sex determination, whereas in mice Cyp26b1 seems to be the dominate form (Rodríguez-Marí *et al.*, 2013). Importantly, Rodríguez-Marí *et al.* (2013) found no homolog of Stra8 in zebrafish and suggested Stra8 may be specific to tetrapods. It is worth noting that in contrast to the mouse, all zebrafish juveniles, regardless of their definitive sex, initially develop an ovary-like gonad with immature oocytes; in females, these oocytes continue to develop and reinforce the differentiation of mature ovaries, but in males, oocytes die by apoptosis and the gonads become testes (Rodríguez-Marí *et al.*, 2013). Therefore there are a number of fundamental differences between the mammalian rodent model and zebrafish in the process of early sexual development. However, it is also well known that not all fish species develop in this way and that genetic diversity among different Classes of fish is quite high.

108. Interestingly, Dong *et al.* (2013) have found Stra8 homologs in Siluriformes (catfish) and Li *et al.* (2016) have demonstrated RA triggers the onset of meiosis via stra8-dependent pathway in Southern catfish, (*Silurus meridionalis*). As with zebrafish it seems Cyp26a1, instead of Cyp26b1, is the key catabolic enzyme involved in meiosis initiation in Southern catfish (Li *et al.*, 2016). Bioinformatics searches have shown that in addition to catfish, Stra8 homologs were identified in Salmoniformes (e.g. rainbow trout) but not in zebrafish, Japanese medaka, pufferfish (fugu, tetraodon), stickleback or Nile tilapia. They suggested two possibilities for this discrepancy, 1) the difficulty identifying Stra8 from other fish species because the overall similarity between fish and tetrapods Stra8 is very low (<30% between fish to humans) or 2) Stra8 was not included in the databases because of incomplete genome sequencing (Dong *et al.* 2013). However, it is possible that some groups of fish may have lost Stra8 and other paralogs may exist in these fish. Recent research has found that although Nile tilapia do not have a homolog of Stra8, retinoic acid still played a vital role in meiotic entry (Feng *et al.*, 2015) via a stra8-independent signalling pathway, where both aldh1a2 and cyp26a1 are still critical. In the Nile tilapia estrogen was found to be vital in regulating sex, for example treatment of XY fish with estrogen (E2) and XX fish with fadrozole (aromatase inhibitor) led to sex reversal, and reversion of meiotic initiation (Feng *et al.*, 2015).

109. To date, less work has been carried out on other lower vertebrate models such as amphibians, birds or reptiles, however Dong *et al.* (2013) have identified Stra8 homologs in species of bird, reptile and amphibian (Figure 1.8).

Figure A.2. Phylogenetic tree of Stra8



Note: The tree was made with the default settings of the ClustalX protein alignment program and visualized using treeview32. The values represent bootstrap scores out of 1000 trials, indicating the credibility of each branch. Asterisks indicate partial sequences. Coloured circles indicate class or order grouping.
Source: Adapted from Figure 2, Dong *et al.2013*.

A.5. Female reproductive organ development, function and health and the role of retinoids

110. The ovary is the site for differentiation and release of mature oocytes for fertilization. It is also where sex hormones (necessary for follicle development, estrous cyclicity, maintenance and function of the reproductive tract) are synthesized and released (Barnett *et al.* 2006).

111. Retinoids are known to play an important role in the reproductive organs of females, including sex differentiation (as previously described in Sections A.1 and A.2), and oogenesis/folliculogenesis during embryogenesis, and potentially also in adult pathologies such as endometriosis.

A.5.1. Oogenesis

112. Oogenesis is the formation of haploid mature gametes from diploid oocytes, and the entire process begins *in utero* with primary oocytes eventually arresting at prophase I (the first phase in meiosis) in primordial follicles, which then can grow to full maturity after puberty (Barnett *et al.* 2006, Teletin *et al.* 2017). In more detail, PGCs differentiate into oogonia upon arrival at the developing female gonad and then undergo mitotic proliferation to create a stock of millions of oogonia that form germ cell nests/cysts, *i.e.*, clusters of cells connected by intercellular bridges (reviewed in Johansson *et al.* 2017). These nests break down when some of the oogonia within the nest undergo apoptosis, thus breaking the connections between the cells and thereby allowing somatic cells to enter and surround the germ cells. This rearrangement results in the creation of primordial follicles each containing a primary oocyte arrested in prophase I and surrounded by a single layer of flattened somatic (granulosa) cells. The breakdown of germ cell nests, constituting the beginning of primordial follicle formation (initiating

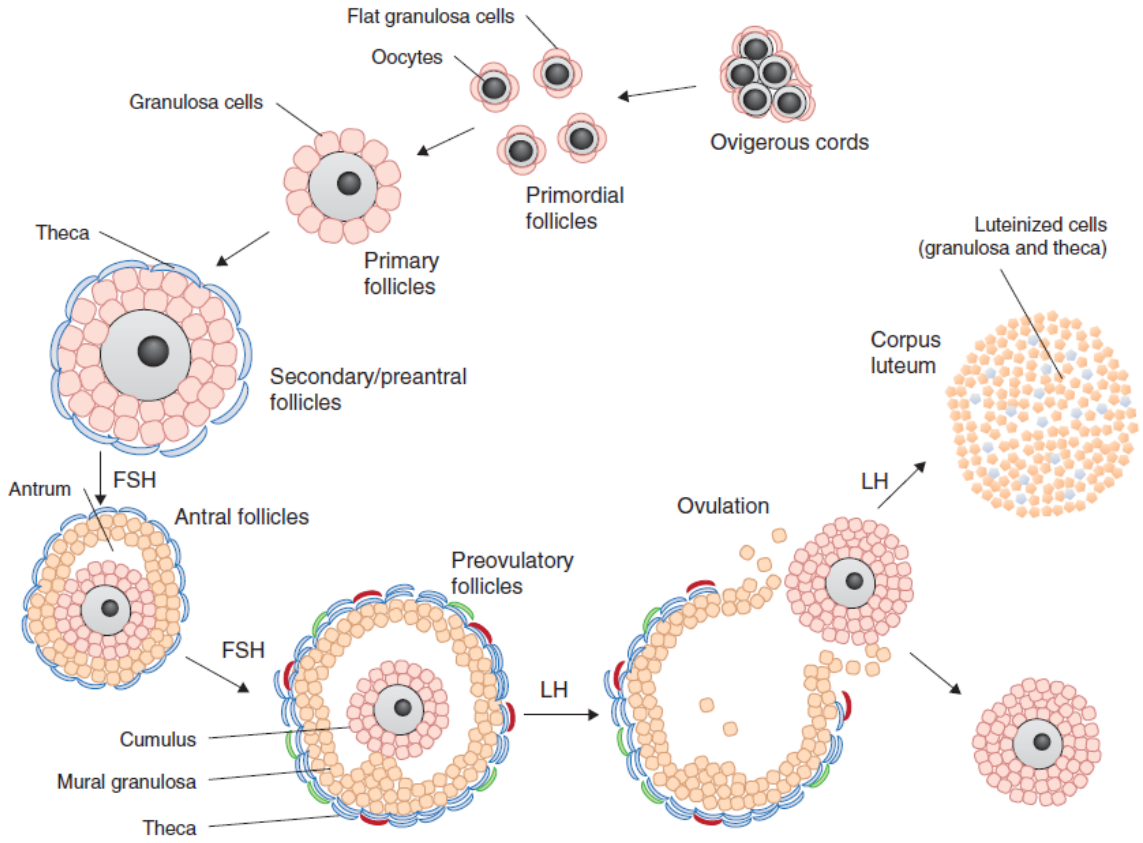
folliculogenesis, see next section), results in a decrease in oocyte number. The oogonia will enter meiosis I around 13.5 dpc in mice, and between 10 and 12 weeks of gestation in humans (Grive and Freiman 2015). By entering meiosis I, the cells can no longer divide mitotically. Thus, when all oogonia have entered the first meiotic division, the size of the ovarian reserve is fixed.

A.5.2. Ovarian somatic cells and folliculogenesis

113. As the genital ridges begin to differentiate into ovaries in female fetuses (at approximately 11 dpc in mice), a subset of gonadal somatic cells will differentiate into granulosa cells, after which meiosis will commence in the oogonia (Minkina *et al.* 2017). The germ cell nests with the proliferating oogonia will break down and somatic cells will enter and surround the germ cells, thereby forming the primordial follicles. The processes of nest breakdown, germ cell death and formation of primordial follicles are similar in mice and humans, but more sequentially organized in mice (Johansson *et al.* 2017). Nest breakdown takes place around the time of birth in mice and begins during mid-gestation (around GW 16) in humans (Grive and Freiman 2015). In addition, follicle assembly takes place prenatally in humans and postnatally in rodents (Johansson *et al.* 2017). These differences may suggest different initiating mechanisms. The primordial follicles, each of which contains an oocyte surrounded by a single layer of somatic pre-granulosa cells, represent the adult ovarian reserve (Grive and Freiman 2015).

114. Folliculogenesis, the process of follicle maturation (See Figure A.3), primarily takes place from the onset of puberty (Johansson *et al.* 2017). The first (primordial) follicles each consist of a single oocyte surrounded by a layer of flat granulosa cells. Granulosa cells progressively form several layers around the oocyte with an outer layer of androgen-producing theca cells. The granulosa cells subsequently convert androgens to estrogens. As granulosa cells continue to proliferate, the antrum is formed. At this stage, selection occurs between growing follicles, so that only one or a limited number of follicles continue growing to the preovulatory stage, while others undergo atresia. After ovulation, luteinized theca cells and mural granulosa cells produce progesterone.

Figure A.3. Folliculogenesis



Note: After breakdown of the germ cell nests (in the figure referred to as ovigerous cords), flat granulosa cells form a single layer around oocytes, thereby forming primordial follicles; this step is considered as the start of folliculogenesis. More layers of granulosa cells form around the oocytes and eventually also an outer layer of theca cells surrounds the follicles. The granulosa cells continue to proliferate and are separated by the antrum into two populations: cumulus and mural granulosa cells. A small selection of these antral follicles grow (while others undergo atresia) and are eventually released by ovulation. Up until ovulation, theca cells synthesize androgens that are metabolized into estrogens by granulosa cells. After ovulation (triggered by FSH; Follicle-Stimulating Hormone, and LH; Luteinizing Hormone), progesterone is produced in the corpus luteum by theca cells and mural granulosa cells.
Source: Georges *et al.* 2014, reprinted with kind permission from the publisher: Bioscientifica Ltd.

115. RA has been considered to be required to maintain ovarian differentiation and development (Minkina *et al.* 2014, Suzuki *et al.* 2015). RA is also believed to regulate mouse ovarian follicle development in the adult; this phenomenon has been studied, *e.g., in vitro* by stimulating granulosa cell proliferation (Demczuk *et al.* 2016)., Using cat ovarian cortices in an *ex vivo* experiment, RA was shown to activate the development of primordial follicles into primary and secondary follicles, possibly *via* differential regulation of matrix metalloproteinases (MMP) (Fujihara *et al.* 2018). It has been shown that mouse theca cells and granulosa cells express the enzymes necessary for conversion of ROH to RA (different forms of ADH and RALDH), or degradation of RA (Cyp26b1). Further, in these experiments by Kawai and co-workers, these enzymes were demonstrated to be differentially regulated after injection of equine chorionic gonadotropin, an analogue of Luteinizing hormone (LH)/Follicle stimulating hormone (FSH) (Kawai *et al.* 2016, Kawai *et al.* 2018). As demonstrated *in vitro*, ovarian *de novo* synthesis of RA is required for follicular expression of the LH receptor in granulosa cells of mouse ovaries and for their ability to respond to the ovulatory LH surge; oocytes appear to negatively regulate RA synthesis in pre-ovulatory follicles, impacting LH receptor expression in follicular somatic cells possibly *via* an epigenetic mechanism (Kawai *et al.* 2016, Kawai *et al.* 2018). Cultured human ovarian cumulus granulosa cells

(obtained during oocyte retrieval during the course of *in vitro* fertilization) produce RA from ROH in the media. RA causes de-phosphorylation of connexin 43, involved in gap junction intercellular communication (GJIC) between the granulosa cells in the cumulus-oocyte complex. De-phosphorylation of connexin 43 increases GJIC, which plays an important role in oogenesis and successful fertilization (Best *et al.* 2015).

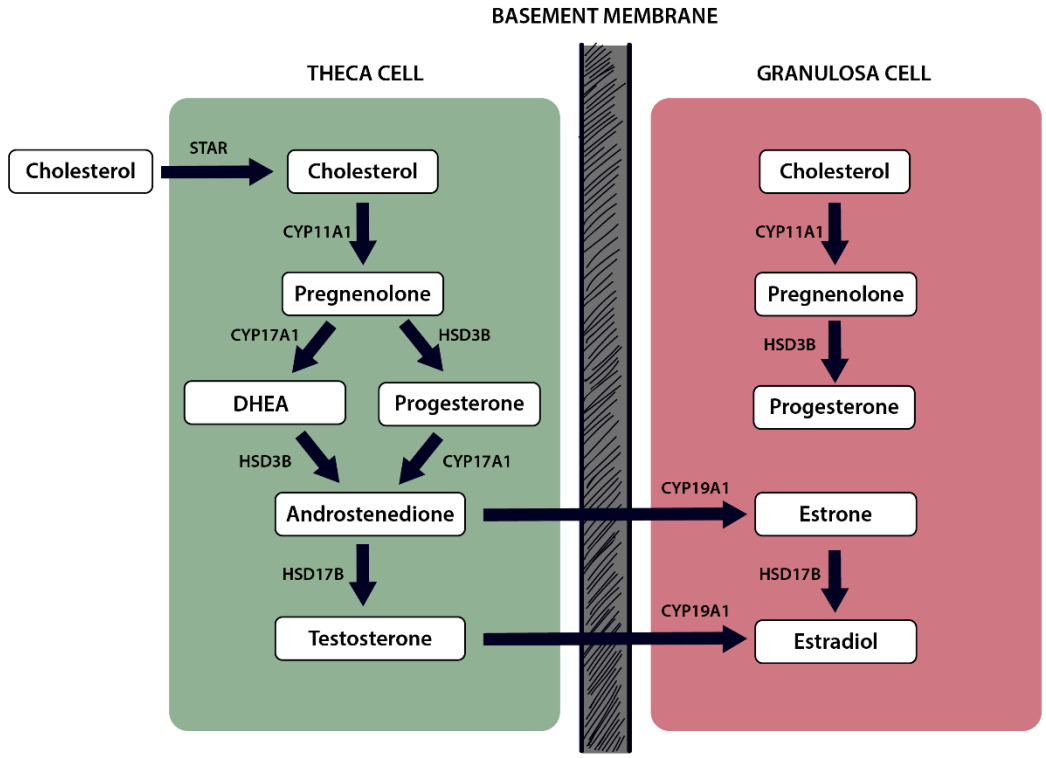
116. Minkina and co-authors however demonstrated that RA may not be required for ovarian granulosa cell specification, differentiation or function. No significant effects were found on ovarian differentiation, follicle development or female fertility in geneticially manipulated mouse models, in which all three RARs were deleted in the female somatic gonad at the time of sex determination (Minkina *et al.* 2017). Nor did the knockout of all three RA-producing aldehyde dehydrogenase genes (*Aldh1a1-3*) at the same timepoint appear to masculinize the mouse ovaries. Additionally, an RAR antagonist (BMS-189453) was capable of blocking meiotic initiation in the germ cells of 10.5 dpc wild-type female mouse gonads cultured *ex vivo,* but had little effect on the expression of markers for either granulosa or Sertoli cells; thus, disruption of RA signaling does not appear to disrupt early somatic differentiation in the female fetus (Minkina *et al.* 2017). Notably, the triple knockout model was based on tamixofen-inducible Cre, whereby the mice are administered tamoxifen to silence the genes; however, tamoxifen is itself an estrogenic compound known to affect gonadal sex differentiation (Patel *et al.* 2017).

117. In mouse ovaries, RBP4 is expressed before puberty but increases significantly in the peripubertal period. In adult mice, RBP4 expression increased at pro-estrous and peaked at estrous and was localized mainly in the granulosa and theca cells of follicles. Expression is also induced by FSH, alone or in combination with LH, while LH alone had no effect (Jiang *et al.* 2018b).

A.5.3. Ovarian steroidogenesis

118. Ovarian steroidogenesis, or the production of sex steroid hormones, is regulated by the pituitary hormones LH and FSH; LH stimulates the ovarian thecal cells to produce androgens and FSH stimulates the granulosa cells to convert these androgens to estrogens (Hannon and Flaws 2015). The process of synthesizing the ovarian steroids from cholesterol is complex, and requires the action of several enzymes (see Figure A.4).

Figure A.4. Ovarian steroidogenesis



Note: Prior to ovulation, enzymatic conversion of cholesterol to estradiol occurs initially in the theca cells and subsequently in the granulosa cells, primarily in the mature antral follicles. After ovulation, conversion of cholesterol to progesterone occurs in the corpus luteum. Hormones: listed in the white text boxes; steroidogenic enzymes: listed adjacent to the arrows.
Abbreviations: DHEA; dehydroepiandrosterone, STAR; steroidogenic acute regulatory protein.
Source: Hannon and Flaws 2015, reprinted under the terms of the Creative Commons Attribution License (CC BY), from frontiers in Endocrinology.

119. Ovarian steroidogenesis is reported to be regulated by RA in several systems (recently reviewed in Damdimopoulou *et al.* 2019). For example, in rats, low levels of RA and ROH stimulate the formation of progesterone (Bagavandoss and Midgley 1987). In a human ovarian surface epithelium cell line, RA significantly induces production of progesterone (Papacleovoulou *et al.* 2009). In women, ROH in plasma is associated with higher estradiol and testosterone levels (Mumford *et al.* 2016). It is likely that RA regulates either the expression or the activity (or both) of the steroidogenic enzymes (Wickenheisser *et al.* 2005). This hypothesis is supported by data from retinoid-deficient rats, in which ovarian activity of the cytochrome P450 cholesterol side-chain cleavage enzyme (P450SCC) decreased along with the progression of retinoid deficiency (Jayaram *et al.* 1973). In addition, RA may modulate the pituitary gonadotropins, which in turn regulate steroidogenesis (Minegishi *et al.* 2000).

120. In the endometrial epithelial cells, estradiol is locally inactivated through conversion to the less potent estrone by the enzyme 17β-hydroxysteroid dehydrogenase type 2 (HSD17B2). RA produced in the stromal cells may act as a paracrine factor capable of stimulating the production of HSD17B2 (Taylor *et al.* 2015, Jiang *et al.* 2018a).

121. A connection between ROH/RA and ovarian function has been hypothesized based on the observation that serum ROH levels in women vary with the stages of the estrous cycle (highest levels in the proestrus and estrus phases), and that RBP4 serum levels are positively correlated with

gonadotropin levels (such as FSH and LH) (Jiang *et al.* 2017). Similarly, ovarian expression of RBP4 mRNA and protein in adult female mice increased at proestrus and peaked at estrus (Jiang *et al.* 2018b). In ovaries from mice treated with FSH, increased levels of RA (and other retinoid forms) and RBP4, as well as increased expression levels of RA-synthesizing enzymes (*Adh1*, *Raldh1*), were observed (Jiang *et al.* 2018b, Liu *et al.* 2018). Similar effects were observed in FSH-treated mouse granulosa cells, in addition to increases in *e.g.* STRA6 (the specific receptor for RBP4) and CRBP1, suggesting that gonadotropin FSH modulates the pathways for ROH uptake and its metabolism to RA in the mouse ovary (Liu *et al.* 2018).

A.5.4. Other female reproductive organs and pregnancy

122. In the female fetus, the Müllerian ducts differentiate into the oviduct, uterus and upper vagina, with the resulting epithelia having distinct and separate organ-specific morphology and function (Nakajima *et al.* 2016). At least in the mouse, RA signaling *via* RAR during embryo development may determine the fate of Müllerian duct stroma into either uterus or upper vagina, as seen *ex vivo* after treatment of Müllerian ducts with RA (leading to uterine epithelial differentiation) or treatment with RAR-inhibitors (leading to vaginal epithelial differentiation) (Nakajima *et al.* 2016).

123. The structure and function of the mucosal inner lining of the uterus, the endometrium, is regulated by the ovarian sex steroid hormones estradiol and progesterone. In humans, the endometrium undergoes major changes during the menstrual cycle. During the proliferative phase, ovarian estrogen stimulate proliferation and growth, and during the secretory phase, progesterone prepares the endometrium for potential implantation. A number of retinoid-related genes (ALDH1A1, ALDH1A2, CYP26A1, CRABP2, RARα, RARγ, and RXRα) are expressed in the endometrium of both rodents and humans, and their expression is regulated by estrogen and/or progesterone (Jiang *et al.* 2017). As reviewed by Jiang and co-workers, expression of these and other retinoid-related genes have been noted in either stromal or epithelial cells (Jiang *et al.* 2017).

124. Following fertilization, the implanting blastocyst first attaches to the epithelial cells and then invades the endometrium by displacing the epithelial cells. It is finally embedded in the endometrium, where formation of the placenta is initiated (Su and Fazleabas 2015). The details of these processes can be species-specific.

125. RA regulates expression of MMPs, which are produced by endometrial stromal cells during decidualization (*i.e.,* the process of endometrial changes in preparation for pregnancy). RARs are expressed in the uterine stroma of mice and uterine epithelium of rats. In rats, RAR protein expression is influenced by ovarian steroids; RAR expression increases under the influence of estradiol, suggesting involvement of retinoids in growth and proliferation of endometrial epithelia. In postmenopausal women taking estrogen, uterine RAR expression is reported to increase. Increased RAR levels have also been observed in premenopausal women during the proliferative phase; a phase which is associated with elevated estradiol levels (Sayem *et al.* 2018).

126. In the mouse uterine epithelium, Cyp26a1 (both mRNA and protein) is expressed during the blastocyst implantation period. Removing or inactivating Cyp26a1 in mice led to a decreased pregnancy rate, and reduced numbers of implantation sites (Han *et al.* 2010). It appears that the Cyp26a1 enzyme, by degrading RA, might prevent the otherwise inhibitory effects of RA on implantation-related genes in the endometrial epithelium during mouse embryo implantation (Ma *et al.* 2012). RA treatment of mouse blastocysts *in vitro* inhibited cell proliferation and caused retarded growth (Huang *et al.* 2005). Following implantation, these RA-treated blastocysts were resorbed to a greater degree than untreated blastocysts (Huang *et al.* 2005). During preimplantation development of the blastocyst, RA signaling appears not to be involved (Rout and Armant 2002).

127. *Via* the PPAR/RXR heterodimer, the retinoid signaling pathway is involved in human placental processes such as the invasion of the uterine epithelium by extraembryonic trophoblasts. Both trophoblasts and decidual cells appear to be capable of synthesizing RA as well as the presumed RXR agonist ligand, 9-*cis*-RA (Tarrade *et al.* 2001). At least *in vitro*, trophoblast invasion is inhibited by PPAR and RXR agonists, while PPAR and RXR antagonists increases invasion. Functional retinoid signaling pathways have also been demonstrated in human amniotic membranes (Marceau *et al.* 2006).

128. RA concentration in follicular fluid (obtained during oocyte retrieval during the course of *in vitro* fertilization) was found to be positively correlated with embryo quality (scored on day 3 after fertilization). RA levels in follicular fluid also correlated moderately with plasma RA. Women with endometriosis had significantly lower concentration of RA in follicular fluid and in plasma than women with no endometriosis (Pauli *et al.* 2013).

129. The genital tubercle is a tissue present during embryonic development of the reproductive system; it later develops into either the glans clitoris or the glans penis in humans. RA appears to be involved in genital tubercle development, along with Rarb, Raldh2 and Cyp26b1 (Liu *et al*. 2012).

A.5.5. Female reproductive pathologies and retinoid involvement

A.5.5.1. Endometriosis

130. Endometriosis is defined as the presence of endometrial tissue outside of the uterus, usually on the ovaries, fallopian tubes, and the peritoneum; a state which can cause severe pain. In addition, endometriosis is associated with fertilization problems (Taylor *et al.* 2015, Jiang *et al.* 2018a).

131. Several studies have demonstrated altered retinoid pathway signaling associated with endometriosis. In human normal premenopausal endometrial tissue, mRNA expression of the RA-catabolizing enzyme CYP26A1 increases substantially during the progesterone-dominated secretory phase (leading to degradation of RA and therefore diminished RA signaling) when compared to the estrogen-dominated proliferative phase, during which RA-synthesizing enzymes such as RALDHs are increased (Deng *et al.* 2003). At least *in vitro*, RA has been shown to inhibit the decidualization of stromal cells; thus, reduced concentration of RA in the endometrial tissue during the secretory phase could be necessary for successful implantation (Deng *et al.* 2003). In the mouse uterus, endogenous RA levels appear to be controlled both by estrogen-dependent expression of RALDH enzymes and by progesterone-dependent expression of CYP26A1 (Fritzsche *et al.* 2007). Expression of CYP26A1 is down-regulated in both the secretory and proliferative phases in endometrial biopsies from women with moderate or severe endometriosis, when compared to healthy women (Burney *et al.* 2007). The availability of RA may therefore be increased in the endometrial tissue of women with endometriosis. In endometriosis, there is evidence for progesterone resistance, and cultured stromal fibroblasts originating from endometriotic lesions have decreased ability to decidualize (which is necessary for a successful blastocyst implantation) (Burney *et al.* 2007).

132. Reduced STRA6, CRBP1 and ALDH1A2 expression have been demonstrated to reduce RA in endometrial stromal cells (Jiang *et al.* 2018a). Transcriptional activation *via* the RA-CRABP2-RAR pathway has been reported to trigger cell cycle arrest and apoptosis; thus, reduced signaling can cause endometrial cells to escape apoptosis and contribute to survival of ectopic cells (Jiang *et al.* 2018a). Since RA appears to stimulate the production of HSD17B2, which inactivates estradiol in the endometrium, an abnormal RA pathway in endometriosis may explain the aberrant HSD17B2 expression and the high local estradiol concentrations in endometriosis. In addition, altered retinoid action may cause decreased expression of gap junctional protein connexin 43 along with decreased gap junctional intercellular communication, reducing the decidualization capacity of the stromal cells in endometriosis, which could contribute to progression of endometric lesions and the associated subfertile uterine phenotype (Taylor *et al.* 2015, Jiang *et al.* 2018a).

A.5.5.2. Polycystic ovarian syndrome

133. Polycystic ovarian syndrome (PCOS) is a heterogenous disorder, characterized both by signs of androgen excess and ovarian dysfunction (such as irregular ovulation and/or polycystic ovarian morphology) (Escobar-Morreale 2018). In PCOS, an increased ovarian androgen production from theca cells, an elevated LH:FSH hormone ratio, and enlarged ovaries containing many antral follicles are often observed (reviewed in Jiang *et al.* 2017). The associated arrest in follicular growth and anovulation can cause subfertility or infertility.

134. When comparing the response to RA, 9-*cis*-RA and ROH in cultured theca cells isolated from normal-cycling women and women with PCOS, only RA led to increased testosterone production (possibly *via* increased expression of 17,20-lyase; *Cyp17*) in normal theca cells, while in the theca cells of PCOS patients, all three tested retinoid variants (RA, ROH, and 9-*cis*-RA) had the same effect (Wickenheisser *et al.* 2005). Thus, in PCOS, theca cells may be sensitized to retinoid signaling stimulation. Moreover, mRNA expression of RA-synthesizing enzymes retinol dehydrogenase (RoDH2) and ALDH6 was increased in PCOS theca cells (Wood *et al.* 2003), and PCOS ovaries show enhanced expression of RoDH2 (Marti *et al.* 2017). These data suggest an increased rate of RA synthesis in the thecal cells of PCOS women (reviewed in Jiang *et al.* 2017).

135. In women of reproductive age with acne and PCOS, treatment with oral isotretinoin (13-*cis*-retinoic acid) decreased ovarian volume (Acmaz *et al.* 2019).

A.5.6. Retinoid deficiency/excess in female reproduction

136. As reviewed in Clagett-Dame and Knutson 2011 (citing publications going back to the 1920's), retinoids are required for successful fertilization, implantation, placentation, embryogenesis and full-term pregnancy: in severely retinoid-deficient female rats, reproduction fails prior to implantation, while in less severe deficiency, fertilization and implantation occur, but embryonic death at mid-gestation is often observed. Retinoid deficiency has adverse effects on placental morphology in rats (Noback and Takahashi 1978). In female pregnant VAD rats, RA supplementation maintains normal implantation and early embryogenesis (White *et al.* 1998), but unless supplementation is sufficiently high by 8.5 dpc, fetuses will be reabsorbed (White *et al.* 2000). Retinoids are also required for the normal onset of meiosis in the developing embryo (discussed in detail in Section A.2), and germ cells in rat embryos with severe retinoid deficiency fail to enter meiosis. This is accompanied by an observed failure of *Stra8* induction. Supplementation of small amounts of RA to dams was sufficient to initiate meiosis (Li and Clagett-Dame 2009). Retinoid-deficient mice have a prolonged estrous cycle, and display a decreased rate of oocyte maturation and number of ovulated oocytes after gonadotropin treatment (Kawai *et al.* 2016).

137. Pregnancy is an absolute contraindication for all oral treatment with retinoid drugs in the EU[7] as they are teratogenic (Lammer *et al.* 1985). The pattern of birth defects that can be observed in several organ systems, however, does not appear to specifically target the female reproductive organs (Azais-Braesco and Pascal 2000, Pennimpede *et al.* 2010), although RA signaling has been shown to be involved in development of the genital tubercle (Liu *et al.* 2012).

A.6. Male reproductive organ development, function and health, and the role of retinoids

138. The testis produces large numbers of gametes (sperm, or spermatozoa) throughout the reproductive life of the male, and is also the primary source of androgens, which are required for

[7] https://www.ema.europa.eu/en/documents/referral/retinoid-article-31-referral-prac-assessment-report_en.pdf, accessed in November 2019.

spermatogenesis and the development and maintenance of male secondary sex characteristics throughout the body (reviewed in Bittman 2015).

139. RA plays several critical roles in the development and/or function of both Sertoli and Leydig cells (Jauregui *et al.* 2018, and reviewed in Lucas *et al.* 2014) as well as in spermatogenesis (reviewed in Mark *et al.* 2015, Griswold 2016, Teletin *et al.* 2017). In addition, RA signaling appears to be necessary for proper development of the testis itself (Spade *et al.* 2019a). As will be discussed below, testicular RA is not derived from the circulation, as a catabolic barrier is formed by Cyp26 enzymes present in the peritubular myoid cells (Vernet *et al.* 2006). The testicular site of RA synthesis (using ROH taken up from the circulation) varies with age (see Spermatogenesis section below). *Stra6* expressed in Sertoli cells may play a role in ROH uptake from the circulation, at least under VAD conditions (Kelly *et al.* 2016).

A.6.1. Testicular somatic cells and steroidogenesis

140. In the adult testis, LH stimulates the production of testosterone in Leydig cells. Steroidogenesis itself takes place in both fetal and adult Leydig cells and depends on several steroidogenic enzymes, such as CYP11A1, HSD3B1 and CYP17A1 (Jauregui *et al.* 2018). Testosterone secreted from the Leydig cells regulates the end of meiosis, the establishment and maintenance of the blood-testis barrier (BTB), and spermiation (Jauregui *et al.* 2018). Sertoli cells both create the BTB (*via e.g.* tight junctions and gap junctions) and play an active role in translocating the male germ cells within the seminiferous tubule epithelium (reviewed in Xiao *et al.* 2014).

141. RA signaling, via RAR/RXR, appears to be necessary for development and function of the Sertoli cells (Lucas *et al.* 2014). In primary rat Sertoli cells isolated on PND 10 and 20, RA suppressed proliferation and initiated tight junction formation (Nicholls *et al.* 2013). Leydig cells express proteins for RA synthesis, breakdown and signaling (Griswold and Hogarth 2018). Based on impairments observed in VAD mice, proper differentiation of Leydig cells appears to depend on sufficient retinoid levels (Yang *et al.* 2018). Fertility studies using conditional transgenic adult mice lacking functional Leydig cell RARα, show that RA signaling via RAR/RXR is required for normal Leydig cell function (Jauregui *et al.* 2018). The same conditional knockouts also had altered steroidogenic enzyme expression levels in Leydig cells, increased BTB permeability, as well as apoptotic pachytene spermatocytes, and were infertile (Jauregui *et al.* 2018). The observed phenotype is similar to mice with low or no levels of testosterone (discussed in Jauregui *et al.* 2018). Increased BTB permeability has also been demonstrated in neonatal mice after treatment with the RALDH2 inhibitor WIN 18,466 (Amory *et al.* 2011, Kent *et al.* 2016). The mechanism behind effects on the BTB, and the possible role of RA on its maintenance, still needs to clarified.

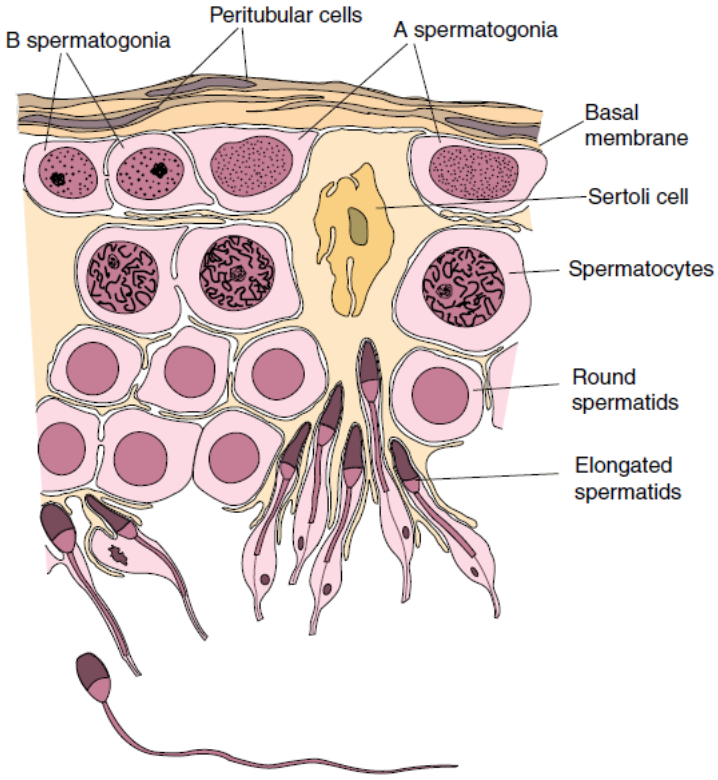
142. In an *ex vivo* model using mouse fetal testes, RA treatment increased testosterone production (Bellutti *et al.* 2019). In contrast, RA has been shown to decrease testosterone production in the developing rat testis (Livera *et al.* 2000a). In *ex vivo* cultured human fetal testes, RA treatment increased testosterone production and expression of steroidogenic enzymes such as cholesterol side-chain cleavage enzyme (P450scc), Cyp17, and steroidogenic acute regulatory protein (StAR) (Lambrot *et al.* 2006). Interestingly, testicular P450scc activity in retinoid-deficient rats decreased concomitantly with the progression of retinoid deficiency (Jayaram *et al.* 1973). Thus, RA appears to be capable of influencing steroidogenesis in both rodents and humans.

A.5.2. Spermatogenesis

143. Spermatogenesis (described in Figure A.5, and in more detail in Figure A.6) is the formation of haploid gametes (spermatozoa) from the diploid stem spermatogonia, and includes the process of spermatogonia differentiation, meiosis, differentiation of spermatids (spermiogenesis) and spermatid

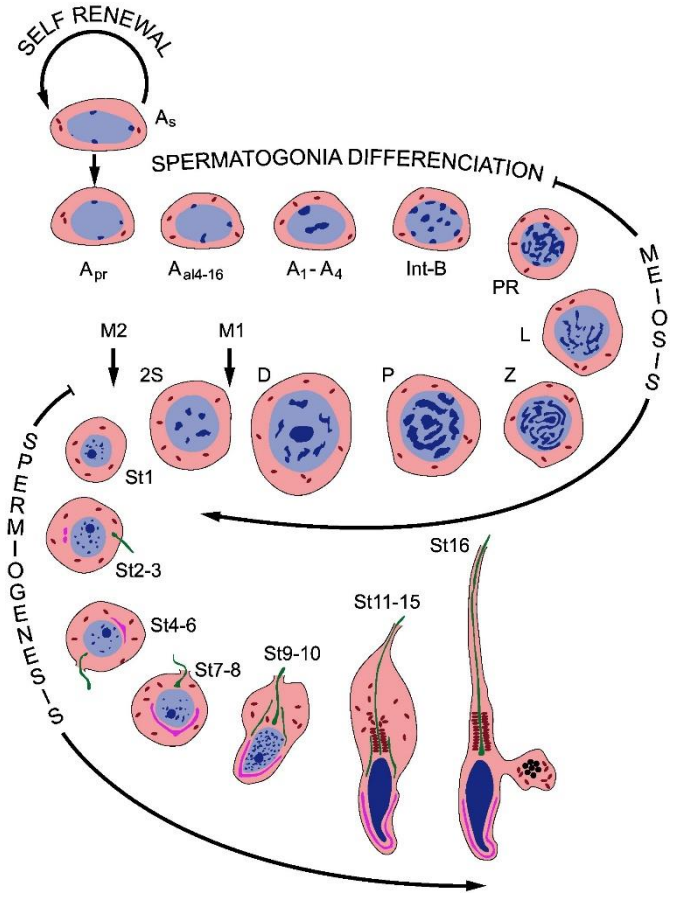
release (spermiation) (reviewed in Mark *et al.* 2015). Spermatogenesis takes place inside the seminiferous tubules in a rigidly structured process ensuring continuous, life-long sperm production (reviewed in Griswold 2016). As will be described below, the process in pubertal animals differs from that in adult animals. The human testis produces sperm in a continuous manner similar to rodents, and it is reasonable to assume that similar regulatory patterns of sperm production also exist in the human testis (Jørgensen *et al.* 2015, Griswold 2016) although more research is needed for a better understanding of human spermatogenesis.

Figure A.5. Schematic of an adult mouse seminiferous tubule, surrounded by peritubular cells.



Note: On the inside of the basal membrane of the seminiferous tubule, the spermatogonia are located, and are completely surrounded by Sertoli cells. The primary spermatocytes and round spermatids are located closer to the lumen. The elongated spermatids will eventually be shed into the lumen as spermatozoa.
Source: de Rooij and Mizrak 2008, reprinted with kind permission from the publisher: The Company of Biologists Ltd., Cambridge UK.

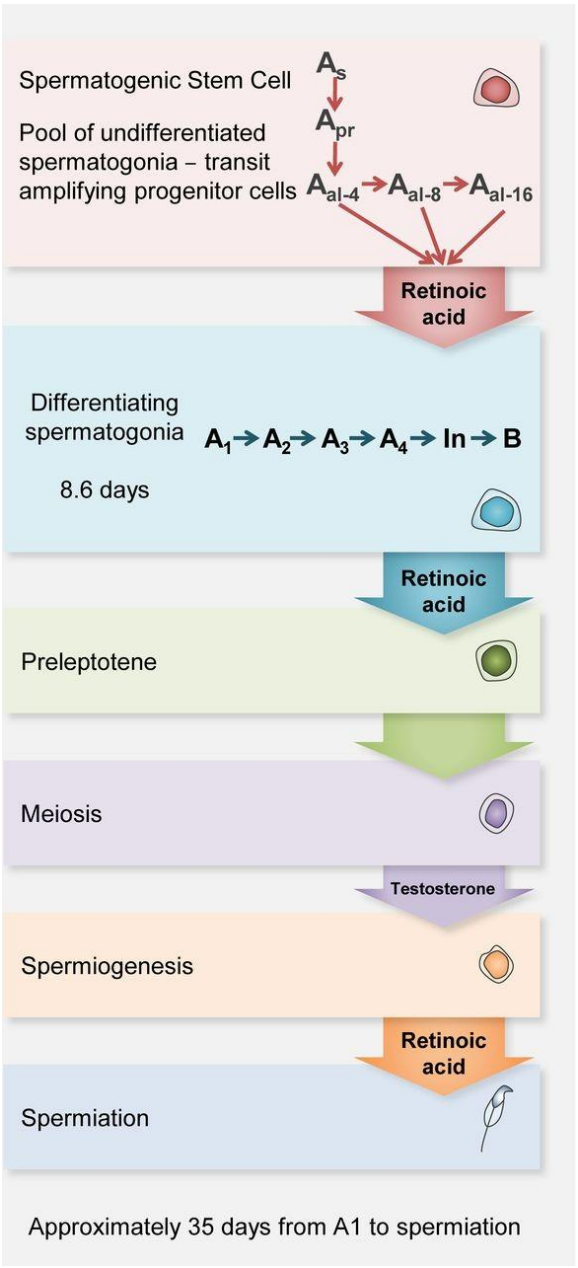
Figure A.6. Spermatogenesis in the adult mouse.



Note: In mice, spermatogonia in the single-cell state (A_S) are considered as the true stem cells of the spermatogenic lineage. A_s spermatogonia divide to maintain a stem cell population (A_s) and expand the population of cells (A_{pr} and $A_{al4–16}$) which enter the differentiation pathway (Stages A_1–A_4, Int and B, collectively referred to as differentiating spermatogonia; the previous stages, A_s – $A_{al16,}$ are collectively also referred to as undifferentiated spermatogonia. In the meiotic phase, the primary spermatocytes undergo recombination and segregation of homologous chromosomes during the meiotic divisions to generate secondary spermatocytes (2S) and subsequently step 1 spermatids (St1). The spermiogenesis phase, is subdivided into 16 steps based on morphological criteria (round spermatids, steps 1 to 8; elongating spermatids, steps 9 to 16). The first 12 steps span the entire cycle of the seminiferous epithelium. Step 16 spermatids are released into the lumen of the seminiferous tubules as spermatozoa, during a process called spermiation.
Abbreviations: PR; preleptotene, L; leptotene, Z; zygotene, P; pachytene, D; diplotene (the stages of the first meiotic prophase), 2S; secondary spermatocytes.
Source: Mark et al 2015. Reprinted with kind permission from the publisher: Elsevier.

144. At least in mice, RA is considered to be critically involved in several steps of spermatogenesis: spermatogonia differentiation specifically during the A_{al}-A_1 transition, for spermiation, and in regulation of the seminiferous epithelium cycle (see Figure A.7 and *e.g.* Teletin *et al.* 2019). RA is indispensable *in vivo* to trigger the A_{al} – A_1 transition (Teletin *et al.* 2019) and is also required for the survival of some A_{undiff} spermatogonia (reviewed in Teletin *et al.* 2017). Synthesis of both mRNA and protein STRA8 occurs exclusively in two distinct phases of spermatogonia differentiation; in differentiating type A spermatogonia (A_1-A_4), as well as in spermatocytes in the preleptotene (PR) and leptotene (L) phase at meiosis entry (Griswold 2016). See section *Meiosis in the post-natal male* below, for further discussion about meiotic regulation.

Figure A.7. Steps in germ cell differentiation and spermatogenesis thought to be controlled by RA in mice.



Note: Once the first/pubertal wave (during which differentiating spermatogonia develop directly from prospermatogonia) has been initiated, the subsequent rounds of differentiating spermatogonia arise from a subset of A single-state spermatogonia (A_s) acting as spermatogenic stem cells. Stem cells divide and form A paired spermatogonia (A_{pr}) that in turn divide and form an aligned cell syncytia of 4, 8, and 16 cells (also called "transit amplifying progenitor cells"), which transition without cell division into A_1 differentiating spermatogonia. After five cell divisions (synchronized to the cycle of the seminiferous epithelium), B spermatogonia are formed, followed by another mitotic division resulting in the formation of preleptotene spermatocytes. The preleptotene spermatocytes proceed through the rest of the meiosis, forming haploid spermatids that are eventually elongated.

Source: Griswold 2016, reprinted with kind permission from the publisher: American Physiological Society, Rockville, MD, USA.

A.6.2. Spermatogonia differentiation and spermiogenesis – first and subsequent waves

145. In the juvenile mouse, the mitotically quiescent gonocytes (also termed pro-spermatogonia) re-enter the cell cycle at approximately PND 1-2, and turn into spermatogonia at PND 3-6 as they migrate to the periphery of the testis cords, where they are surrounded by Sertoli cells (reviewed in Teletin *et al.* 2017 Teletin *et al.* 2019). During this first post-natal week in mice, a sub-population of gonocytes differentiates directly (without passing through the undifferentiated spermatogonia stages) into differentiating spermatogonia that support the so-called first wave of pubertal spermatogenesis. After the first wave of spermatogenesis (completed by PND 35 in the mouse), subsequent waves derive from the undifferentiated spermatogonia that have acquired self-renewal capacity, the spermatogonial stem cells (Yoshida et al 2006).

146. During the onset of puberty in mice, sperm development is initiated by RA produced in Sertoli cells, since these, at this stage, are the only cells in the seminiferous epithelium with RALDH activity (Raverdeau *et al.* 2012). The earliest reported presence of a germ cell derived source of RA in the postnatal mouse is at PND 9 in zygotene spermatocytes (Teletin *et al.* 2019). The RA produced in the Sertoli cells acts in a paracrine manner on the spermatogonia, to regulate the A_{al} – A_1 transition. Spermatogenesis is blocked in juvenile male mice deficient in RDH10 in Sertoli cells and germ cells, leading to a local lack of RA (Tong *et al.* 2013).

147. In juvenile male mice with arrested spermatogonia differentiation, due to either vitamin A deficiency or genetic knockout of Sertoli cell *Aldh1a*, a single injection of RA will resume the A_{al} – A_1 transition, with preleptotene spermatocytes expressing Stra8 and Rec8 appearing synchronously in all seminiferous tubules (reviewed in Griswold 2016). The same effect is observed in mice first treated with the RALDH inhibitor WIN 18,446, and subsequently treated with RA (Hogarth *et al.* 2013). RA administration to vitamin A-deficient mice or rats further results in truncation of the normal 12 (mouse) or 14 (rat) stages of the seminiferous epithelium cycle to only three or four stages (still enabling sperm production). Additionally, the normally asynchronous wave of spermatogenesis is synchronized, leading to a pulsatile rather than continuous sperm production (Teletin *et al.* 2017).

A.6.3. Post-pubertal RA synthesis

148. Once the first wave of spermatogenesis has progressed beyond a certain point, it appears, based on data from mice in which all three *Aldh1a* genes were deleted in Sertoli cells, that Raldh activity in Sertoli cells is no longer needed for spermatogonia differentiation to proceed (Raverdau *et al.* 2012). Thus, Raverdeau suggested that RA must be produced elsewhere, most likely by RALDH2 in spermatocytes and spermatids. Interestingly, Beedle and co-workers reported that in the testis of mice genetically engineered to have a postnatal severe deficiency of *Aldh1a2* gene expression in germ cells (*via Stra8-Cre*), or globally (*via* a tamoxifen-inducible *Cre*), no adverse effects on male fertility or health are noted (Beedle *et al.* 2018). In addition, in other mouse studies, where *Aldh1a1-3* genes have been ablated, it is suggested that spermatocyte-synthesized RA is dispensable for spermatogenesis, and that the Sertoli and spermatocyte RA sources are redundant in maintenance of spermatogenesis (reviewed in Ghyselinck and Duester 2019). Species differences may exist; studies using human testis biopsies suggest that RALDH1 is the predominant form that synthesizes RA in the Sertoli cells, while RALDH2 has the corresponding role in developing sperm (Arnold *et al.* 2015). As mentioned above, blocked spermatogenesis is observed in juvenile male mice deficient in RDH10 in Sertoli cells and germ cells; however, in adult age, these mice appear to have normal spermatogenesis (Tong *et al.* 2013), suggesting that in the adult animal, RDH10 is not important for control of RA availability in the testis.

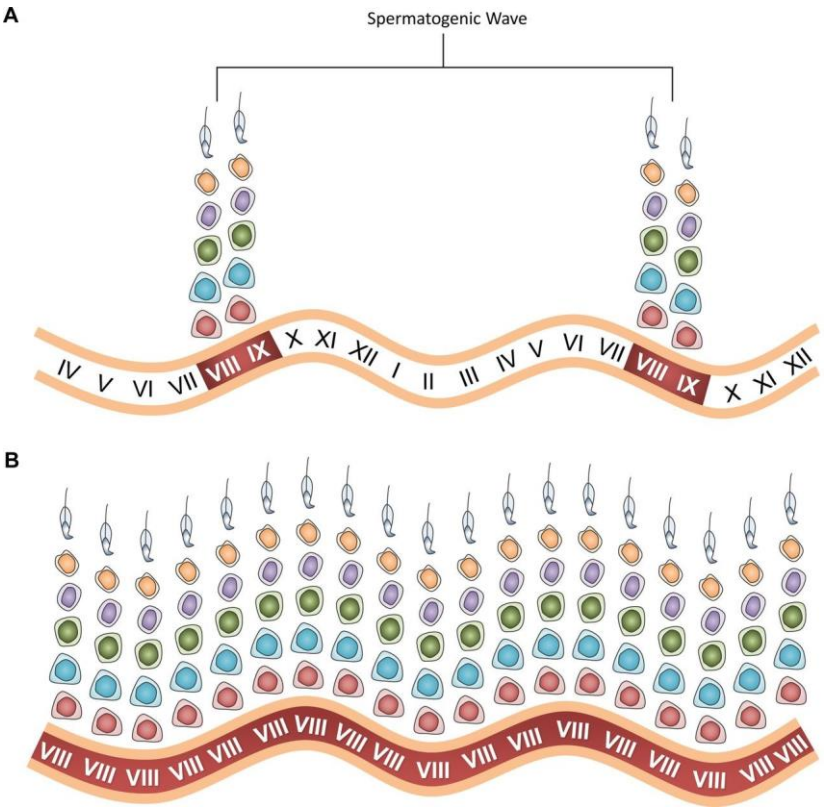
A.6.4 The seminiferous epithelium cycle and the spermatogenic wave

149. The seminiferous epithelium cycle covers the development from spermatogonia to spermatozoa, as they move from the basal compartment of the seminiferous tubule to the lumen. The stages are usually illustrated using Roman numerals (See Figure A.8). The number of stages are species specific; *e.g.*, mice have twelve stages, and humans six. In the mouse, four seminiferous epithelium cycles are required to complete the development from spermatogonia to spermatozoa.

150. The timing of the progenitor cell commitment and subsequent differentiation and maturation of spermatogonia along the seminiferous tubule is staggered, forming a spermatogenic wave (Griswold 2016). The cycle of the seminiferous epithelium is initiated by the precisely timed transition of undifferentiated A_{al} spermatogonia into A_1 spermatogonia, which subsequently will divide to generate successively differentiating A_2, A_3, and A_4 spermatogonia (Figure A.6 and *e.g.* Griswold 2016, Teletin *et al.* 2019).

151. The spermatogonia continue to divide mitotically to produce cells that replenish the stem cell pool and cells that undergo a series of mitotic divisions to increase the number of spermatocytes that subsequently progress through meiotic sub-stages. In addition, in mice, it takes 8.6 days for the A_1 spermatogonia to become preleptotene spermatocytes and enter meiosis and an additional 8.6 days ×3 to form elongated spermatids ready for spermiation. The net result is that once the spermatogenic cycle is fully established, the same cell associations or the same group of cell types appear every 8.6 days (Griswold 2016). The length of the spermatogenic wave is species-specific, *e.g.*, 8.6 days in mice, 13 days in rats and 16 days in humans (reviewed by Bittman 2016, Griswold 2016).

Figure A.8. Overview of the spermatogenic wave. Under normal physiological conditions, spermatogenesis is asynchronous, with a wave generated by RA pulses (red patches, stages VIII and IX in mice) along the tubules.



Note: These RA pulses drive the spermatogonial transition from A_{al} to A_1. **Red**, undifferentiated A spermatogonia; **teal**, differentiating A_1 spermatogonia; **green**, preleptotene spermatocytes; **purple**, pachytene spermatocytes; **orange**, round or elongating spermatids; **blue**, elongated spermatids.
Source: Griswold 2016, reprinted with kind permission from the publisher: American Physiological Society Rockville, MD, USA.

152. During the seminiferous epithelium cycle, pulses of RA are observed at stages VIII-IX (Griswold 2016; see Figure A.8). During these stages the three RA-dependent steps of spermatogenesis is described to occur, namely the transition from undifferentiated to differentiated spermatogonia, meiosis initiation, and spermiation (Griswold 2016). Further, RA may play a role in the initiation of spermatid elongation, which also occurs at stage VIII (Endo *et al.* 2017). The expression of the RA regulated genes *Stra8* (in spermatocytes) and *Stra6* (in Sertoli cells) peak at stages VII-VIII of the seminiferous epithelium cycle (Teletin *et al.* 2017, Griswold and Hogarth 2018). However, at least in adult mice, RALDH enzymes (RALDH1A1-3 and ALDH8A1) were not expressed in a stage-specific manner (Kent *et al.* 2016).

A.6.5. Regulation of RA signaling

153. For the regulation of spermatogonial exposure to RA, two hypotheses have been presented: 1) all spermatogonia are primed to respond to RA, but the exposure to RA is periodic and tightly controlled, and 2) all spermatogonia are exposed to RA but only some can respond (Busada and Geyer 2016). The differential expression of RARγ between spermatogonia subpopulations could explain how the A_s spermatogonia (with no RARγ expressed) remain undifferentiated and maintain self-renewal capabilities even in the presence of high RA concentrations, which induce differentiation in the (RARγ-expressing) A_{al} progeny populations (Teletin *et al.* 2017). Observations from cell-specific conditional knockout mouse studies of RA receptor isomers suggest that the RA signal in spermatogonia is transduced via RAR/RXR heterodimers (Gely-Pernot *et al.* 2015). Another suggestion is that RA signaling also operates via Sertoli cells, as germ cell differentiation can proceed in the absence of functional RAR and RXR isotypes in spermatogonia (Teletin *et al.* 2017). RA may also act via non receptor-mediated pathways, such as *via* kinase signaling (Busada and Geyer 2016).

154. Conditional knockout studies in mice show that despite the critical role of RA in spermatogonia differentiation and in male germ cell meiosis, both differentiation and meiosis can occur in germ cells where RAR or RXR are absent, although a fraction of the A_1 spermatogonia are adversely affected (Gely-Pernot *et al.* 2015). Initiation and progression of meiosis also proceeded in these mice. The conclusion was that the RA signaling pathway was not autocrine, but rather operated in Sertoli cells (Gely-Pernot *et al.* 2015; see also previous sections *Post-pubertal RA synthesis and The seminiferous epithelium cycle and the spermatogenic wave*).

A.6.6. RA metabolism: Role of Cyp26

155. Spermatogonia exposure to RA is also regulated by the presence of the RA-catabolizing CYP26 enzymes; Cyp26a1 and Cyp26b1 form a catabolic barrier against any RA present in the immediate environment of the seminiferous tubules. All three CYP26 isoforms are present in the mouse postnatal testis, although observations after conditional deletion of the Cyp26a1 and/or Cyp26b1 genes in germ and/or Sertoli cells reveal that Cyp26b1 is the critical isoform (Hogarth *et al.* 2015). In the fetal mouse testis, elimination of RA by Cyp26b1 is necessary not only to prevent premature meiosis, but also for normal mitotic arrest of male PGCs and for preventing germ cell apoptosis (Rossitto *et al.* 2015). Cyp26b1 is initially produced by the Sertoli and Leydig cells and/or interstitial somatic cells, although after birth, Cyp26b1 transcripts are confined to the peritubular myoid cells (Rossitto *et al.* 2015). No variations in the expression of Cyp26 enzymes exist across the seminiferous epithelium cycle, unlike several proteins involved in retinoid storage (*e.g.* LRAT) and RA synthesizing Raldh1 in Sertoli cells and Raldh2 in germ cells which are regulated in a periodic manner along the seminiferous tubule (Teletin *et al.* 2017).

A.6.7. Meiosis in the male

156. Just as for oocytes, RA may play a role in the entry of the spermatocytes into meiosis (Raverdeau *et al.* 2012), possibly via control of replication-dependent core histone gene expression necessary for entry into S phase (Chen *et al.* 2016). However, recent findings by Teletin and co-workers suggest that RA may act only as a facilitator in the initiation of meiosis (Teletin *et al.* 2019). See Section A.2 for further discussion on the role of RA in meiosis.

A.6.8. Spermiation

157. Cell-cell junctions between spermatozoa and late-stage spermatids are degraded in a process facilitated by Sertoli cells, and thereby immature spermatozoa (step 16 spermatids) are released into the tubular lumen. The released spermatozoa are subsequently transported by peristaltic movements of the tubule, *via* rete testis to epididymis, where the spermatozoa acquire motility and fertilization potential (reviewed in Xiao *et al.* 2014).

158. In early studies using VAD rats, a delayed release of late spermatids was observed (Huang and Marshall 1983). In the mouse, RA is now known to be required to disengage spermatozoa from the Sertoli cell cytoplasm during spermiation; in male mice genetically modified to lack RALDH1-3 in their Sertoli cells, spermatids were retained in the seminiferious epitithelium (Raverdeau *et al.* 2012). Spermiation failure was also observed in *Rbp4*-null mice rendered VAD (Ghyselinck *et al.* 2006). Similar observations made in Rarα-null mice (Chung *et al.* 2005) suggest that the effect of RA on spermatid release is mediated by RARα.

A.6.9. Secondary male reproductive organs

159. The prostate produces slightly alkaline prostate fluid that makes up approximately 30% of the volume of semen in humans and is critical for male reproductive health (Verze *et al.* 2016). The retinoid signaling pathway has several functions in differentiation and maintenance of secondary male reproductive organs. RA is necessary for prostate formation from the urogenital sinus during sexual differentiation (Vezina *et al.* 2008, Bryant *et al.* 2014). Genetic deletion studies in mice have demonstrated the importance of RA signaling via *Rarγ* in the prostate and in the seminal vesicles (Lohnes *et al.* 1993).

160. RA signaling appears to be necessary for the function of epididymis, into which sperm are released after spermiation, undergo maturation, and are stored until ejaculation (Jauregui *et al.* 2018). In a conditional transgenic model with a dominant negative form of RARα expressed in Leydig cells and in the epididymis, the resulting abnormal epididymis phenotype may contribute to the infertility observed in these mice (Jauregui *et al.* 2018). These observations support previous findings that lack of RA signaling in the epididymis results in squamous metaplastic epididymal epithelium; alternatively, the abnormal epididymal phenotype may be due to lack of testosterone (Jauregui *et al.* 2018).

A.6.10. Male reproductive pathologies

161. Impaired RA signaling may play a role in several male reproductive pathologies. The transition from PGCs to differentiating spermatogonia is impaired in the absence of RA in the testis cords (Teletin *et al.* 2017). PGCs that fail to differentiate into spermatogonia may be the source of carcinoma *in situ*, which in humans may develop into testicular germ cell cancer (Busada and Geyer 2016, Teletin *et al.* 2017).

162. In prostate tumor tissue, RA concentrations are lower than in normal prostate tissue (reviewed in Nelson *et al.* 2013). Since it has been hypothesized that this is due to increased RA catabolism, inhibitors of CYP26 enzymes, called RA metabolism blocking agents (RAMBAs) have been used in the

treatment of prostate cancer (Nelson *et al.* 2013). RAMBA therapy promotes differentiation and inhibits proliferation by increasing endogenous RA in tumors (Denis *et al.* 1998). Some RAMBAs also inhibit estrogen synthesis *via* inhibition of aromatase/CYP19 and testicular androgen synthesis via inhibition of 17, 20-lyase/CYP17 (Bryson and Wagstaff 1996). The RALDH2 enzyme expression is also altered in prostate cancer. The expression of the *ALDH1A1* gene is lower and its promoter region is hypermethylated in epithelia from malignant prostate tumors (Kim *et al.* 2005).

163. Cryptorchidism, or non-descended testis, is a congenital malformation that is associated with an increased risk of testicular cancer and infertility in adult life (Bay *et al.* 2011). Complete testicular descent is necessary for normal testicular function in adult males, and the process of testis descent is regulated by the Leydig cell hormones insulin-like peptide 3 (INSL3) and testosterone (Bay *et al.* 2011). The spectrum of reproductive system malformations caused by VAD in rats includes cryptorchidism (See *et al.* 2008). Both RA levels and *Stra8* expression were significantly lower in the rat cryptorchid testis (induced by *in utero* exposure to the anti-androgen flutamide) compared to the normal testis (Peng *et al.* 2016). In an *in vitro* system, RA upregulated the expression of the gene Relaxin family peptide receptor 2 (*RXFP2*; *LGR8*), which encodes the receptor for INSL3 (Klonish *et al.* 2005).

A.6.11. Retinoid deficiency/excess in male reproduction

164. Animal studies measuring effects of experimentally increased or decreased levels of retinoids have demonstrated the importance of narrowly regulated retinoid levels for normal male reproduction. Adverse effects on the male reproductive system following experimental limitations of RA have been demonstrated in multiple *in vivo* studies (discussed in previous sections of this report, and reviewed in Clagett-Dame and Knutson 2011). In rodents maintained on a vitamin A-deficient diet, testicular degeneration with impaired spermatogenesis and a complete disappearance of all meitotic and postmeiotic cells has been observed (Coward *et al.* 1969, Morales and Griswold 1987, van Pelt and de Rooij 1990). Spermatogenesis is arrested at the preleptotene spermatocyte stage in VAD rats and at the spermatogonia stage in VAD mice; however, spermiation failure is observed in both species under VAD conditions (Ghyselinck *et al.* 2006). Vitamin A deficiency also leads to replacement of normal glandular epithelium in the epididymis, prostate and seminal vesicles, by a stratified squamous keratinizing epithelium, resulting in inhibited seminal fluid production (Wiseman *et al.* 2017).

165. It has been known for almost a century that male rats maintained on a retinol-deficient diet supplemented with RA will be sterile, possibly because the testis cannot take up RA from the circulation (Kurlandsky *et al.* 1995).

166. In human populations with retinoid deficiency, symptoms such as blindness are well-known, but information on reproductive parameters such as sperm production or fertility is lacking (Hogarth and Griswold 2010). However, observations made in testicular tissue samples obtained in different clinical situations, suggest a correlation between adverse reproductive parameters and disturbed retinoid signaling. Significantly lower levels of in *13-cis*-RA was observed in testis tissue biopsies in men with abnormal sperm production undergoing scrotal surgerydue to various benign indications (Nya-Ngatchou *et al.* 2013). In a different study, lower levels of the RALDH2 enzyme in testicular tissue were associated with male infertility (Amory *et al.* 2017). In follow-up studies, treatment with 13-*cis*-RA (used in the treatment of acne) appeared to increase sperm production (Çinar *et al.* 2016, Amory *et al.* 2017).

A.7. Impact on female and male reproduction by compounds acting *via* the retinoid system

167. Exposure to chemicals early in life may affect both female and male reproductive organs and their functions, most likely *via* several mechanisms, with effects *via* estrogen, androgen and/or steroidogenic pathways being the most frequently discussed. A testicular dysgenesis syndrome

(Skakkebaek *et al.* 2001) as well as an ovarian dysgenesis syndrome (Buck Louis *et al.* 2006, reviewed in Johansson *et al.* 2017) have been described. Both dysgenesis syndromes are defined as early (fetal) alterations in testicular or ovarian structure or function that cause an impairment of reproductive parameters in adulthood. One of the mentioned fetal alterations in the ovarian dysgenesis syndrome was disruption of the RA-dependent meiosis initiation (reviewed in Johansson *et al.* 2017). For embryonic males, it has been suggested that testicular toxicity could result from disruption of local RA homeostasis or signaling (Spade *et al.* 2019b). In the adult organism, chemicals may interfere with *e.g*. normal sperm production (Sharpe 2010) and normal function and morphology of female reproductive tissue (reviewed in Johansson *et al.* 2017).

168. Table A.2 summarizes some observed effects and mechanisms by which compounds, or chemicals, could interfere with the retinoid pathway in (and also in *e.g.* Nilsson and Håkansson 2002, Novak *et al.* 2008, Shmarakov 2015). In brief, chemicals have been shown to deplete tissue retinoid levels by affecting retinoid metabolism, also after *in utero* exposure. Chemicals can also cause activation or inactivation of retinoid receptors. As described in this report, the retinoid pathway is important, or even critical, in several aspects of both female and male reproduction and during fetal development. It is thus conceivable that chemicals capable of interfering with the retinoid pathway, as a result, may cause adverse effects on reproductive parameters, assuming that such chemicals reach target cells during critical windows.

169. There were publications retrieved, during the course of this project, describing a general effect by compounds on RA-content and enzyme or receptor expression (*e.g.* in the liver), but none of these animal studies (with the exception of some pharmaceutical compounds) also describe effects on retinoid parameters in reproductive organs, such as RA-synthesizing/metabolizing enzymes. For the male reproductive system, there are *in vivo* studies, where brominated flame retardants causes decreased liver retinoid stores in male Wistar rats, and where slight effects on reproductive organ weights is reported. However, no adverse effects on reproductive organ histopathology, or on reproductive outcome, was found, and no retinoid-related parameters were measured in the reproductive organs (see van der Ven *et al.* 2008, and van der Ven *et al.* 2009 in Table A.2).

170. While most data originate from animal studies, there is human data as well. For example, in the 1960s, it was demonstrated that the pharmaceutical compound WIN 18,446 could reversibly inhibit spermatogenesis in men (Heller *et al.* 1961). More recently, the same compound was shown to inhibit the conversion of retinal to RA, most likely by inhibiting ALDH1A2 (Paik *et al.* 2014), and this finding has led researchers to suggest the use of retinoid metabolism inhibition as an approach to male contraception (Hogarth *et al.* 2011). Acne treatment with 13-*cis*-RA (isotretionin) appeared to increase sperm production (Çinar *et al.* 2016, Amory *et al.* 2017), which is in line with earlier observations of reduced testicular concentrations of 13-*cis*-RA in men with low sperm production (Nya-Ngatchou *et al.* 2013). An important species difference between humans and rodents is that male rodents produce a large surplus of sperm, so decreases in sperm count or quality may not lead to decreased fertility. The situation is very different in humans, where sperm counts/quality are often so low, that any additional decrease would have direct adverse effects on fertility (reviewed in Working 1988).

Table A.2. Examples of chemicals interfering with the retinoid system in different models.

Chemical(s)	Model system	Endpoint	Observed effect	Reference
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	Male rats, single dose	Retinoid levels in several organs, including testes and epididymis	↓Retinyl esters in liver, testes, epididymis ↑Retinyl esters in kidney	Håkansson *et al.* 1991
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	Pregnant rats and PND 7 pups, single dose in utero	Retinoid levels in liver, lung and kidney	↓Retinyl esters in maternal and perinatal liver and lung ↑Retinyl esters in maternal and perinatal kidney	Kransler *et al.* 2007
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	Male rats, single dose	RA and retinyl ester levels and LRAT expression in liver and kidney	↑ RA in liver and kidney ↓Retinyl esters in liver ↑Retinyl esters in kidney ↑ LRAT in kidney	Hoegberg *et al.* 2003
Technical pentabromodiphenyl ether mixture	Female and male rats (enhanced 28d TG407 study)	Retinoid levels in liver	↓Retinyl esters in liver	van der Ven *et al.* 2008[a]
Hexabromocyclodecane	Female and male rats (enhanced 1-gen TG415 study)	Retinoid levels in liver	↓Retinyl esters in liver	van der Ven *et al.* 2009[b]
Fluconazole	Mice (exposed in utero)	mRNA induction of CYP26A1 and CYP26B1 Organ development (not repro)	Upregulation of CYP26A1 and CYP26B1 Abnormal branchial arch development.	Tiboni *et al.* 2009
Ketoconazole	Mouse testis organ cultures	Meiosis markers	Meiosis induction in mouse fetal testes	Bowles *et al.* 2006
Bisphenol A	Mice (exposed in utero)	Retinoid and mRNA levels in liver	↑RA ↓*RXRβ*	Esteban *et al.* 2019
Organochlorine pesticides (chlordane, dieldrin, aldrin, endrin, endosulfan)	Reporter cell lines (RARE & RARα, β, γ)	Activation of RARs	Activation was observed.	Lemaire *et al.* 2005
	CYP26A1	Induction of CYP26A1	CYP26A1 induction was observed.	
543 environmental chemicals	Yeast cells transfected with the human RARγ	RARγ agonistic activity	85 of the 543 chemicals had RARγ agonistic effects (especially monoalkylphenols and styrene dimers).	Kamata *et al.* 2008
309 environmental chemicals	HepG2 cells transfected with *cis*/trans-reporter transcription units for RARα, β, γ	Transcription factor activity	Max responders were lindane, oxadiazon and imazalil	Martin *et al.* 2010
28 environmental and other compounds	Human uterus and prostate cytosol	Chemical displacement; inhibition of ^{3}H-RA binding to cytosol	A number of the chemicals could displace ^{3}H-RA. MEHP most potent.	Paganetto *et al.* 2000
PPARγ agonists rosiglitazone and pioglitazone	HepG2 cells	mRNA induction of CYP26B1 and CYP26A1	mRNA induction of CYP26B1 and (to a lesser extent) CYP26A1	Tay *et al.* 2010
Phenobarbital	Human hepatocytes	mRNA induction of CYP26B1	Weak induction of CYP26B1	Finkelstein *et al.* 2006
Tributyltin	Human and mice ovarian theca cells	Cholesterol homeostasis via RXR pathway	Impaired cholesterol homeostasis	Pu *et al.* 2019
Tributyltin, triphenyl tin	Mouse adipocytes	Cell differentiation into adipocytes	Agonist activity via PPARγ/RXR	Kanayama *et al.* 2005

[a] Also observed in males: decreased weight of epididymis, and an increased weight of the seminal vesicles, however, no histopathological changesRE were noted; in females: induced adrenal activity of the steroidogenic CYP17 enzyme

[b] Also observed in F1-males: decreased weight of testis and prostate (concomitant with a reduction in body weight, however, no histopathological changes were observed). Epididymal sperm count or sperm morphology were not affected, except for the observation of a *decreased* ratio of separated sperm heads. Note that no significant dose–

response effects on endpoints of reproduction, *i.e*. mating success, time to gestation, gestation duration, number of implantation sites and litter size, were observed.

Source: This figure from Retinoids in Mammalian Reproduction, with an Initial Scoping Effort to Identify Regulatory Methods, Nordic Council of Ministers, (Nordic Council of Ministers, 2020), is made available under the Creative Commons Attribution 4.0 International license (CC BY 4.0).

171. It should be pointed out that effects of chemicals on retinoid homeostasis can be indirect, since some metabolic enzymes are used both for detoxification and for retinoid homeostasis, such as RALDH/ALDH class 1 (Alnouti and Klaassen 2008). Induction of *Aldh* isomers, as studied in mouse livers, was isomer- and activator-specific after *in vivo* administration of several activators of different nuclear receptors (such as CAR, PXR, PPAR, AhR) (Alnouti and Klaassen 2008). The well-known induction of CYP enzymes via AhR after exposure to dioxins and dioxin-like polychlorinated biphenyls (PCBs) may be relevant since some of these P450 enzymes are believed to be involved in either synthesis or oxidation of RA (Murphy *et al.* 2007). Likewise, CAR activation has been involved in the disruption of the retinoid system in C57BL/6 male mice by the non-dioxin like PCB153, but not in their Car-null littermates (Shmarakov *et al*. 2019). Such PCB153-related alteration was associated with altered hepatic and adipose tissue target genes involved in the carbohydrate and lipid metabolism which might account for the obesogenic properties of PCB153 (Shmarakov *et al*. 2019). Noteworthy, adipose tissue concentrations of the non-dioxin-like PCBs 138, 153 and 180 were associated with decreased retinol concentrations in adipose tissue and increased RBP4/ratios in serum of participants of the GraMo cohort (Galbán-Velázquez *et al*. 2021). If the end result is altered RA concentrations in fetal or adult reproductive organs, correct development and/or function of these organs could be compromised.

172. Since RXR can heterodimerize with several other nuclear receptors, it is also conceivable (and has indeed been shown; see *e.g.* Tarrade *et al.* 2001) that any interference with the retinoid system can also have effects on other signaling pathways, *via e.g.* PPAR, PXR, CAR and VDR (see earlier section on cross-talk). Also, interactions with AhR signaling pathways on several levels have been shown (Murphy *et al.* 2007, Vezina *et al.* 2008). From a functional/endpoint perspective, cross-talk with the other endocrine systems (estrogen, androgen, thyroid) is important to consider.

A.8. Compounds affecting female reproduction

A.8.1. Analgesics

173. Exposure to the analgesic drugs **acetaminophen** (paracetamol) and **indomethacin** appeared to give rise to delayed entry into meiosis in female rats after *in utero* exposure, with changes in ovarian *Stra8* levels reflecting this delay (Dean *et al.* 2016). Other effects, not currently linked to *Stra8* or RA, included decreased fetal ovarian germ cell numbers and (in adult females exposed *in utero*) reduced ovarian size and female fertility (measured as number of pups per litter) were observed (Dean *et al.* 2016).

174. In a mouse study with *in utero* exposure to **paracetamol**, both fertility, follicle numbers and germ cell numbers (as indicated by decreased mRNA levels of the germ cell marker mouse Vasa homologue; Mvh) decreased (Holm *et al.* 2016). In ovaries dissected at 12.5 dpc and exposed to 100 µM paracetamol for three days in culture, no effect of paracetamol on ovarian Stra8 levels was observed. Paracetamol did not cause the same decrease of the germ cell marker Mvh *ex vivo* as it did *in vivo*; the authors suggested that the sensitive window for the paracetamol effect occurred earlier than 12.5 dpc (Holm *et al.* 2016).

A.8.2. Bisphenol A

175. In mice, *in utero* exposure to bisphenol A has been associated with a delay in meiotic prophase I, hypothesized to be due to a decreased expression of *Stra8* in fetal oocytes (Zhang *et al.* 2012). In the same experiment, it was observed at PND 3, that an increase in bisphenol A dose levels was associated with an increased number of oocytes in germ cell cysts and fewer oocytes in primordial follicles (Zhang *et al.* 2012). Increased DNA methylation may be a mechanism how bisphenol A affects Stra8 expression, meaning that the effect of bisphenol A appears to be independent of any direct effect on retinoid metabolism or homeostasis, although it exerts (one of) its effects on the RA-regulated gene *Stra8* (Zhang *et al.* 2012).

176. Not all data support an effect of bisphenol A on *Stra8*. In human fetal oocytes cultured in bisphenol A-containing media, the expression pattern of *Stra8* was similar to that in control cultures (Brieño-Enriquez *et al.* 2012). In fetal ovaries originating from pregnant mice exposed to bisphenol A, the observed increase in *Stra8* expression did not differ from that in unexposed mice (Lawson *et al.* 2011).

A.8.3. Phthalates

177. Diethyl hexyl phthalate (DEHP) causes a delay in meiosis in mouse fetal germ cells following *in utero* exposure, and the concomitant decrease in mRNA and protein expression of *Stra8* was suggested to be related (Zhang *et al.* 2015). The DNA methylation level of *Stra8* in oocytes of the F1 generation increased as a result of DEHP exposure, and these changes were inherited by the F2 generation (Zhang *et al.* 2015).

178. DEHP may also exert effects *via* PPAR-RXR. *In vitro*, the DEHP metabolite monoethylhexyl phthalate (MEHP) suppressed expression of CYP19, the rate-limiting enzyme for conversion of testosterone to estradiol, *via* activation of PPAR-RXR heterodimers in rat ovarian granulosa cells (Lovekamp-Swan *et al.* 2003).

179. Phthalates can target many aspects of ovarian development and normal function (reviewed by Hannon and Flaws 2015); the exact mechanisms are unknown.

A.8.4. Organotin compounds

180. In marine gastropods, tributyltin, found as a contaminant of dibutyltin in vinyl plastics and also used in antifouling paints for ships and fishing nets, induces imposex (the development of male genitals in females) *via* binding RXR, thereby activating the RXR-RAR heterodimer (Nishikawa *et al.* 2004).

181. In human placental choriocarcinoma cells, trialkyltins stimulate human chorionic gonadotropin production and CYP19/aromatase activity by acting as RXR agonists (Nakanishi *et al.* 2005).

182. The RXR signaling pathway may be involved in the observed increase in progesterone production in human placental cells (*in vitro*) following organotin exposure (reviewed in Macejova *et al.* 2016).

183. No RXR activation was observed in rodent or human placenta tissue in which ng-mg/kg levels of organotin levels were present (de Araújo *et al.* 2018).

184. In ovarian theca cells from humans, mice and other mammalian species, tributyltin stimulated cholesterol extracellular efflux via the RXR pathway (Pu *et al.* 2019).

A.8.5. R115866

185. This triazole-containing molecule is a CYP26 inhibitor, more potent than liarozole, and largely without the inhibitory ability of liarozole on the CYP-dependent formation of estradiol and testosterone (Stoppie *et al.* 2000). R115866 administration leads to increased endogenous levels of RA, with subsequent RA-like effects such as inhibition of vaginal keratinization in estrogen-stimulated rats (Stoppie *et al.* 2000).

A.8.6. Isotretinoin/13-cis-RA

186. This retinoid, used for *e.g.* treatment of acne, has been found to lead to reduced antral follicle count, ovarian volume and levels of anti-Müllerian hormone (AMH; a marker of ovarian follicle number) in humans (Aksoy *et al.* 2015). Similar effects have been observed in rats (Abali *et al.* 2013). The effects appear to be transient both in humans (Çinar *et al.* 2017) and in rats (Korkmaz *et al.* 2017) once treatment ceases. Isotretinoin administered to rats at doses up to five times higher than clinical doses used for acne treatment reportedly had no adverse effects on fertility, conception rate, gestation or parturition, as summarized in an US FDA Pharmacology Review of isotretinoin[8]. The ICH[9] reproductive toxicology guideline for registrations of pharmaceuticals for human use does not require histopathological examination of ovaries in reproductive toxicity studies and therefore it is not possible to draw any firm conclusions on possible effects of isotretinoin on ovarian volume or follicle count from the US FDA summary of this study.

A.9. Compounds affecting male reproduction

A.9.1. Thiocarbamate herbicides

187. Molinate, a known testicular toxicant in the rat, is also an inhibitor of RALDH and has been shown to inhibit the conversion of retinal to RA; decreased testicular levels of RA were observed in rats dosed with molinate (Zuno-Floriano *et al.* 2012).

A.9.2. Conazoles

188. Conazole fungicides are triazole-based compounds used both in agriculture and pharmaceuticals, and which exert their fungicidal effects via broad inhibition of CYP enzymes (reviewed in Sheehan et al. 1999). CYP26 inhibition has been suggested to be specifically involved the in teratogenic effects of triazoles (reviewed in Menegola et al. 2006). Triazole-containing conazoles have been shown to cause decreased hepatic RA levels in mice (Chen et al. 2009), and the ability of the triazole compound flusilazole to modulate RA homeostasis has been hypothesized to be an important mechanism underlying its developmental toxicity (Tonk et al. 2015). Reprotoxic effects have been observed following in vivo exposure (Vickery et al. 1985, Taxvig et al. 2007, Schwartz et al. 2019).

189. In the pharmaceutical industry, structurally related compounds such as liarozole and talarozole have been considered for treatment of some cancers and dermatological diseases (Stevison et al. 2017). One mechanism of action of these compounds is the blocking of RA catabolism via inhibition of CYP26. Transient increased testicular RA levels have been observed in mice given talarozole (Stevison et al. 2017). In VAD mice administered liarozole after a dose of RA, the RA-induced proliferative effects

[8] https://www.accessdata.fda.gov/drugsatfda_docs/nda/2012/021951Orig1s000PharmR.pdf, downloaded Sep 4, 2018.

[9] International Council for Harmonization of technical requirements for pharmaceuticals for Human use; https://www.ich.org/

on A spermatogonia were less than in VAD mice given RA but not liarozole (Gaemers et al. 1997). Ketoconazole was used to suppress Cyp26 activity in fetal mouse testis and thereby preventing degradation of RA; the result was induction of Stra8 expression which was followed by a premature meitotic entry in male germ cells (Bowles et al 2006). Although ketoconazole blocks CYP enzymes without specificity for CYP26B1, the observation that ketoconazole, if used in combination with the RAR antagonist BMS-204493, has no effect on Stra8 expression in ex vivo cultured mouse fetal testis further supports the role of RA for Stra8 induction (Koubova et al. 2006). This indicates that the ketoconazole effect was due to Cyp26b1-inhibition.

A.9.3. Bisphenol A

190. In mice, neonatal exposure to bisphenol A has been shown to cause a decrease in sperm number and damage to sperm motility and morphology in adult mice. The effect was, to some extent, ameliorated by vitamin A supplementation and aggravated under vitamin A-deficient conditions (Nakahashi *et al.* 2001, Aikawa *et al.* 2004). The authors suggested that the development and functional differentiation of the reproductive tract and the gonads may be controlled by a balance between levels of estrogen and retinoids.

191. In mouse embryonic stem cells, bisphenol A was found to upregulate the expression of *Stra8* (amongst other genes) in a manner that appeared to be consistent with a feminizing effect, but bisphenol A seemed to act *via* a non-RA and non-RAR mediated mechanism (Aoki *et al.* 2012).

A.9.4. Organotin compounds

192. Triorganotins are considered endocrine-disrupting compounds and have been shown to bind RXR. Macejova and co-workers reviewed effects of triorganotin compounds on male reproductive organs in rats, but it was unclear if these effects were the result of a disrupted retinoid pathway (reviewed in Macejova *et al.* 2016). Several studies have shown that organotins act via PPARγ/RXR heterodimers (see *e.g.* Kanyama *et al.* 2005, reviewed in Grün and Blumberg 2006). However, since RXR is a silent partner in the PPARγ/RXR heterodimer (Mangelsdorf and Evans 1995), involvement of the retinoid pathway is uncertain.

A.9.5. Phthalates

193. Some phthalate esters are anti-androgenic, and thus considered potential causing agents in the "testicular dysgenesis syndrome" (Skakkebæck *et al.* 2001). These compounds have now been shown to also interfere with RA synthesis, both *in vitro* (Chen and Reese 2016) and *ex vivo* (Spade *et al.* 2019b). It has been suggested that a dual mechanism of both anti-androgen and retinoid disruption may lead to developmental effects in humans and rodents (Chen and Reese 2016).

A.9.6. Hexachlorocyclohexane (the gamma isomer is known under the name of lindane)

194. Lindane administration caused atrophy of the epididymidis and seminal vesicles, along with decreased sperm count in the epididymis and reduced activities of steroidogenic enzymes, in VAD rats but not in rats given a diet with sufficient retinoids (Pius *et al.* 1990).

A.9.7. BMS-189453

195. BMS-189453 is a synthetic RARα-, β- and γ-antagonist. Following oral administration to rats and rabbits for up to one month, BMS-189453 caused testicular degeneration and atrophy (Schulze *et*

al. 2001). In later studies using lower doses, this compound was shown to reversibly inhibit spermatogenesis in rats, without other adverse testicular effects (Chung *et al.* 2016).

A.9.8. WIN 18,446

196. While the BMS synthetic retinoid above inhibits RA signaling, WIN 18,466 (a bisdichloroacetyl diamine) acts by lowering local RA concentration via inhibition of RALDH2 (Kogan *et al.* 2014). WIN 18,466 administration to neonatal mice caused meiotic defects in spermatocytes (Kent *et al.* 2016). In WIN 18,466-treated rodents, the progression of progenitor cells from A spermatogonia into A_1 spermatogonia is blocked (Griswold and Hogarth 2018).

A.9.9. R115866

197. In an *in vitro* dog testis model, treatment with the triazole CYP26 inhibitor R115866 caused an upregulation of *e.g. Stra8* both at the mRNA and protein level (Kasimanickam and Kasimanickam 2014). Increased *Stra8* expression has also been observed in *ex vivo* cultured mouse fetal testes treated with R115866 (Bowles *et al.* 2006).

A.9.10. Ro 23-2895

198. High doses of the synthetic retinoid Ro 23-2895, presumably a RAR agonist, administered to rats caused testicular degeneration, decreased testicular weight, delayed sperm release and retention, and disorganization/desquamination of the tubular epithelium accompanied by reduced numbers of mature elongated spermatids (Bosakowski *et al.* 1991). These effects resemble those caused by vitamin A deficiency. In a parallel study, plasma and testis ROH levels were lower compared to controls, suggesting that Ro 23-2895 caused testicular degeneration by interfering with normal retinoid homeostasis (Bosakowski *et al.* 1991).

A.9.11. Isotretinoin/13-cis-RA

199. Results from a pilot study suggest that treatment with 13-*cis*-RA (used for *e.g.* acne treatment) can increase sperm production (Amory *et al.* 2017). The mechanism behind this possible effect is not known, but it can be noted that reduced testicular concentrations of 13-*cis*-RA have been observed in men with abnormal sperm production (Nya-Ngatchou *et al.* 2013).

A.10. Potential adverse outcome pathways in female and male reproduction

200. Adverse Outcome Pathways (AOPs) form a framework for organizing data on the relationships between a molecular initiating event (MIE) induced by the interaction of a stressor (*i.e.* a chemical) with a molecular target and the resulting sequence of key events (KE), leading to an adverse outcome (AO). AOPs can establish a rationale for the use of particular assays or *in vivo* endpoints, and/or highlight the need for developing assays or exploring existing test guidelines to cover one or more components of an AOP.

201. Currently, there are no AOPs focusing on the disruption of the retinoid pathway published in the OECD series on Adverse Outcome Pathways[10]. In the AOP Wiki database[11]one AOP (Id 297) is under development, linking RALDH inhibition with visual impairment in fish (*not open for citing*). Additional

[10] https://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcome-pathways_2415170x

[11] https://aopwiki.org/aops; accessed in September 2019.

AOPs (AOP Id 37, 107, and 149) mention either “retinoic” or “retinoid”. Retinoid-relevant KE in these AOPs include “retinaldehyde dehydrogenase inhibition”, “retinoic acid (RA) synthesis decreased”, “plasma RA levels decreased”. In addition, the Belgian SPF Santé Publique Sécurité de la Chaîne Alimentaire et Environnement recently published a call for tender to develop an AOP for inhibition of retinol dehydrogenase leading to urogenital and cardiovascular malformations[12]. In the scientific literature, several attempts to build AOPs and other frameworks to understand RA-dependent effects on embryogenesis have been published (Tonk *et al.* 2015, Baker *et al.* 2018, Battistoni *et al.* 2019, Di Renzo *et al.* 2019, Piersma *et al.* 2019[13]), with disruption of CYP26 enzymes and RALDH2 suggested to be important KEs. Previous efforts have not focused specifically on the reproductive system *per se*, even though embryo development of several other organ systems is discussed.

202. Since there is substantial cross-talk between the retinoid system and other nuclear hormone systems (see section 4), there are additional AOPs that may be involved with retinoid pathway effects on reproduction. For example, activation of PPARα may impair steroidogenesis, and could lead to impaired fertility in males (AOP18[14]). RXR is a PPAR heterodimer partner (see section on cross-talk), and it is possible that RXR may be involved in this AOP.

203. The following illustrations are not complete AOPs, neither in terms of how they are constructed, whether or not KEs are measurable, nor in terms of the amount or type of data supporting the AOPs. Rather, they are attempts to visualize possible pathways between effects on retinoid homeostasis and reproductive adversity, as covered in this report. Hopefully, these illustrations can be a starting point for future efforts to develop AOPs in this area.

A.10.1. Proposed AOP for meiosis initiation, oogenesis, folliculogenesis and female fertility, focusing on Stra8

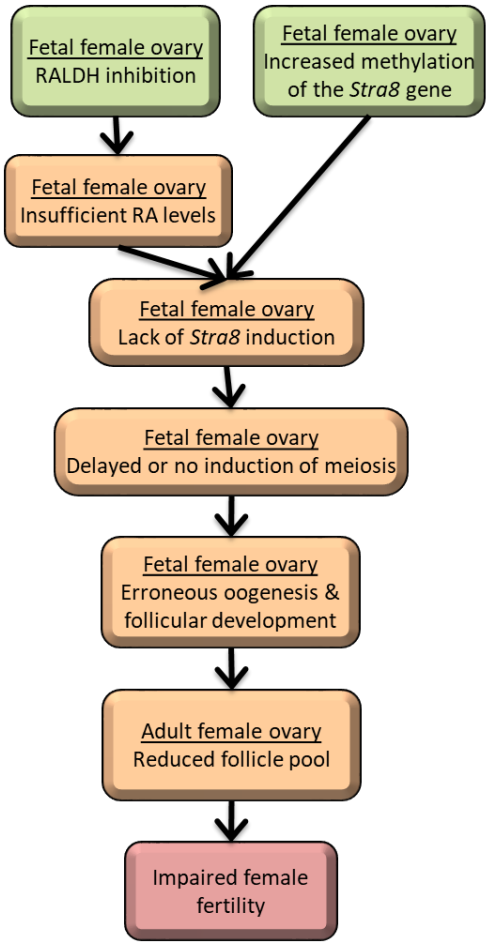
204. The role for RA for meiosis initiation, oogenesis and folliculogenesis in the female fetus is discussed in sections A.2 and A.3. In female fetal offspring of VAD rats, a delay or failure of meiosis initiation was observed in oogonia (Li and Clagett-Dame 2009). Although RA levels were not measured in these VAD rats, the low Stra8 expression suggest lower than normal RA levels in the ovaries. The critical role of Stra8 in meiosis, oogenesis and follicular development is also evident in Stra8$^{-/-}$ female mice, which are infertile and have smaller ovaries with no oocytes or follicles, while heterozygotes are fertile (Baltus *et al.* 2006). Since Stra8 expression is regulated by RA, it is plausible that lack of Stra8 expression could be caused by insufficient fetal ovarian RA levels, which in turn could be the result of a decrease in the synthesis of RA (see Figure A.9 below); alternatively, RAR antagonists could lead to the same effect without affecting RA levels. Animal experiments with bisphenol A and DEHP have connected lack of increased Stra8 expression with impaired female fertility; however, the effects of bisphenol A (Zhang *et al.* 2012) and DEHP (Zhang *et al.* 2015) appear to by-pass the retinoid system and instead affect Stra8 expression via epigenetic mechanisms.

[12] https://enot.publicprocurement.be/enot-war/preViewNotice.do?noticeId=356068&saveSearchParams=true&useWorkingOrganisationId=%66%61%6C%73%65&allLanguages=%66%61%6C%73%65&selectAllChildren=%74%72%75%65&isPopup=&advancedSearch=&publicationDateBDATo=&publicationNumberBDA=&versionReferenceNumber=&tenderSubmissionDeadline=&title=&marketPlaceType=%65%4D%61%72%6B%65%74&publicationDateBDAFrom=%31%35%2F%31%30%2F%32%30%31%39&noticeStatus=%31&purchaseAuthority=&

[13] Abstract SOT 2019: https://cfpub.epa.gov/si/si_public_record_Report.cfm?dirEntryId=345956&Lab=NCCT

[14] https://aopwiki.org/aops/18

Figure A.9. Proposed AOP for absence of ovarian Stra8 induction *in utero* possibly leading to impaired female fertility.



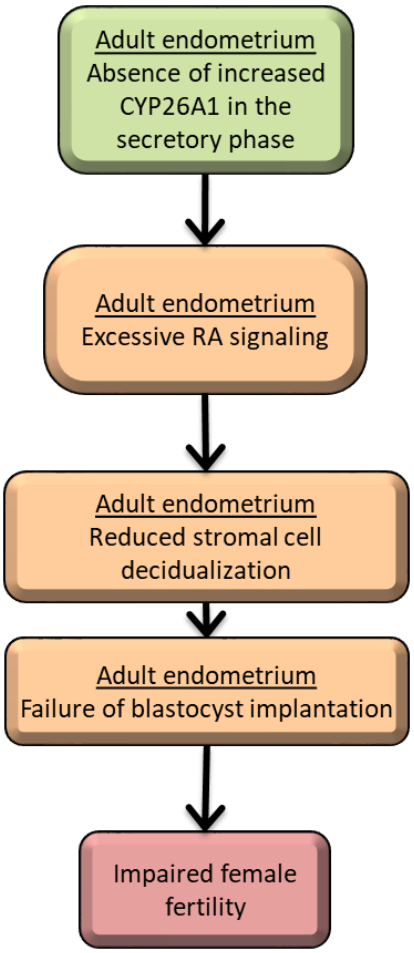
Note: The proposed AOP is an attempt to visualize possible pathways between effects on retinoid homeostasis and reproductive adversity.
Source: This figure from Retinoids in Mammalian Reproduction, with an Initial Scoping Effort to Identify Regulatory Methods, Nordic Council of Ministers, (Nordic Council of Ministers, 2020), is made available under the Creative Commons Attribution 4.0 International license (CC BY 4.0).

A.10.2. Proposed AOP for endometriosis in the adult female, focusing on CYP26A1

205. In endometrial tissue, RA appears to inhibit the decidualization of stromal cells, which is a prerequisite for blastocyst implantation after fertilization; thus, reduced concentration of RA in the endometrial tissue seems to be necessary for successful implantation (Deng *et al.* 2003). As discussed in Section A.3, CYP26A1 catabolizes endometrial RA-and its expression normally increases during the endometrial secretory phase (lowering the RA levels) when compared to the proliferative phase, during which RA-synthesizing enzymes such as RALDHs are increased (Deng *et al.* 2003).

206. When compared to healthy women, expression of CYP26A1 is down-regulated in both the secretory and proliferative phases in endometrial biopsies from women with moderate or severe endometriosis (Burney *et al.* 2007). The availability of RA may therefore be increased in endometrial tissue of women with endometriosis (see Figure A.9).

Figure A.10. Proposed AOP for lack of CYP26A1 increase in the adult human endometrium possibly leading to impaired female fertility.



Note: The proposed AOP is an attempt to visualize possible pathways between effects on retinoid homeostasis and reproductive adversity.
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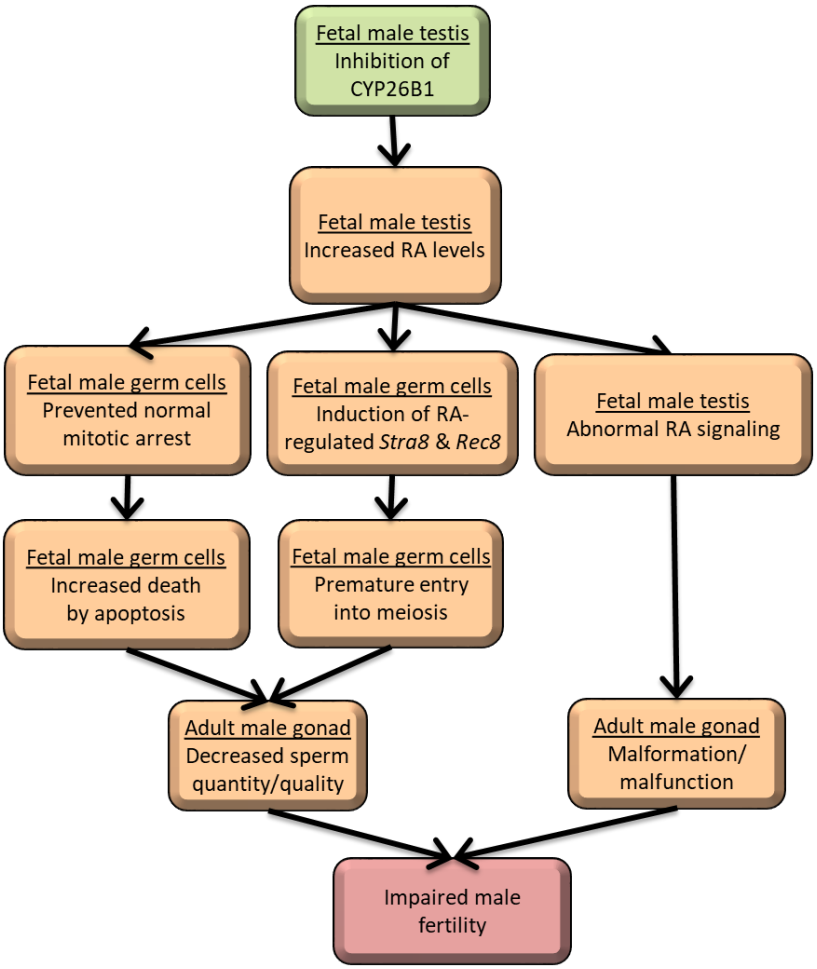
A.10.3. Proposed AOP for in utero CYP26B1 inhibition effects on male fertility

207. As described in Sections A.2 and A.3, temporally and spatially regulated RA concentrations in the fetal gonads appear to play a role both in the proper development of the testis and in the differentiation of gonocytes into either oogonia or spermatogonia. In the fetal testis, perturbed RA catabolism caused by inhibition of Cyp26b1 (putative MIE) could disrupt the normal RA signaling pathway, which would lead to the AO impaired male fertility (See Figure A.11). Disturbed RA-signalling has been observed after exposure of adult mice to chemicals acting as RAR antagonists (Schulze *et al.* 2001) and it is reasonable to assume that RAR agonists would also disturb RA signaling.

208. Following inhibition of the Cyp26b1 enzyme or complete knockout of the *cyp26b1* gene in the male mouse fetus, aberrant meiotic and apoptotic germ cells are observed in the testis (Bowles *et al.* 2006, MacLean *et al.* 2007, Teletin *et al.* 2017), and McLean and co-workers observed that virtually no germ cells are present in testes from neonatal pups (McLean *et al.* 2007). It has also been shown that

ketoconazole can act, at least in an *ex vivo* mouse models, to inhibit Cyp26b1 activity and thereby induce aberrant meiotic entry in fetal testes (Bowles *et al.* 2006). *In vitro*, Cyp26 inhibition prevents the normal mitotic arrest of the male germ cells and induces apoptosis (Teletin *et al.* 2017). Male *cyp26b1*$^{-/-}$ homozygote 13.5 dpc mouse embryos display a mild ovotestis phenotype, with an "ovarian component" at the anterior end of the gonad which is where RA levels are expected to be higher due to the connection to the mesonephric tubules at this end (Bowles *et al.* 2018). In addition, abnormal development of the Leydig cells and of the Müllerian and Wolffian ducts was observed in the same male mouse embryos (Bowles *et al.* 2018). Thus, it is possible that the observed effects on the germ cells and on testis development may potentially lead to adverse effects on spermatogenesis and malfunctioning testes, which would eventually lead to impaired male fertility (see Figure A.11 below).

Figure A.11. Proposed AOP for how Cyp26b1 inhibition in the fetal mouse testis may lead to impaired male infertility.



***Note*: Studies looking at Cyp26b1 inhibition were based on gene deletion or chemical inhibition of the enzyme. The proposed AOP is an attempt to visualize possible pathways between effects on retinoid homeostasis and reproductive adversity.** ** Also in the postnatal testis, prevention of RA-initiated activation of feminizing genes in the Sertolic cells appears to be necessary*

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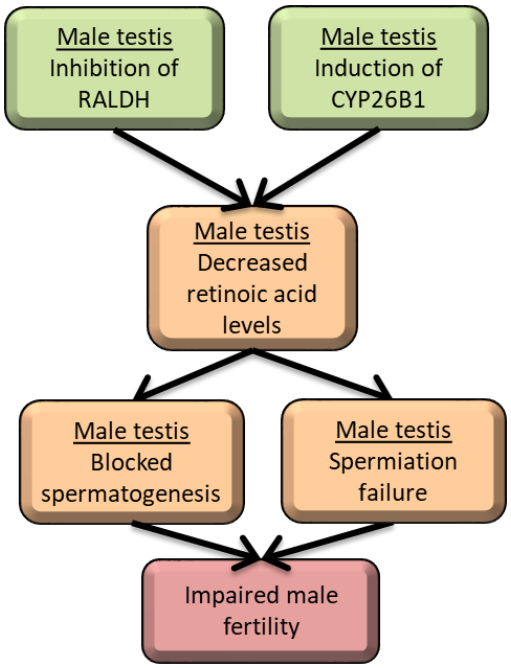
A.10.4. Proposed AOP for effects of RALDH inhibition on male fertility

209. As described in Section A.4, a tight regulation by RA-synthesizing (Raldh) and RA-catabolizing (Cyp26) (putative MIEs) is required for maintaining spermatogenesis, implying that chemicals that affect these enzymes may cause adverse effects on the spermatogenic process leading to the AO impaired male fertility (see Figure A.12 below). It is conceivable that exposure to chemicals acting as RAR antagonists could have the same effect; in such cases, altering of endogenous RA levels would not be needed.

210. At least in the mouse, RA is also required to disengage spermatozoa from the Sertoli cell cytoplasm during spermiation (Spiller and Bowles 2015, Teletin *et al.* 2017). It seems possible that increased RA testis levels *via* chemically-induced CYP26B1 inhibition (putative MIE) could lead to similar adverse effects on spermatogenesis.

Figure A.12. Proposed AOP for how Raldh inhibition in the adult mouse testis may lead to impaired male infertility.



Note: The proposed AOP is an attempt to visualize possible pathways between effects on retinoid homeostasis and reproductive adversity. * Also in the postnatal testis, prevention of RA-initiated activation of feminizing genes in the Sertoli cells appears to be necessary.

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A.11. Initial scoping effort: assays and endpoints for effects of chemicals on female and male reproduction *via* the retinoid system

211. Several existing OECD test guidelines can provide information on endpoints relevant to the estrogen, androgen, steroidogenesis, and thyroid hormone pathways, but currently, no OECD test guidelines include endpoints specifically indicative of retinoid system modulation. Some reproductive parameters already included in existing test guidelines, would also provide information on adverse effects stemming from retinoid disruption. However, due to extensive cross-talk with other pathways, there is no endpoint identified that will give information specifically on disruption of retinoid signaling. The information assembled in this report regarding the role of retinoids in female and male reproduction indicates possible *in vitro*/*ex vivo* assays that could associate adverse reproductive outcomes to disruption of retinoid signaling.

212. Some of the proposed assays are suitable for screening larger numbers of chemicals (*e.g. in silico*/*in vitro*, OECD Conceptual Framework [CF] levels 1, 2), while *in vivo* test methods address more complex mechanistic and organ system effects (CF level 3, 4, 5). One other option could be to include *ex vivo* models.

A.11.1. In silico methods

213. *In silico* methods, such as molecular docking models and quantitative structure-activity relationship (QSAR) models for binding to *e.g.* CYP26, RALDH, RAR and RXR, could, if available, be integrated into CF Level 1. *et al.*Also, homology modeling and computational docking simulations for CYP26A1 and CYP26B1 have been performed to identify inhibitors for these enzymes (Foti *et al.* 2016). Several studies describe QSAR models for binding to and activating PXR (an RXRα-heterodimerizing partner) and CYP3A4 (Rosenberg *et al.* 2017b), CAR (Chinen *et al*. 2020), thyroperoxidase (TPO; Rosenberg *et al.* 2017a) and AhR (Klimenko *et al.* 2019). Similar models for RARs and RXRs would be valuable, and should be developed.

A.11.2. In vitro assays and ex vivo methods

214. Temporal and spatial regulation of local availability of RA is critical for normal reproductive development. Thus, in addition to nuclear receptor activation assays, assays measuring expression and/or activity of the enzymes involved in the metabolism of RA can be considered as candidate *in vitro* assays (Piersma *et al.* 2017; see also section 10).

215. Considering the complex RA modulation of both female and male reproduction, a panel of *in vitro* assays with the following endpoints is suggested:

- CYP26A1, CYP26B1 (induction, inhibition)
- RALDH (induction, inhibition)
- STRA8 (induction)
- RAR/RXR transactivation

216. The proposed top candidate enzyme for fetal exposure to chemicals is the RA-catalyzing **CYP26B1** enzyme. Inhibition of CYP26B1 in the fetal testis (leading to increased RA levels) would cause serious adverse effects on normal development of testicular somatic and germ cells, while induction of the CYP26B1 enzyme in the female fetus could potentially lead to decreased RA tissue levels and subsequent adverse effects on initiation of germ cell meiosis. For evaluating adult females, **CYP26A1** might be the more relevant enzyme, considering its role in the endometrium.

217. Inhibition of the RA-synthesizing **RALDH** enzymes could decrease RA levels, and assays measuring RALDH inhibition may therefore be considered for screening purposes. In the postnatal male, RALDH inhibition and the subsequent decrease in RA levels would interfere with spermatogenesis.

218. **Stra8** is regulated by RA, and is expressed only in germ cells (Oulad-Abdelghani *et al.* 1996, Mark *et al.* 2008). *Stra8* is required for germ cells to enter meiosis (Baltus *et al.* 2006). Therefore, an *in vitro* screening assay for *Stra8* expression could be used to identify substances that, through disturbed RA signaling, affect meiosis initiation.

219. The different **RAR** and **RXR** transcription factors mediate the retinoid signal when activated by RA or 9-*cis*-RA, RXRs can also act as a silent partner, *i.e.* no RXR ligand is necessary for RXR to bind and activate genes. Activation or inactivation of these transcription factors by chemicals could lead to different effects on the cellular level, which may be translated to effects on tissue/organ level.

220. For any *in vitro* assay, the choice of cellular system is important in terms of both relevance and technical considerations. Ovarian stem cell test systems have been described, in which pre-meiotic

oogonia in the post-natal ovary are used (Bhartiya and Patel 2018). However, the existence of such cells is highly controversial (Frydman *et al.* 2017, Wagner *et al.* 2019). For screening large numbers of chemicals, gonadal cell or tissue models (preferably of human origin) would probably be necessary. Given the sex-specific effects of RA pathways, using two model systems in parallel (ovarian/follicular and testicular) is recommended. Any *in vitro* model should be characterized in terms of expression and function of *e.g.* CYP26, RoDH2, and RALDH.

221. *Ex vivo* model systems consisting of rodent follicular cells or whole rodent neonatal ovaries (Hannon and Flaws 2015) could generate valuable information on chemical disruption of the possible association between RA and the formation and maturation of follicles (Minkina *et al.* 2017, Damdimopoulou *et al.* 2019). Pre-meoitic germ cells, which can be obtained from fetal rodent ovaries and cultured *in vitro* (Sun *et al.* 2010, Paczkowski *et al.* 2012) can possibly provide information on chemical effects on RA-mediated meiotic induction. Both human and rat fetal testis cultures have been used to study the effects of hormone disrupting chemicals on the development of human fetal testis (Lambrot *et al.* 2006, Spade *et al.* 2019b).

A.11.3. In vivo endpoints

222. Detection of *e.g.* CYP26, RoDH2, RALDH, and Stra8 expression using immunohistochemistry or *in situ* hybridization could be performed on tissues from *in vivo* studies, for obtaining information on the mechanistic level.

223. Retinoid pathway signaling is highly variable by life stage, tissue type, and sex. Additionally, in animal studies, measuring retinoids in different tissues (including serum) is problematic in many aspects since the polyene chain of retinoids make them vulnerable to light, oxidation, acids and heat (reviewed in Gundersen 2006). In addition, the existence of more relevant endpoints such as the histopathological endpoints mentioned below, and methods that indirectly informs of retinoid levels (*e.g.*, mRNA expression of RARE-controlled genes such as *Stra8*) makes measurements of tissue retinoid levels a lower priority.

224. The detailed histopathological examination of reproductive organs/organ structures that are part of animal studies performed according to OECD reproductive development guideline studies could add valuable information when evaluating a possible retinoid-disrupting impact of the test chemical on these organs (Tables A.3 A.4, A.5, A.6; CF Level 3 or 4). In TGs 421 and 422, evaluating the value of addition of ovary histopathology of pups, could be valuable. Although it is not an endpoint specific for retinoids, retinoids are important for female meiosis, and could thus consequently also affect the size of the ovarian reserve. This is currently a data gap in these screening studies. Serum levels of AMH in the adult female, as a proxy for follicle counting, could potentially be evaluated, although it currently appears unclear if serum AMH levels reflect fertility status in women (see *e.g.* Kahn *et al.* 2019). In addition, measurements of male and female steroid serum levels could potentially add value considering the extensive RXR cross-talk (Jacobs 2005). In females, examined organs should include ovaries, as well as uterus and vagina. In males, parameters such as sperm number and sperm quality, cryptorchidism and the presence of PGC in the adult testis is relevant.

Table A.3. Current OECD TG endpoints which might capture possible retinoid effects on mammalian female reproductive health, at CF level 4 and 5. Also indicated are limitations and data gaps.

Retinoid effects	OECD Test Guidelines	Comments
Reproductive development (female rodents)		
Oogenesis, follicular count	Extended one-generation reproductive toxicity study (TG 443); 2-Generation reproduction toxicity study (TG 416).	TG 443: Quantitative (most sensitive). Histopathological examination should be aimed at detecting a quantitative evaluation of primordial and small growing follicles, as well as corpora lutea, in F1 females. TG 416: only qualitative (limited sensitivity). A quantitative evaluation of primordial follicles should be conducted for F1 females.
Oestrus cycles	Extended one-generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28-day/reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update).	TG 443: Vaginal smears should be examined daily for all F1 females in cohort 1A, after the onset of vaginal patency, until the first cornified smear is recorded, in order to determine the time interval between these two events. Oestrous cycles for all F1 females in cohort 1A should also be monitored for a period of two weeks, commencing around PND 75. TG 421/422: Not included in the offspring and also not possible due to, termination of the offspring on PND 14, *i.e.* before puberty. TG 416: Oestrous cycle length and normality are evaluated in F1 females by vaginal smears prior to mating, and optionally during mating, until evidence of mating is found.

[15] All OECD test guidelines are available via https://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

Vagina, uterus with cervix, and ovaries	Extended one-generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28-day/reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update).	TG 443: Uterus (with oviducts and cervix), ovaries will be weighed (F1). Full histopathology is performed for all high-dose and control F1 animals. All litters should be represented by at least 1 pup per sex. Organs and tissues demonstrating treatment-related changes and all gross lesions should also be examined in all animals in the lower dose groups to aid in determining a NOAEL. TG 421/422: Not included and not possible due to termination on PND 14 (F1). TG 416: Vagina, uterus with cervix, and ovaries (preserved for histopathology (parental F1 animals) determining a NOAEL. TG 421/422: Not included and not possible due to termination on PND 14, although follicular counts can be made at this age (F1). TG 416: Vagina, uterus with cervix, and ovaries (preserved for histopathology (parental F1 animals).
Adult exposure (female rodents)		
Vagina, uterus with cervix and ovaries (histopathology)	Extended one-generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28-day/reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update).	TG 443: Uterus (with oviducts and cervix), ovaries will be weighed. Full histopathology is performed for all high- dose and control P animals. Organs demonstrating treatment-related changes should also be examined in all animals at the lower dose groups to aid in determining a NOAEL. Additionally, reproductive organs of all animals suspected of reduced fertility should be subjected to histopathological evaluation. TG 421/422: optional: paired ovaries (wet weight) and uterus (including cervix) in females (P). TG 416: Examination of the ovaries of the P animals is optional.

Oestrus cycles	Extended one-generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28-day/reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update).	TG 443: Normally the assessment of oestrous cyclicity (by vaginal cytology) will start at the beginning of the treatment period and continue until confirmation of mating or the end of the 2-week mating period. TGs 421/422: Oestrous cycles should be monitored before treatment to select study females with regular cyclicity. Vaginal smears should also be monitored daily from the beginning of the treatment period until evidence of mating. TG 416: Oestrous cycle length and normality are evaluated in P females by vaginal smears prior to mating, and optionally during mating, until evidence of mating is found.

Source: Adapted from draft 2017 DRP prepared by Brunel University and Technical University of Denmark.

Table A.4. Current OECD TG endpoints which may detect retinoid effects on non-mammalian female reproductive health.

Retinoid effects	OECD Test Guidelines (TG)	Comments
	Reproductive development (non-mammalian)	
Dysgenesis of internal reproductive ducts	Reproductive ducts are collected for histopathology in The Larval Amphibian Growth and Development Assay (LAGDA) (TG 241)	TG 241 Oviduct Duct Stage scored (1-4 as per histology guidance document 228). Maximum 40 juveniles (mixed sex) sampled per treatment at end of experiment.
Oogenesis (ovary histopathology)	Ovary collected for histological analysis during the final sampling (juvenile) in The Larval Amphibian Growth and Development Assay (LAGDA) (TG 241). Ovary collected for histological analysis during the final sampling in Fish Sexual Development Test (TG234) F1 ovary collected for histological analysis in Medaka Extended One Generation Reproduction Test (MEOGRT) (TG 240)	TG 241 Gonad phenotype (ovary, testis, intersex) and genetic sex used to calculate phenotypic/genotypic sex ratios. Ovary development scored from 'Undifferentiated' (1) to 'consists almost entirely of vitellogenic oocytes' (5), germ cell degeneration and mononuclear cell infiltrates also scored. Maximum 40 juveniles (mixed sex) sampled per treatment at end of experiment. TG 234 Histopathology is primarily used to focus on sex ratio (male, female, intersex or undifferentiated), staging ovary is optional but can be investigated. TG 240 Histopathology of gonads in F1 generation only. Histopathology is primarily used to focus on sex ratio, in conjunction with genetic sex markers (male, female, intersex). Additional assessment of the ovary is optional.
	Adult exposure (non-mammal)	
Oogenesis (ovary histopathology)	Ovaries are collected for histology at the end of the 21-day adult exposure in Fish Short Term Reproduction Assay TG 229.	TG 229 histopathology of gonads is optional. When assessed endpoints include decreased yolk formation and perifollicular hyperplasia.

Source: Adapted from draft 2017 DRP prepared by Brunel University and Technical University of Denmark.

Table A.5. Current OECD TG endpoints which might capture possible retinoid effects on mammalian male reproductive health, at CF level 4 and 5. Also indicated are limitations and data gaps).

Retinoid	OECD Test Guidelines (TG)	Comments
Reproductive development (male rodents)		
Hypospadias, dysgenesis of external reproductive organs, cryptorchidism	Extended one-generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28- day/reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update); Prenatal Developmental Toxicity Study (TG 414).	TG443: Most sensitive design due to 20 litters per group, 2 or more males per litter and termination after puberty. TG 421/422: Limited sensitivity due to limited group size (n~8-10) and termination before puberty (hypospadia should be detectable at birth). TG416: Sensitive design due to 20 litters per group and termination after puberty, but only 1 male per litter decreases the sensitivity compared to TG443. TG 414: Sensitive design due to 20 litters per group, but limited/unknown sensitivity as the offspring is terminated before birth (although *e.g.* hypospadia should still be detectable).
Testes development (weight and histopathology)	Extended one-generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28- day/reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update).	TG443: Most sensitive design due to 20 litters per group and 2 adult male offspring per litter. TG 421/422: Not included but could be assessed at termination on PND 14. Limited sensitivity due to limited group size (n~8-10) and termination well before puberty. TG416: Sensitive design due to 20 litters per group and termination after puberty, but only 1 male per litter decreases the sensitivity compared to TG443.

[16] All OECD test guidelines are available via https://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

Spermatogenesis (sperm quality and testicular histology	Extended one-generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28-day/reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update).	TG443: sensitive design due to 20 litters per group, 1 adult male offspring per litter examined. TG 421/422: Not included and not possible due to termination on PND 14. TG416: Sensitive design due to 20 litters per group, 1 adult male offspring per litter examined (only included in the most recent update in 2001).
Adult exposure (male rodents)		
Spermatogenesis (sperm quality, testis weight and testicular histopathology)	Extended one- generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28- day/reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update); Repeated Dose 90-day Oral Toxicity Study in Rodents (TG 408); Repeated Dose 28-Day Oral Toxicity Study in Rodents (TG 407).	TG 443: Sperm parameters assessed in around 20 parental males unless there is existing data to show that sperm parameters are unaffected in a 90-day study. TG 421/422: Limited sensitivity due to limited group size (n~8-10) and assessment of sperm quality is not required; TG416: Sperm parameters assessed in around 20 parental males (sperm quality only included in the most recent update in 2001). TG 408, 90-day study: Limited sensitivity due to limited group size (n~10) and assessment of sperm quality is not required. TG407, 28 day study: Very limited sensitivity due to limited group size (n~5), assessment of epididymal sperm parameters are optional.

Source: Adapted from draft 2017 DRP prepared by Brunel University and Technical University of Denmark.

Table A.6. Current OECD TG endpoints which may detect retinoid effects on non-mammalian male reproductive health.

Retinoid effects	OECD Test Guidelines (TG)	Comments
Reproductive development (non-mammalian)		
Dysgenesis of internal reproductive ducts (histopathology)	Reproductive ducts are collected for histopathology in The Larval Amphibian Growth and Development Assay (LAGDA) (TG 241).	TG 241 Wolffian Duct Stage scored (1-4 as per histology guidance document 228). Maximum 40 juveniles (mixed sex) sampled per treatment at end of experiment.
Testes development and spermatogenesis (testicular histopathology)	Testes collected for histological analysis during the final sampling (juvenile) in The Larval Amphibian Growth and Development Assay (LAGDA) (TG 241). Testes collected for histological analysis during the final sampling in Fish Sexual Development Test (TG 234) F1 Testes collected for histological analysis in Medaka Extended One Generation Reproduction Test (MEOGRT) (TG 240)	TG 241 Gonad phenotype (ovary, testis, intersex) and genetic sex used to calculate phenotypic/genotypic sex ratios. Testes development scored from 'Undifferentiated' (1) to 'All stages of spermatogenesis evident' (5), development of tubule lumen, germ cell degeneration and mononuclear cell infiltrates also scored. Maximum 40 juveniles (mixed sex) sampled per treatment at end of experiment. TG 234 Histopathology is primarily used to focus on sex ratio (male, female, intersex or undifferentiated), staging testis is optional but can be investigated. TG 240 Histopathology of gonads in F1 generation only. Histopathology is primarily used to focus on sex ratio (male, female, intersex) additional assessment of the testes is optional.
Adult exposure (non-mammalian)		
Spermatogenesis (testicular histopathology)	Testes are collected for histology at the end of the 21-day adult exposure in Fish Short Term Reproduction Assay TG 229.	TG 229 histopathology of gonads is optional. When assessed endpoints include the presence of testicular oocytes (intersex), Leydig cell hyperplasia and increased spermatogonia rather than assessment of particular spermatogenic stages.

Source: Adapted from draft 2017 DRP prepared by Brunel University and Technical University of Denmark.

A.12. Concluding remarks

225. Potential endpoints for studying effects on female and male reproduction caused by chemical exposure, possibly affecting the retinoid pathway, have been identified and are described in this report.

226. *In silico* methods, such as QSAR and molecular docking models for *e.g.* CYP26, RALDH, RAR, RXR could be integrated into OECD CF Level 1. A small number of *in silico* tools for retinoid signaling pathways (all focusing on CYP26) have been developed (Battistoni *et al.* 2019, Foti *et al.* 2016). Such tools exist for other signaling pathways (Rosenberg *et al.* 2017a, Rosenberg *et al.* 2017b, Klimenko *et al.* 2019), which suggests that they could be developed further also for the retinoid pathway. It should be recognized that large amounts of data are needed to build QSAR models.

227. Some *in vitro* assays are available (See Section A.12.1) that measure retinoid-relevant endpoints such as RAR/RXR activation. Other assays (*e.g.* CYP26, Stra8, RALDH) that might be suitable for integration into OECD CF Level 2 remains to be developed (see Section A.12 for more information). The CYP26 and RALDH isomers appear to be the best candidates at present, since they play a critical role in regulating RA concentrations in several reproductive tissues in both males and females. RAR antagonists and agonists could lead to the same effects as altered RA concentrations. CYP26 isomer expression and activity appears to be well studied in many laboratories, suggesting that these assays might be mature enough for further development. Assays focusing on steroidogenesis should also be considered. However, a more comprehensive analysis has to be performed in order to identify the most suitable candidate assay. Recent development of models such as the human female reproductive tract-on-a-chip (Xiao *et al.* 2017) needs to be evaluated for possible regulatory use in the future.

228. Regarding current mammalian *in vivo* OECD TG studies, no endpoint specifically relevant for retinoid-disruption have been identified that could be added to already existing test methods (such as TG 443 which has the most sensitive design). One exception could be evaluating the value of addition of ovary histopathology of pups in OECD TGs 421/422, although not an endpoint specific for retinoids, retinoids are important for meiosis, and this could consequently also affect the size of the ovarian reserve. This is currently a data gap in these screening test guidelines. Serum levels of AMH in adult females, as a proxy for follicle counting, could potentially also be evaluated, although it is currently unclear if serum AMH levels reflect the fertility status in women. Certain already existing endpoints (in studies covering sensitive windows; see Table A.1) could be informative regarding retinoid disruption. For females, ovaries uterus and vagina are of particular interest, and for males, testes and sperm parameters. Serum level measurements of steroids could add value considering the extensive RXR cross-talk. However, no endpoints that would provide information specifically on retinoid disruption have been identified, since the control of male and female reproduction involves many other endocrine pathways.

229. The initial scoping effort in this report highlights that there are several challenges before *in silico* or *in vitro* screening assays, for identifying retinoid disruption, could be added to the test guidelines programme, or *in vivo* endpoints added to already existing test guidelines:

- Due to extensive cross-talk, it is difficult to distinguish between adverse fertility effects caused specifically by retinoid disruption, from effects caused by disruption of other endocrine signaling pathways important for proper reproduction.
- More research is needed to not only to clarify the role of RAR- or RXR-mediated influence on cross-talk in retinoid homeostasis and its role in normal reproduction, but also to further clarify the spatial and temporal control of RA availability in reproductive tissues.

- The scientific approach used in the reviewed publications did not evaluate retinoid homeostasis (in relevant organs) concomitantly with recording adverse effects on reproduction. This is a major data gap. Consequently, no suitable reference chemical, known to affect reproduction specifically via the retinoid pathway, has been identified (except a very small number of specific RA-disrupting compounds originating from development of pharmaceuticals). Thus, validation of potential endpoints and assays described herein will be challenging.

230. Finally, the pathway visualizations in the proposed AOPs of this report point to similar early events, *i.e.*, dysregulated RA levels in target reproductive tissues. RA levels are controlled by a set of enzymes under physiological conditions, but it should be recognized that other enzymes capable of affecting RA levels could become relevant after exposure to chemicals. While it is currently unclear which degree of RA dysregulation that is necessary for adverse effects to occur, the suggestion of these KEs can be useful for guiding future work. Currently, the level of knowledge on cross-talk in RA-signaling is limited, and thus RAR/RXR activation was not included in the proposed AOPs in the present report. When the understanding of cross-talk in RA-signaling has matured, RAR/RXR activation should be taken into account when AOPs are developed.

A.13. References (Introduction and Appendix A)

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A.14. Appendices

A.14.1. Assay methods and availability:

- RA levels:
  - Can be measured in tissues (organs, blood) or cells with *e.g.* liquid chromatography methods coupled to ultraviolet or mass spectrometric detection (a multitude of publications reviewed in the D-DRP).
  - Can also be measured with ELISA kits (available for several species)
  - https://www.mybiosource.com
- CYP 26:
  - The different isomers can be studied on mRNA and protein level using commercially available probes and antibodies (see *e.g.* Topletz *et al.* 2012).
  - ELISA kits for CYP26A1, B1 and C1 for several species are available.
  - https://www.mybiosource.com
  - Enzyme activity can be measured in microsomes as conversion of RA to its metabolites (Thatcher *et al.* 2010 analyzed human liver microsomes).
- RDH 10:
  - ELISA kits for several species are commercially available.
  - https://www.mybiosource.com
- Stra8:
  - mRNA and protein levels can be measured (Mark *et al.* 2008).
  - cDNA and antibodies for multiple species are commercially available, as are ELISA kits
  - https://www.mybiosource.com,https://www.labome.com, https://www.biocompare.com
- RARs, RXRs:
  - Several RAR/RXR activation assays are commercially available, many use HepG2 cells:
  - http://alttox.org/application-of-medium-and-high-throughput-screening-for-in-vitro-toxicology-in-the-pharmaceutical-industry/
  - http://www.attagene.com (used in ToxCast™)
  - https://ncats.nih.gov/tox21/projects/assays
- AhR:
  - AhR mRNA induction or activation can be measured in *e.g.* human HepG2 cells; commercially available.
  - http://www.attagene.com (Attagene used in ToxCast™)
  - https://ncats.nih.gov/tox21/projects/assays (Tox21 used in ToxCast™)

- CYP1A1:
    - CYP1A1 activity can be measured in microsomes
    - A multitude of assays (mRNA, protein, activity) are commercially available.
    - A description of a cell-based method (using mouse pluripotent P19 cells) for identifying chemicals that disrupt RA signaling pathways was published by Chen and Reese in 2013. It has been used to test phthalate esters (Chen and Reese 2016).

A.14.2. PubMed search strategy – retinoid parameters, chemical names, female/male reproduction parameters

231. Retinoid parameters were combined with chemical names (see Appendix A Table 1, below) and with either female or male reproduction search terms (see Appendix A Table 2, below). No cut-offs (for *e.g.* year of publication) were used. The searches yielded several hundred hits, however, only a small percentage was deemed relevant. In fact, a larger number of relevant articles was found via non-structured searches (using *e.g.* references in articles, citing articles, etc).

232. Retinoid parameters: (retinoic OR retinoid OR retinol OR retinyl OR retinal). Inclusion of the term “vitamin A” rendered the exact same number of hits as when this term was excluded.

233. The selected chemicals are a combination of a) chemicals handpicked by Nancy Baker and co-workers at EPA, from ToxCast™ and other sources; b) ToxCast™ chemicals with activity on RARs/RXRs and CYP1A1 (information found in tables in the draft-DRP); c) CYP26 inhibitors found via text mining (information found in table in the draft-DRP); and finally, d) chemicals classified as Persistent Organic Pollutants by the Stockholm Convention.

Appendix A. Table 1. Chemicals searched for preparation of Annex A.

"ser 2–7" (methyl 3-(1H-imidazol-1-yl)-2,2-dimethyl-3-(4-(naphthalen-2-ylamino)phenyl) propan-oate
1-(6-tert-Butyl-1,1-dimethyl-2,3-dihydro-1H-inden-4-yl)ethanone
1,2,3-Trichlorobenzene
1,3,5-Triisopropylbenzene
2,2-dimethyl-3-(4-(naphthalen-2- ylamino)phenyl)-3-(1,2,4)triazol- 1-yl-propionic acid methyl ester
2,4,5-Trichlorophenol
2,4,6-Tris(tert-butyl)phenol
2,4-Bis(1-methyl-1-phenylethyl)phenol
2,6-Di-tert-butyl-4-ethylphenol
2,6-Di-tert-butyl-4-methoxyphenol
2-Mercaptobenzothiazole
2-Naphthylamine
3-(6-(2-dimethylamino-1- imidazol-1-yl-butyl)naphthalen-2- yloxy)-2,2-dimethyl-propionic acid
4,4'-Sulfonylbis[2-(prop-2-en-1-yl)phenol]
4-Nonylphenol
4-Nonylphenol, branched
4'-octyl-4-biphenylcarboxylic acid
AC-41848 hydrate
Acitretin
Adapalene
Aldrin
all/trans-Retinoic acid
Alpha-hexachlorocyclohexane
AM580
AR7
Azinphos-methyl
Bensulide
Benzyl alcohol
Beta-hexachlorocyclohexane
Bifenazate
BMS 493
BMS-189453
BMS-195614
Butylated hydroxytoluene
CD437
Chlordane
Chlordecone
Chlorthal-dimethyl
Citric acid
Clorophene
Coumaphos
CP-532623
DDT OR Dichlorodiphenyltrichloroethane

Dieldrin
diethylaminobenzaldehyde
Difenoconazole
Diniconazole
Dioxin OR dibenzofuran
Dodecyl gallate
Dysprosium(III) chloride
Econazole nitrate
Endosulfan
Endrin
EPN OR ethyl phenylphosphonothioate
Esfenvalerate
Fludioxonil
Gentian Violet
Heptachlor
Hexabromobiphenyl
Hexabromocyclododecane
Hexabromodiphenyl ether and heptabromodiphenyl ether
Hexabromodiphenyl ether OR heptabromodiphenyl ether
Hexachlorbenzene
Hexachlorobutadiene
Imazalil
Imidazole
Isoniazid
Isopentyl benzoate
LE 135
Liarozole
Lindane OR gamma-hexachlorocyclohexane
Mepanipyrim
Methoprene acid
methyl 3-(4-(6-bromopyridin-3- ylamino)phenyl)-3-(1H-imidazol- 1-yl)-2,2-dimethylpropanoate
Mirex
N,N-Dimethylformamide
Nicotine
N-Phenyl-1,4-benzenediamine
Organotin
Pentachlorobenzene
Pentachlorphenol
Perfluorooctanesulfonic acid OR perfluorooctanesulfonyl fluoride
Phosalone
polychlorinated AND (naphtalene OR naphtalenes OR napthalene OR napthalenes)
Polychlorinated biphenyls OR PCBs
Polychlorinated AND (dibenzo-p-dioxins OR dibenzofurans)
Prochloraz
Pyraclostrobin
R115866 OR rambazole

R116010
SR271425
Symclosene
Talarozole
Tetrabromodiphenyl ether and pentabromodiphenyl ether
Tetrabromodiphenyl ether OR pentabromodiphenyl ether
Tetrabutyltin
Toxaphene
Tributyltin benzoate
Tributyltin chloride
Tributyltin methacrylate
Triflumizole
Triphenyltin hydroxide
Triticonazole
TTNPB

Source: This table from Retinoids in Mammalian Reproduction, with an Initial Scoping Effort to Identify Regulatory Methods, Nordic Council of Ministers, (Nordic Council of Ministers, 2020) is made available under the Creative Commons Attribution 4.0 International license (CC BY 4.0).

Appendix A. Table 2. Search terms used in Annex A.

Search terms for female reproduction	Search terms for male reproduction
"female genitalia"	Aspermia
"female gonad"	Asthenozoospermia
"Female reproduction"	Azoospermia
"Granulosa cells"	Blood-Testis Barrier
"Polycystic ovarian syndrome"	Cryptorchidism
"Theca cells"	Ejaculation
Abortion	Epididymis
Cervix OR cervical	Epididymitis
Corpus Luteum	Erectile
Decidua OR decidualization	Fertilization
Eclampsia	Hemospermia
Embryo OR embryonic	Hypospadia
Endometriosis	Impotence
Endometrium OR endometrial	Leydig
Estrous	male infertility
Fallopian	Oligospermia
Fertility OR infertility	Orchitis
Fetus OR fetal	Penile
Folliculogenesis	Prostate
Gestational	Prostatic
Lactation	Prostatitis
Litter	"Rete Testis"
Luteal	Scrotum
Maternal	Seminiferous
Menopause	Sertoli
Menstrual OR menstruation	Sperm
Oocyte	Spermatid
Oogenesis	Spermatocele
Oogonia	Spermatocyte
Ovary OR Ovarian	Spermatogenesis
Ovulation	Spermatogonia
Placenta OR placental	Spermatozoa
Pregnancy OR pregnant	Testes
Prenatal OR postnatal	Testicular
Puberty	Testis
Trimester	
Uterus OR uterine	
Vagina OR vaginal	

Source: The content of this table from Retinoids in Mammalian Reproduction, with an Initial Scoping Effort to Identify Regulatory Methods, Nordic Council of Ministers, (Nordic Council of Ministers, 2020), is made available

Annex B. Retinoid Signaling in Skeletal Development: Scoping the System for Predictive Toxicology

B.1. Annex preface

B.1.1. Scope and Rationale of this Annex

234. All-trans retinoic acid (ATRA), the biologically active form of vitamin A, is instrumental in regulating normal embryonic growth and developmental programs of vertebrate species (Duester 2008; Ghyselinck and Duester 2019). Infants born to mothers exposed to excess ATRA and other retinoids during pregnancy have a 25-fold increased risk for malformation (Williams, Mear et al. 2004), indicating a direct or indirect disruption of these programs and the importance of windows of exposure in the context of increased risk for malformations of various organ systems. Although concentration-dependent effects of ATRA on morphogenesis led to its early characterization as a 'morphogen' that can influence skeletal patterning (Thaller and Eichele 1987), our current understanding of its cellular consequences on the embryo indicate direct interactions with morphoregulatory pathways that pattern growth and differentiation of different organ systems, including the skeleton (Riddle, Johnson et al. 1993). This annex provides a review of the major concepts that support current understanding of the embryology and toxicology of retinoid signaling and its impact on normal and induced abnormal skeletal morphogenesis. In considering pathogenesis of chemical disruption to retinoid signaling, a common theme is the regulation, homeostasis, and physiology of ATRA as a paracrine signal that collaborates with cell-cell signaling networks driving patterning of the embryo. A developmental role was first observed in experimental studies showing severe eye reduction defects in pigs born to dietary maternal vitamin A deficiency (VAD) (Hale 1935). A "vitamin A deficiency syndrome" in pregnant rats fed a retinol-deficient diet revealed numerous defects of varying penetrance and severity (eye, genitourinary tract/kidneys, diaphragm, lung, aortic arches, and heart), but did not report defects in skeletal development (Kalter and Warkany 1959 , Kaiser, Merrill et al. 2003). However, striking teratogenic activity of exogenous ATRA on murine limb and palatal development was noted by Kochhar (Kochhar 1973), and Kessel and Gruss showed 'homeotic transformations' in mouse fetuses exposed to teratogenic doses of ATRA (Kessel and Gruss. 1991). Adverse outcomes evaluated in a prenatal developmental toxicity study conducted for regulatory assessment may include altered growth trajectories, functional and behavioral deficits, structural abnormalities that include both malformations (permanent structural changes that may adversely affect survival, development, or function (sometimes

referred to as teratogenicity) and variations (used to indicate a divergence beyond the usual range of structural constitution that may not adversely affect survival or health). A translational challenge in scoping this review is to determine which endpoints currently included in a guideline prenatal developmental toxicity study (e.g., OECD TG 414) are relevant for retinoid-based disruption of skeletal development. From a regulatory developmental toxicity perspective, it is not clear how any skeletal defects found will be strictly attributable to or exclusively to an ATRA-signaling-related mechanism. The reconstruction of toxicity pathways in early embryonic development sets the stage for future perspectives on the potential susceptibility for retinoid system associated modulations on bone health and bone-related disease over the life-course.

235. Development of the vertebrate skeleton commences during gastrulation with genes that pattern the distribution and proliferation of mesenchymal cells from cranial neural crest, somite-derived sclerotomes, and lateral plate mesoderm. During organogenesis, mesenchymal cells condense at sites of future skeletal elements and subsequently differentiate to chondroblasts or osteoblasts to form cartilages and bones (Olsen, Reginato et al. 2000). Research from over 60 years has led to current understanding of retinoid signaling as a critical player in spatial patterning of the major body axes (e.g., anterior-posterior, dorsal-ventral, right-left) and temporal processes underlying the specification of individual organ rudiments (e.g., cranio-facial bones during neurodevelopment; segmentation of the vertebral column during somitogenesis; proximal-distal determination of the appendicular skeleton during limb-bud outgrowth). Common themes and diverse strategies have emerged from experimental models across multiple vertebrate species leading to substantial understanding of retinoid metabolism, transport and homeostasis for most skeletal domains. Elucidating Adverse Outcome Pathways (AOPs) for each major system (craniofacial, axial, appendicular) is a long-term vision, although the DRP will utilize case examples to demonstrate how diverse information can be integrated to inform regulatory test method development (or identify gaps that need to be filled to get there). Retinoid pathway assays are expected to facilitate early screening of chemicals for tiered testing of developmental toxicity and to enhance existing test guidelines (e.g., OECD TG 414 prenatal developmental toxicity). Opportunities exist for refining and supplanting current developmental toxicity testing protocols using in vitro data and in silico models in the design and review of revolutionary alternatives to animal testing by experts in the field, alongside their independent validation (Scialli, Daston et al. 2018).

236. The term ‘new approach methodologies’ (NAMs) has been recently adopted in reference to any technology, methodology, approach, or combination thereof that avoids the use of intact animals and can be used to provide information on chemical hazard and risk assessment (EPA 2018). Special emphasis will be given here to NAMs for profiling *in vitro* data from high-throughput screening (HTS) data in repositories such as ToxCast/Tox21 as a source of NAM data, with advanced computational *in silico* models that address chemical read-across, bioactivity profiles, and tissue dynamics in response to defined levels of perturbation (EPA 2018). A case study approach will link chemicals that perturb skeletal development (in vivo) to the retinoid signaling pathway (in vitro). This aligns with the published strategy plan for using NAMs in the Toxic Substance Control Act (TSCA) new chemicals program (EPA 2018). Given the availability of some in vitro assays for key elements in retinoid signaling, the case studies (existing data) would be expected to inform tools for implementation within three years. This will require a collaboration with experts who can guide the modelers and determine how true to reality the models are.

B.2. Abbreviations relevant to Annex B

AC50	concentration that inhibits an *in vitro* activity by 50%
AP axis	anterior-posterior axis, rostral-to-caudal
AER	apical ectodermal ridge
9-cisRA	9-cis retinoic acid, physiological ligand of RXRs
CI	computational intelligence
CNC cell	cranial neural crest cell
CYP26 a1, b1, c1	isoforms of the Cyp26 family of cytochrome P450 oxidoreductases
DART	developmental and reproductive toxicity
DV axis	dorsal-ventral axis, back-to-front
GD	gestation day
HTS	high-throughput screening
IATA	integrated approaches to testing and assessment
IM	intermediate mesoderm
IVIVE	*in vitro* to *in vivo* extrapolation
LOPAC	Library of Pharmacologically Active Compounds
LPM	lateral plate mesoderm
NAMs	new approach methodologies
NCC cells	neural crest cells in general
PSM	presomitic mesoderm
RALDH 1,2,3	isoforms of retinaldehyde dehydrogenase
RAR α/β/γ	retinoic acid receptor isoforms alpha, beta, gamma
RXR α/β/γ	rexinoid receptor isoforms alpha, beta, gamma
SAM	subridge apical mesoderm of the limb-bud, also known as the progress zone
SHH	sonic hedgehog
SMRT	Silencing Mediator of Retinoid and Thyroid hormone receptor
SNC	spinal neural crest
TTNPB	synthetic retinoid 8-tetrahydro-5,5,8,8-tetramethyl-2-napthalenyl-1-propenylbenzoic acid

B.3. Introduction

B.3.1 Skeletal development

237. Current developmental toxicity testing adheres largely to protocols suggested in 1966 involving the administration of test compound to pregnant laboratory animals. The skeleton is routinely examined in standard developmental toxicity bioassays (e.g., OECD 414) and has proven to be sensitive to a wide variety of chemical agents (Knudsen, Martin et al. 2009; Theunissen, Beken et al. 2016). The human skeleton, for example, develops with 270 bones at birth that decrease to ~206 in adults due to various fusions. One might find more bones in experimental animals due to tail structures, minor ossification patterns or supernumerary (accessory) structures. Anatomically, the skeleton is comprised of an axial skeleton (vertebral column, ribs, and skull) and paired appendages (upper, lower extremities). Dysmorphogenesis in the skull, vertebral column and ribs, and appendicular skeleton has been described in both human populations and in laboratory animals used to assess potential adverse developmental effects of drugs and chemicals. Delayed ossification is a common observation for fetal evaluation and is sometimes considered a transitory variation, rather than a permanent abnormality (Solecki, Rauch et al. 2015).

238. Multiple fetal anomalies occur in vitamin A-deficient animals as well as in retinoic acid receptor gene 'knockout' mice, indicating that ATRA (an active metabolite of vitamin A) performs some essential functions in normal development (Kam, Deng et al. 2012; Rhinn and Dolle 2012), which will be discussed in Section B.2. Generally, the work in retinoid signaling reflects cellular concepts for axis formation across three skeletal domains (craniofacial, postcranial axial, and appendicular). This entails early patterning of the primary body axis (neuraxis) and neural tube. Craniofacial development is closely linked to changes in the neural tube associated with brain development, where the craniofacial skeleton is largely derived from cranial neural crest (CNC) cells emigrating from the anterior neural tube. Segmentation of the vertebral column has its origin in sclerotome cells that migrate medially from the somites around the postcranial neural tube and elsewhere to form the ribs and sternum. In both axial domains (e.g., skull and vertebral column) a common target cell in retinoid-sensitive structures is migrating mesenchymal cells (CNC, sclerotome). Although the developing individual may be vulnerable to disruption of retinoid signaling at later gestational stages (Williams, Kondo et al. 2009; Zuo and Wan 2017; Conserva, Anelli et al. 2019), this annex will focus on the embryonic period where the skeletal patterning is most sensitive to retinoid signaling (Kundsen, Thomas et al. 2021)

B.3.2. The retinoid system

239. Retinoid signaling has a conserved ancestry from gastropods to humans, with endogenous ATRA functioning during major organ system development (Bushue and Wan 2010). Recent publications suggest that signaling by ATRA may be an ancestral feature of bilaterians rather than a chordate innovation; however, there is no conclusive evidence showing that a retinoid is required for development of non-chordates. Details on the regulation and function of retinoids during early development of vertebrate embryos have been recently reviewed (Ghyselinck and Duester 2019). The retinoid system has dual status as both vitamin (nutritional dependence) and nuclear receptor ligand (Piersma, Hessel et al. 2017). ATRA is a metabolic derivative from dietary vitamin A, existing in isomers (9-cis, 13-cis, all-trans). Apart from retinal (which is involved solely in the visual cycle), in mammals ATRA is the best known endogenous active metabolite of vitamin A and is considered the only endogenous ligand for RARs. Many groups with expertise in retinoid analysis failed to detect endogenous 9-cisRA in humans and other mammals and questioned its endogenous existence as well as its physiological significance (Krezel, Ruhl et al. 2019). 13-cis RA does not bind efficiently to RAR or RXR. Since it acts only weakly in transactivation assays, it is generally assumed that one of the functions of 13-cisRA is to serve as a precursor for the formation of ATRA (Blaner 2001). Its indispensable

developmental role was first recognized by experimental studies showing severe eye reduction defects in pigs born to maternal vitamin A deficiency (VAD) [Hale 1935], and as a teratologic experimental model in rats showing over 90% ocular and urogenital anomalies, 50% diaphragmatic hernias, and 17% congenital heart defects (Wilson, Roth et al. 1953).

240. During pregnancy, vitamin A ingested by the mother is conveyed to the placenta via the blood, where it circulates bound to the retinol-binding protein (RBP) and freely enters the embryonic compartment. The main, central vitamin A stores available for the rest of the organism is the maternal liver where it is mobilized as needed (Blaner, Li et al. 2016). In peripheral cells, vitamin A is locally stored as vitamin A esters generated through transesterification of lecithin retinol acyltransferase (the most potent vitamin A-esterifying enzyme) and liberated via retinyl ester hydrolases (Teletin, Vernet et al. 2017). Unlike endocrine hormones, the active ligand is not produced by a specific gland but instead bioactivated (dehydrogenases) and degraded (oxidases) locally (Mey 2017; Ghyselinck and Duester 2019). This pattern is similar to many peptide hormones (proteolytic cleavage), paracrine active receptor ligands, and lipid signaling molecules.

241. An early site of ATRA production in the embryo is the presomitic mesoderm (GD 7.5 in mouse). Cells can initiate ATRA synthesis form maternal retinol by retinol dehydrogenase-10 (RDH10) that oxidizes retinol to retinaldehyde (RAL). RAL dehydrogenases (RALDH1, 2, and/or 3) then generate ATRA. Excessive RA accumulation is prevented in part by the reverse conversion of RAL back to retinol, a reaction catalyzed by at least one enzyme (DHRS3) that interacts with RDH10 (Kam, Shi et al. 2013). Local ATRA is a short-range (paracrine) signal, although there is evidence for autocrine effects in cells that synthesize it (Teletin, Vernet et al. 2017). Spatial gradients of ATRA are established in target cell fields by regional expression of dehydrogenases (retinol → RDH → RALDH → ATRA). ATRA formation is buffered in part by reversion of retinaldehyde to retinol, a reaction catalyzed by at least one enzyme (DHRS3) that interacts with RDH10 (Kam, Shi et al. 2013). ATRA is rapidly degraded to inactive forms by cytochrome P450 monooxygenases, resulting in its short (~1 h) half-life (Shimozono, Iimura et al. 2013). The CYP family includes three genes (Cyp26a1, Cyp26b1, Cyp26c1) that are differentially expressed and act with different substrate preferences on ATRA, 9-cisRA, and 13-cisRA (Isoherranen and Zhong 2019). The regional patterns of RDH10/ RALDH2 and CYP26A/B/C expression set up ATRA morphogen gradients that restrict signaling to short-range paracrine or autocrine kinematics (Teletin, Vernet *et al.* 2017).

242. Once inside the cell, ATRA's canonical activity is mediated by specific nuclear receptors (RARs) through genomic (canonical) or non-genomic cascades (Mey 2017). Just as most nuclear hormone receptors, RARs exhibit a modular structure composed of 6 conserved domains (designated A-F), wherein the highly conserved 'C' domain confers sequence-specific DNA binding (Bastien and Rochette-Egly 2004). In mouse, the canonical genomic response is mediated by liganded retinoic acid receptors (RARα, RARβ, RARγ) that heterodimerize with rexinoid receptors (RXRα, RXRβ, RXRγ) to transactivate (or repress) genes harboring a DNA sequence known as the RA response element (RARE). Most RARE sites consist of two hexameric motifs, 5'-(A/G)G(G/T)TCA-3' arranged as palindromes, direct repeats (DR), or inverted repeats (IR) (Balmer and Blomhoff 2005). The classical DR for RAR/RXR binding has a 5-nucleotide spaced direct repeat (referred to as DR5); however, RAR/RXR heterodimers also bind to direct repeats separated by 1 nucleotide (DR1) or 2 nucleotides (DR2). Theses RAREs bind the heterodimer with specific polarities. For example, at DR1 elements for the mouse *Crbp2* gene (5'-AGGTCA *c* AGTTCA-3') the 5'-half site is recognized by RAR and the RAR/RXR heterodimer acts as a transcriptional repressor. In contrast, at DR5 elements for the mouse Cyp26a1 gene (5'-AGTTCA cccaa AGTTCA-3'), the RAR occupies the 3'-half site and the RAR/RXR heterodimer acts as a transcriptional activator (Zhang, Wang et al. 2015). Progressively fewer spacers may favor RXR heterodimers with other nuclear receptors (e.g., TR, VDR, and PPAR) (Mangelsdorf 1994; Zhang, Wang et al. 2015), and some RAR/RXR heterodimer-occupied sites in embryoid bodies or F9 embryonal carcinoma cells show non-canonical half-site nucleotide spacing (Moutier, Ye et al.

2012). RARE binding complexes with nuclear receptor coactivator (NCOA) or nuclear receptor corepressor (NCOR) proteins to activate or repress, respectively, target gene expresion. In the absence of ligand, the RAR/RXR complex is associated with histone deacetylase-containing (HDAC) complexes tethered through corepressors that dissociate upon RAR liganding, allowing the recruitment of coactivators (Bastien and Rochette-Egly 2004).

243. An important question is what happens in the nucleus after retinoid-activated RARs have recruited the transcription machinery. RAR chromatin immunoprecipitation studies have reported 13,000-15,000 potential RAREs in the mouse genome; however, most are not likely functional. Over 500 genes are known to be regulated by ATRA based on ligand involvement, receptor dimerization, DNA binding, and the resulting transcriptional modulation of the gene (Ghyselinck and Duester 2019). It is important to note that the control of RAR/RXR transcriptional control is subject to further regulation by phosphorylation. This may occur in response to G-protein coupled receptors (GPCRs) acting through second messengers (e.g., cyclic AMP, calcium), or receptor tyrosine kinase (RTK) signals acting through downstream kinases (e.g., phosphatidylinositol 3-kinase, PI3K; extracellular signal-related kinase, ERK; mitogen-activated protein kinase, MAPK). Site-specific phosphorylation of RARs modulates cofactor recruitment associated with the general transcription machinery (Bastien and Rochette-Egly 2004). Finally, a non-genomic mechanism of RAR signaling has been characterized during neurogenesis, leading to rapid activation of the PI3K and MAPK pathways without new transcription or protein synthesis (Al Tanoury, Piskunov et al. 2013; Evans and Mangelsdorf 2014; Khatib, Marini et al. 2019). The non-genomic effects in the CNS will not be discussed in this annex since there is no information on its role in skeletogenesis.

B.3.3. Effects of altered ATRA signaling

244. Several lines of study have attempted to establish functional analysis for retinoid signaling during pregnancy and development. One line of experimentation addresses the developmental consequences of retinoid deficiency, caused either by (i) dietary deficiency in vitamin A, (ii) inhibition of ATRA synthesis by functional inactivation of genes encoding retinaldehyde or alcohol dehydrogenases, (iii) administration of RALDH inhibitors, (iv) deletion of genes encoding RARs, or (v) administration of RAR antagonists. These manipulations are interesting from a phylogenetic-ontogenetic perspective because they eventually provide information on the physiological functions of ATRA during normal skeletal development (Ghyselinck and Duester 2019). The second line of experimentation addresses an increase in retinoid signaling with teratogenic effects that can be observed after (i) administration of pharmacological doses of vitamin A or its derivatives (natural and synthetic retinoids), (ii) inactivation of genes coding for CYP26 enzymes, or (iii) exposure to CYP26 (and related) chemical inhibitors. Teratogenic effects resulting from systemic administration of exogenous retinoids to embryos, or from increase of endogenous retinoid levels through genetic manipulations, do not necessarily reflect the physiological roles of endogenous ATRA in the corresponding developmental processes. For example, the teratogenic effect of excess ATRA on lumbosacral truncation is transduced by RARγ, the function of which is dispensable for normal development of the lumbosacral vertebrae (Lohnes, Kastner et al. 1993). Species differences also are known; for example, zebrafish possess two homologs of RARA (encoded by raraa and rarab) and two homologs of RARG (encoded by rarga and rargb), but lack an RARB ortholog (Ghyselinck and Duester 2019). In evaluating the embryonic origins of defects related to retinoid disruption of skeletal development, this annex focuses first on the mammalian (e.g. rodent) models, then data from other animal models (chick, zebrafish, xenopus) and finally what is known in humans or can be extrapolated to humans. Table B.1 lists existing endpoints in OECD TG that may detect chemicals interfering with retinoid pathway signaling in the craniofacial and skeletal development of non-mammalian species.

Table B.1. Existing endpoints in OECD TG that may detect chemicals interfering with retinoid pathway signaling in the craniofacial and skeletal development of non-mammalian species.

Retinoid effects	OECD Test Guidelines (TG)	Comments
Craniofacial and skeletal systems (non-mammalian)		
Status of craniofacial and skeletal systems	OECD 206: Avian reproduction test	Gross necropsy
Development of craniofacial and skeletal systems, structural abnormalities	OECD 210: Fish: early life-stage toxicity test	Gross morphological abnormalities
Development of craniofacial and skeletal systems, structural abnormalities	OECD 231: The amphibian metamorphosis assay (AMA)	Hind limb and snout development, gross morphological abnormalities
Development of craniofacial and skeletal systems, structural abnormalities	OECD 241: The larval amphibian growth and development assay (LAGDA)	Gross morphological abnormalities

Source: 2017 draft DRP prepared by Technical University of Denmark/Brunel University.

245. The PubMed Abstract Sifter (Baker, Knudsen et al. 2017) was used to mine the literature with queries and filters that synopsize the concepts aggregated for Sections B.3, B.4, and B.5 of this annex (Table B.1). The tool is an Excel front end for PubMed and delivers the same results as a query in PubMed, but in a format that can filter for specific terms found in the article's title, abstract, or key words. The broad query returned a catalogue of 5,903 publication records with the query "(vitamin A or retinoid or retinol or retinal or retinoic acid or tretinoin) and (embryo or fetus) and (development)" (accessed January 2020) broadly annotated for retinoids, embryos, and development. The corpus was sifted by terms specific to each of the three structural domains reviewed in Sections B.3 (craniofacial skeleton), B.4 (postcranial axial skeleton), and B.5 (appendicular skeleton). Those records were subsequently narrowed to find the most relevant concepts for this review, and especially for AOP elucidation (Table B.2).

Table B.2. Literature Records Selected

QUERY [1] (PubMed)	*CORPUS (# records)*	*COVERAGE (year)*	*CURATED [3] (# titles)*	*REFERENCED (# currently cited)*
vitamin A or retinoid or retinol or retinal or retinoic acid or tretinoin	336,456	--	--	--
(vitamin A or retinoid or retinol or retinal or retinoic acid or tretinoin) and (embryo or fetus)	12,180	--	--	--
(vitamin A or retinoid or retinol or retinal or retinoic acid or tretinoin) and (embryo or fetus) and (development)	5,903	1950 - 2019	--	--
SKELETAL DOMAINS (Abstract Term) [2]			***SECTIONS B.3, B.4, or B.5***	
retino* and (crani* or skull or facial or palatal or neural crest or pharyngeal or branchial)	405	1980 - 2019	210 (B.3)	55 (B.3)
retino* and (*axial or axes or vertebral or vertebrae or somit* or primitive streak)	460	1971 - 2019	142 (B.4)	45 (B.4)
retino* and (limb or append*)	349	1973 - 2019	255 (B.5)	72 (B.5)
TERATOGENESIS (Abstract Term) [2]			***SECTION B.6***	
retino* and (terato* or *toxic* or deficien* or _chemical)	1244	1968 – 2019	216 (B.3-5)	52 (B.3-5)

[1] PubMed queried using the AbstractSifter tool (Baker, Knudsen et al. 2017). Simple queries returned a corpus of publication records based on MeSH curation. The # records returned (accessed January 2020) and coverage by years of publication are indicated.
[2] Corpus (5,903) sifted by specific terms in the article's title, abstract or keywords appropriate to skeletal domains discussed in Sections B.2-5 of this annex; asterisk (*) indicates wild-card character(s).
[3] Records curated manually to generate titles most relevant to each section and finally cited.

B.4. ATRA Signaling in Craniofacial Development

246. **Key Section points:**

- endogenous ATRA is essential for development of the facial bones and branchial arches
- retinoids influence positional information of premigratory CNC cells during gastrulation
- ATRA specifies pharyngeal endoderm that secretes factors permissive to CNC migration
- CNC cells migrating from hindbrain to branchial arches sensitive to retinoid excess

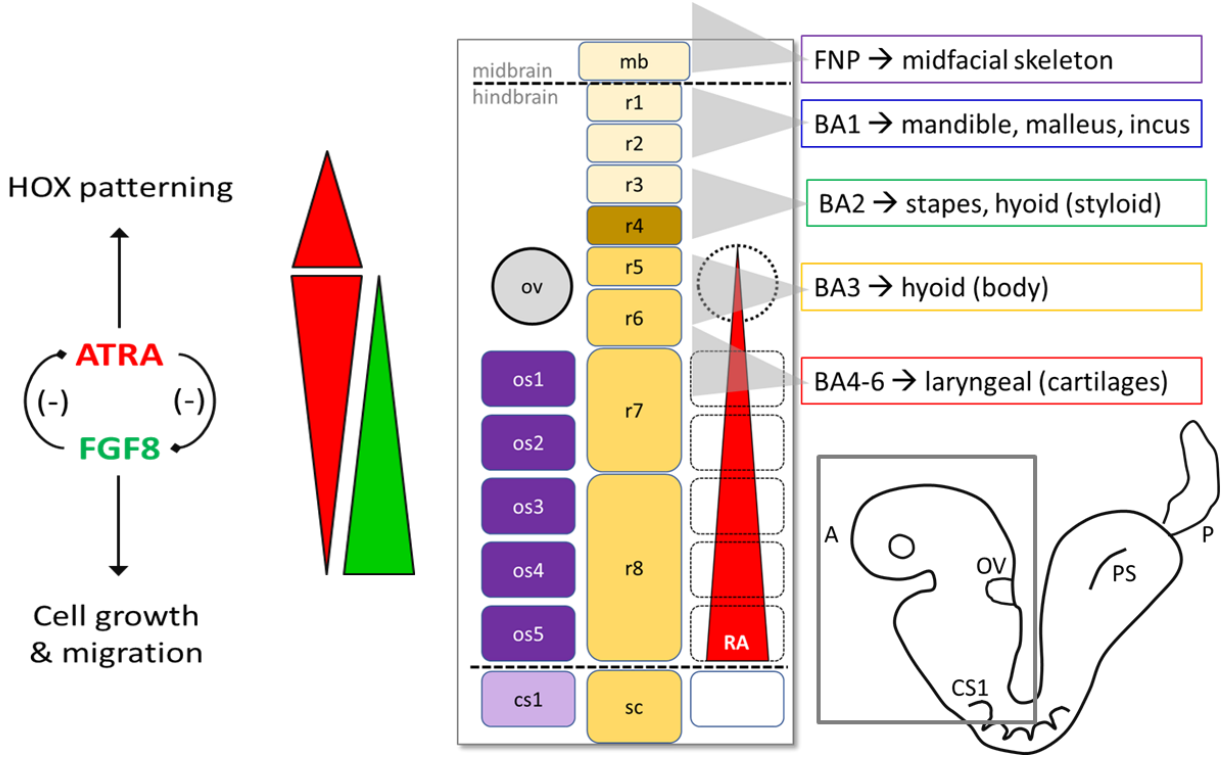
B.4.1. ATRA signaling in craniofacial development

247. Endogenous ATRA is essential for development of the facial bones and branchial arches. Craniofacial malformations induced by retinoid excess, including those of *Cyp26(-/-)* null mutant mice, have been linked to disruption of craniofacial mesenchyme primarily affecting the formation of bones in the midface. It may also be the case for the malformation of bones derived from the caudal branchial arches under conditions of 'functional ATRA deficiency' in *Rar(-/-)* null mtnat mice. Two migratory cell lineages populate the embryonic head: CNC cells, and cells from the paraxial mesoderm (Noden and Trainor 2005). Their distinct migratory streams have been shown in transgenic mice carrying an X-Gal neural crest cell lineage reporter (Wnt1-Cre/R26R) along with DiI-labeling to reveal mesodermal cells (Jiang, Iseki et al. 2002). In mouse, bones of the facial skeleton (viscerocranium) derive primarily from CNC precursors whereas those of the cranial vault (neurocranium) derive from both migratory lineages, depending on the bone. The frontal bone, for example, is primarily of CNC origin whereas the occipital bone is primarily mesodermal.

248. Regional differences in CNC sensitivity to retinoid signaling reflect their position in the developing anterior neural tube. As to the situation of functional ATRA deficiency, it is well established that ATRA controls anteroposterior patterning of the hindbrain through at least the 5- to 11-somite stage, notably through controlling homeobox genes (Begemann and Meyer 2001; Mark, Ghyselinck et al. 2006; Dupe and Pellerin 2009). CNC cells initially form at the level of the midbrain and hindbrain regions (Figure B.1). They start emigrating from the neuroepithelium at the 5-somite stage (~GD 8.5 in the mouse) to populate the frontonasal process and branchial arches. CNC cells arising furthest anteriorly form frontonasal structures (e.g., frontal bone). Hindbrain CNCs from the more rostral segments populate the 1st and 2nd branchial arches, and the postotic (caudal) cells the 3rd – 6th branchial arches (Morriss-Kay, Ruberte et al. 1993; Clouthier, Garcia et al. 2010; Dupe and Pellerin 2009).

249. The impact of endogenous retinoid signaling on hindbrain patterning is stage-dependent and determined in part by the threshold response to local retinoid concentrations (Begemann and Meyer 2001). That has been demonstrated with a pan-RAR antagonist (BMS493) in the chick, but the concept applies to functional retinoid deficiency in mouse as well. RAR antagonism at the 5-6 somite stage results in anteriorization of rhombomeres r5 – r8, presumably by blocking the 'posteriorizing' influence of retinoid signaling. BMS493 progressively loses the capacity to invoke posterior regression as the embryo advances to the 11-somite stage. This effect can be phenocopied by different combinatorial RAR null mutant backgrounds. Retinoid deficiency during this window (GD 8.5 in mouse and week 4 of human gestation) would, therefore result in misprogramming the positional information of premigratory CNC cells destined to populate the caudal branchial arches.

Figure B.1. Retinoid signaling during morphogenesis of the craniofacial skeleton.



Functional inactivation of *Rdh10* in mouse manifests in severe anterior defects (facial malformation, ear and eye deficiency, and loss of forelimb) due to inability of the embryo to metabolically convert retinol → retinoic acid (Rhinn, Schuhbaur et al. 2011). Endogenous ATRA is required, but at different threshold levels, for normal development of the midface and branchial arches. Positional information of premigratory hindbrain CNC cells is determined by threshold ATRA levels coming from the paraxial mesoderm/occipital somites. ATRA signaling through RAR/RXR 'posteriorizes' the hindbrain and is essential for specification of rhombomeres r5-r8 (5- to 11-somite stage). ATRA also specifies pharyngeal endoderm, which in turn secretes factors that create an environment permissive to (but not required for) postotic CNC migration. Abbreviations: ov, otic vesicle; os, occipital somite 1-5; cs1, first cervical somite; r1 – r8, rhombomeres 1-8; FNP, frontonasal process; BA, branchial arches 1-6; PS, primitive streak. Dotted lines indicate midbrain/hindbrain and hindbrain/spinal cord junctions. Figure from (Knudsen, Thomas et al., 2021), used here with the kind permission from the publisher, Elsevier.

250. CNC cells emigrating from the hindbrain region follow a pattern of segmentation that is sensitive to different threshold levels of ATRA along the anteroposterior axis (Begemann and Meyer 2001). ATRA is likely produced in the paraxial mesoderm (somites) of which the occipital somites 1 to 5 are in physical register with rhombomeres r7 to r8. Rhombomere r4 is a conserved signaling center for FGF8 production and therefore may contribute to regulation of the ATRA gradient from the otic vesicle (low end) to the caudal extent of rhombomere r8 (high end) (Figure B.1). Unique programming and integration of CNCs within the frontonasal, maxillary and mandibular prominences generate skeletal structures in the midface and rostral branchial regions of the embryo, but only malformations of bones/cartilages derived from the more caudal branchial arches may eventually be accounted by altered positional information of premigratory CNC cells with functional retinoid deficiency (Wendling, Ghyselinck et al. 2001). There is however, evidence that the pharyngeal endoderm, not CNC cells (or their precursors in the hindbrain neurectoderm) is the primary target for ATRA signaling. Treatment of mouse embryos with BMS493 induced a lack of caudal branchial arches and disturbed the paths of postotic CNC cell migration. Like in the chick embryo, this treatment was effective only during a narrow window of development that did not coincide with the period of postotic CNC cell migration. Thus, migrating CNC cells destined to populate the caudal branchial arches may not represent primary targets of ATRA action (Begemann and Meyer 2001). However, BMS493 altered endodermal expression of patterning genes, indicating that ATRA signaling is required to specify the pharyngeal endoderm, which provides a permissive environment for CNC migration through secretion of specific paracrine factors (Mark, Ghyselinck et al. 2006).

B.4.2. Retinoid homeostasis

251. ATRA is produced from metabolism of retinol to retinaldehyde by retinol dehydrogenase (RDH), followed by metabolism of retinaldehyde to ATRA by retinaldehyde dehydrogenase (RALDH) (Chatzi, Cunningham et al. 2013). Embryos from oviparous species can obtain retinaldehyde by metabolizing carotenoids stored in the yolk; however, mammalian embryos rely on retinol transferred from the maternal circulation and locally convert it to retinaldehyde. In mouse, ATRA synthesis is first detected at the late primitive streak stage coincident with the expression of RALDH2. This occurs in the posterior mesoderm during gastrulation and later in the craniofacial mesenchyme during neurulation (Ang, Deltour et al. 1996). Advanced techniques for endogenous ATRA detection and quantification have advanced our understanding of the kinetics of ATRA metabolism, kinematics of RA signaling, and downstream gene expression (reviewed in (Dubey, Rose et al. 2018)).

252. In mouse embryos, retinol → retinaldehyde is catalyzed as the first step in the tissue-specific regulation of retinoic acid synthesis by RDH10, a gene identified from N-ethyl-N-nitrosourea (ENU)-mutagenesis screens as being responsible for a spectrum of abnormalities reminiscent of ATRA-deficiency phenotypes (Sandell, Sanderson et al. 2007). Developmentally, *Rdh10* is expressed in regions of active retinoid signaling andcolocalizes with *Raldh2* expression (Cammas, Romand et al. 2007). Functional inactivation of *Rdh10* (Rhinn, Schuhbaur et al. 2011) disrupted endogenous ATRA synthesis and caused severe craniofacial defects in the *Rdh10*-nullizygous *c*ondition, demonstrating the requirement of RDH10 for ATRA synthesis and downstream function. In another *Rdh10(-/-)* murine line, the craniofacial defects were rescued by exogenous retinaldehyde on GD 7-9 confirming the requirement for retinol metabolism in anterior patterning (Chatzi, Cunningham et al. 2013). Although three cytosolic isoforms (*Raldh1, Raldh2, Raldh3*), only RALDH2 is indispensable for normal mouse development (Niederreither, Fraulob et al. 2002). *Raldh2(-/-)* mouse embryos kept alive with maternal ATRA supplementation GD 7.5 to 8.5 showed rudimentary development of the 3rd-6th branchial arches (Niederreither, Vermot et al. 2003). In zebrafish, the 'neckless' mutation (*nls*) inactivates RALDH2 leading to truncation of anterior structures in a manner that can be partially rescued with exogenous retinoid, suggesting the phenotype results from a primary defect in endogenous ATRA signaling (Begemann, Schilling et al. 2001). Citral (3,7-dimethyl-2,6-octadienal), an inhibitor of retinol and

retinaldehyde dehydrogenases as well as other alcohol and aldehyde dehydrogenases, reduced teratogenic effects of exogenous retinol on neural crest cells (Schuh, Hall et al. 1993). Taken together, these findings demonstrate the importance of endogenous RA synthesis for proper anterior patterning of the early embryo.

253. The appropriate regulation of ATRA homeostasis also depends on its breakdown. A novel cytochrome P450 (P450RA, later identified as CYP26A1) was identified that specifically metabolized biologically active retinoids to 5,8-epoxy retinoids but did not act on retinol or retinal; this enzyme was proposed to help establish ATRA gradients in the gastrulating embryo (Fujii, Sato et al. 1997). Three functionally redundant paralogs of the CYP26 gene family (*Cyp26a1, Cyp26b1, Cyp26c1*) expressed at the anterior end of the gastrulating mouse embryo cooperatively regulate anterior-posterior patterning of the neural tube. Whereas functional inactivation of *Cyp26c1(-/-)* did not appear to affect mouse embryonic development, double knockouts for both *Cyp26a1(-/-)* and *Cyp26c1(-/-)* failed to produce migratory CNC cells in the prospective forebrain and midbrain presumably due to excessive ATRA accumulation (Uehara, Yashiro et al. 2007) where it is normally maintained at low threshold concentrations for retinoid signaling (Figure B.1). In contrast, *Cyp26b1(-/-)* mice do not display abnormalities in hindbrain segmentation but instead show phenotypes linked to defective formation of Meckel's cartilage as a consequence of misdirected migration of hindbrain CNC to locations outside of the 1st branchial arch (Maclean, Dolle et al. 2009). In the gastrulating mouse embryo, *Cyp26b1* expression is first observed at GD 8.0 in prospective rhombomeres r3 and r5, then expands to rhombomeres r5 and r6 by GD 9.5 (MacLean, Abu-Abed et al. 2001). As noted earlier, CNC cells start emigrating from the preotic hindbrain (r3-r5) at the 5-somite pair stage (~GD 8.5) and follow a pattern of segmentation that is sensitive to different threshold levels of ATRA (Begemann and Meyer 2001). Loss of CYP26B1 function in r3/5 (GD 8.0) and r5/6 (GD 8.5) expression domains could be expected to elevate ATRA at a critical time before posterior regression of signaling activity ceases around the 11-somite stage (refer to Figure B.1).

254. Defects in hindbrain segmentation, CNC programming and migration, and craniofacial abnormalities show the importance of 'retinoid homeostasis' in patterning the anterior neuraxis during gastrulation-neurulation. Severe craniofacial defects result from loss of endogenous ATRA production (*Rdh10* deficiency), whereas caudal defects follow loss of ATRA breakdown (*Cyp26a1* deficiency) (Figure B.1). Furthermore, retinoid homeostasis in early embryos is dependent on the localized expression of various retinoid binding proteins. For example, early mouse embryos cultured at a presomitic or early somite stage, when deprived of retinol by yolk-sac injection of antisense oligodeoxynucleotides to knockdown the retinol binding protein (RBP), displayed cranial neural tube dysmorphogenesis in a manner that was rescued with exogeneous ATRA; this is consistent with a disruption of precursor retinol to retinoid target tissue (Bavik, Ward et al. 1996). Additionally, in the GD 8.5 mouse embryo ATRA and its congeners co-localize with a CRABP-immunoreactive protein (CRABP I) at the transitional zone between surface ectoderm and neuroepithelium, from where neural crest cells emanate (Dencker, Gustafson et al. 1991).

B.4.3. Retinoid receptors

255. The crucial role of ATRA in patterning mesenchymal structures derived from the neural crest cells that migrate through, or populate, the frontonasal process and branchial arches raises the important question of the role played by the retinoid nuclear receptors. RAR and RXR families of nuclear receptors each comprise three subtypes (alpha, beta and gamma) that have been individually knocked out in the mouse. Defects displayed by RAR alpha, beta or gamma singular null mutant mice are confined to a small subset of the tissues normally expressing these receptors, or are not observable (Mark, Ghyselinck et al. 1998). Furthermore, singular RAR knockout mice do not display VAD defects, whereas compound RAR mutants die *in utero* or at birth from severe developmental defects that, aside from the 1st branchial arch, include a spectrum of malformations belonging to the fetal VAD-induced

syndrome (Lohnes, Mark et al. 1995). This suggests a degree of functional redundancy in the cellular retinoid response in mice (Mark, Ghyselinck et al. 2009), although morpholino knockdown of RARs in zebrafish show the need for all subtypes in patterning the rhombomeres (Linville, Radtke et al. 2009).

256. *Rara*-, *Rarb*-, or *Rarg* null mice and their compound mutants also exhibit congenital abnormalities that were not described in Hale's and Warkany's classical VAD studies, including defects of the neurocranium and viscerocranium. This occurrence of non-VAD defects is most likely due to the difficulty to achieve, by dietary deprivation, a state of severe VAD compatible with pregnancy. In fact, almost all these non-VAD defects have been subsequently duplicated in rodent embryos deficient in vitamin A, but supplemented with ATRA; or that lack RALDH3 (which specifically display defects in the nasal ethmoturbinates and agenesis of the choana) and were treated with synthetic retinoids having RAR antagonistic activities (Mark, Ghyselinck et al. 2006). Craniofacial defects that are strikingly similar to those observed in RAR knockout mice are also present in rat embryos treated with the specific retinaldehyde dehydrogenases inhibitor Win 18,446 (a bis(dichloroacetyl) diamine) (Taleporos, Salgo et al. 1978), as well as in mice lacking *Rdh10* (refer to Figure B.1). Therefore, the dysregulation of RARs can explain the adverse effects resulting from altered endogenous ATRA levels on craniofacial development.

257. To overcome the early embryonic lethality of compound RAR mutants, mutant mice have been generated in which RARs were specifically ablated in neural crest lineage using somatic mutagenesis. Ablation of all three RARs in the neural crest lineage did not affect their specification and migration, nor the formation of the branchial arches (Dupe and Pellerin 2009). Finally, mice carrying targeted knock-in mutations of the corepressor Silencing Mediator of Retinoid and Thyroid hormone receptor (SMRT) display defects in CNC-derived structures and posterior homeotic transformations of axial vertebrae; SMRT-dependent repression of RAR signaling can modify the Hox code via epigenetic marking (Hong, Fang et al. 2018). It is worth noting that RARs have been instrumental to the evolution of the cranial skeleton (Mark, Ghyselinck et al. 2009). In addition to the dramatic craniofacial skeletal deficiencies affecting *Rara/g*-null mutants, subtle defects that often alter the shape of an individual skeletal element are observed in several *Rar*-null mice: a cartilaginous or osseous connection between the incus (middle ear bone) and the alisphenoid bone (e.g., the pterygoquadrate element); a cartilage separating the trigeminal ganglion from the brain (the pila antotica); and an agenesis of the rostral ethmoturbinate and maxillary sinus. The pterygoquadrate element and the pila antotica, which were lost during evolution from reptiles to mammals, represent atavistic features. Ethmoturbinate bones and maxillary sinus are typical mammalian features not present in reptiles, and their agenesis in *Rar*-null mutants also reflects an atavistic condition. As such, the presence of atavistic features in *Rar*-null mutants supports the notion that changes in temporal or spatial patterns of *Rar* expression provided a general mechanism for modifying the number and shape of individual cranial skeletal elements during vertebrate evolution.

B.4.4. Craniofacial teratogenesis

258. Many teratogenic effects of exogenous retinoids described in laboratory animal models and in humans reflect alterations to tissues derived from CNC cells (Williams, Mear et al. 2004). Clinical observations following isotretinoin (13-cisRA, Accutane) exposure during pregnancy in humans (Irving, Willhite et al. 1986; Pratt, Goulding et al. 1987) and nonhuman primates (Yip, Kokich et al. 1980) have shown a spectrum of malformations including craniofacial defects linked to hypoplasia of the 1st (mandibular) and 2nd (hyoid) branchial arches. Isotretinoin has a low affinity for RARs and RXRs relative to ATRA but may be converted intracellularly to more active metabolites. Similar branchial arch defects can also be invoked with exogenous ATRA in pregnant rodents (hamsters, rats, mice) via delayed or disorganized patterns of cell migration and excessive cell death in the CNC (Lorente and Miller 1978; Wiley, Cauwenbergs et al. 1983; Sulik, Cook et al. 1988; Granstrom and Kullaa-Mikkonen 1990; Webster and Ritchie 1991; Wise, Xue et al. 2010). These findings from teratological observations

showed that CNC cells were a likely target in dysmorphogenesis of the craniofacial skeleton linked to retinoid excess.

259. A direct, quantitative effect of retinoids on facial morphogenesis has been demonstrated in experimental models monitoring CNC cell functions prior to endochondral or perichondral differentiation. Most of this work is from two lines of investigation, one using controlled-release bead carriers soaked with retinoids and applied to different locations of the developing chick embryo, and the other with *in vitro* (whole embryo culture, WEC) studies on rodent embryos. Carrier beads soaked with ATRA (or the stable synthetic retinoid TTNPB) applied to the facial primordia of stage-20 chick embryos induced frontonasal dysmorphogenesis in a dose-dependent and time variable manner; these embryos lacked the upper beak whereas the lower beak was normal (Wedden and Tickle 1986). Exposing facial mesenchyme cultured from different regions of the chick embryo (e.g., frontonasal mass, mandibular mesenchyme) under micromass conditions conducive to cartilage differentiation showed no regional specificity to retinoid inhibition, indicating that retinoids do not produce the specific facial defect by directly interfering with cartilage differentiation (Wedden, Lewin-Smith et al. 1987). Furthermore, fragments of the frontonasal mass give rise to typical upper-beak structures (e.g., central rod of cartilage) when transplanted to the wing bud, and the extent of cartilage is dependent on inclusion of the surface ectoderm. Heterotypic tissue recombinations (retinoid-ectoderm x control mesoblast versus control ectoderm x retinoid mesoblast) pinpointed frontonasal mesenchyme as the sensitive CNC population, which *in situ* is dependent on epithelial interactions for upper beak dysmorphogenesis (Wedden 1987). Specific effects on the upper beak and outgrowth and cartilage differentiation in the frontonasal mass with 13-cis linked to disruption of CNCs (not SNCs) by decreasing cell-substratum adhesion *in vitro* (Smith-Thomas, Lott et al. 1987). Direct exposure of GD 8 mouse embryos to ATRA (0.1 µM) or 13-cisRA (2 µM) in WEC within 6 h led to a dramatic reduction of CNC cell migration to the first and second branchial arches, where the CNC cells either did not leave the neuroepithelium or aggregated nearby due to alterations in the cell surface (Goulding and Pratt 1986 , Pratt, Goulding et al. 1987). CNC cells destined to the second branchial arch are most sensitive in rat embryos exposed to threshold levels of 13-cisRA (Webster and Ritchie 1991). The dysmorphogenesis observed upon direct exposure of chick and rodent embryos to retinoids is consistent with the teratological phenotypes observed *in vivo* and confirm the CNC cell population as the likely target of retinoid teratogenesis.

B.4.5. Determinants of CNC sensitivity

260. Early mouse embryos exposed to exogenous ATRA on GD 7.5 – 8.0, just before differentiation of the cranial neural plate and hindbrain segmentation, showed a shortened hindbrain on GD 9 lacking metameric patterning (Morriss-Kay, Murphy et al. 1991). This dysmorphology correlated with ‘posteriorization’ of the hindbrain with respect to Hox-2.9 (normally confined to rhombomere 4) at the expense of Krox-20 (rhombomere 3) (some Krox-20 positive CNC cells that emigrated from the hindbrain remained close to the neural tube) (Morriss-Kay, Murphy et al. 1991). With exogenous ATRA other CNC cells migrated incorrectly to yield ectopic differentiation, such as Meckel’s cartilage in maxillary region (Morriss-Kay, Ruberte et al. 1993).

261. Similar to mouse, early rat embryos on GD 9.5 exposed to 0.1 µM ATRA in WEC for 6 h displayed reduced size and shape of the first and second branchial arches due to altered regional identity of the hindbrain crest cells (Lee, Osumi-Yamashita et al. 1995). Therefore, although CNC cells form a majority of the facial skeleton and are primary target cells in the retinoid-induced craniofacial defects their reprogramming may occur prior to emigration from the neuroepithelium. This implies a critical effect on neural tube patterning that is realized after CNC cells migrate from the segmented neuroepithelium but prior to reaching their destination to form neural crest-derived skeletal elements. In this regard, endogenous retinoids have been shown to function as ‘posteriorizing factors’ on rat hindbrain development, with the caudal hindbrain region being most sensitive to retinoid insufficiency (White, Highland et al. 2000). In Xenopus, CNC cells also acquire positional information in the neural

tube prior to migration. A 30 min exposure to ATRA at the gastrula stage caused a dose-dependent truncation of the body axis in tadpoles, resulting in progressive loss of anterior structures between 0.5 and 5.0 µM, to complete loss of the head at 10.0 µM (Papalopulu, Clarke et al. 1991).

262. Microarray analysis of the mouse neural crest at 6- to 48 h following *in vitro* exposure to teratogenic (1 µM) levels of ATRA revealed that more than one-third of all differentially expressed genes participated in pathways linked to developmental regulation over time (6 to 48 h), including canonical and noncanonical WNT signaling pathways, cell adhesion and cell cycle regulation (Williams, Mear et al. 2004). Those findings are consistent with the notion that retinoid signaling reprograms genomic determinants of migratory neural crest function. In the chick embryo, premigratory neural crest cells have limited programming about the lower jaw whereas the upper jaw and facial midline are specified later by local tissue interactions. A transcriptomic analysis of the maxillary prominence 16 h after respecification by ATRA-beads showed effects on the retinoid, bone morphogenic protein (BMP) and WNT signaling pathways, as well as cross-talk with Noggin, a diffusible extracellular signal that antagonizes BMP4 and activates the retinoid pathway (Nimmagadda, Buchtova et al. 2015). *Msx1* and *Msx2* transcripts are rapidly down-regulated in upper beak primordia (where outgrowth is inhibited by ATRA) but remain largely unchanged in lower beak primordia (where outgrowth is unaffected) (Brown, Robertson et al. 1997). Both homeobox genes function in the transcriptional regulation of craniofacial development and are likely mediators of the adverse consequences of exogenous retinoid signaling on cleft palate through upstream fibroblast growth factor (FGF) signaling that up-regulates *Msx1/2* expression in the maxillary prominence. Both *Msx1/2* are down-regulated by the application of ATRA to the maxillary mesenchyme (Shimomura, Kawakami et al. 2015) as is *Fgf4* in the frontonasal mesenchyme following teratogenic doses of RA (Munoz-Sanjuan, Cooper et al. 2001). Taken together, these findings point to crosstalk between the retinoid system and BMP-, WNT- and FGF-signaling on the craniofacial mesenchyme largely derived from CNC cells.

263. Interestingly, ATRA exposure in mice at GD 8.5 caused homeotic transformation of the lower jaw into upper jaw-like structures preceded by down-regulation of *Fgf8* expression in the 1st branchial arch ectoderm as an indication of reorganization of mandibular arch reprogramming (Abe, Maeda et al. 2008). Endothelin-1 (EDN1) is one of the primary signals that establish the identities of CNC cells within the mandibular portion of the first branchial arch: mice lacking the *Edn1* gene or its cognate receptor (*Ednra*) display homeotic transformation of the lower jaw to an upper jaw like phenotype like that seen with ATRA exposure (Clouthier, Garcia et al. 2010). Perhaps EDN1 is one of the aforementioned paracrine factors secreted by the pharyngeal endoderm to provide a permissive environment for CNC migration (Mark, Ghyselinck et al. 2006). Interestingly, recent teratogenic experiments using ATRA in excess have led to similar conclusions with regards to CNC cells colonizing the 1st branchial arch (Vieux-Rochas, Coen et al. 2007). A single dose of 25 mg/kg ATRA administered to pregnant mice by gavage at GD 8.5–8.75 resulted in a pattern of craniofacial defects affecting most of the 1st branchial arch. The developmental stage at which ATRA treatment resulted in craniofacial malformations equates to embryos of 9 to 14 somites and corresponds to the window of time during which CNC cells from rhombomeres r1 and r2 reach the 1st branchial arch. This evidence showed that ATRA acts on the signaling epithelium of the 1st branchial arch, gradually reducing the expression of EDN1 and FGF8 (Vieux-Rochas, Coen et al. 2007).

B.5. Retinoids in Postcranial Axial Skeleton Development

264. Key Section points:

- ATRA signaling is involved in three morphogenetic process organizing the trunk:
  - mesoderm segmentation (somite size and bilateral registration)
  - axial elongation (posterior extension)

- anterior-posterior identity of individual segments (regionalization)

265. Trunk organization in *Vertebrata* involves the establishment of a metameric primary body axis leading to formation of the neural tube and paraxial mesoderm (somites). ATRA signaling participates in the organization of both systems (neural tube, vertebral column) although only the vertebral system is considered here. The onset of ATRA signaling in the mouse embryo, based on expression of RDH10, RALDH2, is GD 7.5; however, ectopic or excessive retinoid signaling can disrupt three morphogenetic processes organizing the trunk during early gastrulation (GD 6.5) and well into organogenesis (GD 9.5).

1. Mesoderm segmentation: ATRA signaling determines the size of the somite precursors forming from the presomitic mesoderm (PSM) and their right-left alignment in the trunk region. This occurs at the somite 'determination front' from the early headfold stage (GD 8.0) through hindlimb initiation stage (~GD 9.5 in mouse) and covers the 'pharyngula stage' when the body plan is very similar across diverse Vertebrate species. For example, *Raldh2*(−/−) mouse embryos that completely lack ATRA activity in the paraxial mesoderm form trunk somites approximately half their normal size ((Rhinn and Dolle 2012) and they often exhibit fewer somites on one side (Kaiser, Merrill et al. 2003).

2. Axial elongation: a somite pair is added to the early mouse embryo every 2 hours in rostral (e.g., cervical) to caudal (e.g., sacral) order drawing cells from a posterior growth zone in the 'nodal region' at the anterior extent of the primitive streak. This occurs continuously in the pharyngula-stage embryo (GD 7.5 to 8.0) and later in the tail bud region (GD 8.0 through at least GD 10.0). ATRA signaling plays a permissive role in this process by opposing FGF8 and Wnt signaling. For example, *Cyp26a1*(-/-) mouse embryos that lack the capacity to breakdown endogenous ATRA may display caudal regression due to premature cessation of posterior elongation caused by excessive retinoid signaling (Rhinn and Dolle 2012).

3. Regional identity: ATRA signaling is involved in the early determination of individual vertebral identities through co-regulation of Hox gene expression (together with FGF8 and other signals) (Lohnes, Mark et al. 1994;Kaiser, Merrill et al. 2003). Four clusters of Hox genes (*Hoxa* - *Hoxd*) occur in mammals, individually numbered 1-13 by their sequential position (3' → 5') in each genomic cluster. Precise control of ATRA thresholds in the PSM and nodal region is critical for proper regulation of Hox genes that specify regional identify in the somite precursors. Alterations in ATRA produce skeletal phenotypes that ultimately reflect anterior-posterior patterning and defects in Hox regulation.

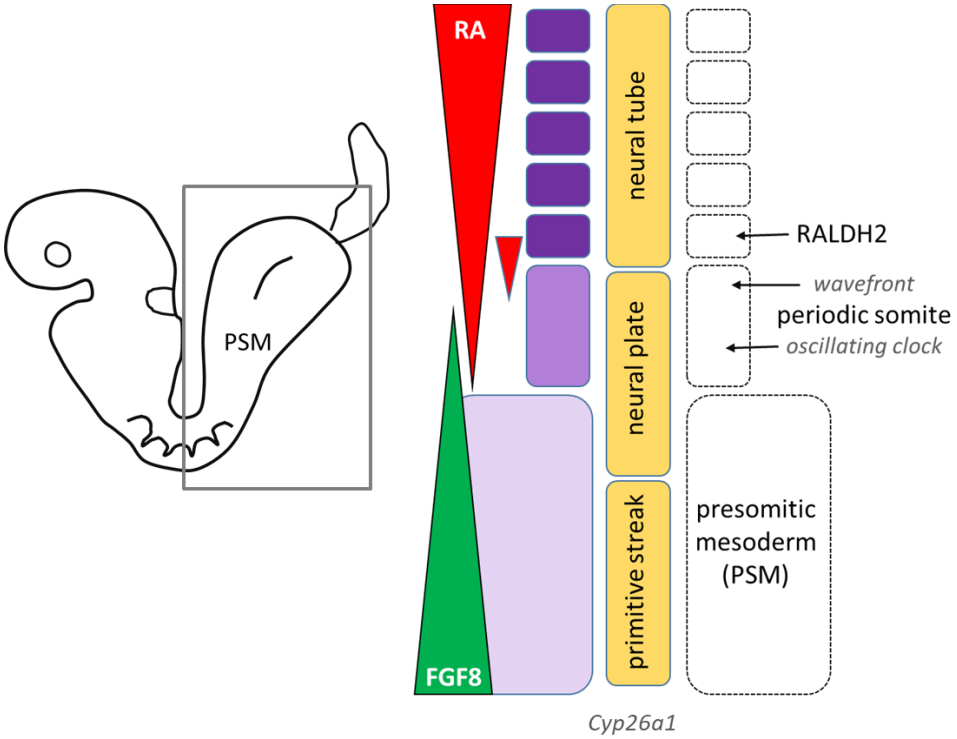
B.5.1. ATRA signaling and mesoderm segmentation

266. Somites form by epithelialization from whorls of precursor cells in the PSM (somitomeres) and are continuously added to the caudal end of the left and right somite columns (Wilson, Olivera-Martinez et al. 2009). The so-called 'determination front' is controlled by a clock-and-wavefront scenario. In this model, a periodic signal controls timing of the nascent somite from its somitomere through lateral inhibition of the Notch-Delta pathway in crosstalk with an ATRA-FGF8 signaling. ATRA is highly concentrated in newly formed somites via RALDH2 expression and dampens FGF8 signaling via repressive interaction with RARE in the *Fgf8* regulatory region that, in turn, is maintained by posterior Wnt-8a and Wnt-3a signals (Aulehla and Pourquie 2010; Cunningham, Brade et al. 2015; Hubaud and Pourquie 2014; Cunningham, Kumar et al. 2015). Consequently, WNT-FGF signals primarily set the posterior boundary of a newly formed somite in conjunction with the segmentation clock, and ATRA plays a permissive role by suppressing the posterior FGF8 wavefront (Figure B.2).

267. A key aspect of this signaling front pertains to the nature of ATRA and FGF8 distribution in the PSM (Deschamps and van Nes 2005). Sources of *Fgf8* expression include the node region of the primitive streak during gastrulation and the tail bud post-gastrulation (Iimura, Denans et al. 2009). FGF8

is released into the extracellular milieu as a diffusible signal that facilitates cell growth and migration. It also induces and maintains *Cyp26a1* expression for ATRA breakdown. As epiblast cells migrate through the anterior primitive streak and into the PSM, ATRA coming from the paraxial mesoderm suppresses *Fgf8* expression. Consequently, the FGF8 gradient and CYP26A1 activity wanes at the determination front. Unlike FGF8, the non-peptidic structure of ATRA makes it difficult to directly image and map ATRA gradients. It is generally accepted that differential expression of RALDH2 and CYP26A1 produce ATRA thresholds that elicit specific cellular behaviors. However, questions remain as to the precise ATRA distribution and topological range in the PSM and how, as short-range paracrine signals, ATRA and FGF8 interact with the determination front (Cunningham and Duester 2015).

Figure B.2. Retinoid signaling in metameric organization of the paraxial mesoderm during somitogenesis



A molecular oscillator (clock) delivers a periodic signal controlling somite production from the presomitic mesoderm (PSM). During axis elongation the signal is displaced posteriorly by a system of traveling signaling gradients (wavefront) that depends on RALDH2 in newly formed somites (rostral) and FGF8 coming posteriorly (Iimura, Denans et al. 2009 , Rhinn and Dolle 2012 , Shimozono, Iimura et al. 2013). ATRA antagonizes the FGF8-mediated growth front (based on (Strate, Min et al. 2009)). *Cyp26a1* expression is highest posteriorly; functional inactivation manifests as severe posterior defects in the mouse (loss of hindlimb, caudal regression) due to premature cessation of posterior elongation (Rhinn and Dolle 2012). ATRA signaling modulates somite size in the trunk region, but not the tail region. ATRA thresholds control bilateral symmetry of the left and right somite columns and determine vertebral identity through RARE-dependent Hox genes (gastrulation). Retinoid excess disrupts PSM growth and caudal extension in the posterior region. Figure from (Knudsen, Thomas et al., 2021), used here with the kind permission from the publisher, Elsevier.

268. To address the dynamics of diffusible ATRA gradients during somitogenesis, different zebrafish reporter lines have been generated to express from a family of genetically-encoded probes engineered to respond to different ATRA thresholds (Shimozono, Iimura et al. 2013). That study engineered the ligand-binding domain of mouse RARs to incorporate cyan-emitting and yellow-emitting variants of *Aequorea* green fluorescent protein (GFP). Using the principle of fluorescence resonance energy transfer (FRET), alterations in the conformation of the ligand-binding domain of the RAR in response to ATRA binding converted into changes from blue to yellow fluorescence that could be mapped by live-cell imaging. Three such reporter lines were generated in which variations in the RAR ligand-binding domain affinities to ATRA were adjusted to produce a yellow FRET signal in response to low (dissociation constant, K'_d = 2 nM), medium (K'_d = 4 nM), or high (K'_d = 50 nM) intracellular ATRA concentration and with negligible sensitivity to retinol or retinaldehyde. These studies revealed a two-tailed linear ATRA gradient across the head-to-tail axis of the zebrafish embryo at the 5-somite stage. The bimodal gradient showed a steady, symmetrical rise and fall of ATRA peaking 6 nM in the mid-trunk and declining to <1 nM anteriorly (head) and posteriorly (tail). Computer simulation of gradient dynamics during growth and development, based on simple diffusion of ATRA (10 μm^2 per sec) over a signaling region of 200 µm, created a linear gradient in about 10 min (Shimozono, Iimura et al. 2013). Importantly, these findings support a conventional 'source-sink' arrangement established by cell-specific expression of *raldh2* and *cyp26s* at a much faster time scale than that of embryonic growth, indicating stability of the gradient.

269. In mouse, the RALDH2 expression domain is strong posterior in the early headfold stage (presomitic) embryo and becomes restricted to the paraxial and lateral mesoderm as rostral expansion advances to the 5-somite stage (Hochgreb, Linhares et al. 2003). Although the ATRA signal generated by RALDH2 in the somitic mesoderm normally travels throughout the trunk mesoderm (Molotkova, Molotkov et al. 2005), the mutual antagonism between ATRA-FGF8 may only be realized over short distances of a few cell diameters at the periodic somitic wavefront (Mallo, Vinagre et al. 2009).

270. Furthermore, coordination between neural and mesodermal patterning is important to align spinal-vertebral segments. For example, at the head-trunk transition of the early embryo, anatomical segments specified in the posterior hindbrain (e.g., rhombomere 7) and anterior spinal cord (e.g., cervical segment 1) must align with those for the occipital bone of the neurocranium and first vertebra (atlas). ATRA signaling coordinates axial position of neural structures relative to paraxial mesoderm at the head-trunk transition in zebrafish (Lee and Skromne 2014). Like in mice (Kaiser, Merrill et al. 2003), this synchrony entails mutual antagonism with Wnt, FGF (and/or Notch-Delta) signaling pathways (Kawakami, Raya et al. 2005). Bipotent axial stem cells generate both lineages (Mallo, Vinagre et al. 2009).

271. An evolutionary scenario has been described for the recruitment of ATRA into the somitic gene regulatory network. Amphioxus, the closest living invertebrate relative of the vertebrates, has a persistent notochord, segmental axial musculature, a dorsal hollow nerve cord that can be posteriorized by exogenous ATRA. This species lacks neural crest cells (Escriva, Holland et al. 2002) but expresses neural crest genes (used for other purposes) that have been co-opted into neural crest formation in higher chordates (Yu, Meulemans et al. 2008). Like vertebrate species, anterior-posterior patterning in Amphioxus is regulated by Hox genes and a posterior FGF signal; however, unlike vertebrate species, this species develops relatively simple somites, has no PSM, and disruption of ATRA signaling does not affect somitogenesis (Bertrand, Aldea et al. 2015). As such, mutual antagonism between ATRA-FGF8 signaling in higher chordates was concurrent with the advent of the PSM and a more complex control of somitogenesis and specialization of somites along the body axis. While some of the control mechanisms governing ATRA-mediated patterning are well conserved between vertebrates and invertebrate chordates, such as amphioxus, less is known about its roles in non-chordates (e.g., echinoderms). A putative RARE (DR5 element) has been found in close proximity to the Hox1 gene in

sea urchin suggesting that at least some components of the ATRA signaling cascade were probably already present in the last common ancestor of deuterostomes (Ferdinard, Marlétaz et al. 2006).

B.5.2. ATRA signaling and axial elongation

272. Posterior elongation extends the developing trunk through a mesodermal stem cell growth zone maintained caudally at the node of the primitive streak at the pharyngula-stage embryo (GD 7.5 to 8.0) and later in the tail bud region (GD 8.0 through at least GD 10.0 (Figure B.3). Posterior elongation is at least partly dependent on an ATRA-depleted environment because *Cyp26a1(-/-)* null mouse embryos that are deficient in the capacity to degrade ATRA display severe truncation of posterior structures (Rhinn and Dolle 2012). ATRA signaling plays a permissive role in this axial elongation by opposing FGF8 and Wnt signaling. ATRA-sensitive anterior transformations of axial vertebrate have been also been reported in mice genetically deficient in growth differentiation factor 11 (GDF11), a member of the TGF-β superfamily that is involved in axial patterning. The *Gdf(-/-)* phenotype includes caudal regression from diminished *Cyp26a1* expression in the tailbud and can be rescued with the pan RAR antagonist, AGN 193109 (Lee, McPherron et al. 2010). In zebrafish embryos, *cyp26a1* expression is further regulated by the miR-19 family microRNAs during vertebral axis formation; miR-19, in turn, is suppressed by ATRA and the adverse consequences of ATRA on somitogenesis can be phenocopied with antisense morpholinos that ablate miR-19 function (Franzosa, Bugel et al. 2013). Hyperossification of axial bones and fusions of vertebral primordia have been reported in zebrafish embryos rendered *cyp26b1*-deficient by genetic mutation (*stocksteif*) or by exposure to R115866, a pharmacological inhibitor of CYP26B1 expressed in osteoblast cells (Laue, Janicke et al. 2008; Spoorendonk, Peterson-Maduro et al. 2008).

273. Similarly, ATRA and FGF8 signaling influence posterior axial development in *Xenopus* at multiple points of interaction (Shiotsugu, Katsuyama et al. 2004). Experimental suppression of FGF8 signaling was shown to downregulate RALDH2, CYP26, and RARα2 expression whereas a constitutively active RARα2 rescued downstream Hox gene expression (XCAD3, HOXB9). These findings suggest that anteroposterior patterning of the primary body axis is driven by a series of mutually interactive feedback loops among FGF, Wnt, RAR signals in concert with Hox expression. The constellation of Hox expression foreshadows the type of vertebra that forms during anterior-posterior patterning and specification of vertebral identity (Diez del Corral and Storey 2004), mediated by the caudal (CDX1/2/4) family of Hox regulatory genes (Deschamps and van Nes 2005).

B.5.3. ATRA signaling and regional specification of vertebral segments

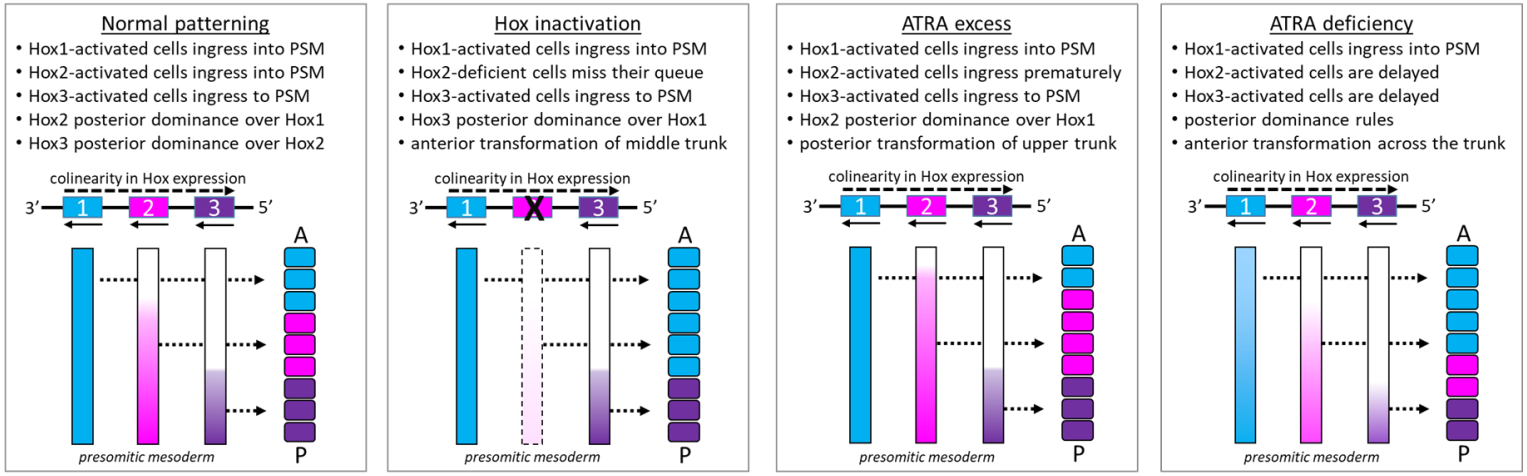
274. ATRA produces concentration-dependent defects on regional phenotypes of the vertebral segments that reflect regulation of Hox gene expression patterns in the PSM. The critical biology begins as the bilaminar disc embryo (epiblast, hypoblast) reorganizes into three primary germ layers (ectoderm, mesoderm, endoderm) that give rise to all tissues in the body. This process, gastrulation, commences in mice at the egg cylinder stage (GD 6.5) when cells from the posterior region of the epiblast converge on the midline and undergo epithelial-mesenchymal transition (EMT) to form a rod-shaped 'primitive streak' that progressively advances ('streaks') anteriorly (Williams, Burdsal et al. 2012). The streak persists as a conduit through which cells undergoing EMT pass. It reaches the distal tip of the egg cylinder proper at GD 7.5. Mesodermal cells so induced then express RALDH2; consequently, the mouse embryo first acquires its capacity to generate ATRA production between the late primitive streak stage (GD 7.2) and early headfold stage (GD 7.7) (Deschamps and van Nes 2005). CYP26A1 is already highly expressed by virtue of FGF8 signaling from the underlying hypoblast. Rostral expansion is characterized by a nodal growth zone that adds tissue to the caudal end of the embryo proper from GD 7.7 through axis elongation stages.

275. Hox gene expression (e.g., *Hoxb1*) is first observed in the posterior streak by GD 7.2; however, this does not concern the future paraxial structures because fate maps show that these cells contribute to the extraembryonic mesoderm. Rather, epiblast cells migrating through the anterior streak form the likely source of Hox activation for anterior-posterior vertebral identity because these are the precursor cells for the paraxial mesoderm. Whereas the primary germ layers are primed to express Hox genes early in gastrulation, Hox gene expression is not activated until the primitive streak in mouse is fully extended during late gastrulation (Forlani, Lawson et al. 2003). Positional information determined by the Hox code is fixed into the PSM as mesodermal cells emerge from the streak (Tam and Beddington 1987). PSM retains its Hox signature identity even when transplanted at an ectopic axial level in the chick embryo.

276. Hox genes exhibit sequential activation from 3' → 5', reflecting their numbered arrangement along the Hox cluster (temporal colinearity). Selective pressure has kept the Hox genes tightly packed, and changes in chromatin structure (Deschamps and van Nes 2005; Iimura and Pourquie 2007). For example, in the PSM *Hoxd4* first unpacks, followed in turn by *Hoxd8-9, Hox10*, and *Hoxd11-12*. This colinear activation is also reflected in the anterior-posterior location of somites (spatial colinearity). Because the PSM acquires its positional information as cells emerge from the primitive streak (Tam and Beddington 1987), early structures formed during gastrulation are given an anterior identity with 3′ Hox genes as key determinants; progressively, later structures express more 5′ Hox genes and acquire a more posterior identity (Krumlauf 1994). Epiblast cells, before migrating through the streak, are a mosaic of different Hox signatures (Iimura and Pourquie 2007). Cells of a similar Hox signature ingress through the streak at a certain time, sorting themselves into the metameric lineages in the PSM. The anterior boundary of expression for each gene in the Hox cluster thus moves anteriorly with rostral expansion as posterior tissues are added to the body axis (Figure B.3). Dynamic temporal pairing of 3'→5' Hox activation with the timing of ingression through the anterior streak migration thus lays out the spatial pattern of Hox expression along the trunk (Deschamps and van Nes 2005 , Iimura and Pourquie 2007). 5' Hox overrides more 3' Hox phenotypes (posterior dominance). Breaking temporal colinearity by disrupting an upstream gene regulatory element in the HoxD cluster (as a model system) produces posterior homeotic transformations coincidentally with an earlier activation of *Hoxd* genes (Kondo and Duboule 1999).

277. ATRA is a potent, global activator of Hox gene expression and regional patterning (Dolle, Ruberte et al. 1990; Ruberte, Dolle et al. 1991). Although RAR/RXR liganding may transactivate Hox genes, this effect is not responsible for colinearity (Diez del Corral and Storey 2004). That is known from the observation of a relatively normal pattern of Hox gene activation in *Raldh2(−/−)* mouse mutants (Niederreither, Subbarayan et al. 1999). On the other hand, perturbations of ATRA signaling have been shown to alter Hox gene expression and induce stage-dependent homeotic transformations of vertebral segments. For example, ATRA excess during gastrulation (GD 7.3) induced posterior homeotic transformations affecting upper vertebral segments (Kessel and Gruss 1991). This can be explained by premature entry of 5' Hox cells into the streak, resulting in the formation of somites with a more posterior character than normal (Figure B.3). The effect was reminiscent of the above-mentioned model where colinearity in the HoxD cluster was broken by depletion of an upstream gene regulatory element (Kondo and Duboule 1999). This implies RARE-derepression of colinear Hox gene activation. It is also reminiscent of findings in vitamin A deficiency (VAD) where fetuses from pregnant VAD rats showed anteriorization of vertebral identity along the entire vertebral column. Shifts in Hox expression domains foreshadowed these transformations, and the window for rescue by exogenous ATRA was late gastrulation (equivalent to GD 7.5 mouse) (Kaiser, Merrill et al. 2003). These findings indicate that a precise distribution of ATRA in PSM is critical for proper regulation of the Hox clock, although ATRA is not obligate to temporal colinearity.

Figure B.3. Ontogeny of Hox-mediated axial patterning and its regulation by retinoid signaling.



Drawn after Iimura and Pourquie (2007)

Hox-patterning decoded during somitogenesis in spatial and temporal waves of transcription to determine relative positions in the vertebral column at which the paralogs are expressed during development (Luo, Rhie et al. 2019). Emergence of the somitic column is depicted from PSM based on the 'posterior dominance' model (redrawn from (Iimura and Pourquie 2007)). Somite colors reflect chromosomally linked Hox genes (simple representation of only 3 genes) in temporal colinear expression. Newly formed somites are morphologically similar across the trunk but are fixed with regards to future vertebral identity. Normal patterning (left) and phenotype with Hox gene inactivation (right) and potential consequences of ATRA excess imposed during late gastrulation (GD 7.3 mouse) and ATRA deficiency.

B.5.4. Nature of the RAR response

278. Exogenous ATRA can induce posterior neural tube defects (spina bifida) in the mouse at GD 8.5 although *RARγ(-/-)* embryos are resistant to this effect (Iulianella, Beckett et al. 1999); however, *Raldh2(-/-)* mouse embryos exhibit axial shortening due to a block in ATRA synthesis (Niederreither, Subbarayan et al. 1999). Studies in *Xenopus* showed that RARβ (*Rarb2*) participates in the control of somite number and size, and restriction of the PSM anterior border, among other effects, during somitogenesis (Janesick, Tang et al. 2017). This is the RAR subtype most upregulated in response to liganding, and its localization in the trunk somites positions it at the right time and place to respond to ATRA during somitogenesis. In contrast, RAR*γ*2 is the major RAR subtype expressed in the caudal stem cell zone; in the absence of ATRA liganding, RARγ2 is a transcriptional repressor and this state maintains the pool of caudal progenitor cells. When liganded, RARγ2 is a transcriptional activator of caudal gene expression that facilitates somitogenesis but can prematurely terminate body axis extension if uncontrolled. This effect is seen when early *Xenopus* embryos are treated with the RARγ selective agonist NRX204647 at a concentration of 0.1 µM; in contrast, the inverse-agonist NRX205099 had no effect at that concentration (Janesick, Nguyen et al. 2014). By activating RARγ, the agonist (NRX204647) relieves repression of posterior Hox gene expression and markers of PSM creating posterior truncations. In RAR/RXR complexes, polycomb group (pcG) proteins interact with non-liganded RAR/RXR to recruit NCORs and silence gene expression. In contrast, trithorax group (trxG) proteins interact with liganded RAR/RXR to recruit NCOAs and activate gene expression (Iimura and Pourquie 2007).

279. Reductions in somitogenesis and axial length with the RARγ-specific agonist in zebrafish were associated with loss of *hoxb13a* expression, suggesting RARγ maintains stem/progenitor cells during embryonic development in its nonligated state (Wai, Kawakami et al. 2015). In *Xenopus*, a switch in RARγ signaling from repressor to activator states could be invoked not only by the RARγ-selective agonist (NRX204647), but also by overexpressing a constitutively active RARγ (VP16-RARγ2) or a dominant-negative nuclear corepressor (c-SMRT, Silencing Mediator of Retinoid and Thyroid hormone receptor) (Janesick, Nguyen et al. 2014). SMRT-dependent repression of RAR is also critical to establish and maintain the somitic Hox code and segmental identity in mice. This involves epigenetic marking of loci (Hong, Fang et al. 2018). Mice deficient in the Polycomb homolog (M33) show homeotic transformations of the axial skeleton, sternal and limb malformations and an aggravation of the skeletal malformations when treated with ATRA on GD 7.5 suggesting M33 plays an epigenetic role in defining access to RAREs in some Hox genes (Core, Bel et al. 1997). Taken together, these findings are consistent with roles for both RARβ and RARγ during somitogenesis: liganded RARγ facilitates expression of RARβ (Janesick, Tang et al. 2017) in the caudal region until required to facilitate body axis cessation when somitogenesis is nearing completion and the progenitor cell pool is exhausted (Olivera-Martinez, Harada et al. 2012). In general, CDX (1/2/4) is a central integrator of FGF/WNT/ATRA signals on Hox gene activation and expression, and may therefore be a key node in the regulatory network integrating the molecular control over segmentation, posterior axial elongation, and specification of vertebral identity (Deschamps and van Nes 2005).

B.6. Retinoids in Appendicular Development

280. Key Section points:

- ATRA signaling essential for initiation of forelimb bud (but not hindlimb bud)
- ATRA enters limb-bud proximally and is degraded by CYP26B1 distally
- antagonistic ATRA-FGF8 dual morphogen system permissive to polarized outgrowth

- retinoids modulate chondroblast differentiation and interdigital cell death

281. The appendicular skeleton (upper and lower extremities in bipeds, forelimb and hindlimb in quadrupeds) is defined in three segments along the proximodistal axis: stylopod (humerus, femur), zeugopod (radius-ulna, tibia-fibula), and autopod (hand, foot). A secondary axis defines anterior-posterior asymmetry (e.g., digits I through V in mouse and humans) and a tertiary axis dorsal-ventral asymmetry. The rudimentary 'limb-bud', composed of surface ectoderm and a mesoderm derived from the lateral plate (LPM), forms as outcroppings of the flank (forelimb bud, hindlimb bud). Development and patterning of the appendicular skeleton has been a general paradigm for understanding embryogenesis, and the consequences of physiological (ATRA) and teratological (chemical) disruption of retinoid signaling on limb development has been a subject of much interest and controversy over the decades (Kochhar 1973; Tickle, Alberts et al. 1982; Thaller and Eichele 1987; Lewandoski and Mackem 2009; Tabin and Wolpert 2007 ;Ghyselinck and Duester 2019). The physiological functions of ATRA reviewed here primarily focus on mouse limb development, while also addressing key concepts from experimental embryology in zebrafish, amphibians, and avian species, and for retinoid teratogenicity in rodents, nonhuman primates, and humans.

B.6.1. ATRA in the initiation of the forelimb bud

282. Limb-bud induction in the LPM is triggered by a cascade of signaling events involving FGF, WNT, and ATRA. Retinoid signaling is required for initiation of the forelimb bud (but not hindlimb bud) (Zhao, Sirbu et al. 2009 , Cunningham, Zhao et al. 2013). For example, the *RDH*(*trex/trex)* mouse fetuses lacking the capacity to generate retinaldehyde and subsequently ATRA (as shown by RARE-lacZ reporter transgenes and rescue with exogenous ATRA) display stunted forelimbs and apparently normal hindlimbs, reminiscent of *Tyrannosaurus rex* (Cunningham, Chatzi et al. 2011). Forelimb development is also disrupted in the *Raldh2(-/-)* mouse embryo (Niederreither, Subbarayan et al. 1999).

283. T-box transcription factors TBX5 and TBX4 have critically important roles in specifying forelimb and hindlimb identity, respectively, through FGF signaling. These are the primary initiators of limb-bud outgrowth and the earliest genes expressed in the prospective limb fields. Forelimb expression of *Tbx5* in the mouse is initiated in the anterior flank at GD 8.5, and *Tbx4* mirrors this pattern posteriorly with hindlimb-specific expression at a half-day lag (Naiche and Papaioannou 2003). The failure to initiate forelimb development in association with ATRA deficiency is reflected in loss of *Tbx5* and can be alleviated with exogenous ATRA administration to the pregnant dam on GD 8 (Mic, Sirbu et al. 2004). *Tbx5* activation is suppressed by FGF8 signals; consequently, *Fgf8* expression must be downregulated both in the anterior (cardiac) and posterior (caudal) regions to enable limb initiation. ATRA signaling is a major factor as demonstrated in RALDH2- and RDH10-deficient mouse mutants (Zhao, Sirbu *et al.* 2009; Cunningham, Zhao et al. 2013). This points to a permissive role for ATRA signaling in conditioning the microenvironment of the prospective limb-bud field for *Tbx5* expression through antagonism of FGF8 signaling, similar in concept to craniofacial and axial patterning. Other studies proposed that ATRA might directly activate *Tbx5* via a RARE located in intron 2 (Nishimoto, Wilde et al. 2015); however, subsequent enhancer knockout experiments using CRISPR/Cas9 gene editing showed that this RARE is not required for forelimb bud initiation (Cunningham, Lancman et al. 2018). Thus, the most parsimonious model in mice is that ATRA is permissive to forelimb bud initiation by alleviating FGF8 signaling, which then allows another factor (perhaps Wnt) to activate *Tbx5* expression.

284. ATRA is also required for pectoral fin (forelimb homolog) initiation in the zebrafish embryo (Begemann, Schilling et al. 2001). This phenotype is observed in the *nof* (no-fin) and *nls* (neckless) mutations in the zebrafish *raldh2* locus (Grandel, Lun et al. 2002) (Gibert, Gajewski et al. 2006). A signaling cascade has been proposed where physiological ATRA signals originating in the somitic mesoderm → *wnt2b* in the intermediate mesoderm → *tbx5* in the lateral plate mesoderm → *prdm1* in the forelimb bud → FGF10 (Mercader, Fischer et al. 2006). FGF10, in turn, activates *Fgf8* expression

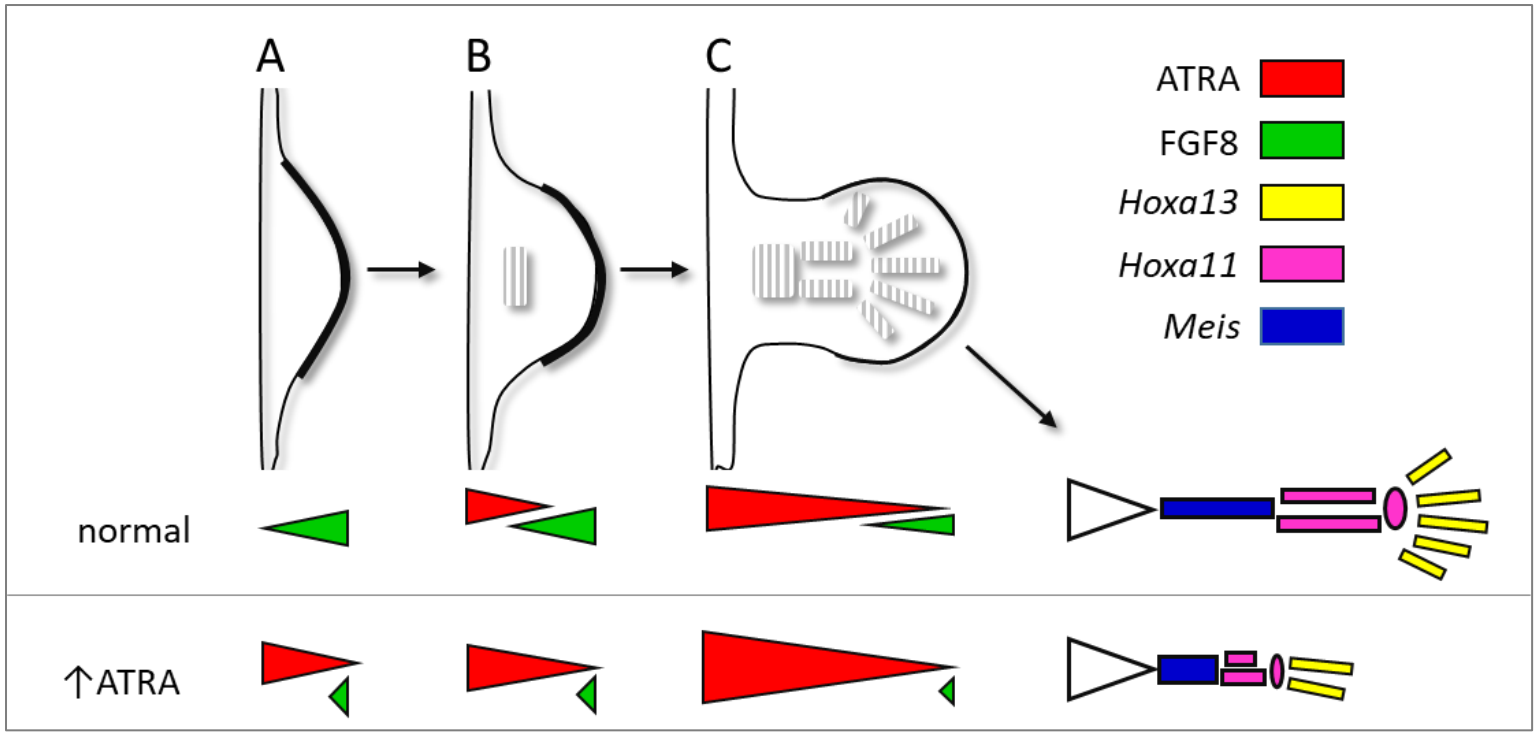
in the distal ectoderm and thickening that drives polarized outgrowth of the limb-bud. This is consistent with the notion that ATRA antagonizes early axial FGF8 signals that otherwise inhibit the limb field.

B.6.2. ATRA signaling and limb patterning

285. In addition to mice lacking RALDH2 and RDH10, malformed limbs are observed in mice lacking RARα and RARγ receptors, lacking CYP26B1, or lacking CRABP2 (Cunningham and Duester 2015). These phenotypes reflect the continuing physiological role for ATRA signaling during limb-bud outgrowth and appendicular development; however, the system is complex and data from the literature sometimes contradictory with regards to its involvement in shaping skeletal elements specified along the primary (proximodistal) axis (e.g, stylopod, zeugopod, and autopod) and the secondary (anteroposterior) axis (e.g., number and morphology of the digits). In both cases, polarized outgrowth and patterning is dependent on two major organizing centers of the early limb-bud (Figure B.4): the Apical Ectodermal Ridge (AER) distally and the Zone of Polarizing Activity (ZPA) posteriorly (Zakany, Zacchetti et al. 2007).

286. ATRA synthesized by RALDH2 in the flank enters the limb-bud proximally and like in the trunk has an opposing distribution to FGF8 signals, here coming distally from the AER (Yashiro, Zhao et al. 2004). Although concentration-dependent effects of ATRA on morphogenesis led to its early characterization as a ‘morphogen’ that can instruct skeletal patterning (Thaller and Eichele 1987), our current understanding indicates the instructional role is perhaps species-specific and that permissive interactions may be more the case for murine development (Riddle, Johnson et al. 1993). Our current understanding again points to a permissive role for ATRA signaling in the mouse via mutual antagonism with FGF8 as is consistent with other organizing centers of the embryo (Lewandoski and Mackem 2009; Tabin and Wolpert 2007; Ghyselinck and Duester 2019; Zuniga 2015).

Figure B.4. ATRA signaling during early development of the appendicular skeleton.



TOP: Mouse forelimb from early outgrowth (A, GD 9.5) to precartilage induction (B, GD 10.5) to precartilage pattern (C, GD 11.5); corresponding stages in the hindlimb are delayed by a half day. The precartilage pattern is laid down in proximo-distal fashion for the stylopod (humerus, femur), zeugopod (radius-ulna, tibia-fibula), and autopod (digits of fore- and hind paw) (Zuniga 2015). BOTTOM: permissive role ATRA signaling on proximo-distal patterning (Uzkudun, Marcon et al. 2015). ATRA (from RALDH2) enters the proximal limb-bud and is degraded distally by CYP26B1 induced by FGF8; cells leaving the ATRA-free distal mesenchyme have positional values determining regional identity for stylopod (*Meis*), zeugopod (*Hoxa11*), and autpod (*Hoxa13*). Gradients represent two-signal model for ATRA and FGF8 signal inputs; a one-signal model (not shown) was also simulated wherein the time exposed to FGF8 alone determined regional identity. Figure adapted from (Aimee, Zuniga 2015)

B.6.2.a. Proximodistal patterning

287. Progressive specification of the limb proximodistal segments is based on a timing mechanism in the distal 'progress zone'. This mesodermal growth zone measures ~300 µm in length and elongates the limb-bud in response to FGF8 elaborated from the AER (Summerbell 1974; Niswander, Tickle et al. 1993; Fallon, Lopez et al. 1994; Mariani, Ahn et al. 2008). Under this model, the fate of mesodermal cells becomes progressively more distal depending on time spent in the progress zone exposed to FGF8 (Figure B.4). While the timing mechanism is not fully understood (Uzkudun, Marcon et al. 2015), cells falling out of the progress zone condense into precartilage skeletal rudiments. In particular, SOX9 controls the mesenchyme to chondrogenic differentiation (Reinhardt, Gullotta et al. 2019). A one-signal FGF-driven progress zone model for limb proximodistal patterning has been proposed, based on mouse genetic studies, coupled with colinear Hox gene activation. In this model, Hox activation does not require ATRA to specify proximodistal fate but requires ATRA degradation distally to maintain the progress zone and prevent teratogenesis from excess ATRA (Lewandoski and Mackem 2009). ATRA entering the limb-bud from the flank is an indirect 'proximalizing factor' of the limb-bud through transactivation of the proximal determinant *Meis* genes (*Meis1, Meis2*). This activity earmarks stylopod specification, which is dependent on Hox9 and Hox10 genes in the forelimb and Hox10 genes in the hindlimb, and is counteracted by FGFs from the AER (Mercader, Leonardo et al. 2000; Rosello-Diez, Ros et al. 2011). Like somitogenesis, the antagonistic relationship between ATRA-FGF signals are realized during early limb-bud outgrowth between ATRA (proximalizing factor) and FGF8 (distalizing factor).

288. In the one-signal model of limb-bud outgrowth, continued FGF8 signaling from the AER activates *Cyp26b1* expression in the progress zone and therefore drives limb-bud elongation by maintaining distal mesenchyme in a proliferative state. Excessive ATRA signaling would otherwise terminate *Fgf8* expression in the progress zone and lead to a precocious involution of the AER before the precartilaginous skeleton completely forms. *Cyp26b1(-/-)* mice show abnormal distal expansion of ATRA signaling in both the forelimbs and hindlimbs, with excessive apoptosis and cartilage disruption that correlates with meromelia (limb truncation) and other severe limb deformities reminiscent of retinoid teratogenesis (Yashiro, Zhao et al. 2004). *Cyp26b1* is markedly expressed in the AER and distal mesoderm (MacLean, Abu-Abed et al. 2001) and is likely to create an 'ATRA-free' progress zone. This reflects a complex signaling module whereby AER-FGF and CYP26B1-ATRA regulate distal progression of limb-bud outgrowth (Probst, Kraemer et al. 2011). Quantitative computer simulation of the ratiometric signaling (Figure B.4) show that high ATRA and low FGF8 activate Meis1/2 in the stylopod (humerus, femur); ATRA and FGF4/8 at intermediate levels promote Hoxa11 in the zeugopod (radius-ulna, tibia-fibula); and low ATRA and high FGF4/8 promote Hoxa13 in the autopod (hand, foot) (Uzkudun, Marcon et al. 2015). While these findings do not provide evidence that endogenous ATRA signaling is a direct morphogen for proximodistal patterning of the limbs (Duester 2008), they are consistent with a two-signal model observed in the chick whereby ATRA participates in the establishment of proximal fate.

B.6.2.b. Anteroposterior patterning

289. Numerous studies have investigated the potential for ATRA to influence anteroposterior patterning of the limb-bud, or so-called 'polarizing activity' that determines, for example, the numbers, sizes and identities of the digits. Some of the most striking findings have followed a line of investigation where carrier beads are soaked with different concentrations of retinoids and then locally applied to various regions in the chick embryonic limb bud, permitting a detailed evaluation of skeletal phenotypes resulting from the timing and position of ATRA-soaked bead application.

290. Sonic hedgehog (SHH) emanating from the ZPA is the polarizing signal responsible for organizing anteroposterior patterning of the digits (Riddle, Johnson et al. 1993). The ZPA occupies the distal-posterior aspect of the early limb-bud (Figure B.4). When ATRA-soaked beads were implanted to the opposite (anterior) margin of the early chick limb-bud, dose-dependent effects were observed such

as supernumerary digits (low dose), mirror image digital duplications (medium dose), and truncations (high dose) (Summerbell 1983; Helms, Thaller et al. 1994). The lower dose effects were reminiscent of ectopic ZPA transplantation. When the ZPA region was surgically removed before bead implantation to the anterior margin, ATRA reprogrammed the distal mesenchyme to restore digit formation, but in reverse polarity with respect to anterior-posterior symmetry similar to an ectopic ZPA (Eichele 1989). Beads soaked with synthetic retinoid analogs also caused concentration-dependent duplications when applied to the anterior margin (10- to 100 µg/ml), and truncations at very high concentration (1 mg/ml) (Tamura, Kagechika et al. 1990) or when implanted proximally (Tickle and Crawley 1988). Together, the chick bead findings demonstrate the capacity of ATRA signaling to reprogram mesenchyme to an ectopic ZPA. *Shh* expression is not, however, directly dependent on ATRA in the physiological range (10 µg/ml) (Niswander, Tickle et al. 1994; Helms, Thaller et al. 1994; Chen, Dong et al. 1996). The permissive effect of ATRA on polarizing activity appears to be conserved from fish to mammals (Ogura, Alvarez et al. 1996; Akimenko and Ekker 1995) and may involve ATRA-regulated Hoxd genes that lie genetically upstream to *Shh* (Lu, Revelli et al. 1997; Pickering, Wali et al. 2017).

B.6.3. ATRA in interdigital cell death

291. ATRA signaling promotes the naturally occurring zones of interdigital cell death that separate the developing digits. Double mouse mutants lacking both the *Rarg* gene and one or both alleles of *Rarb* display a severe and fully penetrant interdigital webbing (Dupe, Ghyselinck et al. 1999). *Rdh10(trex/trex)* mutants also display interdigital webbing, where the hindlimb skeletal pattern was not affected but interdigital cell death was absent at GD 1.5 (soft tissue syndactyly) (Cunningham, Chatzi et al. 2011). These findings demonstrate that ATRA, although unnecessary for limb patterning, is required later for interdigital tissue loss. This is an important point indicating that deficiency in signaling by ATRA could be a cause of (cutaneous, osseous) syndactyly in humans (Dupe, Ghyselinck et al. 1999).

B.6.4 Retinoid-induced limb teratogenesis

292. Kochhar in 1973 was among the first to identify teratogenic effects of exogenous ATRA on the mouse limb, demonstrating dose-dependent phocomelia following a single dose in the range of 1- to 100 mg/kg administered to the pregnant dam (Kochhar 1973). The window of vulnerability in mice (GD 10-12) coincided with the formation of precartilaginous mesenchymal condensations. ATRA caused dose-dependent phocomelia and digital defects when administered to pregnant mice at 20- to 80 mg/kg on GD 11, where the deficiencies to precartilage presented before overt cartilage differentiation (Kwasigroch, Skalko et al. 1984). Higher teratogenic dosages (e.g., 200 mg/kg) on GD 12 also disrupted joint formation in the hindlimbs (Abu-Hijleh and Padmanabhan 1997). Similar teratological phenotypes have been reported in rats (Yu, Gonzalez et al. 2003) and hamsters (Wiley 1983) indicating generalized susceptibility in rodents. Beyond phocomelia, more severe limb reduction defects/deficiencies have been reported with teratogenic exposures to retinoids at earlier gestational stages. For example, exposure on GD 6 induced ectopic or supernumerary hindlimbs as part of a lower body duplication syndrome (Liao and Collins 2008) and on GD 9 resulted in complete loss of the hindlimbs as part a caudal regression syndrome (Padmanabhan 1998). Discounting broader syndromes where limb malformations are secondary to primary body axis determination, GD 10-12 is the window of vulnerability in mouse for and related deformities induced with teratogenic retinoid exposures.

293. Susceptibility of limb development to retinoid teratogenesis coincides with stages at which limb development is undergoing polarized outgrowth, and with the formation of the precartilaginous skeleton. For example, a fully phocomelic exposure to ATRA in the mouse (100 mg/kg on GD 11) led to the early appearance of excessive cell death in the central prechondrogenic core of the forelimb (Zhou and Kochhar 2004). In fact, expansion of several naturally-occurring zones of physiological cell death in the limb-bud have been observed following ATRA exposure on GD 10 (Bynum 1991). In exposed hamsters

(Wiley 1983) a disorganized vasculature was found to encroach on areas of mesenchymal condensation prior to overt differentiation (Wiley 1983). These findings on the cellular basis of retinoid teratogenesis are consistent with disruption of skeletogenesis with or before development of precartilage rudiments of the long bones. It is worth noting that increased bone resorption in the mature skeleton is a well-documented effect of hypervitaminosis A (Conaway, Henning *et al.* 2013). This effect is related to an imbalance of osteoclast activity in post-menopausal women. Cellular effects of retinoids on bone homeostasis have been observed in neonatal mouse calvarium culture (and other *in vitro* models), perhaps through RARalpha-dependent effects on the osteoclast signal RANKL (TNFSF11) that functions in bone and cartilage development. For additional information on ATRA and skeletal homeostasis the reader is referred to excellent reviews by (Conaway, Henning *et al.* 2013, Henning, Conaway *et al.* 2015).

294. Clinical observations with various retinoid congeners have suggested that the major teratogenicity is due to direct effects on the embryo. GD 10 mouse embryos directly exposed to ATRA in whole embryo culture (WEC + 48 h) showed palatal and limb deficiencies, and inhibition of cell proliferation as low as 0.1 µM ATRA (Watanabe and Pratt 1991). When GD 8 mouse embryos were cultured in the presence of isotretinoin (13-cis retinoic acid, also known as Accutane), dysmorphogenesis was first observed in branchial arches I/II at 2 µM followed by the limb-buds at 20 µM (Goulding and Pratt 1986). Isotretinoin, a known human teratogen (NIH, 2019) causes primarily heart and craniofacial malformations; however, this retinoid congener, as well as a number other natural and synthetic retinoids, do cause limb dysmorphogenesis in rat WEC +48 h. Differences between *in vitro* and *in utero* susceptibilities for the retinoid congeners could, on one hand, reflect maternal or placental effects (Steele, Marlow et al. 1987). On the other hand, an analysis of five retinoids tested in rat WEC showed dysmorphologies resembling the *in vivo* phenotypes. The no observable adverse effective concentrations (NOAECs) ranged from 0.03 nM to 29.7 µM and correlated well with the teratological lowest observable adverse effect levels (LOAELs) observed *in vivo* (Bechter, Terlouw et al. 1992). As such, the teratogenic action of retinoids on the embryo can be largely attributed to direct effects on the embryo and in the absence of maternal factors. Further evidence that the limb-bud itself is a direct target of retinoid teratogenicity comes from *in vitro* models such as whole organ culture, where precartilage rudiments differentiate into cartilage rudiments that inform organ-level phenotypes, and high-density micromass cultures, which enable chondrogenesis to occur *de novo* from dissociated mesenchymal cells informing tissue-level phenotypes. For example, isotretinoin, ATRA and synthetic retinoids (Ro 10-1670, Ro 11-1430) showed a persistent and dose-dependent inhibition of chondrogenic differentiation in both systems (Zimmermann and Tsambaos 1985).

B.6.5. ATRA homeostasis in the developing limb

295. Limb reduction defects induced by a high teratogenic dose of ATRA (200 mg/kg on GD12.5 mouse) correlated with elevated retinoids within 30 min, peaking at 2 hr then falling sharply by 4 hr (Ward and Morriss-Kay 1995). Analysis of GD 11 mouse limb buds found retinol as the predominant retinoid, although ATRA was enriched over the rest of the embryo for several hours after maternal dosing. The elevation in ATRA content peaked 50-fold over basal (endogenous) levels following a weak teratogenic dose of 10 mg/kg ATRA, and 300-fold after a fully phocomelic dose of 100 mg/kg (Satre and Kochhar 1989). Because teratogenic doses of exogenous ATRA (10 to 100 mg/kg) may result in many-fold higher elevations in ATRA versus endogenous levels, the equivalence of exogenous retinoid teratogenesis to physiological retinoid signaling has been cautioned (Ghyselinck and Duester 2019).

296. Labeled ATRA was shown to differentially accumulate in regions of the embryo that express cellular retinoic acid binding protein (CRABP), including the developing limb (Hatori, Shigematsu et al. 1991). CRABP function has been implicated in buffering ATRA availability in tissues where ATRA activity is required to be low (Dolle, Ruberte et al. 1990) or where ATRA gradients are important in patterning (Vaessen, Meijers et al. 1990). Indeed, the potency of various retinoid congeners to cause anterior-

posterior pattern respecification correlated well with their ability to bind CRABP in both limb development (chick) and regeneration (axolotl) (Maden, Ong et al. 1989). Both CRABP-I and CRABP-II are structurally conserved phylogenetically and subject to cell-specific regulation ontogenetically in the chick limb-bud (Maden, Ong et al. 1988 , Maden 1994) and regenerating amphibian limb (Scadding and Maden 1994). *CrabpI* transcript abundance inversely correlated with tissue-specific susceptibility to retinoid-induced teratogenesis, consistent with a buffering role (Ruberte, Friederich et al. 1992). Due to a RARE sequence the *Crabp1* locus is ATRA-inducible (Kleinjan, Dekker et al. 1997). However, homozygous mice carrying deletions of *CrabpI* alleles are normal, indicating that CRABP-I does not play a crucial role in ATRA signaling (Gorry, Lufkin et al. 1994). This is in contrast to functional loss of CRABP-II, where *CrabpII(-/-)* mice show a developmental defect of the forelimb, specifically an additional, postaxial digit at an incidence that varies with genetic background, from 10% in outbred CD1 mice to 30% and 50% for inbred strains C57BL/6 and 129Sv, respectively (Fawcett, Pasceri et al. 1995). Aside from this minor defect (polydactyly), mice deficient in CRABP-II (or in both CRABP-I and -II) are otherwise normal. Furthermore, *CrabpI(-/-)* and *CrabpII(-/-)* double knockouts are not more sensitive than wild-type embryos to ATRA excess treatment. Thus, CRABP-I and CRABP-II are dispensable to mouse development and none of the functions previously proposed for CRABPs are important enough to account for their evolutionary conservation (Lampron, Rochette-Egly et al. 1995).

B.6.6. ATRA kinematics and receptor biology

297. The concentration of free ATRA calculated from kinematic coefficients and dissociation constant (Kd) of murine nuclear retinoic acid receptors (RARs) suggests a small dynamic range in the mouse limb-bud, perhaps as low as two-fold with regards to physiological function (Scott, Walter et al. 1994). Endogenous retinoids were measured in the GD 10.5 mouse embryo both before and 3h after maternal exposure to a fully teratogenic dose of ATRA (100 mg/kg) (Horton and Maden 1995). Those results, when compared to endogenous ATRA levels, showed increases of >100-fold in the spinal cord (0.12 → 21.3 µM after treatment); >400-fold in the limb-buds (0.03 → 12.5 µM); and >1000-fold in the forebrain (<0.01 → 10.0 µM). As such, these embryos treated to induce malformations experienced transitory exposures to massive doses of ATRA. Based on affinity constants RARs would be saturated throughout the limb-bud during both normal and teratological extremes. The more important regulator may be the AER-FGF-CYP26B1 module that maintains an ATRA-free distal zone for FGF-driven outgrowth (noted earlier).

298. Also as noted in earlier sections, the phenotypic abnormalities observed in compound null mutants of RARs indicate functional redundancy between RARα, RARβ and RARγ (Lohnes, Mark et al. 1995). Although limb defects have not been described in VAD rats, they do appear in RARα/RARγ double null mutant mice (Lohnes, Mark et al. 1994). Those phenotypes include reductions in the scapula and radius (preaxial hemimelia), with weaker sensitivity of the corresponding hindlimb elements, and may reflect the need for ATRA signaling to control the proper amount of limb mesenchyme. It is noteworthy that the limb phenotypes displayed by RARα/RARγ double null mutant mice and by RARβ/RARγ double null mutant mice recapitulate what is observed in (vitamin A sufficient) fetuses lacking RDH10.

299. During mouse limb development RARα and RARγ (not RARβ) are uniformly expressed at GD 10 (Dolle, Ruberte et al. 1989). Overexpression of RARα in transgenic animals caused appendicular skeletal defects by blocking chondroblast differentiation of the precartilage anlagen, which could be released with pharmacological antagonists of retinoid signaling (Weston, Rosen et al. 2000). A constitutively active RARα transgene in mice resulted in marked limb defects that recapitulated many features of exogenous retinoid teratogenesis, pinpointing defective chondroblast differentiation as a failure to properly downregulate RARα (Cash, Bock et al. 1997). RARγ transcripts in the developing mouse limb were uniformly distributed through GD 11.5, then found in precartilage condensations (GD 12.5) and cartilages (GD 13.5 onward) (Ruberte, Dolle et al. 1990). An antisense oligodeoxynucleotide

to knockdown RARγ function promoted chondrogenesis in limb micromass culture (Motoyama and Eto 1994). These results indicate that RARα and RARγ play a role in the formation of precartilage rudiments and are downregulated during overt chondrogenic differentiation. This is consistent with the teratological findings pinpointing the critical effect of exogenous ATRA to the stage of overt chondrogenic differentiation *in vivo* and *in vitro.*

300. In contrast, the role of RARβ is more confined to regions of the embryo that are susceptible retinoid teratogenesis. Administration of a fully phocomelic dose of ATRA to pregnant mice on GD 11 induced RARβ transcripts to a greater extent in the limbs versus RARα and RARγ, or versus the embryo proper (Harnish, Jiang et al. 1992). Adverse effects on skeletal pattern correlated with the timing of retinoid exposure and the sequence of mesenchymal condensation. Ectopic RARβ promoter activity was detected within 2 h of exposure to ATRA and preceded alterations in the precartilage anlagen (Wood, Ward et al. 1996). Forelimb buds cultured from GD 12.5 mouse embryos in the presence of a RARγ-selective agonist (BMS-189961) showed reduction of chondrogenic progression and increased RAREβ2-lacZ reporter proximally (Galdones and Hales 2008). Together, these findings indicate that RAR-ATRA complexes play a role in position-dependent patterning of the limb skeleton during normal development, where RARβ isoforms block limb mesenchymal cells from expressing their inherent chondrogenic bias (Jiang, Soprano et al. 1995). While these findings support the notion that retinoid-induced RARβ expression plays a unique role in teratogenesis, other findings have shown that induction of RARβ alone is not sufficient to explain the phenotypes, given redundancy in the RAR system (Luo, Pasceri et al. 1995), and that several isoforms of the RARβ gene are independently regulated during limb development (Smith, Kirstein et al. 1995).

301. Homeobox genes are RAR targets and key regulators of outgrowth and pattern formation during limb development and regeneration. Several studies have shown ATRA-induced dysregulation of Hox gene expression in the developing limb. For example, VAD rat embryos harvested at the 35-somite stage showed reduced expression of *Hoxd12* and *Hoxd13* (Power, Lancman et al. 1999), and mouse embryos exposed to ATRA at a similar stage showed alterations in *Hoxd11* and *Hoxd13* expression prior to a delay in the formation of digital rudiments (Wood, Ward et al. 1996). Reciprocal changes in *Hoxd13* and *RARβ* expression have been observed with ATRA-soaked beads applied to chick limb-buds, again affecting distal skeletal elements (Hayamizu and Bryant 1994). Finally, the synthetic retinoids TTNPB (RAR agonist) and LG69 (RXR agonist) both induce *Hoxb6, Hoxb8* and RARβ in the chick limb-bud (Lu, Eichele et al. 1997). Although normal functions of ATRA cannot be directly inferred from pharmacological studies alone, the evidence from normal and teratological studies implicates ligand bound RAR/RXR as key molecular determinants in both scenarios.

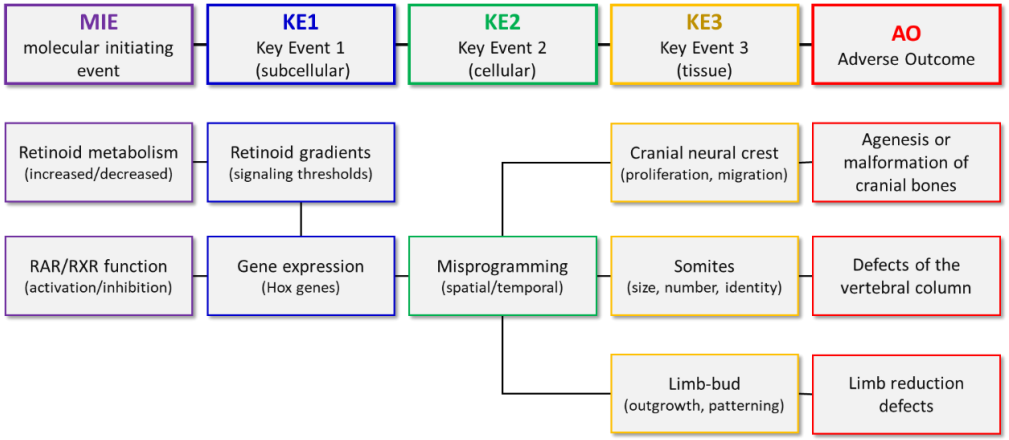
302. Polycomb-group genes in *Drosophila* maintain appropriate Hox codes, and mice null for a polycomb-group gene (*M33*) showed homeotic transformations of the axial skeleton and limb malformations aggravated by exogenous ATRA at GD 7.5 (Core, Bel et al. 1997). These results suggest that M33 defines access to RAREs in the regulatory regions of several Hox genes. Mathematical modeling and simulation show that ATRA forms a mutual antagonistic loop with repressive polycomb group factors in the distal forelimb bud as transcriptional repressors that mediate epigenetic gene silencing by chromatin modification (Yakushiji-Kaminatsui, Kondo et al. 2018).

B.7. Adverse Outcome Pathway (AOP) framework

303. Performance-based models that address the regulation, homeostasis, and biological activity of the retinoid signaling pathway will be useful for predictive toxicology based on alternative (non-mammalian) tests. An AOP framework is necessary to organize the relevant data, information and knowledge on molecular initiating events (MIEs) that reflect a disruption in retinoid signaling at critical stages of gestation, and the ensuing cascade of key events (KEs) and their relationships (KERs) that lead to adverse outcomes (AOs) of regulatory value in toxicology. Developmental defects in the craniofacial, axial, or appendicular skeletal systems would qualify, as they are readily observed in a traditional prenatal developmental toxicology test guideline study (e.g., OECD TG 414).

304. Both too little and too much vitamin A can have negative consequences on patterning the skeleton. Human VAD is an endemic nutrition problem throughout much of the developing world (West 2003). Recently, the gut microbiome has been identified as a new player in vitamin A metabolism. Commensal and pathogenic bacteria may drastically alter the patterns of vitamin A uptake in the host gut (Iyer and Vaishnava 2019). Genetic errors, synthetic retinoids, or environmental factors can perturb the transport, metabolism, or utilization of endogenous ATRA during critical times in development. Humans and animals may be exposed to compounds in the natural environment that can invoke AOPs for the retinoid system that mimic a state of ATRA deficiency or excess. The putative list of AOPs may be neither complete nor comprehensive but point to the relevant developmental biology from which the mechanistic toxicology can be better understood. Three examples are shown to represent diverse MIEs and specific phenotypes affecting the facial, vertebral, or appendicular skeleton. (Figure B.5).

Figure B.5. Framework and examplels of potential AOPs for skeletal dysmorphogenesis linked to disruption of retinoid signaling.



REGION	*MIE*	*KE1*	*KE2*	*KE3*	*KE4*	*KE5*	*AO*
Anterior Neural Tube	Inhibition of CYP26A1 enzymatic activity	Local increase in endogenous ATRA levels	Hyperactivation of the RAR/RXR heterodimer	Repression of *Fgf8* limits FGF8 signaling	Mis-specification of CNC cell fate and behavior	Maxillary arch dysplasia alters palatal outgrowth	Cleft palate
Paraxial Mesoderm	Reduction in RDH/RALDH2 activity	Local decrease in endogenous ATRA levels	Hypoactivation of the RAR/RXR heterodimer	Overextension of FGF8 signaling	Disruption of the periodic somitic wavefront	Altered somite number, shape, and alignment	Hemivertebra
Limb-Bud Mesoderm	Hyperactivation of the RAR/RXR heterodimer	Underextension FGF8 signaling from the AER	Dysregulation of *Meis1/2* and *Hox* gene expression	Proximalization of the limb-bud mesenchyme	Mis-specification of precartilage blastema	Malformed cartilaginous bone rudiment	Phocomelia

TOP: General framework. MIE, Molecular Initiating Event; KE, Key Events upstream to downstream; AO, Adverse Outcome. BOTTOM: Specific examples for three skeletal domains. MIEs may include, for example: vitamin A deficiency, chemical inhibitors of RALDH2 or CYP26 enzymatic activity/expression; pharmacological agonists/antagonists of RAR or partner receptor signaling pathways (e.g., RXR/PPARγ); agents that modulate RAR/RXR binding to RARE sites. Subcellular KEs may be reflected in critical imbalances to local ATRA concentration or threshold responses leading to changes in Hox patterning and other molecular determinants of cell lineages. Cellular KEs entail developmental programming of undifferentiated progenitors of body axis determinants, depending on the stage of gestation and region of the embryo affected. Tissue KEs reflect the collective behavior of target cells directed by heterotypic interactions for rudimentary organs. AOs reflect the phenotypes resulting form stage and positional alterations in the fetal skeleton. Figure from (Knudsen, Thomas et al., 2021), used here with the kind permission from the publisher, Elsevier.

B.7.1. Candidate AOPs for retinoid-induced skeletal defects

305. Subtle disturbances in ATRA homeostasis *in vivo* dramatically alter fetal morphology. This has been widely demonstrated in genetically engineered mice (Chambon 1996), in animal models of developmental toxicity (Kochhar 2000), and in human subjects (Lammer, Chen et al. 1985). Table B.3 lists estimates for ATRA levels across different developmental systems, indicating the potential ranges of perturbation experimental perturbations.

306. Whereas VAD animals and RAR gene 'knockout' mice indicate that ATRA performs essential functions in skeletal development, different AOP structures will be specific to the stages and sites in the embryo where a presence of endogenous ATRA is critical. One strategy is the intervention in RAR-dependent MIEs at specific developmental stages by means of synthetic retinoids that act as highly effective RAR agonists (e.g., AGN 190121) or antagonists (e.g., AGN 193109). For example, the following putative AOP can explain midfacial defects in the mouse linked to functional retinoid deficiency induced with 1 mg/kg AGN 193109 on GD 8.0 (Kochhar, Jiang et al. 1998): RARα/γ antagonism (MIE) → ↓RARE-driven gene expression (KE1) → altered CNC-patterning (KE2) → frontonasal dysplasia (KE3) → midfacial defects (AO). Alternatively, RAR antagonism or antagonism of retinoid function may take a different path with KE's on CNC cells migrating to the caudal branchial arches that would explain the defects observed with the pan-RAR inverse agonist, BMS493 (Mark, Ghyselinck et al. 2006).

Table B.3. Tissue levels of ATRA reported across different experimental systems

ATRA Dosimetric	ATRA conc.	Indication	Reference
baseline ATRA (5 somite zebrafish embryo)	< 1 nM	non-morphogenetic	Shimozono *et al.* 2013
maternal serum (animal study)	1.7 nM	non-teratogenic	Daston *et al.* 2014
devTOX^{qp} assay (pluripotent hESC)	3.0 nM	teratogenic threshold	Zurlinden *et al.* 2020
normal plasma concentration	5.0 nM	physiological (adult)	Napoli *et al.* 1991
axial gradient (5 somite zebrafish embryo)	6.0 nM	morphogenetic signal	Shimozono *et al.* 2013
endodermal differentiation (h-iPSC)	17 nM	toxicological tipping point	Saili *et al.* 2019
devTOX^{qp} assay (pluripotent h-iPSC)	19 nM	DevTox threshold	Palmer *et al.* 2017
genetic perturbation (mouse)	30 nM	altered homeostasis	Helms *et al.* 1994
maternal serum (animal study)	30 nM	teratogenic potential	Daston *et al.* 2014
limb-bud (GD 10.5 mouse embryo)	30 nM	physiological (embryo)	Horton and Maden 1995
pharmacological kinetics	1,000 nM	efficacious (therapeutic)	Helms *et al.* 1994
limb-bud (GD 11 mouse embryo)	1,500 nM	weakly teratogenic dose	Satre and Kochhar 1989
limb-bud (GD10.5 mouse embryo)	12,500 nM	fully teratogenic dose	Horton and Maden 1995

307. Other putative AOPs are needed to explain adverse outcomes linked to non-retinoid disruption of ATRA homeostasis. Citral, an inhibitor of retinol and retinaldehyde dehydrogenases as well as other alcohol and aldehyde dehydrogenases, induced a specific loss of derivatives from the maxillary prominences in the chick embryo that may in part result from disruption of endogenous ATRA synthesis (Shimomura, Kawakami et al. 2015). Ethanol, as a competitive inhibitor of RALDH2, may invoke ATRA deficiency causing phenotypes of the midface in the chick embryo (Kiecker 2016), and in rodent models for human Fetal Alcohol Spectrum Disorder (FASD) (Petrelli, Bendelac et al. 2019), through acetaldehyde formation (Shabtai, Bendelac et al. 2018). Glyphosate-based herbicides increased endogenous ATRA activity in a reporter assay and induced cranial defects in *Xenopus* embryos observed at the tadpole stage. These defects were ameliorated with RAR antagonists and infer a disruption of CNC development following elevated endogenous ATRA content (Paganelli, Gnazzo et al. 2010); however, it is unclear whether thyroid hormone could also have played a role in this phenomenon. Tributyltin, an antifoulant widely found industrially as a contaminant of dibutyltin in vinyl plastics, binds the RXR and causes retinoid-related craniofacial skeletal deformities in fish embryos (Zhang, Zuo et al. 2012); however, there are deficiencies in this putative AOP because tributyltin has no activity on RARs and the RAR-RXR heterodimer is not permissive to RXR activation.

308. Inhibition of ATRA degradation has been suggested as a mechanism to explain the dysmorphogenic effects of some triazole fungicides on hindbrain segmentation and branchial arch development in cultured rodent embryos that has been hypothesized to result from a local increase of endogenous ATRA (Menegola, Broccia et al. 2006). For example, rat embryos exposed to flusilazole or fluconazole at the 1-3 somite stage displayed ATRA-like dysmorphogenesis of rostral branchial arches (1^{st}, 2^{nd}, 3^{rd}) with concentrations above 6 µM and 60 µM, respectively (Menegola, Broccia et al. 2001). The antifungal properties of azole-derivatives is based on interference with the iron-porphyrin group in cytochrome P450 (CYP51, lanosterol C14 alpha-demethylase) that is required in the synthesis of fungal cell walls; however, this inhibition is not restricted to fungi and also occurs in mammalian CYP450-dependent activities, including CYP26 isozymes that are expressed in the embryo at the sensitive time (Menegola, Broccia et al. 2006). Evidence for a CYP26-mediated degradation effect was shown in cultured rat embryos exposed to fluconazole, where the incidence and severity of branchial arch defects was attenuated with citral (Di Renzo, Broccia et al. 2007). Since the postulated mode of action of azoles is inhibition of CYP enzymatic activity, and some ATRA-responsive patterning genes and proteins (e.g., *Msx1, TGFβ1*) were down-regulated in the branchial arch (Di Renzo, Corsini et al. 2009 , Di Renzo, Rossi et al. 2011), a likely AOP is shown in Figure B.5 (Di Renzo, Metruccio et al. 2019).

309. Congenital vertebral malformations occur in 5 to 10 per 10,000 live births (White and Goldberg 2018). Many are caused by genetic syndromes or intrauterine exposures such as hyperglycemia, carbon monoxide, or antiepileptic drugs. These phenotypes may be classified as a failure of complete vertebral formation (e.g., hemivertebrae), a failure of segmentation, or both. The former is generally attributed to asymmetric vertebral body formation often related to neural tube defects (e.g., spina bifida). Failures of segmentation are characterized by bony fusions between adjacent vertebrae or cranial-caudal border shifts and may also involve changes in ribs and sternum for the associated vertebra. An AOP framework for neural tube and axial defects via modulation of ATRA homeostasis has been outlined as a general mechanism that, when perturbed, may result in manifestations of developmental toxicity that cover a large part of malformations known to occur in humans and experimental animals (Tonk, Pennings et al. 2015). An AOP such as shown in Figure B.5 includes key genes in the regulation of retinoid homeostasis, as well as marker genes of neural tube and axial patterning, and tested against existing data of flusilazole exposure in the rat WEC, the zebrafish embryotoxicity test, and the embryonic stem cell test.

310. Some data are available regarding chemical effects on craniofacial malformations in non-mammalian species mediated through altered retinoid signaling. For example, the triazole fungicide, triademfon, has been shown to induce craniofacial malformations and specifically, alterations at branchial arch-derived cartilages, in *Xenopus laevis* embryos (Papis *et al.*, 2006). Increased expression of CYP26

was found in triadimefon treated embryos and the developmental effects were reduced by co-treatment with the ATRA synthesis inhibitor, citral (Papis *et al.* 2007). Table B.4 provides a brief summary of craniofacial and skeletal endpoints in non-mammalian species.

311. Retinoid-induced limb malformations vary in scope and severity with dose and time of exposure. For example, retinoid teratogenicity at early stages of limb-bud growth (GD 9.5 mouse) are primarily restricted to missing digits (ectrodactyly) with varying sensitivities for different inbred mouse strains (Lee, Cantor et al. 2005). A full genome scan localized the susceptibility loci to chromosome 11 (near D11Mit39) syntenic to human Meckel syndrome that includes digital phenotypes. Reports of hindlimb malformations in frogs across North America suggested a likely consequence of retinoid disruption (Degitz, Kosian et al. 2000). Subsequent findings, however, linked the etiology to natural parasites (trematodes) in combination with agricultural runoff (e.g., triazine herbicides, organophosphate insecticides, and synthetic pyrethroids) that decreased the host tadpole's ability to resist parasitic infection (Kiesecker 2002). As such, the AOP was not initiated by retinoid disruption but in combination with a parallel AOP linked to changes in immunity. Similarly, France in 2018 launched a nationwide investigation into incidents of babies born with missing or malformed forelimbs. The outcome of the investigation is not known (France24, 2018). In the absence of known etiology (genetics, retinoids, other), the forelimb-specific limb reduction defects seen in *Rdh(-/-)* mutant mice may indicate a shared downstream KE linked to retinoid deficiency.

312. Target cell populations in an AOP will vary based on stage of development, timing and degree of MIE perturbation required to invoke a change. The window of vulnerability for retinoid-induced phocomelia (e.g., GD 10-12) follows later in gestation than for axial defects (e.g., GD 6.5 - 8.5). This temporal factor is further dependent on the type and nature of adverse outcomes. Of importance is the notion that an ATRA-FGF8 antagonistic gradient is a common KE to many potential AOPs. A potential AOP for phocomelia is shown in Figure B.5.

313. AOP frameworks are an important tool for developmental hazard evaluation because several chemically distinct classes of developmental toxicants may invoke features of a Fetal Retinoid Syndrome, including anticonvulsants (e.g., valproic acid), some triazole fungicides (e.g., fluconazole), organic metals (e.g., tributyl tin), organophosphates (e.g., aldrin), and flame retardants (e.g., PBDEs). Given the complex relationships noted earlier, a generalized AOP framework for retinoid biology and teratogenesis is highly desirable, but seriously constrained by the complexity of the system and its crosstalk with other morphogenetic signaling pathways and cellular behaviors. Despite the abundance of information on some chemicals, only two literature reports refer to an AOP for the retinoid system (Tonk, Pennings et al. 2015 , Baker, Boobis et al. 2018) and searching the AOPWiki[17] for 'retinoic acid' or 'retinoid' returned key events for only 5 AOPs (#ID = 43, 297, 7, 37, 38) at the present time.

B.7.2. New Approach Methodologies (NAMs)

314. Opportunities exist for refining and supplanting current developmental toxicity testing protocols using *in vitro* data and in silico models in the design and review of revolutionary alternatives to animal testing by experts in the field, alongside their independent validation (Scialli, Daston *et al.* 2018). New Approach Methodologies (NAMs) is the term adopted as a broadly descriptive reference to any technology, methodology, approach, or combination thereof that can be used to provide information on chemical hazard and risk assessment that avoids the use of intact animals (EPA 2018). NAMs would include methods that evaluate hazard (human health and environmental), exposure, and environmental fate as well as integrative approaches for decision making such as AOPs and integrated approaches to testing and assessment (IATA). Technology platforms include, for example automated *in vitro* assays for high-throughput screening (HTS) and *in silico* computational toxicology models to reconstruct complex pathways. For example, 8 *in vitro* HTS assays for some components in the retinoid system are included in

[17] https://aopwiki.org/

the US ToxCast/Tox21 program (Figure B.6). Briefly, the ToxCast NovaScreen assay (NVS_ADME_hCYP1A1) is a cell-free biochemical assay using a type of enzyme reporter to measure loss-of-signal enzymatic activity (AC50) as it relates to human cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1). Loss of fluorescence intensity signals produced from an NADP-dependent enzymatic reaction involving a fluorogenic substrate for cytochrome P450-linked enzymes substrate, Resorufin Benzyl Ether. There are high-priority deficiencies in the portfolio. For example, assays described in the literature but lacking in ToxCast/Tox21 include dehydrogenases for retinol bioactivation (RDH, RALDH2) (Schindler, Berst et al. 1998; Koppaka, Thompson et al. 2012; Thomas, de Antueno et al. 2016 , Attignon, Distel et al. 2017) and oxidoreductases for retinoic acid breakdown (CYP26 a/b/c) (Mulvihill, Kan et al. 2006). As shown earlier, these are critically important for anterior (RALDH2) and posterior (CYP26) development of the fetus and may predict susceptible stages for developmental toxicity. Although HTS assays are not available for CYP26a/b/c in ToxCast/Tox21, CYP1A1 effectively degrades ATRA in a cell free biochemical surrogate assay. The Attagene (ATG) assays track changes in transcription factor activity utilizing a library of multiplex reporter transcription unit constructs. In this case, RAR/RXR heterodimers bind to their cognate gene response element, RARE (retinoic acid receptor elements) in this case. Individual transcription units are essentially barcoded to particular receptor. The HepG2 human hepatoma cell line used in this platform retains the potential for Phase I and Phase II metabolic responses to xenobiotics (e.g., expression of various Cytochrome P450s) at levels similar to mature hepatocytes. Finally, DR5 is a cis-regulated assay for the direct repeat element in RARE binding sites and is broadly responsive to chemical changes that increase RARE-dependent transcription. As a cautionary principle, use of these and other HTS data must consider the methodological, platform, reagent differences, cytotoxicity, and data quality flags. Individual ToxCast assay results provide only one piece of a complex puzzle and must be considered within the larger ToxCast/Tox21 data context in order to advance understanding of potential chemical hazards and their mechanisms (Houck, Judson *et al.* 2017). Less complete information is available on disruption of retinoid transport, such as serum transporters (e.g., TTR), molecular transporters (e.g., STRA6), and intracellular binding proteins (e.g., CRABP).

315. A preliminary analysis of 1983 ToxCast Phase I/II chemicals across the 8 assay systems identified 97 as having an AC50 <2 µM in one or more of these assays (Figure B.6). Among those on the list were persistent organic pollutants that preferentially activated RARs (e.g., aldrin, dieldrin) and tert-butyl compounds and organotins that preferentially activated RXRs (e.g., butylphenol, tributyltin). These findings are consistent with results from targeted studies in the literature (Lemaire, Balaguer et al. 2005; Le Maire, Grimaldi et al. 2009). Other examples include retinoids themselves (ATRA, retinol), anticonvulsants (valproic acid), triazoles (fluconazole), and flame retardants (PBDEs) (Baker, Boobis et al. 2018). In addition, some mitochondrial disrupters displayed activity on DR5 (e.g., strobins, rotenone) that may reflect a dependence of the response on bioenergetics (Le Maire, Grimaldi et al. 2009). Note that the chemicals may have activity over multiple types of assays that are not necessarily related to retinoid receptors or signaling.

Figure B.6. Case examples in the class distribution of ToxCast chemicals available in the current HTS assay portfolio for profiling the retinoid system.

DSSTOXID	PREFERRED_NAME	CYP1A1 (72)	RARa (65)	RARb (17)	RARg (49)	RXRa (69)	RXRb (299)	RXRg (0)	DR5 (250)
DTXSID7021239	all-trans-Retinoic acid	1.317	NA	NA	NA	NA	1.036	NA	0.006
DTXSID1040619	Bexarotene	NA	7.539	NA	2.655	0.009	0.009	NA	0.014
DTXSID3023556	Retinol	NA	0.076	NA	0.227	2.142	0.464	NA	0.197
DTXSID1020807	2-Mercaptobenzothiazole	0.164	NA	NA	NA	NA	NA	NA	NA
DTXSID2040363	Diniconazole	0.674	NA	NA	NA	NA	NA	NA	NA
DTXSID0032655	Triticonazole	0.793	NA	NA	NA	NA	NA	NA	16.741
DTXSID8024151	Imazalil	1.413	0.908	NA	NA	NA	NA	NA	5.888
DTXSID4032372	Difenoconazole	1.459	NA	NA	NA	NA	NA	NA	2.124
DTXSID3023897	Triademifon	2.085	41.462	NA	NA	NA	NA	NA	11.223
DTXSID7029871	Clotrimazole	2.306	NA	NA	NA	NA	NA	NA	NA
DTXSID3024235	Flusilazole	3.704	8.155	NA	NA	NA	NA	NA	7.718
DTXSID2032500	Triflumizole	4.134	1.453	NA	NA	NA	NA	NA	0.298
DTXSID0021337	Thiabendazole	4.721	NA	NA	NA	NA	NA	NA	NA
DTXSID8024280	Propiconazole	9.010	23.801	NA	NA	NA	NA	NA	6.253
DTXSID9020453	Dieldrin	NA	0.770	NA	1.679	NA	22.531	NA	0.579
DTXSID9037539	Endosulfan I	NA	1.384	NA	NA	NA	NA	NA	1.827
DTXSID6020561	Endrin	NA	NA	1.606	1.698	NA	24.982	NA	0.806
DTXSID1020560	Endosulfan	NA	NA	NA	NA	NA	NA	NA	0.894
DTXSID7020267	Chlordane	NA	NA	NA	6.878	71.470	21.422	NA	1.784
DTXSID7042065	Isodrin	NA	NA	NA	1.077	NA	NA	NA	2.111
DTXSID8020040	Aldrin	NA	NA	NA	0.912	NA	7.167	NA	3.085
DTXSID3042500	Triphenyltin fluoride	NA	NA	NA	NA	0.004	0.001	NA	0.655
DTXSID5034981	Tributyltin benzoate	NA	NA	NA	NA	0.005	0.036	NA	0.023
DTXSID9044796	(Acryloyloxy)(tributyl)stannane	NA	NA	NA	NA	0.015	0.026	NA	0.022
DTXSID2040733	Triphenyltin chloride	NA	NA	NA	NA	0.081	0.037	NA	0.356
DTXSID9035204	Tributyltin methacrylate	NA	NA	NA	NA	0.147	0.025	NA	0.005
DTXSID3027403	Tributyltin chloride	NA	NA	NA	NA	0.176	0.078	NA	0.003
DTXSID4022153	Tetrabutyltin	NA	NA	NA	NA	0.741	0.033	NA	0.279
DTXSID1021409	Triphenyltin hydroxide	NA	NA	NA	NA	NA	0.013	NA	NA
DTXSID9040712	Triethyltin bromide	NA	NA	NA	NA	4.029	0.252	NA	NA

Distribution from chemical hits (n=261) having AC50 < 2 µM in one or more of the 8 ToxCast assays mapping to potential nodes in ATRA signaling (Baker, Boobis et al. 2018). Each assay target is indicated with the number of chemical his registered in the EPA CompTox Chemicals Dashboard[18] (last accessed October, 2020). CYP1A1: NVS_ADME_hCYP1A1. RARs: ATG_RARa_TRANS_up, ATG_RARb_TRANS_up, ATG_RARg_TRANS_up. RXRs: ATG_RXRa_TRANS_up, ATG_RXRb_TRANS_up, ATG_RXRg_TRANS_up. DR5: ATG_DR5_CIS_up. As might be expected the DR5 assay lights up several potential RAR/RXR combinations. First group: vitamin A (retinol) and retinoid ligands (ATRA/RAR, Bexarotene/RXR) references. Second group: Triazole effects on biochemical activity of CYP1A1 as a surrogate for CYP26 isoforms; malformations, vertebral transformations, and caudal regression are linked to CYP26 inhibition (Menegola, Broccia et al. 2001, Kamata, Shiraishi et al. 2008, Tonk, Pennings et al. 2015). Third group: several organochlorine pesticides of a persistent nature have weak RARg-agonist activity and can transactivate retinoid-responsive genes (e.g., CYP26A1) via RARE (Lemaire, Balaguer et al. 2005, Kamata, Shiraishi et al. 2008). Fourth group: several organotin biocides are known to preferentially bind RXRs with nM affinity, but forms a non-permissive RAR/RXR heterodimer (Grun, Watanabe et al. 2006, Brtko and Dvorak 2015).

[18] https://comptox.epa.gov/dashboard

316. The complex nature of the retinoid system means that a complete battery of *in vitro* assays capturing all potential points of retinoid disruption, biological information, and pathway-level crosstalk is unlikely to emerge in the near term. Computational modeling will be necessary to build data-driven models for predictive toxicology and quantitative simulation, similar to what has been done for the estrogen signaling pathway (Browne, Judson *et al.* 2015, Judson, Magpantay *et al.* 2015, Kleinstreuer, Ceger *et al.* 2017);(Juberg, Knudsen *et al.* 2017).. A multistep, performance-based computational model has been developed for 18 estrogen receptor (ER) targets in ToxCast/Tox21 that sufficiently discriminate estrogenic bioactivity from assay-specific interference and cytotoxicity. These assays quantify ER binding, dimerization, chromatin binding, transcriptional activation, and ER-dependent cell proliferation (Judson, Magpantay et al. 2015). In pilot studies, the ER model was validated using 43 reference chemicals based on the uterotrophic assays (30 active, 13 inactive). Next, the ER agonist/antagonist potential of 1812 commercial and environmental chemicals was quantified. Recent publications (Browne, Judson et al. 2015 , Kleinstreuer, Ceger et al. 2017) demonstrate that the ToxCast ER model performed as well or better than the US EPA's Endocrine Disruptor Screening Program (EDSP) Tier 1 ER binding, ER transactivation assay (ERTA) and uterotrophic assays, with a reported accuracy of 84–93% or better, if inconclusive scores are excluded from the dataset (Browne, Judson et al. 2015; Judson, Magpantay et al. 2015; Kleinstreuer, Ceger et al. 2017). This comparison benefitted from positive effects in the uterotrophic assay being largely ER-mediated.

317. Retinoid signaling-related modeling may prove to be more challenging due to the levels of complexity involved. However, several findings show promising outcomes in a predictive model for retinoid signaling:

a RAR was top weighted feature in an early ToxCast (phase I) predictive model for ToxRefDB (Sipes, Martin et al. 2011)

b ATRA ranked #1 in potency across 1065 chemicals tested in a human embryonic stem cell platform (PoD = 10 nM) with up to 84% concordance to animal models for human developmental toxicity (Zurlinden, Saili et al. 2020)

c RNAseq profile of human iPSC cell differentiation provides a detailed molecular characterization of a toxicological tipping point for retinoid signaling and endodermal toxicity (Point of Departure = 17 nM) (Saili, Antonijevic et al. 2019); and

d the retinoid signaling pathway also turned up in ToxCast signatures for male reproductive development (Leung, Phuong et al. 2016), cleft palate (Baker, Sipes et al. 2019), and digital paw defects (Ahir, DeWoskin et al. 2019).

318. Translation of exposure-based hazard predictions can utilize *in silico* prediction tools such as P&G's DevTox 'SAR decision tree' (Wu, Fisher et al. 2013) and the pregnancy HTTK model and chemotype information (Lumen, Chang et al. 2020). For these models, it is useful to have knowledge of ATRA concentrations/levels in the various systems (Napoli, Posch et al. 1991; Helms, Thaller et al. 1994; Shimozono, Iimura et al. 2013; Daston, Beyer et al. 2014; Palmer, Smith et al. 2017; Saili, Antonijevic et al. 2019; Zurlinden, Saili et al. 2020).

B.8. Conclusions

319. This review underscores the importance of ATRA homeostasis to patterning and differentiation of the fetal skeleton. These pathways are complex and connected directly or indirectly to morphogenetic signaling. A critical role of ATRA signaling during gastrulation and early organogenesis influences regional specification and fate of precursor cell populations in the cranial neural crest, paraxial mesoderm, and lateral plate mesoderm. To date, no OECD test guidelines specifically capture the retinoid system for toxicity screening and evaluation. While retinoid disruption may contribute to any number of skeletal endpoints observed in a regulatory guideline developmental toxicity study (e.g., OECD TG 414), related AOP tracks could also explain similar outcomes. This raises the question of whether the ATRA system is a primary target, secondary affiliate, or unrelated to the outcome. Unraveling this complexity will be important for NAMs that may use *in vitro* data and in silico models for predictive DART. Perturbations evaluated in a prenatal developmental toxicity study conducted for regulatory assessment (Table B.4) include a number of adverse outcomes, such as: altered growth trajectories, functional and behavioral deficits, and structural abnormalities. These abnormalities encompassed both malformations (permanent structural changes that may adversely affect survival, development, or function, sometimes referred to as teratogenicity) and variations (used to indicate a divergence beyond the usual range of structural constitution that may not adversely affect survival or health). The regulatory guidelines further state that distinguishing between variations and malformations is difficult, given the continuum of response from the normal to the extremely deviant. In addition, xenobiotics can affect multiple molecular pathways so it is not clear how any skeletal defects found will be strictly attributable to or exclusively to an ATRA-signaling-related mechanism. This classic ‘one-to-many problem’ in bioinformatics (e.g., one MIE can diverge into many adverse outcomes; many MIEs may converge onto one adverse outcome) inspires several types of modeling activities for integration and synthesis of retinoid endpoints (including measurement of retinoids and chemical dosimetry).

320. An AOP framework will be a central resource for informing AOP-based integration of the biology-toxicology and subsequent parameterization of performance-based models for predictive toxicology of the retinoid system and skeletal development. Some information is already available for profiling the retinoid system *in vitro* (derived from ToxCast/Tox21 datasets) and building performance-based models to predict skeletal dysmorphogenesis *in vivo* (derived from the literature and ToxRefDB_v2 databases). There is a need to further develop and/or select assays that reflect the known biology of the retinoid signaling system and can be reproduced across laboratories as a fundamental requirement of validation. Such assays should also require corroboration by independent experts.

Table B.4. Endpoints in existing OECD Test Guidelines that may detect chemicals that alter retinoid pathway signalling.

Retinoid effects	OECD Test Guideline (TG)	Comments
Craniofacial and skeletal systems (mammalian)		
Status of craniofacial and skeletal systems	OECD 407-413: Repeated dose toxicity studies OECD 451-453: Chronic toxicity and carcinogenicity studies	Hematology, clinical chemistry, gross necropsy, histopathology
Development of craniofacial and skeletal systems, structural abnormalities	OECD 414: Prenatal development toxicity study	Detailed skeletal examination
Development and status of craniofacial and skeletal systems	OECD 415: One-generation reproduction toxicity study	Gross necropsy
Development and status of craniofacial and skeletal systems	OECD 416: Two-generation reproduction toxicity study	Gross necropsy, optional histopathology
Development and status of craniofacial and skeletal systems	OECD 421: Reproduction/developmental toxicity screening test	Gross necropsy, optional clinical chemistry and histopathology
Development and status of craniofacial and skeletal systems	OECD 422: Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test	Gross necropsy, hematology, clinical chemistry and histopathology
Development and status of craniofacial and skeletal systems	OECD 443: Extended one-generation reproductive toxicity study	Gross necropsy and histopathology
Craniofacial and skeletal systems (non-mammalian)		
Status of craniofacial and skeletal systems	OECD 206: Avian reproduction test	Gross necropsy
Development of craniofacial and skeletal systems, structural abnormalities	OECD 210: Fish: early life-stage toxicity test	Gross morphological abnormalities
Development of craniofacial and skeletal systems, structural abnormalities	OECD 231: The amphibian metamorphosis assay (AMA)	Hind limb and snout development, gross morphological abnormalities
Development of craniofacial and skeletal systems, structural abnormalities	OECD 241: The larval amphibian growth and development assay (LAGDA)	Gross morphological abnormalities

Note: In addition, there are also non-OECD TGs such as the 'Frog Embryo Teratogenesis Assay-Xenopus' (FETAX) test which include craniofacial, early developmental endpoints.
Source: Taken from 2017 draft DRP prepared by Brunel University/Technical University of Denmark

B.9. References

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Annex C. Retinoids and Developmental Neurotoxicity

C.1. Annex Preface

321. Retinoic acid (RA) is a critical signalling molecule involved in multiple aspects of mammalian central nervous system (CNS) development. RA, and other retinoids, bind to RAR/RXRs, leading to expression of molecules which control cellular processes, (e.g., morphogenesis, patterning, differentiation) critical for the formative steps of CNS development (e.g., hindbrain formation). Due to the importance of strict control in these processes, imbalances in retinoid signalling (excess or reduction) can lead to developmental neurotoxicity (DNT). Studies in human and mammalian/non-mammalian models suggest that RA effects are highly dependent on several factors, including compound specificity, dose, exposure duration, timing of exposure, and species tested. Environmental chemicals may cause DNT by disrupting retinoid signalling, and testing for these effects is inadequate under the current regulatory guidelines. Due to the importance of the retinoid signalling pathway for CNS development, and its implications for DNT, guidance is necessary to define the mechanisms which lead to perturbations on cellular and organ levels that translate into adverse effects. In response to the growing need to reduce animal usage and develop animal-free alternatives, *in vitro* and in silico approaches have been established to evaluate chemicals in their ability to cause DNT, and several retinoids have been evaluated in this context. Here, using a case-study approach, we review evidence of retinoid exposures and DNT in humans and animal/*in vitro* models. We provide a provisional adverse outcome pathway (AOP) for imbalances in RA embryonic bioavailability and hindbrain formation, as well as alternative models that may be used to evaluate a chemical's ability to disrupt retinoid signalling pathways and contribute to DNT.

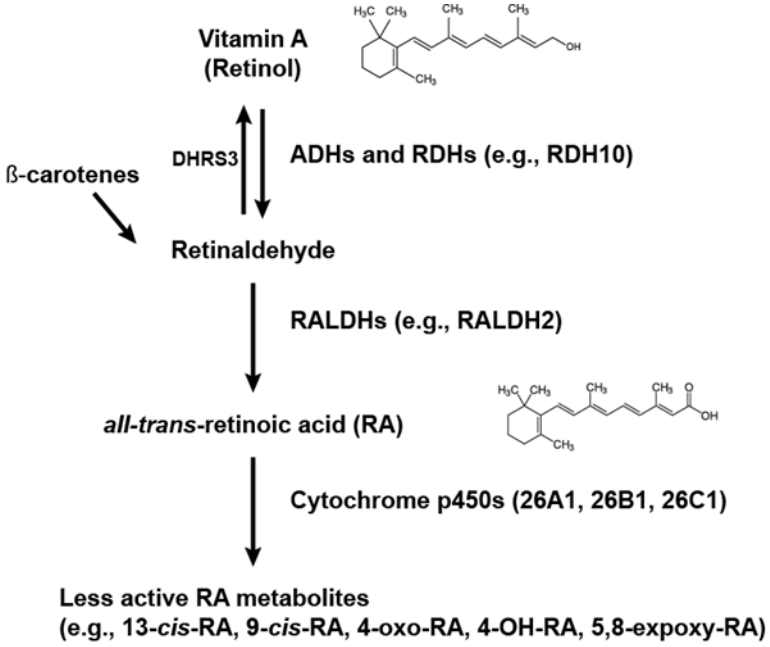
C.2. Abbreviations relevant to Annex C

13-cis-RA	Isotretinoin or 13-cis-retinoic acid
9-cis-RA	Alitretinoin or 9-cis-retinoic acid
CNS	Central nervous system
CRABP	Cellular retinoic acid binding protein
DE	Differentially expressed
DNT	Developmental neurotoxicity
hESC	Human embryonic stem cell
HOX	Homeobox
KE	Key event
mESC	Mouse embryonic stem cell
MIE	Molecular initiating event
NTD	Neural tube defect
Zf	Zebrafish

C.3. Retinoid Signaling and Metabolism in CNS Development

322. Retinoic acid (RA), also known as all-trans-retinoic acid, is required for central nervous system (CNS) development and is the active derivative of retinol (Vitamin A). Mammals are unable to synthesize RA de novo and require intake of vitamin A or other retinoid precursors through food sources which can be transformed into RA (Figure C.1; (Marceau, et al., 2007). Vitamin A is transformed into RA in two steps. First, retinol conversion to retinaldehyde is mediated by two classes of oxidizing enzymes (alcohol dehydrogenases and retinol dehydrogenases). In mouse, retinol dehydrogenase-10 (RDH10) is necessary for conversion of retinol to retinaldehyde during early embryogenesis (Sandell, et al., 2012) (Chatzi, et al., 2013). Enzymes such as Short-chain Dehydrogenase/ Reductase 3 (DHRS3) facilitate the reverse conversion of retinaldehyde to retinol (Adams, et al., 2014) (Feng, et al., 2010). In the second step, retinaldehyde is irreversibly oxidized to form RA by aldehyde dehydrogenases (ALDHs). Specifically, ALDH1A2, also known as retinaldehyde dehydrogenase 2 (RALDH2), is critical for RA synthesis during early CNS development (Niederreither, et al., 1999). The cytochrome p450 26 subfamily enzymes (CYP26A1, CYP26B1, and CYP26C1) are also necessary in regulating RA levels in the embryo and catalyzing reactions to reduce RA bioavailability by converting RA to 9-cis-RA, 4-OH-RA and other oxidized, less active metabolites (Abu-Abed, et al., 2001). Polar RA metabolites undergo glucuronidation (phase 2 metabolism) to form water-soluble metabolites which can be eliminated by the body (Samokyszyn, et al., 2000). Although less defined, other enzymes, including other CYPs (CYP1B1) (Ross & Zolfaghari, 2011) (Chambers, et al., 2007), may play secondary roles in RA metabolism during early CNS development.

Figure C.1. Retinoic acid metabolism



Note: The availability of RA is tightly controlled in the mammalian CNS. Mammals are unable to synthesize RA *de novo* and require intake of vitamin A or other precursors (β-carotenes) from food sources. Vitamin A is converted to retinaldehyde by alcohol dehydrogenases (ADHs) and retinol dehydrogenases (RDHs). In mouse, RDH10 is necessary for conversion of retinol to retinaldehyde in the developing embryo (Sandell, et al., 2012). Enzymes such as Short-chain Dehydrogenase/Reductase 3 (DHRS3) facilitates the reverse transformation of retinaldehyde to retinol (Adams, et al., 2014) (Sham, et al., 1993). Retinaldehyde is further oxidized to form RA by aldehyde dehydrogenases (ALDHs or RALDHs) in an irreversible step. RALDH2 is critical for RA synthesis during early CNS development (Niederreither, et al., 1999). Cytochrome p450 26 subfamily enzymes regulate RA levels in the embryo and catalyze reactions to reduce RA bioavailability by converting RA to 4-OH-RA, 4-oxo RA, and other oxidized, less active metabolites (Abu-Abed, et al., 2001). These metabolites undergo glucuronidation which promote elimination pathways (Samokyszyn, et al., 2000). Adapted from (Chen, et al., 2020):

323. Cellular binding-proteins such as CRABP1 and CRABP2 facilitate RA transfer to the nucleus and also play roles in RA uptake, metabolism and function (Napoli, 2017). RA acts as a transcriptional activating ligand by binding to nuclear receptors known as retinoic acid receptors (RARs) alpha, beta, and gamma (Brand, et al., 1988) (Krust, et al., 1989) (Petkovich, et al., 1987) and retinoid X receptors (RXRs) alpha, beta, and gamma (Mangelsdorf, 1994). RAR/RXRs are expressed in the embryo, in a regional- and age-dependent manner during embryogenesis (Table C.1). In general, RARA, RXRA, and RXRB are ubiquitously expressed, while RARB, RARG, and RXRG are expressed in a more restricted profile (Dolle, 2009). The primary endogenous ligand for RARs is RA, while 9-cis-RA readily binds to both RARs and RXRs (Mangelsdorf, et al., 1992) (Allenby, et al., 1994). RARs form heterodimer complexes with RXRs and bind to motifs known as RA response elements (RAREs). In contrast to RARs, RXRs are not specific to retinoids or binding of RARs and form homodimers and heterodimers with other nuclear receptors. While only a subset may be critical for development (Cunningham, et al., 2018), over 13,000 potential RAREs have been identified in mouse embryonic stem cells (mESCs; (Moutier, et al., 2012)). Though the co-factors involved in RA-mediated CNS development remain undefined, transcriptional activation is influenced by binding of RAR/RXR heterodimer complexes, leading to the release of factors (e.g., SMRT (Jepsen, et al., 2007), histone deacetylases) that restrict chromatin access and the recruitment of co-activators (e.g., CBP/p300 (Lee, et al., 2009)) and co-repressors (e.g., LCoR (Fernandes, et al., 2003) (Koide, et al., 2001)). In summary, RA levels and ligand-activity is tightly regulated in the developing CNS by a combination of molecules, including RAR/RXRs, co-factors, binding proteins, and metabolizing enzymes.

324. While existing OECD Test Guidelines include endpoints that measure some aspects of CNS development in mammals (e.g. TG 426, Developmental Neurotoxicity Study; TG 443 the Developmental Neurotoxicity (DNT) cohort of the Extended One-Generation Reproductive Toxicity Study (EOGRTS)), the ability to identify chemicals that alter CNS development is highly dependent on the type of effect. Endpoints included in OECD Test Guidelines for non-mammalian species (e.g. TG 236, Fish Embryo Acute Toxicity (FET) Test) are limited to very basic measures such as lack of somite formation. Effects specifically related to alterations in retinoid signalling are not currently covered in OECD Test Guidelines (see Table C.3 for more detail).

Table C.1. Expression of RA Metabolic Enzymes and Signaling Members in Early Embryogenesis.

Expression of molecules during the onset of gastrulation to early organogenesis. References are summarized for chick (C) and/or mouse (M).

Source: Adapted from (Rhinn & Dolle, 2012).

Gene	Function	Expression in CNS and embryo proper	Ref.
Rdh10	Conversion of retinol to retinaldehyde	Ventral neural plate (hindbrain, spinal cord); mid hindbrain isthmus; optic vesicle, otocyst (ventral); nasal epithelium; dorsal somitic mesoderm, mesonephros, lateral plate mesoderm; foregut mesenchyme and endoderm, branchial pouches; proximal limb bud mesoderm	(Reijntjes, et al., 2010) (C); (Cammas, et al., 2007) (Sandell, et al., 2007) (M)
Raldh1	Conversion of retinaldehyde to RA	Ventral midbrain; optic vesicle, dorsal retina and lens; thymus primordium (3rd branchial pouch) (M); posterior foregut and midgut endoderm (C); mesonephros	(Suzuki, et al., 2000) (C); (Suzuki, et al., 2000) (Haselbeck, et al., 1999) (Li, et al., 2000) (M)
Raldh2	Conversion of retinaldehyde to RA	Primitive streak, node, posterior embryonic mesoderm; anteriormost neural plate and optic vesicle (transient); presomitic and somitic mesoderm, mesonephros, lateral plate mesoderm; posterior branchial arches and foregut mesenchyme; posterior heart tube	(Blentic, et al., 2003) (Swindell, et al., 1999) (C); (Haselbeck, et al., 1999) (Niederreither, et al., 1997) (Ribes, et al., 2006) (Ribes, et al., 2009) (M)
Raldh3	Conversion of retinaldehyde to RA	Node (C); head ectoderm, nasal epithelium, optic vesicle, dorsal and ventral retina; otocyst (dorsal); mid-hindbrain isthmus; Rathke's pouch (pituitary anlage)	(Suzuki, et al., 2000) (Blentic, et al., 2003) (Grun, et al., 2000) (C); (Suzuki, et al., 2000) (Li, et al., 2000) (Mic, et al., 2000) (M)
Cyp26a1	Conversion of RA to 4-OH-RA	Anteriormost epiblast and neural plate (transient); primitive streak and posterior mesoderm; (pre)rhombomeres 2 (M), 3 and rostral spinal cord (C); posterior hindbrain and branchial arch mesenchyme; heart endocardium; caudal neural plate and tail bud; distal limb bud ectoderm	(Blentic, et al., 2003) (Swindell, et al., 1999) (C); (Sirbu, et al., 2005) (MacLean, et al., 2001) (Fujii, et al., 1997) (M)
Cyp26b1	Conversion of RA to 4-OH-RA	(Pre)rhombomeres 3, 5, 2-4 (ventral) (M), 1, 4, 6 (C); posterior (2nd, 6th) branchial arch ectoderm/endoderm; distal limb bud mesenchyme; midhindbrain isthmus; heart and vascular endothelia; tail bud (transient)	(Reijntjes, et al., 2003) (C); (Sirbu, et al., 2005) (MacLean, et al., 2001) (M)
Cyp26c1	Conversion of RA to 4-OH-RA	Rostral head mesenchyme (transient); (pre)rhombomeres 2, 4 (M), 2, 3, 5 (C); hindbrain mesenchyme (facing r3, and post-otic); first branchial arch and pouch; otocyst (ventral)	(Reijntjes, et al., 2005) (C); (Sirbu, et al., 2005) (Tahayato, et al., 2003) (M)
Rara	RA receptor	Widespread, weaker in forebrain neuroepithelium; upregulated in rhombomeres 4, 7 and spinal cord	(Ruberte, et al., 1991) (Ruberte, et al., 1993) (M)
Rarb	RA receptor	Rhombomere 7 and spinal cord; head and branchial arch mesenchyme (except mandibular arch); foregut endoderm and mesenchyme; mesonephros, lateral plate mesoderm; proximal limb bud mesenchyme	(Smith & Eichele, 1991) (C); (Ruberte, et al., 1991) (Ruberte, et al., 1993) (Dolle, et al., 1989) (M)
Rarg	RA receptor	Primitive streak; frontonasal and 1st branchial arch mesenchyme; limb bud mesenchyme; trunk and caudal neural plate (transient), presomitic and tail bud mesoderm; precartilaginous cell populations	(Ruberte, et al., 1993) (Dolle, et al., 1989) (Ruberte, et al., 1990) (Abu-Abed, et al., 2003) (M)
Rxra	RA receptor	Widespread/ubiquitous; upregulated in posterior hindbrain and dorsal spinal cord (C)	(Hoover & Glover, 1998) (C); (Dolle, et al., 1994) (M)
Rxrb	RA receptor	Widespread/ubiquitous	(Dolle, et al., 1994) (M)
Rxrg	RA receptor	Cranial and peripheral nervous system neural crest (C); myotomes, developing muscle (M)	(Rowe & Brickell, 1995) (C); (Dolle, 2009) (M)
Crabp1	Facilitates transfer of RA to nucleus	Rhombencephalon (localized to rhombomeres 2, 4, 5 and 6), branchial arches, neural crest cells, optic stalk (M)	(Ruberte, et al., 1992) (Momoi, et al., 1990) (Maden, et al., 1992) (Means & Gudas, 1997) (M)
Crabp2	Facilitates transfer of RA to nucleus	Hindbrain. More widely-expressed as compared to Crabp1.	(Ruberte, et al., 1992) (M)
Cyp1b1	Conversion of retinol to RA; xenobiotic metabolism	Posterior primitive streak, somites, mid-hindbrain boundary, hindbrain (rhombomere 2), neural crest-derived cells, otic, dermomyotome, notochord, lens vesicle and neural retina, heart, pharyngeal arches; forelimb bud (C)	(Chambers, et al., 2007)(C)

C.4. Retinoic Acid and Mammalian CNS Development

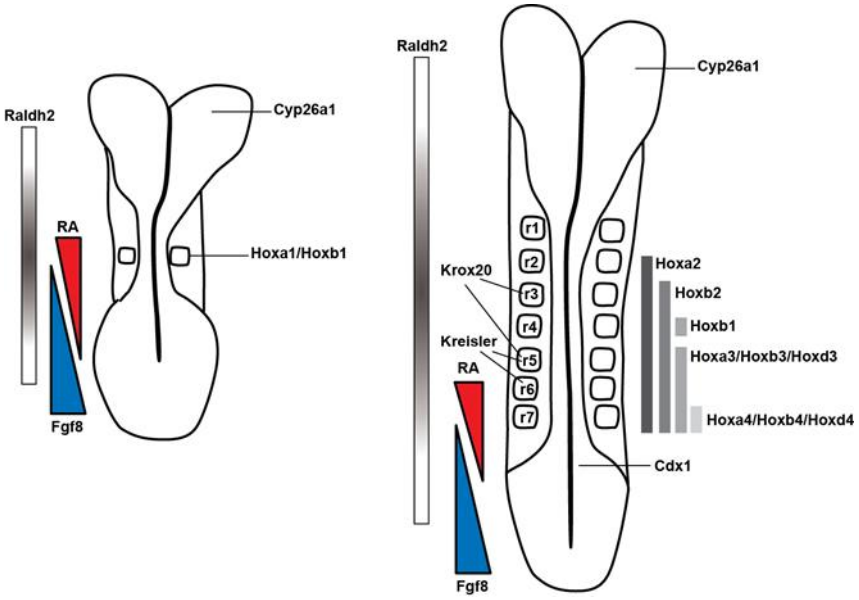
325. Many aspects of early CNS development are conserved across vertebrates. Neural induction (as reviewed, (Nikolopoulou, et al., 2017)) is initiated by the release of factors from notochord mesoderm to neighboring ectoderm cells overlying the notochord, giving rise to neuroectoderm, which forms the neural plate along the dorsal region of the embryo. Thickening of neural plate tissue and elevation at the lateral edges of the neural tube results in neural folds which eventually merge at the dorsal midline and establish the neural tube, forming the backbone for the anterior (brain) and posterior (spinal cord) CNS. Subsequently, during primary vesicle development (as reviewed, (Chen, et al., 2017)), the brain subdivides into three distinct regions from anterior to posterior location: forebrain (telencephalon and diencephalon), the midbrain (mesencephalon) and the hindbrain (rhombencephalon). The forebrain will serve as the structural basis for the cerebral cortex, basal ganglia and hippocampus, thalamus, hypothalamus and pineal gland and optic vesicles. The midbrain will give rise to centers of sensory and motor control. The hindbrain will develop into the cerebellum, pons and medulla, and include control centers for motor activity, respiration, sleep, blood circulation and receive/process inputs from auditory, precerebellar and vestibular systems.

326. RA is a key signaling molecule during embryonic development and its role in neural patterning of the developing CNS is well-established. Broadly, RA induces the 1) patterning of the CNS and 2) differentiation of neurons within these regions. While neural patterning has been documented and reviewed thoroughly (Cunningham & Duester, 2015) (Maden, 2007) (Maden, 2002), aspects of this complex process will be briefly summarized here.

C.4.1. Hindbrain and anterior/posterior axis formation

327. Early in development, RA contributes to the antero-posterior and dorso-ventral patterning of the neural plate and neural tube via a tightly-regulated temporal and spatial concentration gradient (Niederreither, et al., 2002) (Duester, 2008), a phenomena that has been visualized (Shimozono, et al., 2013). This RA gradient arises from the differential distribution of endogenous and synthetic "sources" and "sinks" of RA. These sources and sinks are primarily represented by the spatial domains of RALDHs in the posterior and CYP26 enzymes in the anterior ends of the embryo, respectively.

Figure C.2. Retinoic acid and anterior-posterior axis formation



Note: During the formative stages of CNS development, retinoic acid (RA) is regionally restricted and induces posterior embryonic growth. CYP26A1 is exclusively expressed in anterior tissues leading to the breakdown of RA and inactivation of RAR/RXR-signaling. Growth factors (Fgf, Wnt) which promote a posterior phenotype inhibit CYP26A1 and promote RALDH2 biosynthesis of RA, contributing to a gradient of RA availability in the intermediate zone (e.g. grey indicates higher expression), enabling RAR/RXR activation of HOX genes and other molecules which promote expansion of the posterior domain.

Source: Modified from Original information based on reviews (Bayha, et al., 2009) (Cunningham & Duester, 2015).

328. During the presomite stages, the presence of CYP26A1 in the anterior neuroepithelium (Abu-Abed, et al., 2001) restricts diffusion of RA from the posterior trunk regions originating from RALDH2 and RDH10 expression in the paraxial mesoderm after gastrulation (Molotkova, et al., 2005) (Sandell, et al., 2007). Consequently, excess RA signaling results in a posteriorization of the mid- and forebrain (Conlon & Rossant, 1992). Meanwhile RA, via activation of FGF and WNT, represses expansion of the CYP26A1 domain to the posterior region to prevent its degradation (Kudoh, et al., 2002). The hindbrain lies between the gap of these two regions, its development controlled by this RA gradient.

329. During hindbrain formation, transient metameric units called rhombomeres develop (Figure C.2). The hindbrain is composed of 7 rhombomeres and each segment generates a specific repertoire of genes that confers its distinct regional identity. RA regulates the formation of rhombomeres through interactions with RAREs on *Hox* genes. The function of *Hox* genes and their targets in establishing rhombomere identity has been thoroughly reviewed elsewhere (Alexander, et al., 2009) (Svingen & Tonissen, 2006) (Gavalas & Krumlauf, 2000). Briefly, RA regulates expression of Hox genes 1-4 (Hunt, et al., 1991) during hindbrain segmentation, with the highest concentrations of RA beginning at the caudal end and shifting throughout development (Sirbu, et al., 2005). In general, Hox expression varies within one- or two-segment rhombomere boundary increments. *Hoxa1* and *Hoxb1* are induced early at the presomite stage and are critical for r4 patterning (Studer, et al., 1998) (Barrow, et al., 2000). *Krox20* (Egr2), a zinc finger transcription factor, is subsequently activated and regulates the expression of *Hoxa2* and *Hoxb2* in r3 and r5 (Sham, et al., 1993) (Nonchev, et al., 1996). *Hoxa2* expression is required to maintain r2 identity (Gavalas, et al., 1997), while the interactions of *Hoxa2* and *Hoxb2,* in combination with *Hoxa1* and *Krox20*, are necessary for r3 (Davenne, et al., 1999) (Helmbacher, et al., 1998). *Kreisler* (Mafb) targets *Hoxa3* and *Hoxb3,* Hox genes important for the r5 and r6 segments (Manzanares, et al., 1997) (Manzanares, et al., 1999). While no direct interaction between RA and *kreisler* is known to us, indirect interactions could affect its

expression. *Krox20* and *kreisler* synergize to regulate *Hoxb3* expression (Manzanares, et al., 2002), and *Cdx1,* which prevents hindbrain patterning in the spinal cord region (Skromne, et al., 2007), is activated by RA and represses *kreisler* (Sturgeon, et al., 2011). *Hoxa4/ Hoxb4/Hoxd4* maintain the r6/r7 boundaries respectively and all contain RAREs (Niederreither, et al., 2000).

330. Deciphering downstream effectors of Hox genes could provide additional insight into how RA signaling during rhombomere specification establishes neuronal identity in the hindbrain. For example, transcriptomic analyses of individual rhombomeres from embryonic mice suggest that while certain genes are expressed across all segments, including neurofilament genes such as *Nef3* and *Ina*, molecules such as CYP1B1 at r4 could be a RALDH-independent RA source (Chambers, et al., 2007) (Chambers, et al., 2009). Other potential effectors include *GATA2* and *GATA3*, transcription factors associated with noradrenergic neuronal differentiation (Tsarovina, et al., 2004), which are also regulated by *Hoxb1* expression in r4 (Pata, et al., 1999). *GBX2,* a r1 marker that is necessary for formation of the cerebellum (Wassarman, et al., 1997), is repressed by Hox genes (McNulty, et al., 2005), potentially via *Hoxa2* (Carapuco, et al., 2005). NCAM1, which contains Hox binding domains, is also a downstream effector and potentially mediates cell segregation during rhombomere formation (Edelman & Jones, 1995) (Jones, et al., 1992). However, much work remains in characterizing *Hox* targets.

331. In contrast to the hindbrain, the role of RA synthesis in initial forebrain development is conflicting. The optic (Mic, et al., 2004) and olfactory domains (Kane, et al., 2008) generate, and require RA for their specification, but the forebrain itself produces little RA early in development (Wagner, et al., 1992). Thus while RA has a critical role in forebrain patterning (Ribes, et al., 2006) (Schneider, et al., 2001), studies show it is not required for the overall development of the embryonic forebrain (Molotkova, et al., 2007) (Chatzi, et al., 2013). Rather, it is responsible for the localized differentiation of neurons in that region.

332. The prospective spinal cord is produced from a stem zone in the caudal tail bud. Neuron specification along this dorso-ventral axis is mediated by the complex interplay of four signaling gradients: Sonic hedgehog (Shh) from ventral notochord and floor plate, bone morphogenic proteins (BMPs) from the dorsal ectoderm and roof plate, FGF from the underlying stem zone, and RA from the differentiating adjacent somites (Wilson & Maden, 2005). Deficits in RA signaling in this region is associated with disruption of various markers of neuronal differentiation and motor neuron specification (e.g. *Dbx1, Ngn2, Pax6*) (Lee, et al., 2009) (Wilson, et al., 2004). Thus the maintenance of a RA gradient is critical for the development of both the posterior hindbrain and the anterior spinal cord**.**

C.4.2. Neurite outgrowth and elongation

333. Proper neurite outgrowth during development is a critical determinant of neuronal connectivity and experimental evidence *in vitro* suggests that RA can serve as a signaling and/or chemotactic molecule during this process. Mean neurite length of dorsal root ganglia (DRG) derived from neonatal mice increases with RA exposure at concentrations as low as 1nM, without increasing overall neurite number (Quinn & De Boni, 1991). In the same study, fetal murine and human spinal cord explants display similar levels of neurite extension with RA supplementation, though inhibition was seen at higher concentrations (10μM). Whether retinoid sensitivity differs between neuron types remains to be further explored; however, RA's inhibitory effects have been seen in other sympathetic neurons. RA can suppress the length and number of dendrites induced by BMP7 in cultured rat superior cervical root ganglia at concentrations as low as 3nM without affecting axon outgrowth, potentially via RAR and RXR activation (Chandrasekaran, et al., 2000). Similar observations were seen in PC12 cells, which possess sympathetic neuron-like characteristics after NGF exposure. PC12 cells showed inhibition of neurite length at 30nM RA, concentrations independent of cytotoxicity (Radio, et al., 2008).

334. The mechanisms underlying RA's ability to regulate neurite outgrowth remains unclear and may include genomic and non-genomic-based mechanisms. Application of RA in culture can induce neurite outgrowth in human neuroblastoma lines and chick DRG explants in a manner similar to nerve growth

factor (NGF); (Haskell, et al., 1987), and likewise, high affinity NGF receptors are induced with RA in sympathetic neurons (Rodriguez-Tebar & Rohrer, 1991). Combinatorial treatment of RA and NGF does not cause additive or synergistic changes, further suggesting the two share common pathways (Canon, et al., 2004). Activation of kinase cascades, such as the ERK pathway (Canon, et al., 2004) (Li, et al., 2006) (Lu, et al., 2009) and protein kinase C/PI3K (Miloso, et al., 2004) (Lopez-Carballo, et al., 2002), have been suggested as potential non-genomic targets of RA-induced neurite outgrowth.

335. RA may also be a chemotactic, or guiding, agent for neurite extension. Neurites from chick neural tube cells not only extend in response to nM concentrations of RA, but also display a directionality preference when cultured in a Dunn chamber with a RA gradient (Maden, et al., 1998). However, while RA's chemotactic role has been further elucidated with invertebrate neurons (Dmetrichuk, et al., 2006) (Farrar, et al., 2009) (Carter, et al., 2010), to our knowledge no further work has been done in other developmental vertebrate neuronal models.

C.4.3. Neurobehavior and synaptic plasticity

336. Due to the role of RA in the maintenance and differentiation of neurons, alterations in RA signaling during development manifests postnatally in neurobehavioral outcomes. For instance, murine models with knockouts of Rarb, Rxrg, or both develop without gross abnormalities and structurally normal, functioning synapses, yet perform poorly in the Morris water maze (Chiang, et al., 1998). Similarly, mice that were Vitamin A-deprived throughout development showed normal hippocampal development and synaptic response but possess impairments in long-term potentiation (LTP) and long-term depression (LTD) (Misner, et al., 2001). *Rxrg*-null mice also show deficits in novel object recognition at intermediate delay (Wietrzych, et al., 2005). As described in preceding sections, sites of high RA signaling during development, such as the nigrostriatal system, are especially sensitive to disruption. The striatum is disproportionately affected compared to the hippocampus and cortex in Vitamin A-deprived rats, as measured by neurogranin and neuromodulin transcription levels (Husson, et al., 2004). Likewise, dopaminergic signaling in the striatum is compromised in Rarb-Rxrb, Rarb-Rxrg, and Rxrb-Rxrg knockout mice (Krezel, et al., 1998). RA itself can also modulate synaptic transmission, as exogenous RA treatment in primary rat hippocampal culture increases synaptic scaling and activity blockade increases neuronal RA synthesis (Aoto, et al., 2008). Subsequently mice chronically treated with RA show more depressive behaviors, with longer durations of immobility in tail suspension and forced-swim tests, though hippocampal volume, which is associated with depressive behaviors, was not assessed (O'Reilly, et al., 2006).The weight of evidence to date regarding neurological/cognitive impacts of altered RA signalling are further reviewed in Kubickova et al (2021). Whilst the causal link between aberrant retinoid signalling and neurological disease is currently weak, the biological plausibility of the association is high: RA is a morphogen during early development and is strongly involved in shaping the CNS, including differentiation and maturation of neurons. Despite the significant role during development, the role of RA signalling in the adult or postnatal brain is less clear. Still, the conserved mechanisms of RA signalling are most likely to act also in the adult brain, though the effects may be less evident due to the multitude of parallel processes and potential influencing factors. Also, it is difficult to simulate and assess the many hues of neurological disease in animal models that are distinctly different from humans and it is not possible to assess behavioural changes in in vitro systems.

C.5. Genetic Defects in RA Signaling and Adverse CNS Outcomes

337. Due to the strict control of RA and RA pathway members during embryonic development, genetic modulation of RA metabolic enzymes, receptors, and downstream targets are linked with perturbations in embryonic patterning, neural tube closure, and CNS morphology (Table C.2). Regulation of RA

bioavailability is particulary important during hindbrain formation and AP patterning; enzymes critical for RA biosynthesis, expressed in this particular region of the early CNS, are necessary for proper development. For example, in mice, loss of Rdh10 results in diminished RA synthesis/activity and defects in hindbrain patterning and eye development (Chatzi, et al., 2013). Meanwhile Raldh2(-/-) embryos display reduced capability to synthesize RA and hindbrain/axial defects (Niederreither, et al., 1999). Effects of loss of Rdh10/Raldh2 function can be rescued via retinaldehyde (Chatzi, et al., 2013) or RA (Niederreither, et al., 2002) supplementation, respectively. Cyp26a1(-/-) embryos exhibit increased hindbrain defects, NTDs (exencephaly and spina bifida) and caudal truncation (Sakai, et al., 2001) (Abu-Abed, et al., 2001). The knockout phenotype is sensitive to subtoxic concentrations of RA (Ribes, et al., 2007) and rescued by eliminating embryonic expression of Rarg (Abu-Abed, et al., 2003). While Cyp26c1(-/-) do not display an overt phenotype, combined loss of Cyp26a1(-/-) and Cyp26c1(-/-) magnifies the severity of the Cyp26a1(-/-) phenotype, suggesting a role in RA metabolism during this period in embryogenesis.

338. Altered expression of RAR/RXR, transducers of RA-signalling are also linked with abnormalities of the CNS, related to the timing and their localized expression pattern. Rarg is expressed prior to closure of the neural tube, while Rarb and Rara are expressed as the neural tube folds adjoin (Smith & Eichele, 1991) (Ruberte, et al., 1991) (Chen, et al., 1995). Rara(-/-):Rarg(-/-) double null mouse mutants (Lohnes, et al., 1994) have significantly higher levels of exencephaly, while Rarg(-/-) are less susceptible to abnormalities in the neural tube (Lohnes, et al., 1993) (Iulianella & Lohnes, 1997). In addition, genetic loss in expression of downstream targets of RA, including Hoxa1 (Mark, et al., 1993) (Carpenter, et al., 1993), Hoxb1 (Goddard, et al., 1996) (Studer, et al., 1996), Krox-20 (Egr2) (Schneider-Maunoury, et al., 1993) and Kreissler (Mafb) (Cordes & Barsh, 1994) (McKay, et al., 1994) are associated with abnormalities in hindbrain development.

339. Not all components of the RA signalling pathway may be essential. In mouse, single or double genetic knockouts of RA binding proteins, Crabp1 and/or Crabp2, are phenotypically normal and not as sensitive to supplemented RA as compared to wildtype (Gorry, et al., 1994) (Lampron, et al., 1995). In contrast, in zebrafish, Crabp2a is essential for hindbrain development and posterior patterning, and hypersensitive to exogenous RA (Cai, et al., 2012). These results suggest that other RA binding proteins may be of higher critical importance in rodent. In summary, genetic studies in mouse as well as other vertebrates (e.g., zebrafish; (Samarut, et al., 2015); xenopus (Carron & Shi, 2016)) suggest that loss-of-function in several components of the RA signalling pathway is linked with adverse CNS outcomes.

Table C.2. Adverse CNS outcomes associated with genetic defects of RA metabolism and signalling in mouse models.

Models include single/double knockouts or mutants.

Genetic Mouse Model	Examples of Observed Adverse CNS Outcomes	Ref.
Rdh10(-/-)	Shortened anteroposterior axis, irregular somitogenesis	(Chatzi, et al., 2013)
Raldh2(-/-)	Defects in hindbrain (abnormal patterning) and forebrain, altered axial patterning (truncation)	(Niederreither, et al., 1999)
Raldh2(-/-); Raldh3(-/-)	Forebrain, nasal region, optic vesicle defects	(Halilagic, et al., 2007)
Cyp26a1(-/-)	Caudal region (severely truncated with spina bifida), anterior hindbrain (abnormal patterning); sensitive to excess RA	(Abu-Abed, et al., 2001) (Ribes, et al., 2007) (Abu-Abed, et al., 2003)
Cyp26a1(-/-): Cyp26c1(-/-)	Forebrain, midbrain, branchial arches (reduced), hindbrain (expanded), cranial neural crest (deficient)	(Abu-Abed, et al., 2003)
Rara(-/-): Rarb(-/-)	Hindbrain (abnormal patterning)	(Chen, et al., 1995)
Rara(-/-): Rarg(-/-)	Hindbrain (abnormal patterning)	(Lohnes, et al., 1994)
Rxra(-/-): Rxrb(-/-)	Caudal region (truncated), nasal region, posterior defects	(Chen, et al., 1995)
Hoxa1(-/-)	Hindbrain defects (5th rhombomere missing, 4th abnormal), altered neural crest cells derived from 2nd branchial arch	(Mark, et al., 1993) (Carpenter, et al., 1993)
Hoxb1(-/-)	Altered identity of neurons in 4th rhombomere	(Goddard, et al., 1996) (Studer, et al., 1996)
Hoxa1(-/-): Hoxb1(-/-)	Hindbrain defects (deletion of r4 and r5), loss of 2(nd) branchial arch	(Rossel & Capecchi, 1999)
Krox-20(-/-)	Hindbrain defects (altered or loss of r3 and r5)	(Schneider-Maunoury, et al., 1993)
Kreisler(*kr*)	Defects of hindbrain defect (loss of r5 and r6) and inner ear	(Cordes & Barsh, 1994) (McKay, et al., 1994)

Source: Adapted from (Rhinn & Dolle, 2012).

340. Human studies also suggest that genetic variants in RA metabolism and signalling are important for neurodevelopment. In case-control investigations, genetic variants in enzymes controlling RA synthesis and degradation are associated with NTDs (as reviewed, (Greene, et al., 2009)). For example, Deak *et al.* (Deak, et al., 2005) identified a significant association between genetic polymorphisms (three total) in RALDH2 and lumbosacral myelomeningocele (n=230 cases), a severe form of spina bifida, but not with CYP26A1, CYP26B1, CRABP1, and CRABP2. Rat *et al.* (Rat, et al., 2006) found a functional genetic deletion (g.3116delT) on CYP26A1 to be linked with spina bifida in an Italian/Caucasian population. Li *et al.* (Li, et al., 2018) discovered variants in exonic and upstream regions of RA binding motifs, i.e., RA receptor elements (RARE) of CYP26A1, CRABP1, ALDH1A2, to also be associated with NTDs. While limited, epidemiological evidence supports observations in animal models that the function of critical RA pathway members in regulating RA is essential for early CNS development.

C.6. Retinoid Imbalance and Adverse CNS Outcomes in Humans

341. Despite the critical importance of Vitamin A (retinol) in embryonic growth and development (Clagett-Dame & Knutson, 2011) (Maden, 2006) (Duester, 2008), too much or too little exposure is associated with fetal malformations and adverse neurodevelopmental outcomes (McCaffery, et al., 2003) (Gutierrez-Mazariegos, et al., 2011). Guidelines for Vitamin A supplementation are commonly provided as Retinol Equivalents (RE) where 1 RE = 1µg retinol, 6µg β-carotene and 12µg other provitamin A carotenoids, or as International Units (IU) where 1 RE = 3.33IU retinol (Medicine, 2001) (Trumbo, et al., 2001). However, data proposing that the conversion of dietary β-carotene to retinol is half as efficient as previously suggested has led to the establishment of the Retinol Activity Equivalents (RAE) unit of measurement, where 12 RAE β-carotene = 1µg retinol.

342. Although RAE accounts for the current understanding of the differing bioactivities of retinol and other Vitamin A precursors (e.g., β -carotenoids), opinions on dietary intake or values within food composition tables may still be expressed in units of RE or IU. For example, the United States Institute of Medicine suggests a recommended dietary allowance (RDA) for women aged 19 years and older of 700µg RAE/day, increasing to 750µg RAE/day during pregnancy and 1,200µg RAE/day during lactation. While the European Food Safety Authority (EFSA) suggests a population reference intake for women aged 18 years and older of 490µg RE/day, increasing to 540µg RE/day during pregnancy and 1,020µg RE/day during lactation (Medicine, 2001) (European Food Safety Authority (EFSA) Panel on Dietetic Products and Allergies (NDA), 2015). Currently, the recommended tolerable upper limit is set at 3,000µg RAE (10,000 IU)/day. In general, consumption of retinoids during pregnancy in line with these guidelines are generally safe and not significantly associated with toxicity (Mills, et al., 1997) (Shaw, et al., 1997). However, at higher exposure levels (>10,000 IU/day), the health risks remain undefined. In a case-control study, women who consumed >10,000 IU versus <5,000 IU of supplemented Vitamin A per day during pregnancy were found to have 4.8 times higher risk of having an offspring with a cranial-neural crest defect defined as irregularities of the thymus, craniofacial deformities, and CNS defects (Rothman, et al., 1995). An association between adverse pregnancy outcomes and Vitamin A concentrations in amniotic fluid during mid-pregnancy (second trimester, n=106) was revealed in a prospective cohort study; pregnancies resulting in a neural tube defect (NTD) had approximately 1.5 times greater concentrations of Vitamin A in amniotic fluid than normal pregnancies (Parkinson, et al., 1982). In contrast to these positive associations, the prevalence of pregnancies resulting in offspring with NTDs was not significantly different between women who consumed >8,000 or >10,000 IU per day of preformed Vitamin A during the periconceptional period versus women who did not (Mills, et al., 1997). Furthermore, retinol levels in maternal serum during early pregnancy (1st trimester) were not associated with NTD risk in a case-control study examining NTD-affected (n=89) and normal pregnancies (n=178; (Mills, et al., 1992)). Although these studies found no association between periconceptional Vitamin A levels and increased risk for NTDs, several cases which oppose these findings suggest that contraindication of excessive Vitamin A intake during pregnancy and lactation remains warranted.

343. In addition to dietary supplementation, synthetic retinol derivatives such as Isotretinoin and Tretinoin are widely-used for treatments against severe acne. Tretinoin is a naturally occurring Vitamin A derivative synthesized from β-carotene. Percutaneous absorption of Tretinoin into the circulatory system following topical application is low. Approximately 2% of Tretinoin is absorbed in patients after short-term therapy (1-28 days) and ~1% in patients following long term therapy (>1 year) of dermal daily application of 0.05% Tretinoin (Latriano, et al., 1997). In a variety of study designs (e.g., case-control, retrospective and prospective cohort), dermal and oral exposures to retinol derivatives during early pregnancy have been associated with congenital malformations, including CNS malformations such as microcephaly (Lammer, et al., 1985) (Stange, et al., 1978), mid/hindbrain malformations (e.g., absent cerebellar vermis (Hansen & Pearl, 1985), cerebellar vermis hypoplasia (Merlini, et al., 2014) or brainstem abnormalities (Hansen & Pearl, 1985)) and spontaneous abortion (Bigby & Stern, 1988). For example, examination of

154 Isotretinoin-exposed pregnancies, (118 observed retrospectively and 36 observed prospectively) was associated with 21 malformed infants (18 of which developed anomalies of the CNS). All malformations were associated with mothers who were exposed to Isotretinoin on or before the 28th day of gestation (Lammer, et al., 1985), suggesting a severe level of risk to retinoid exposures during vulnerable periods in early embryogenesis.

344. While cumulative evidence in humans suggests imbalances in Vitamin A consumption or retinoid exposures to be developmentally (neuro)toxic, defining guidelines for Vitamin A consumption or retinoid exposure during pregnancy remains unclear due to inconsistencies in reported risk associations, the analysis of small sample sizes, and the reliance of case-control study designs that commonly include recall bias due to the retrospective nature of the study. Furthermore, research is needed to understand additional factors, including genetics, diet, and maternal stress (Stover & Caudill, 2008) (Geraghty, et al., 2015) (Lindsay, et al., 2017), that may influence variation in response to RA excess or insufficiency, and the role of retinoids in neurobehavior, including cognition (Grodstein, et al., 2007) and activity.

C.7. Retinoids and Adverse CNS Outcomes in Mammalian Models

345. In animal models, including mouse (Tibbles & Wiley, 1988), rat (Colakoglu & Kukner, 2004), hamster (Shenefelt, 1972), and monkey (Hendrickx, et al., 1998), excessive exposures to retinoids during prenatal life results in CNS malformations and altered neurodevelopmental outcomes. Common defects associated with excess retinoid exposures include: anterior (exencephaly or anencephaly) and posterior (spina bifida) NTDs, microcephaly, irregular somitogenesis, delayed or reduced caudal elongation, vascular damage, notochord defects, and irregularities in the neural folds. On a cellular level, morphological perturbations are associated with changes in proliferation, differentiation, viability (apoptosis), and migration, and on a molecular level, altered expression of metabolic enzymes, and RA-mediated gene targets (Luijten, et al., 2010) (Robinson, et al., 2012). Severity of outcomes may depend on several factors such as dose, time, exposure route, timing of exposure, and genetic background.

346. RA is the most active and potent retinoid (Turton, et al., 1992), though other retinoids such as retinol and 13-*cis* RA, also produce -at excessive levels- similar pathological phenotypes (Tembe, et al., 1996). In general, the most sensitive windows to retinoid-induced gross CNS malformations appears when excess retinoid exposures occur during neurulation and early organogenesis (Adams, 1993) (Cunningham, et al., 1994). A comprehensive assessment of an RA-induced Sprague Dawley rat model of myelomeningocele (60 mg/kg) demonstrates the numerous CNS malformations that can emerge, including lumbosacral defects, exencephaly, and caudal regression syndrome (Danzer, et al., 2005). In rodents, CNS malformations due to RA show sensitivities between gestational days (GDs) 8-13 (Adams, 2010). While GDs 11-13 appears to be a more vulnerable period for deficiencies in brain weight and potentially postnatal survival (Nolen, 1986), RA exposures between GDs 8-10 are more prone to be associated with CNS malformations (Holson, et al., 1997), as reviewed elsewhere (McCaffery, et al., 2003) (Adams, 2010). For example, single oral gavages of RA (10 mg/kg) in pregnant dams at GD 9 in Sprague Dawley rats can elicit exencephaly in the offspring (Nolen, 1972). Similarly, Wistar rats treated subcutaneously or orally with 5 mg/kg RA at GD 9 display skull, thoracic, and sternal abnormalities (Tzimas, et al., 1997). Lumbosacral NTDs are also observed in Wistar rats exposed at GD 10 to RA at 135 mg/kg (Cai, et al., 2007) (White, et al., 2000). Deficiencies in RA during early CNS development also associate with CNS defects and alterations in embryonic patterning. Embryos of Vitamin A-deficient (VAD) pregnant rats (Sprague Dawley), display loss/disorganization of cranial nerves, loss of caudal hindbrain segmentation, expansion of rhombomeres R3-4 and otic vesicle abnormalities (White, et al., 2000).

347. Later periods in CNS development may be vulnerable windows to excess or deficits in RA, resulting in altered neurodevelopmental and neurobehavioral outcomes that manifest later in life. For example, RA-exposed juvenile rats display changes in basal serum corticosterone concentrations, increased thickness of the adrenal cortex, and anxiety-like behaviours (Cai, et al., 2010); while gestational

deficiency in Vitamin A is linked with impaired learning and memory (Zhang, et al., 2017) as well as autistic-like behaviours due to Rarb-CD38-oxytocin suppression in the hypothalamus (Lai, et al., 2018). Wistar rats deprived of retinoids post-weaning display degeneration in motor neurons and accumulation of axonal neurofilaments in the lumbar cord (Corcoran, et al., 2002). In summary, RA is critical for vertebrate development, and imbalances during specific windows of development are linked with CNS defects and potentially, developmental alterations that underlie neurobehavioral adverse outcomes.

C.8. Alternative Assays to Assess RA Developmental Neurotoxicity

348. Traditionally, chemicals are evaluated for neurotoxicity ((OECD), 1997) (Agency, 1998), and more specifically, developmental neurotoxicity (DNT) (Agency, 1998) (Development, 2007) (Makris, et al., 2009) usingstandardized *in vivo* tests based on guidelines developed both in the US and Europe (e.g., OECD TG 426), which provide extensive information onchemical exposures in utero and/or during postnatal life. In testing for DNT, chemicals are evaluated from GD6 to postnatal day 10 or 21 (in the mother) to assure that exposures extend across vulnerable windows (in utero and lactation) in prenatal and postnatal development. Endpoints related to DNT that are typically evaluated include: altered growth, functional and behavioral deficits, and structural abnormalities that include both malformations and variations (Agency, 1991). While informative and predictive in identifying potential developmental neurotoxicants (Makris, et al., 2009), *in vivo* assessments are costly and time-consuming. Furthermore, a limited number of the thousands of compounds used in industrial applications and consumer products, many of which are identified in human maternal and fetal biological matrices, have been thoroughly tested for their potential to cause DNT (Miodovnik, 2011) (Grandjean & Landrigan, 2006). In line with strategies to integrate the 3R principles (replacement, reduction, refinement) into toxicological testing, efforts to use animal-free/reduced testing approaches are advancing. *In vitro* and in silico models are emerging as viable replacements for *in vivo* animal studies for the purposes of assessing DNT (Bal-Price, et al., 2018). Sponsored research by several agencies, including the United States Environmental Protection Agency, National Institutes of Environmental Sciences (e.g., Tox21/ToxCast (Richard, et al., 2016)) and the European Commission (Worth & Patlewicz, 2016), has promoted efforts towards rapid development of an animal-free/reduced testing framework to screen industrial chemicals for toxicological assessments. Due to the biological complexity of human neurodevelopment, multiple models may be needed to accurately predict DNT. Here, we review specific alternative models used to evaluate retinoids in the context of DNT.

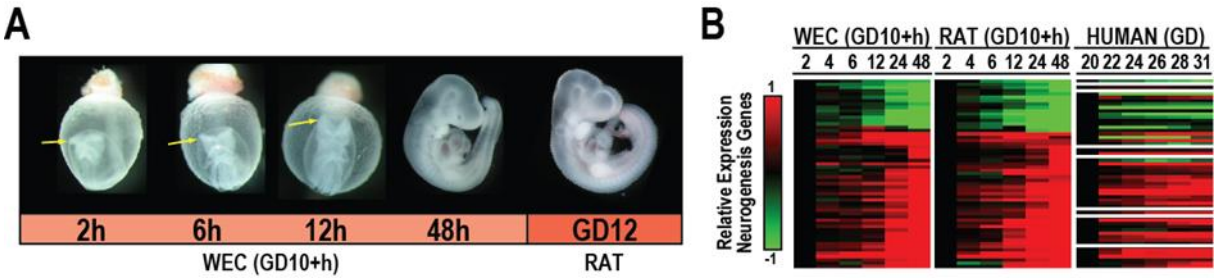
C.8.1. Rodent Whole Embryo Culture

349. The rodent whole embryo culture (WEC) model is an established tool to study the influence of both environmental and genetic factors on early embryonic development, including molecular and cellular changes within the neuroepithelium and surrounding embryonic tissues that support neurulation and early organogenesis of the brain and spinal cord. Although the WEC is not an animal-free assay, compared to traditional whole rodent toxicological studies the model involves a reduction in animal usage and cost, shortened experimental time, and control over dosage and compound specificity (Augustine-Rauch, et al., 2010) (Webster, et al., 1997) (Lee, et al., 2012). Using a variety of proposed scoring methods (Zhang, et al., 2010) (Anon, 1999), embryos can be evaluated for developmental progression of the CNS and other morphological embryonic features.

350. Due to these attributes, WEC has been proposed as a predictive toxicological assay to identify developmental toxicants (Spielmann, et al., 2006). In a study organized by the European Centre for Validation of Alternative Methods (ECVAM), the WEC (in combination with 3T3 cytotoxicity tests) was able to successfully predict developmental toxic compounds (n=20) at an accuracy of ~80% and distinguish strongly embryotoxic compounds with high precision (100%; (Piersma, et al., 2004) (Genschow, et al., 2002) (Genschow, et al., 2004)). Additional investigations have supported the general utilization for this assay in hazard identification; however, success has been mixed in accurately distinguishing weak versus

non-embryotoxic compounds and for specific classes of compounds (Thomson, et al., 2011). Limitations of the WEC model for DNT testing include: 1) limited metabolic capacity; 2) examination of a limited developmental timeframe, i.e., neurulation and early organogenesis, and inability to evaluate manifestations which may appear outside developmental window (e.g., neurobehavioral); and 3) complexity of readout for screening (multiple morphological parameters).

Figure C.3. Rat postimplantation whole embryo culture.



Note: (**A**) Cultured embryos undergo neural tube closure (arrows) and the initial stages of CNS morphogenesis in a similar manner to *in vivo* embryogenesis. (**B**) Neurogenesis gene expression patterning in WEC correlates with rat (*in vivo*) and human (*in vivo*) embryos during neurulation and early organogenesis.

Source: Adapted from (Robinson, et al., 2012).

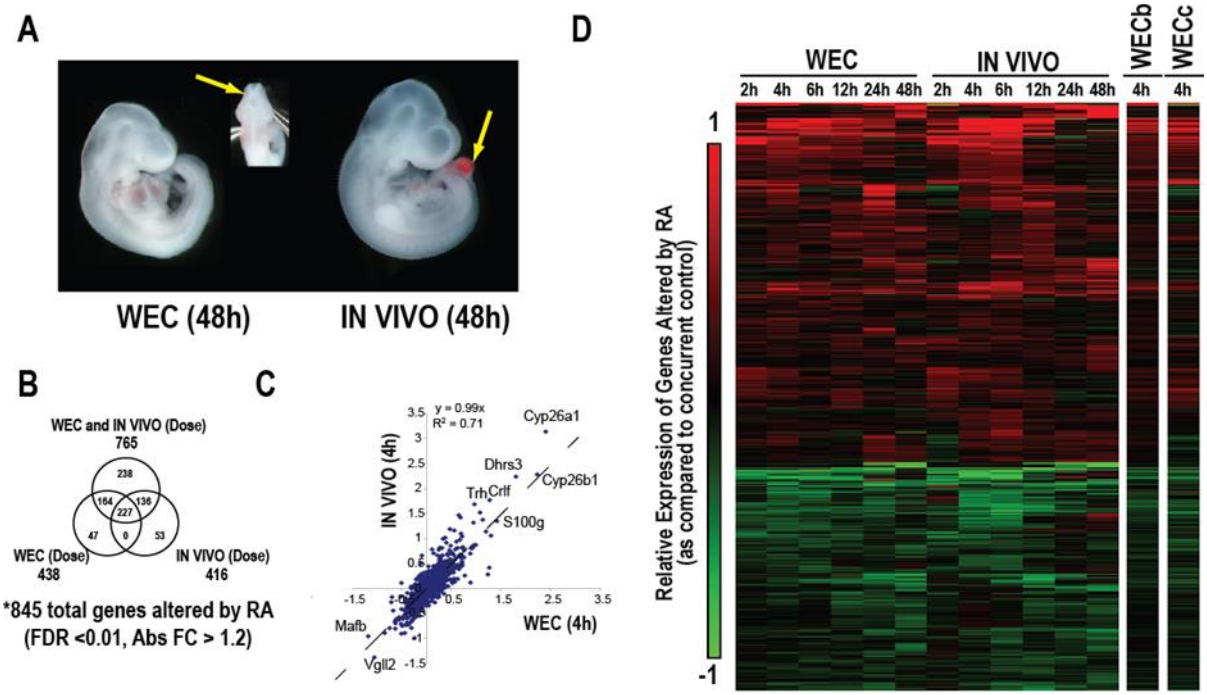
351. In brief, rat (Brown & Fabro, 1981) (Harris, 2019) or mouse (Culshaw, et al., 2019) embryos are isolated (1-5 somites) with the ectoplacental cone intact and cultured during the initial stages of neural tube formation (GD 8, mouse; GD 10, rat). Embryos, under optimal conditions (e.g., increasing oxygen, steady temperature, rodent serum-containing media, rolling-bottle culture system), successfully progress through neurulation and early organogenesis, parallel to the transitions which occur *in vivo* on morphological (Figure C.3A) and gene expression levels (Figure C.3B; (Robinson, et al., 2012)). Morphological development of the neural tube (e.g., cranial or caudal NT closure; primitive fore-, mid-, hindbrain, and spinal cord formation) can be evaluated for abnormalities or growth delays in the context of compound exposure.

352. The WEC has proven to be a valuable model to evaluate teratogenic potency of retinoids, timing of exposure, and mechanisms underlying developmental toxicity. Morris and Steele (Morriss & Steele, 1977) demonstrated the use of the WEC model to profile the teratogenic effects of excess retinol and RA exposures during early organogenesis. These initial studies validated *in vivo* observations that elevated exposures to retinoids during neurulation result in defects of the neural tube as well as malformations of the palate and limb. Subsequent studies by Steele *et al.* (Steele, et al., 1987) and Klug *et al.* (Klug, et al., 1989) using the WEC demonstrated the metabolite, 13-cis RA, to also cause CNS defects and impair embryonic growth. These studies suggest the ability of the WEC to evaluate retinoid exposures and teratogenic endpoints relevant to the *in vivo* condition.

353. Recent studies examining RA effects in WEC have incorporated transcriptomic approaches to identify global changes in gene expression that may underlie and precede morphological alterations. Luijten *et al.* (Luijten, et al., 2010) used transcriptomic profiling to identify early responses to teratogenic levels of RA (0.5µg/mL) in single cultured rat embryos initially exposed at 2-4 somites. RA affected 260 genes after a 4h exposure duration, including genes involved in embryonic patterning and CNS development such as Gbx2 and Otx2, as well as genes linked with RA metabolism (Cyp26a1, Cyp26b1, Dhrs3) and RA gene activation (Rarb). Gene expression changes due to RA were similar across embryos of 2-4 somites or between embryos cultured in rat serum vs. a cheaper alternative consisting of rat (10%)-bovine (90%) serum mixture. In a study by Robinson *et al.* (Robinson, et al., 2012), RA effects on morphological and transcriptomic levels were compared between the rat postimplantation WEC model and

embryos *in vivo* undergoing neurulation. In both models, RA (0.5 µg/ml or 50mg/kg BW oral gavage) caused developmental toxicity, including growth reduction (size, somites) and defects of the neural tube (Figure C.4A). On the transcriptomic level, RA response was evaluated across six time points (GD10 + 2-48h) using generalized linear models to identify differentially expressed (DE) genes due to RA independently in each model (and in combination). In total, RA perturbed expression of 845 genes in a time-dependent manner (Figure C.4B). Consistent with Luitjen *et al.* (Luijten, et al., 2010) perturbations in expression were enriched for genes involved in CNS and embryonic development as well as neuronal differentiation. Based on significance alone, more than 50% of identified DE genes in either model overlapped with the other system. In terms of directionality and general significance, similar patterns in gene expression due to RA exposure were observed between embryos *in vivo* and WEC (Figure C.4C, D). Differences in the timing of perturbations to select genes related to RA metabolism (e.g., Cyp26a1, Cyp26b1, Dhrs3) and embryonic patterning (e.g., Pbx1, Pbx2, Gli1, Dlx1, Hoxa9, Hoxa10, Hoxc10, Hoxd10) associated with significant differences in the propensity of NT defects to be anterior (WEC) vs. caudal (*in vivo*; Figure C.4A), suggesting toxicokinetics, and developmental timing of RA exposure to be critical factors in DNT outcomes and RA molecular response.

Figure C. 4. Retinoic acid induces CNS defects in rat whole embryo culture and rat embryos *in vivo*.



Note: (**A**) Examples of CNS defects (yellow arrows) caused by retinoic acid (RA) exposure in WEC (0.5ug/mL; open neural tube in midbrain region) and rat embryos (50mg/kg (BW); open caudal neural tube). (**B**) Comparison of genes identified to be differentially expressed (DE) due to RA exposure (RA-DE genes) in WEC, *in vivo,* or the two models combined. (**C**) Cross-scatter plot displaying fold change response (in comparison to concurrent control) of RA-DE genes at 4h in WEC vs. rat. Responses to RA at 4h post-exposure were most similar between the two models on the gene expression level in terms of magnitude and direction ($y=0.99x$; $R^2=0.71$). (**D**) Relative expression of RA-DE genes in WEC and rat embryos *in vivo* across time (Robinson, et al., 2012). Trends in RA response (in WEC and *in vivo*) correlated with each other and previously generated transcriptomic profiles of RA-response in embryos cultured in rat serum (WECb) or rat/bovine serum (WECc) containing media (Luijten, et al., 2010).

354. Overall, the WEC has proven to be an acceptable model to examine RA-induced defects during neurulation and early CNS organogenesis. Excessive RA exposure leads to molecular changes that can be evaluated in the context of embryo morphogenesis. Despite the usage of animals, the WEC may be considered a valid system to test retinoids and other environmental compounds in their effects on RA signalling in the context of early CNS development.

C.8.2. Embryonic Stem Cells (Mouse and Human)

355. ESCs derived from the inner cell mass of the mammalian blastocyst possess an unlimited potential for self-renewal and the capacity to differentiate into multiple somatic cell types, including neurons, oligodendrocytes, glial cells and astrocytes. Using defined protocols, mouse or human ESCs can be used as models of early CNS development. Biological processes critical for neurogenesis and neurulation (e.g., self-renewal, proliferation, differentiation, morphological patterning) can be interrogated *in vitro* in the context of environmental exposure (Figure C.5A). ESC model systems have been used to screen compounds for their potential to induce DNT. While both systems may be applicable for toxicological screening, hESC models may provide advantages over the murine system due to interspecies extrapolations (Ginis, et al., 2004). While the predictive value of ESCs for DNT screening has yet to be fully defined (Bal-Price, et al., 2018), early investigations suggest that these model systems can be used to study a wide range of risk factors on multiple aspects of neurogenesis (Bal-Price, et al., 2018) (Fritsche,

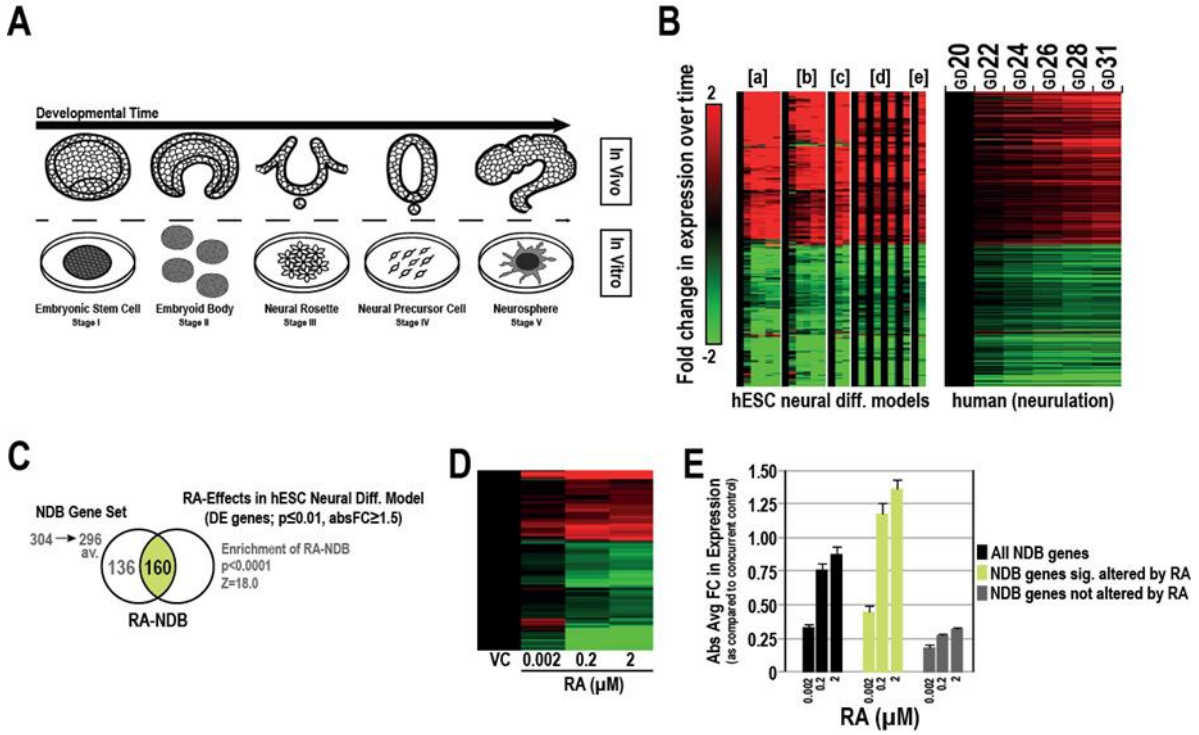
et al., 2018) (Singh, et al., 2016), including retinoids and other environmental chemicals that cause neurotoxicity by perturbing RA-signalling pathways via multiple mechanisms.

356. Due to its well-established role in promoting neuronal differentiation (Maden, 2001) (Janesick, et al., 2015), RA or Vitamin A is commonly added in ESC cultures to induce neural differentiation. Using a transcriptomic approach at four time points during mESC-embryoid body differentiation, Akanuma *et al.* (Akanuma, et al., 2012)demonstrated the stage-dependent sensitivity to RA at two concentrations (0.01 or 0.1µM). These analyses provided information regarding gene networks influenced by RA exposure at 0, 2, 8, and 36 days of mESC differentiation in parallel with cell morphological changes (increased growth and neuronal differentiation). They also identified particular targets (e.g., Gfap, Gbx2*)* that may amplify RA-signalling and neuronal development. In a protocol developed by Theunissen *et al.* (Theunissen, et al., 2011), mESCs differentiated towards a neuronal fate with the addition of RA, at a subcytotoxic concentration (0.5µM), led to significant downregulation in expression of Cyp26a1 and upregulation in expression of Aldh1a2 and patterning molecules, Hoxa1 and Hoxa2 as well as Pax6, a key transcription factor involved in early neuroectodermal differentiation.

357. The mESC model has also been used in a variety of ways to evaluate chemical toxicity. In recent studies, transcriptomic approaches were used to profile the global molecular effects of suspected neurodevelopmental toxicants, methylmercury (Theunissen, et al., 2011), valproic acid and triazoles (Theunissen, et al., 2012) on neural differentiation, demonstrating a wide-range of perturbations on the gene expression level. Specific to RA-signaling disruption in the context of mESC cardiomyocyte differentiation, 12 unique azoles─proposed inhibitors of CYP26─were observed to upregulate expression of Dhrs3 and inhibit expression of Cyp26a1, suggesting disruption in RA bioavailability/metabolism and signalling (Dimopoulou, et al., 2018).

358. The effect(s) of RA has also been examined in various hESC models in relation to neurodevelopment and DNT *in vitro*. For example, in a study using a hESC-based neural rosette (NR) model, a proposed screen to identify toxicants that disrupt early CNS morphogenesis and apical neuroepithelial organization of the neural tube, and toxicogenomics, the concentration-dependent effects on cell morphology, function and mRNA expression were evaluated (Colleoni, et al., 2011). Exposures to RA cause increased cytotoxicity (2µM), disorganized NR morphology (≥0.2µM), and perturbed expression of molecules critical for CNS patterning (HOXA1, HOXA3, HOXB1, HOXB4) and neural differentiation (FOXA2, FOXC1, OTX2, PAX7)─ suspected targets of RA *in vivo* (Bally-Cuif, et al., 1995) (Balmer & Blomhoff, 2002)─at concentrations as low as 0.02µM. These findings suggest that the hESC-NR *in vitro* model may be used to assess representative early CNS morphology, i.e., neural rosette formation, and gene expression relevant to RA-induced DNT.

Figure C.5. Human embryonic stem cell neural differentiation and response to retinoic acid.



Note: (A) Example schematic of transitions of human neurogenesis and hESC neurodevelopment (Chen, et al., 2019). hESC models can be used to evaluate representative aspects of human development *in vitro*. (B) Genes (304 total) commonly differentially expressed (DE) during hESC neuronal differentiation and human embryos undergoing neurulation, categorized as the NeuroDevelopmental Biomarker (NDB) gene set (Robinson, et al., 2016). NDB gene expression patterns over time strongly correlated between *in vitro* models and human embryos (C) Over 50% of NDB genes were identified to be significantly altered by RA in a hESC-neural rosette model. (D) Heatmap displaying concentration-dependent changes in expression of NDB genes. VC=vehicle control. (E) Absolute average fold change in expression of NDB genes and significant/non-significant subsets with RA exposure in hESCs.

359. Robinson *et al.* (Robinson, et al., 2016), performed a meta-analysis of publicly available transcriptomic datasets to define biomarkers for DNT assessments (Figure C.5B-E). First, genes DE commonly across five independent studies examining hESC neural differentiation were identified. These analyses suggested a core group of genes to be commonly changing as cells transformed from pluripotent hESCs to neurons. Secondly, genes identified *in vitro* were matched with whole human embryo transcriptomic datasets evaluating three unique developmental time periods: blastocyst formation, neurulation, and organogenesis. Based on expression profiles, the transition of hESCs to neural progenitors significantly correlated with human embryos undergoing neurulation/early organogenesis (3rd-4th gestational week; Figure C.5B). Genes in common between the two systems were termed the NeuroDevelopmental Biomarker (NDB) gene set which contained 304 genes. NDB genes were significantly enriched for biological processes involved in neurogenesis and CNS development. NDB genes were examined in independent datasets to demonstrate the utility of the NDB biomarker set for toxicological studies. RA was shown to significantly alter over 50% of the NDB set in a concentration-dependent manner in a hESC neurodevelopmental model (Figure C.5C-E). Other suspected compounds (valproic acid, carbamazepine) also affected NDB expression (not shown).

360. ESCs, isolated from either human or rodent, may provide a capable screening system for retinoids and other environmental chemicals. RA is commonly used as a supplement to induce ESC neural differentiation, and excessive amounts lead to perturbations in neuronal development, including alterations in molecules which regulate neurogenesis and neuronal differentiation and patterning in mammals.

C.8.3. Immortalized Cell Lines

361. Immortalized mammalian cell lines have provided significant insight in RA-mediated differentiation and represent viable model systems to evaluate retinoid-induced effects and DNT. The embryonal carcinoma (EC) cell lines, as surrogates for normal stem cells, have been thoroughly studied (Soprano, et al., 2007) (Bain, et al., 1994). The P19 mouse EC stem cell line, originally derived from an teratoembryocarcinoma, can be used to assess chemical effects and mechanisms of action related to neurogenesis and gliogenesis, including morphology, proliferation, differentiation, and neuronal specification as well as the functionality of ion channels and metabotropic receptors (Negraes, et al., 2012). Like ESCs, RA induces neuroectodermal differentiation in ECs (Jones-Villeneuve, et al., 1982). For instance, homeodomain transcription factors that are regulated by RA such as Pbx proteins are increased in P19 cells with RAR activation (Qin, et al., 2004), observations shared in mESCs (Kashyap, et al., 2013). Given their sensitivity to RA, P19 cells have been used to cross-evaluate the sensitivity of retinoids (i.e., RA, 13-cis-RA and 9-cis RA), in inducing neuronal differentiation (Adler, et al., 2005) and DNT (Solari, et al., 2010).

362. While the P19 line is the most comprehensively characterized, other immortalized cell lines may be candidates for incorporation in a DNT testing battery with contextual use, especially prior to differentiation (Cheung, et al., 2009). For example, the NE-4C neuroectodermal line, established from the cerebral vesicles of 9-day-old mouse embryos lacking functional p53 (a master transcriptional regulator of proliferation/apoptosis (Resnick, et al., 2005)), is highly sensitive to RA and RA precursors, retinal and retinyl acetate, and thus may also have promise in toxicity testing (Orsolits, et al., 2013). Other cell lines are sensitive to RA-DNT. For instance, the immortalized human neuroprogenitor cell line, RenNCell CX, shows significant changes in neurite outgrowth with RA exposure (Harrill, et al., 2011), and the EC line, NT2, is sensitive to chemical toxicants during RA-induced differentiation (Taylor, et al., 2019).

C.8.4. Zebrafish

363. The zebrafish (Zf) embryo model has been proposed as a rodent-alternative high-throughput screening system for toxicology (Hermsen, et al., 2011) (Selderslaghs, et al., 2009). The Zf embryo develops rapidly, completing organ development by ~120 hours post fertilization (hpf), and the effects of compounds on CNS morphology can be externally evaluated. Initial reports suggest that the Zf embryo model may be predictive in identifying developmental toxicants (Ball, et al., 2014) (Brannen, et al., 2010). Similar to mammals, RA signalling is critical for patterning of the anterior-posterior axis, and development of the hindbrain and spinal cord (Costaridis, et al., 1996) (Grandel, et al., 2002) (Lee & Skromne, 2014), and the Zf is also highly sensitive to RA-induced DNT (Papalopulu, et al., 1991). While many aspects of Zf development are comparable to mammals, the Zf may diverge from mammals in neural tube formation (e.g., folding of the neural plate instead of cavitation of the neural rod; (Lowery & Sive, 2004)), and the need of RA for body axis extension (Berenguer, et al., 2018). Furthermore, much like mammals, conserved elements involved in regulating RA embryonic distribution, including RA-metabolizing enzymes (e.g., cyp26a1 (White, et al., 2007) (Emoto, et al., 2005)), raldh2 (Begemann, et al., 2001), dhrs3 (Feng, et al., 2010), and RA binding proteins (crabp2a; (Cai, et al., 2012)) are suspected to control RA levels and signalling pathways as well as prospective HOX signalling and the organization of the Zf hindbrain/spinal cord (as reviewed (Samarut, et al., 2015)).

364. Using the Zf, several investigations have examined the relationship between excess or deficiencies in RA and DNT. In a concentration-dependent manner, Holder and Hill (Holder & Hill, 1991) demonstrated RA (0.001-1µM) to cause CNS and tail malformations. Concentrations of 0.1-1µM RA produced loss of the mid-hindbrain border, rhombomere abnormalities and reduced neurons within the anterior lateral line and ganglia. Follow-up studies by Herrmann et al (Herrmann, 1995) demonstrated the utilization of the Zf to compare potencies of nine retinoid compounds. Similar to differential potencies described in rodents, RA was identified to be most toxic retinoid - followed by 9-cis RA, 13-cis RA, tretinoin,

3,4-didehydro and 4-oxo – causing malformations, including defects of the CNS (anencephaly, microcephaly, anophyalmy). Concentrations of RA during embryogenesis which do not cause morphological defects may also cause DNT, including neurobehavioral changes (e.g., hyperactivity) in juveniles (Wang, et al., 2014).

365. In Zf, alterations in RA levels leads to similar changes in molecular targets as mammals. Global transciptomic analyses of Zf embryos exposed to ethanol (vehicle) vs. RA (0.1µM), during early stages of embryogenesis, suggest hundreds of genes to be disrupted by RA as early as 4h, including cyp26a/b1, dhrs3a/b, aldh1a2 (raldh2), WNT pathway members, Hox genes, transcription factors (sox, fox, meis), and estrogen receptors (Samarut, et al., 2014). In the same study, using translational-blocking morpholinos, the authors demonstrated that the expression levels of a subset of genes (~27%; 85 genes) altered by RA are dependent on rar expression. Additionally, like in mammals, genetic knockdowns of metabolic enzymes which promote biosynthesis (rdh10 (D'Aniello, et al., 2015); raldh2 (Retnoaji, et al., 2014)) or breakdown (cyp26a1 (Emoto, et al., 2005), dhrs3 (Billings, et al., 2013)) of RA as well RA binding proteins (crabp2; (Cai, et al., 2012)), are associated with CNS malformations, providing additional evidence of the relevancy of RA regulation in the Zf embryo.

366. In general, these studies suggest that the Zf may be used as a surrogate to rodents and be an appropriate model to evaluate RA effects on early CNS development, providing advantages over cell-based models due to the ability to assess both CNS morphogenesis and related conserved neurodevelopmental pathways.

C.8.5. In Silico Models

367. Computational *(in silico)* approaches may be used in many aspects of predictive toxicology. It includes diverse tools such as (1) structure activity modelling, e.g., QSAR; (2) chemoinformatics; (3) database repositories; (4) data mining, analysis, and prediction models (Loiodice, et al., 2019) (Parthasarathi & Dhawan, 2018). Due to vast experimental data regarding mechanisms related to RA signalling, generated data inputs have been incorporated into *in silico* applications to help define and predict outcomes in normal and abnormal development. In particular, adverse outcome pathways (AOPs) provide a framework to link environmental exposures to perturbations on molecular, cellular and functional levels that contribute to changes to DNT and disease (Ankley & Edwards, 2018). Molecular initiating events (MIE) and subsequent key events (KE) are defined based on scientific evidence. AOPs can be implemented in *in vitro*, *in silico* and *in vivo* toxicological testing strategies aimed at determining potential hazards. AOPs and other *in silico* models (*e.g.,* IVIVE modelling (Louisse, et al., 2015)) may be used to assist in: 1) predicting toxicological effects and related adverse outcomes; 2) guide the implementation of *in vitro*/*in vivo* assessments or data gaps in toxicological studies; and 3) interpreting common and unique RA effects across model systems. Tonk *et al.* (Tonk, et al., 2015) proposed an AOP connecting perturbations in RA signalling and neural tube/axial patterning defects. Studies of molecular interactions focused on the balance in RA and enzymatic family members which regulate RA bioavailability (Adh, Rdh, Dhrs3, Raldh, and Cyp26). Cellular effects (proliferation, migration, and differentiation) were proposed to be driven by RA and interactions with Wnt3a (rostral) and Fgf8 (caudal). Also described were suites of gene and gene families proposed to be regulated by RA involved in neural tube (Hox family, Krox20, Kreisler, Hnf1b, Meis2, Gbx family, Otx family, Grfs1, Zic family, and Crabp family) and axial patterning (Hox family, Cdx family, T, Notch, Lnfg, Axin2, Msgn1, Mesp2, Hes family, Dll family, Lef1, Tch15, Sfrp1, Tshz1, Fox family, Crabp family). Based on genetic evidence from mouse models, the authors provided key examples of enzymes involved in RA metabolism (Rdh10, Raldh2, Cyp26a1, Cyp26c1) where diminished function is known to be linked with imbalances in RA and defects in the neural tube, axial patterning and other malformations such as craniofacial and cardiac defects. As a proof of principle, the AOP framework was validated for hazard prediction using pre-existing experimental data of flusilazole exposures—an antifungal agent and proposed RA-signalling disruptor—in rat WEC, Zf, and ESC models.

Based on transcriptomic signatures, the authors presented AOP targets disrupted by flusilazole in the three model systems.

C.8.6. Brain patterning and neuron differentiation

368. Regional cell patterning and neuronal differentiation are dependent on bioavailability of RA controlled by the coordinated expression of metabolic enzymes (Table C.1). RALDH expression in the developing rodent telencephalon is well-defined (Smith, et al., 2001). RALDHs show a sequential activation pattern: *RALDH3* is detectable in the telencephalon by E11 and peaks early at E14 followed by a decline, whereas *RALDH2* shows a steady increase in expression starting from E12.5 with a peak shortly before P5. With regards to region-specific expression, RA seems to be critical in areas required for differentiation of neurons in the nigrostriatal system. After E11, *RALDH3* expression shifts to the lateral ganglionic eminence (LGE) and extends to the piriform cortex and septum (Li, et al., 2000). The LGE serves as local site of RA during striatal neurogenesis throughout development, with production originating from *RALDH3* expressing cells (Li, et al., 2000) (Waclaw, et al., 2004) and glia (Toresson, et al., 1999). LGE cells and neuronal subpopulations migrating through the LGE require RA signaling for GABAergic differentiation (Chatzi, et al., 2011). RA production in the LGE is mediated by *Gsh2* (Waclaw, et al., 2004), though *Gsh1* also plays a role in maintaining proliferation of LGE progenitors (Toresson & Campbell, 2001). *RALDH2* and *Rdh10* are also found in the surrounding meninges (Zhang, et al., 2003), and RA synthesis from that region is critical for cortical neurogenesis (Siegenthaler, et al., 2009).

369. As the CNS matures, downstream effectors of RA signaling are expressed in similar early regions of RA synthesis with increased spatial restrictions (Thompson Haskell, et al., 2002). This suggests that RA is involved in continued maintenance of these neurons after maturation (Zetterstrom, et al., 1999). For example, in the postnatal rodent brain the highest levels of endogenous levels of RA show similar regional distinctions as seen early, such as in the striatum, when measured with mass spectrometry (Kane, et al., 2008) (Kane, et al., 2005). *Meis* induction via RA, important for limb development (Mercader, et al., 2000), is also involved with maintenance of intermediate neurons such as those in the LGE (Marklund, et al., 2004) (Rataj-Baniowska, et al., 2015).

370. Accordingly, various *in vitro* models demonstrate that RA supplementation in culture regulates patterning factors in a manner similar to their *in vivo* counterparts (e.g., *HOXB4, LBX1, OTX1, GSX2*) (Merrill, et al., 2004) (Nadadhur, et al., 2018). Stem cell sensitivity to RA is mediated by *HOXA1*, as *HOXA1*-null cells express significantly lower levels of neuronal markers (Martinez-Ceballos & Gudas, 2008). RA can be used to differentiate cells towards numerous identities, including serotonergic, GABAergic, glutamatergic, and dopaminergic neurons. Transcriptional regulation of these different differentiation processes is context-dependent, based on factors such as RA levels (Okada, et al., 2004) and positional cues (Carcagno, et al., 2014). This can be illustrated by the differentiation programming of cells from the ventral p3 hindbrain domain, which can become glutamatergic V3 spinal interneurons or serotonergic hindbrain neurons despite originating from the same progenitor pool. RA signaling can influence the fate of these progenitors through dose-dependent attenuation of *ASCL1* levels via the Notch pathway, with areas of low RA/high *ASCL1* being permissive of serotoninergic differentiation and RA repression of *ASCL1* inducing a glutamatergic V3 identity (Jacob, et al., 2013).

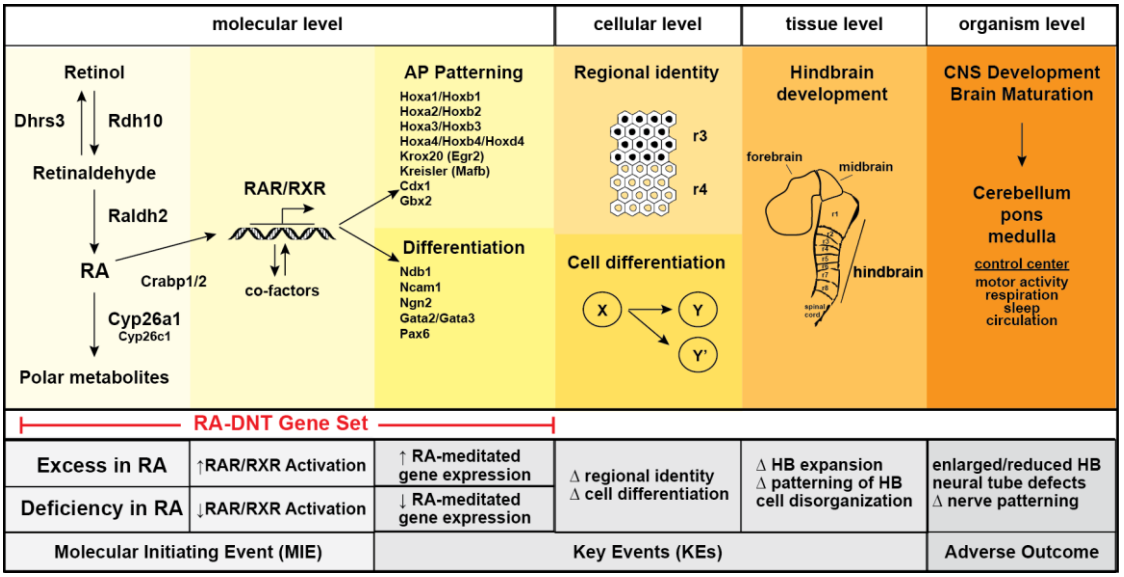
371. RA transcriptionally regulates dopaminergic differentiation via activation of the dopamine autoreceptor D2 at a RARE in its promoter region (Samad, et al., 1997) *RALDH1* is expressed in neurons of the ventral midbrain from E9.5 in rodents (Wallen, et al., 1999) and becomes restricted to the dorsal retina and dopaminergic neurons in the substantia nigra and ventral tegmentum (McCaffery & Drager, 1994) in adulthood. *RALDH1* expression in these regions is transcriptionally regulated by *Pitx3,* as evidenced by loss of the enzyme in the meso-diencephalic dopaminergic neurons of *Pitx3* knockout models and restoration with RA (Jacobs, et al., 2007). In those same regions, midbrain dopamine neurons can co-release GABA and dopamine without *Gad67* upregulation, instead utilizing an alternative *Aldh1a1*

(*RALDH1)* synthesis pathway (Kim, et al., 2015). *Gad67* upregulation is also a demonstrated effect of RA, though the mechanism is unclear as no RARE has been identified in the promoter region (Chatzi, et al., 2013).

C.9. A Provisional Adverse Outcome Pathway of RA-induced DNT

372. A preliminary AOP of RA-induced DNT, which describes the mechanistic links between excessive or deficiency in RA bioavailability and defects in hindbrain development, is outlined in Figure C.6.) (Chen, et al., 2020). Initially, we connected evidence supporting the role of RA in hindbrain formation/patterning and neuronal differentiation on the molecular, cellular, tissue, and organism level. The initial development and segmentation of the hindbrain occurs within the period of neurulation and early organogenesis, approximately between the 3rd and 4th weeks of human pregnancy, GD7.5-10 in mouse, and GD9.5-12 in rat. RA bioavailability is regionally controlled by a suite of enzymes. Rdh10 and Raldh2 are expressed in the paraxial mesoderm and promote biosynthesis of RA and diffusion to proximal tissues. Cyp26a1 is highly expressed in the anterior region and strictly limits RA bioavailability in this region. Nuclear transfer of RA and activation of RAR/RXR leads to the recruitment of co-activators and co-repressors which mediate specificity of transcription. In a tightly regulated sequence, segments of the hindbrain are formed due to RA bioavailability and subsequent RA-induced gene expression. RA regulates the regional expression of several genes and signalling molecules that underlie hindbrain segmentation and regional identify, including Krox20(Egr2), MafB, and Hox family members (Tumpel, et al., 2009) as well as target genes involved in the differentiation of cells within each region.

Figure C.6. A provisional adverse outcome pathway for RA and hindbrain development.



Note: Under normal conditions during the initial stages of CNS development, RA is regionally restricted by enzymes that promote biosynthesis (Rdh10, Raldh2) or elimination (Cyp26a1, Cyp26c1) of RA. Binding proteins (e.g., Crabp1/2) facilitate RA transfer to the nucleus. RA binding of RAR/RXRs leads to recruitment of co-activators and co-repressors which mediate specificity of transcription. Other key signalling molecules (*e.g.,* Fgf, Wnt) influence RA bioavailability and RAR/RXR activation. RA mediates expression of multiple gene and gene families involved in patterning and differentiation. On a localized level, changes in expression lead to cell specification (patterning) and cell differentiation, and underlie expansion of the hindbrain and maturation of the CNS. The hindbrain serves as the basis for the cerebellum, pons, and medulla. A prospective MIE that alters RA availability and/or activation of RAR/RXR can lead to KEs that change the expression of genes responsible for regulating cell regional identify and differentiation. These altered pathways underlie perturbations in hindbrain expansion and cell organization, which manifest as adverse outcomes in brain development and structure. Molecular signatures linked with RA-induced DNT may serve as as predicitive indicators of RA signalling disruption. Adapted from (Chen, et al., 2020):

373. Alterations in bioavailability of RA, in excess or deficiency, can result in severe malformations and/or variants in hindbrain development and posterior segmentation. These changes can occur on the molecular, cellular, and tissue level in the CNS, and depend on the timing/duration of RA exposure, dose, and factors such as genetic variants in RA metabolic enzymes (reviewed in Section C.5). In mammals, imbalances in RA (excessive or deficiency) can result in DNT, including changes on the molecular, cellular, and tissue level in the CNS, depending on the timing/duration of exposure, dose, and many other factors (see Sections C.6 and C.7). Excess RA results in hyper activation of *RAR/RXR*-mediated gene expression, whereas deficiency in RA, leads to lower activation of *RAR/RXR*-mediated gene expression.

374. As a result, MIEs may be initiated via multiple mechanisms, including by: 1) altering the bioavailability of RA via interference with enzymes that regulate spatiotemporal gradients of RA; or 2) interacting with RAR/RXR and/or its cofactors, thus, modifying RA-mediated gene expression. Subsequent KEs may include dysregulation of RA-mediated target genes critical for regional cellular identity (*e.g., Hox* family members, *Krox20, MafB, Gbx2, Ncam1)* and neuronal differentiation (*e.g., Ngn2, Dbx1, Pax6*). Perturbations in these pathways are suspected to underlie disruption of hindbrain expansion and disorganization on the tissue level. Postnatally, defects may appear as irregularities in CNS morphology (abnormal segmentation), NTDs, and/or changes in brain patterning and size. Disruptions in the development of these regions can potentially manifest in, or increase the risk of, adverse neurobehavioral outcomes such as deficiencies in motor control or sleep on the organismal level. While multiple targets have been proposed in the event of excess or deficiency in RA, the direction in which changes on the gene level may occur are complex and highly dependent on the concentration of RA and the point in

developmental time when the embryo is exposed. Thus, in the preliminary framework, we propose that alterations in either direction in specific gene/gene families may be biomarkers of changes in RA status.

375. Traditional *in vivo* rodent toxicological tests provide information regarding chemical effects after exposure in utero during critical windows of neurodevelopment relevant for the proposed AOP. Effects of chemical exposures in utero which alter RA signalling pathways can be evaluated on gross morphological levels in the CNS as well as functional and neurobehavioral outcomes in the offspring. *In vitro* models, including systems reviewed in this document (WEC, ESCs, cell lines, and Zf) may be used as screens to identify compounds that cause DNT by perturbing RA signalling at multiple steps within the context of the provisional AOP, including the disruption of RA metabolic enzymes, carrier proteins, RAR/RXRs, and downstream targets involved in hindbrain formation, cell identity and differentiation. Uniquely, in comparison with cell models, WEC and Zf provide information regarding effects of compounds on relevant morphological endpoints (e.g., hindbrain patterning and neural tube formation) linked with perturbations in RA. Other *in vitro* or *in vivo* model systems may be used as is, or modified (e.g., adding additional molecular readouts) to evaluate KEs within the provisional AOP framework, including non-mammalian *in vivo* assays currently recommended or considered to identify potential endocrine disrupting compounds by OECD (Table C.3).

Table C.3. Potential OECD non-mammalian *in vivo* assays to screen for RA-DNT

OECD Test Guidelines (TG)	Endpoints related to AOP	Other RA DNT endpoints
OECD TG 210: Fish, Early-life Stage Toxicity Test	Appearance (morphology)	Behaviour, and survival
OECD TG 234 Fish, Sexual Development Test	Appearance, morphological abnormalities	Behaviour
OECD TG 241 The Larval Amphibian Growth and Development Assay	Growth (morphology)	Mortality, behaviour
OECD TG 206 Avian Reproduction Test	Appearance (morphology)	Viability
OECD TG 240 Medaka Extended One Generation Reproduction Test	Appearance (morphology); histopathology	Behaviour
The Draft TG Zebrafish Extended One Generation Reproduction Test	Appearance (morphology); histopathology	Behaviour

C.10. Toxicogenomic Response to RA in Neurodevelopmental Models

376. The recent use of genomic approaches, including transcriptomic, proteomic, and methylomic profiling, has increased our ability to identify molecular targets of environmental chemicals in association with DNT. The effects of retinoids have been profiled in diverse relevant models, including: *in vivo* (rat), WEC, mESC, hESC, cell lines, and zebrafish (Table C.4). In general, these studies have shown that excess RA disrupts expression of many genes, including molecules involved in RA metabolism, embryonic patterning and neurodevelopmental pathways. The integration of toxicogenomic datasets related to RA exposures, combined with genetic studies in animal/cell model systems, may assist in defining additional molecules involved in RA signalling and predictive biomarkers of RA response.

Table C.4. Examples of toxicogenomic studies assessing effects of RA exposures in animal and *in vitro* models.

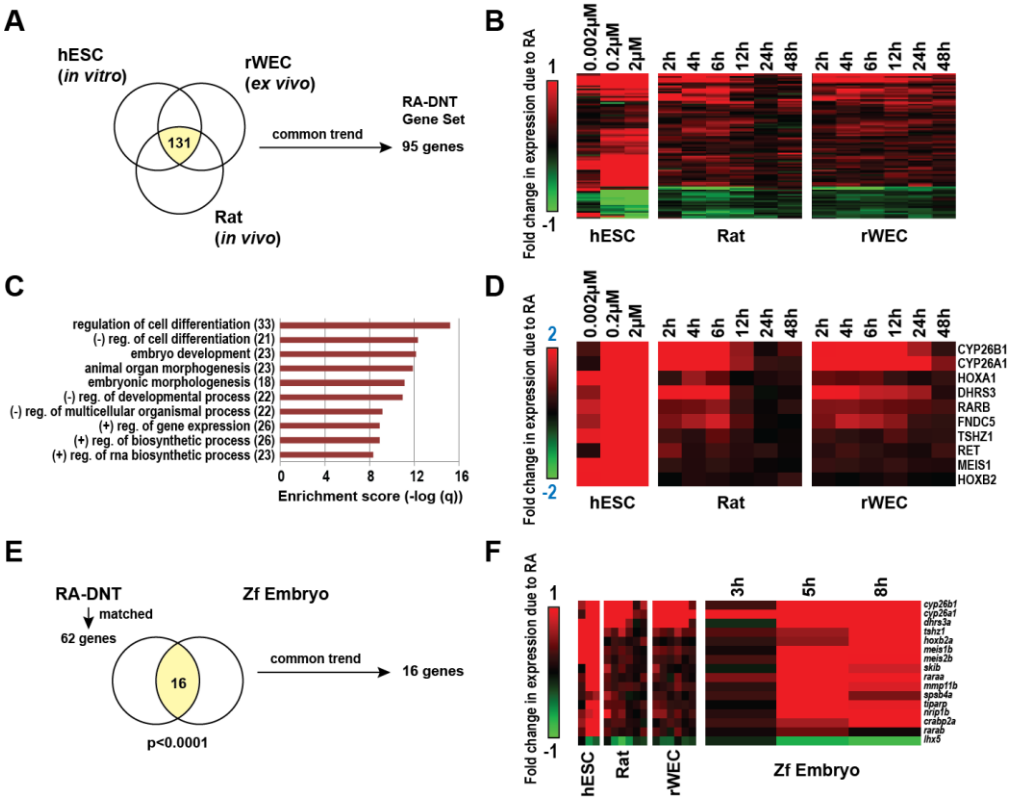
NCBI/NCI (Geodata) and EMBL/EBI (Arraytrack) data repositories were searched for relevant datasets assessing retinoic acid exposure *in vitro* or *in vivo*. References (Ref) are noted (if available). Table adapted from (Chen, et al., 2020)

Dataset	Species	Model	Design	Ref
Datasets used to identify a RA-DNT Gene Signature in humans and rodent (see below)				
EMEXP3577	Homo sapiens	hESC (HUES1); neural rosette model	Concentration-dependent response, ~7 day exposure (0.02-2µM)	(Colleoni, et al., 2011)
GSE33195	Rattus norvegicus	*In vivo*	RA (50mg/kg), exposed on GD10; 6 timepoints	(Robinson, et al., 2012)
GSE33195	Rattus norvegicus	WEC (*ex vivo*)	RA (50mg/kg), exposed on GD10; 6 timepoints	(Robinson, et al., 2012)
Other datasets				
GSE104173	Homo sapiens	hESC (H1, H9, HUES1); neural differentiation model	Time-dependent response	NA
GSE1596	Mus musculus	Neuroblastoma (Neuro2a)	Time-dependent response	NA
GSE4075	Mus musculus	mESC; embryoid body	Time-dependent response in embryoid bodies	(Aiba, et al., 2006)
GSE28834	Mus musculus	*in vivo*	Spinal cord of GD18 fetuses	NA
GSE57639	Mus musculus	mESC	RA-response (single concentration)	NA
GSE43755	Danio rerio	Zf	Time-dependent response	(Weicksel, et al., 2013)
GSE135781	Danio rerio	Zf	Vagus motor neurons at 38 hours post fertilization	NA
GSE41335	Danio rerio	Zf	Exposed to RA or 9-cis RA, evaluated at 24/72h	(Oliveira, et al., 2013)
GSE16264	Danio rerio	Zf	Exposed to RA or RA antagonist	(Feng, et al., 2010)

377. As a proof of principle, we defined a mammalian gene signature associated with excessive levels of RA during early neurodevelopment conserved across *in vitro*, *ex vivo* and *in vitro* models. We identified three unique datasets from two studies: E-MEXP-3577 and GSE33195, assessing the: 1) concentration-dependent response of RA in hESCs differentiating towards neural cells (neural rosettes) or 2) time-dependent response of a toxic concentration of RA in cultured embryos (WEC) or *in utero* during neurulation and early organogenesis (Table C.4). As previously described (Robinson, et al., 2016), datasets were retrieved and independently processed, i.e., log2 transformed, robust multi-array (RMA) normalized, and annotated. DE genes due to RA were identified via generalized linear models (ANOVA), and datasets were compared to identify common targets across all three models. In total, we observed

131 DE genes ($p<0.005$; Figure C.7A). Within this gene subset, 95 genes displayed common trends in RA response (either all up or downregulated) in all three models (Figure C.7B).

Figure C.7. Common RA-responses in models assessing developmental neurotoxicity.



Note: (**A**) In total, we identified 131 genes to be commonly differentially expressed (DE) in association with DNT across *in vitro*, *ex vivo* and *in vitro* models of neurodevelopment (p<0.005, ANOVA). (**B**) Within this subset, 95 genes displayed common trends in regulation, of which 74 were upregulated, 21 were downregulated. We defined this subset of gens as the RA-DNT gene set. (**C**) Enriched GO Biological Processes within the RA-DNT gene set ($q<1*10^{-10}$). (**D**) RA-DNT genes with an absolute average fold change greater than 2-fold (log 2 scale; RA vs. control). (**E**) We matched RA-DNT genes to 62 homologues in the Zf embryo in a published dataset. (**F**) Within this subset, we identified 16 genes to be altered with RA in the Zf embryo model. All 16 genes trended in similar fashion to RA-exposed mammalian model systems.

Source: Adapted from: (Chen, et al., 2020); . Referenced datasets: E-MEXP-3577 (Colleoni, et al., 2011), GSE33195 (Robinson, et al., 2012) and GSE43755.

378. We performed enrichment analysis of the defined gene subset, "RA-DNT" using Gene Set Enrichment Analysis (GSEA; (Subramanian, et al., 2005)). RA-DNT genes were significantly enriched for processes related to regulation of cell differentiation, embryo development, embryonic morphogenesis and regulation of gene expression (q<1*10^-10; Figure C.7C). Upregulated targets identified through this analysis included molecules involved in RA metabolism (CYP26A1, CYP26B1, DHRS3), RA signalling (RARA, RARB), RA binding (CRABP2), patterning (HOXA1, HOXB2) and differentiation (MAFB, MEIS1, MEIS2, PBX1, SIX, SKIL; Figure C.7D). We propose that this gene set may be used to identify potential compounds that cause DNT via RA-mediated pathways within the provisional AOP framework (Figure C.6). For example, RA-DNT genes were significantly overrepresented in genes altered by 10/13 azoles in rat WEC, suggesting RA-signaling disruption by the majority of tested azoles. Furthermore, trends in dysregulation by azoles generally matched gene expression profiles associated with excess RA (Chen, et al., 2020).

379. Another application of the RA-DNT gene set could be used to compare across mammalian and non-mammalian model systems for common/unique mechanisms of RA-induced DNT. In a preliminary

comparison, common patterns in dysregulated expression of RA_DNT genes were observed between mammalian models and Zf embryos exposed to RA (100nM) (Figure C.7E); 16 genes were commonly dysregulated, including: *crabp2a*, *cyp26a1*, *cyp26b1*, *dhrs3a*, *hoxb2a*, *lhx5*, *meis1b*, *meis2b*, *mmp11b*, *nrip1*, *raraa*, *rarab*, *skib*, *spsb4a*, *tiparp*, and *tshz1* (Figure C.7F), supporting conservation of specific targets across vertebrate models in the context of RA exposure and DNT (Weicksel, et al., 2013).

C.11. Proposed Developmental Neurotoxicants and Altered RA Signaling

380. Environmental chemicals are hypothesized to cause DNT via multiple defined (Rock & Patisaul, 2018) and unknown mechanisms of action (MOA). Many compounds may induce DNT by perturbing RA signalling as a direct or secondary MOA. Here, we review a subset of diverse compounds proposed to alter RA signalling in association with DNT in animal and/or *in vitro* model systems.

C.11.1. Triazoles

381. Azoles represent a diverse class of compounds (e.g., triazoles, imidazoles) which contain a five-membered ring comprised of at least one nitrogen atom and at least one other non-carbon atom. Azoles are widely-used as antifungal agents due to their ability to impair ergosterol formation, a necessary component of fungal cell-wall integrity, via inhibition of lanosterol 14α-demethylase (CYP51A1). In rodents, zebrafish, and xenopus, select azoles induce CNS malformations similar to observations as RA (at excess levels) (Menegola, et al., 2006), and are suspected to alter RA signalling pathways by inhibiting CYP26 (Menegola, et al., 2006). In the rat WEC model, flusilazole, in a concentration and time-dependent manner, induces NTDs, compromises CNS morphology, and alters expression of RA metabolic enzymes (Cyp26a1, Cyp26b1, Dhrs3) and signalling molecules critical for hindbrain/spinal cord development (e.g.,. Gbx2, Cdx1) (Dimopoulou, et al., 2016) (Robinson, et al., 2012). Using benchmark concentration modelling and transcriptomics, Robinson *et al.* demonstrated that perturbations in gene expression related to RA metabolism and hindbrain development were observed at significantly lower concentrations (~1 order of magnitude lower) than alterations in CYP51A1 and genes regulating cholesterol biosynthesis (Robinson, et al., 2012). Furthermore, in this same study, the authors found high similarities in transcriptomic profiles across flusilazole, cyproconazole and triadimefon exposures, including upregulated expression of enzymes which reduce RA bioavailability (Cyp26a1, Dhrs3). These particular targets were shown to be conserved across WEC, mESC, and Zf model systems in response to triazole exposures (Robinson, et al., 2012), suggesting that they may be used as biomarkers in relation to RA-effects.

C.11.2. Alcohol (Ethanol)

382. Fetal alcohol spectrum disorder (FASD) shares characteristics with RA insufficiency, including craniofacial defects, microcephaly, and cognitive deficiencies (Jones & Smith, 1973). Accordingly, a potential mechanism of FASD is competitive inhibition of ethanol (EtOH) against RA synthesis (Pullarkat, 1991) (Deltour, et al., 1996). In EtOH treated *Xenopus* embryos, RA signalling is decreased at the early gastrulation stages as measured with the RAREZ reporter, as are genes such as *hoxb1* and *hob4*, with overexpression of *raldh2* partially rescuing these phenotypes (Kot-Leibovich & Fainsod, 2009). Decreases in other Hox genes, such as *hoxa2*, *hoxb3*, *hoxb4*, and *hoxb9* were also observed with EtOH treatment in *Xenopus* embryos, and the teratogenic effects of excess retinol or retinal can be mitigated with EtOH (Yelin, et al., 2005). In Zf, alcohol disrupts RA signalling, leading to defects of the mid/hind brain boundary, and endogenous RA can rescue these effects (Zhang, et al., 2015). However, observations within the brain proper suggest that EtOH also increases RA production. EtOH administered to Sprague Dawley pups prenatally from embryonic day 13-19 significantly induces cortical and hippocampal RA levels (Kane, et al., 2010), and early postnatal (P7) exposures to RA alters the expression pattern of cerebellar RA receptors with associated reductions or increases in the DNA binding potential of Rara/g and Rxrg,

respectively (Kumar, et al., 2010). Taken together, this is sufficient evidence to demonstrate that EtOH affects RA signalling homeostasis, though further research is needed to determine precise molecular mechanisms.

C.11.3. Organochlorine pesticides

383. The agnostic potential of organochlorine pesticides towards RARs has been previously documented, suggesting that developmental exposure to these chemicals can mediate DNT via disruption of RA signaling (Kamata, et al., 2008). Wnuk *et al.* (Wnuk, et al., 2016) identified that dichlorodiphenyldichloroethylene (DDE) induces cytotoxicity in primary murine cultures of hippocampus, cortex, and cerebellum at μM levels, with equivalent concentrations upregulating Rxra and Rxrb expression. While pharmacologic antagonism of RXR with HX-531 can modestly ameliorate DDE-induced cytotoxicity at lower concentrations, genetic knockdown of Rxra or Rxrb significantly reduced caspase-3 and lactate dehydrogenase (LDH) release, suggesting DDE can compromise RA signaling to induce cytotoxic effects (Wnuk, et al., 2016). Though other organochlorine pesticides have been identified as Rara and Rarg activators, to our knowledge few other studies exist evaluating their role as disrupters of RA signaling, in the context of neurotoxicity (Lemaire, et al., 2005).

C.11.4. Flame retardants

384. In Zf embryos, acute exposure to DE-71, a common flame retardant mixture of polybrominated diphenyl ethers (PBDEs), reduces retinol and RA levels, and upregulates transcription of raldh2, crabp1a, crabp2a, and raraa, suggesting a disruption of RA homeostasis. Alterations are in association with defects in eye development of fish larvae (Xu, et al., 2013).

C.11.5. Isoflurane

385. Isoflurane, a common anesthetic, impairs mouse fetal growth and development (*in vivo*, exposed pre-neurulation) and alters mESC self-renewal (*in vitro*) and RA-signalling pathways (Liu, et al., 2015). In the same study, *in vitro*, Vitamin A supplementation was observed to attenuate effects of isofluorane, suggesting toxicity to occur through RAR-mediated pathways.

C.11.6.Summary

386. RA is a signalling molecule vital for CNS development in vertebrates, including rodents and humans. Retinoids and other environmental chemicals may target RA signalling pathways leading to DNT. While unresolved, mechanisms regulating RA and mediated-effects have been delineated at a detailed level, including molecular pathways mediated by RA that control cellular processes critical for the initial steps of CNS development. Here, we propose a variety of *in vitro* and in silico approaches that may be used to screen the ability of chemicals to cause DNT. Future *in vivo* and *in vitro* analyses aimed at defining the role of RA in CNS development, including neural differentiation and neurobehavioral pathways, may lead to improved definition of AOPs, biomarkers and targets that may be used to evaluate a chemical's ability to disrupt RA signalling pathways and contribute to DNT.

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Annex D. Summary Record from November 2019 Meeting of the Expert Group on Retinoid Pathway signalling

D.1. Opening of the meeting and adoption of the draft Agenda

388. The meeting co-chairs Anne-Lee Gustafson (Sweden) and Patience Browne (OECD secretariat) welcomed participants to the meeting. The meeting was attended by 19 experts from 5 member countries, the European Commission, and BIAC. A list of meeting participants is provided in Annex 1.

389. To provide background and context for the project, the meeting was opened with a short presentation from Sweden and the secretariat. The project to develop a draft Detailed Review Paper (DRP) on Retinoid Pathway Signalling was discussed in several meetings of the Endocrine Disruptor Testing and Assessment (EDTA) Advisory Group. The EDTA recommended focusing on the male and female reproductive systems, craniofacial and skeletal system, and central nervous system (CNS) where information is available regarding effects of retinoids. The draft DRP is organised as a main section that describes the biology of the retinoid system, and effects of retinoids on each of the selected organ systems is described in a separate annex to the main document. In each annex, chemicals with adverse effects on the respective organ systems are described, along with potential targets that may mediate chemical effects on the retinoid signalling pathway. *In vitro* and *in vivo* endpoints are identified, and in some cases, candidate assays (existing or for potential development) are mentioned.

390. A draft of the DRP was circulated for written commenting in September 2019. The aim of the meeting is to get input on specific comments and recommendations for next steps from the expert group.

D.2. Overarching biology of the retinoid section

391. Charlotte Nilsson (Sweden) presented an overview of the main body of the DRP that describes the biology of the retinoid system and highlights key molecules involved in retinoid pathway signalling.

392. Some specific challenges regarding laboratory analyses of retinoids were highlighted. For example, due to double bonds in the isoprene unit of natural and synthetic retinoids, biochemical quantification requires that samples are protected from natural light and there is still some controversy regarding the biological activity of specific retinoid metabolites. Retinoid limitation is simulated with the vitamin A deficient (VAD) rodent model, however, it is difficult to study effects on offspring as severe vitamin

A deficiency is associated with pre-implantation reproductive failure and less severe deficiency is associated with mid-gestational embryonic death.

393. The complexity of the retinoid synthesis, local tissue metabolic activation and inactivation, gradient effects within a tissue were all highlighted. Receptor-ligand signalling is also complex; retinoids bind to the nuclear retinoic acid receptor (RAR) which forms heterodimers with the retinoid X receptor (RXR). RXR does not require a ligand to be active, and it heterodimerises with a variety of other nuclear receptor partners.

394. The group discussed the genetic models that may completely knock out a gene involved in retinoid signalling, but for examining potential toxicological effects of environmental chemicals on retinoid signalling, more subtle changes are of interest. While knock-out models can be helpful for investigating the physiological effect of gene/proteins, it was also pointed out that in order to examine effects of environmental chemicals, pharmaceutical manipulation to alter retinoid pathway signalling may provide different and complementary information.

D.2.1.Cross-talk and epigenetic effects of retinoid signalling

395. On the last day of the meeting Miriam Jacobs (Public Health England, UK) provided an overview of sections describing cross-talk and epigenetic effects of retinoids that are forthcoming and will be added to the overview of retinoid biology. Some information on epigenetic effects of retinoids is coming from the work evaluating microRNAs in the context of developing an integrated approach to testing and assessment (IATA) for non-genotoxic carcinogenicity. Transcriptomics data from cell transformation assays may also be informative. Literature on the topic has been collected and will be reviewed for the next draft.

396. The group noted that this is an important topic and recent publications on retinoid effects on stem cell differentiation could be considered in the context of epigenetics. Further, there is some information on retinoid interactions with RAR/RXR that changes DNA methylation patterns which will also be included in the upcoming draft.

D.3. Reproductive system

D.3.1. Female reproduction

397. Charlotte Nilsson (Sweden) presented an overview of the female reproduction section and comments received in the WNT/Expert Group commenting round. The section includes a description of the role of retinoids in the formation of female reproductive tissues, germ cell differentiation and meiosis, development and function of female reproductive tissues, and alterations to female reproduction via chemicals acting through the retinoid system.

398. There are conflicting data regarding the requirement of retinoids for meiosis in the mammalian ovary. In support of the role of retinoic acid (RA) in the mouse ovary, retinoid-synthesising enzymes (RDH and RALDH) are present in the foetal mesonepheros, coelomic epithelium and foetal ovary. CYP26B1 is detected in the female mouse foetus at earlier stages but not around the time of initiation of meiosis, supporting the hypothesis that levels of retinoid acid required for induction of Strat8 and Rec8 may be sufficient. Treatment of cultured foetal mouse ovary with an RAR antagonist inhibited meiosis, and meiosis initiation was inhibited in the rat VAD foetal ovary. Experimental treatment of the foetal testis also support the role of RA in germ cell meiosis. Under normal conditions, meiosis is not initiated in the foetal mouse testis, however, treatments results in increased RA (either directly or due to inhibition of CYP26B1 inhibition) are associated with meiosis in the gonad of XY individuals. In contrast, normal meiosis occurred in the foetal mouse ovary lacking enzymes necessary for RA synthesis (RALDH2 and RALDH3) and foetal

mice lacking functional RARs. From these studies it is not clear if RA plays an obligate or facultative role in ovarian germ cell meiosis or if differences may be due to the various experimental models evaluated.

399. The group discussed these conflicting results. The input was that there was more experimental data supporting the requirement of RA in ovarian germ cell meiosis, and data supporting the lack of RA requirement for ovarian germ cell development, namely normal meiosis in the RAR knock-out model, may be due to non-genomic effects. The experts noted there are differences between the physiology of mice and humans (e.g., in mice, ovarian *Cyp26b1* expression is known to be downregulated prior to meiosis initiation, while such downregulation is not observed in the human ovaries) and if the aim is to develop retinoid assays that protect humans, it may be helpful to develop separate AOPs for humans and for the various animal models.

400. The group commented that new studies regarding the RA effects in males and effects on somatic (e.g. granulosa) cells in females may provide further insight and the references will be added to the updated draft. The issue of potential cross-talk between the retinoid system and other systems was acknowledged as an important topic by the group. The group further noted that oestrogen induces RAR and the use of the SERM (Selective Estrogen Receptor Modulator) tamoxifen in some of the foetal studies should be considered with this in mind.

401. With regard to the role of retinoic acid in the development and function of the ovary, RA is believed to be required. RA treatment of somatic cells within the gonad has a feminising effect *in vivo*, stimulates granulosa cell proliferation *in vitro*, and activates primordial follicular development *ex vivo*. Experimental mouse models with disrupted RAR somatic cell signalling during a specific time frame of sex differentiation had normal ovarian differentiation and fertility.

402. In the adult mammalian ovary, retinoid signalling seems to be involved in folliculogenesis and steroidogenesis. Retinoids also have differential effects on the endometrium during the follicular and luteal phases of the ovarian cycle that may be mediated by changes in RAR/RALDH expression upregulated by oestrogen during the follicular phase and CYP26A1 regulated by progesterone during the luteal phase. Decreases in RA appear to be necessary for successful embryonic implantation.

403. Chemicals with potential impact on the female reproductive system were identified from systematic and non-systematic searches of the published literature. Few chemicals were identified that were studied for both retinoid and reproductive effects, but those that were identified were included in the section. The AbstractSifter tool (discussed below) can help to identify additional chemicals based on refined search criteria. Two “provisional” AOPs were proposed evaluating retinoid effects on foetal ovarian meiosis and endometriosis. The FREIA project, which is part of the European Commission Horizon 2020 EURION cluster (https://eurion-cluster.eu/) and includes members of the Retinoid EG, is working to develop putative AOPs for female reproductive toxicity and may be referenced as they become available. While the AOPs presented for this section are very preliminary drafts, the group agreed these may be helpful for organising information and identifying strengths/weaknesses in data. Despite the preliminary draft nature of these AOPs, the Expert Group commented that it would be helpful to enter these in the AOP wiki as the work to review the literature supporting the key event relationships had been done to develop DRP.

404. The group remarked that a recent paper from Denmark updated what is known regarding ovarian dysgenesis syndrome and may add additional insight. Denmark also noted that Brunel University contributed information on the effects of retinoids in wildlife to a previous draft of the DRP and it would be helpful to include the information in a future update of the DRP draft.

D.3.2. Male reproductive systems

405. Charlotte Nilsson (Sweden) presented an overview of the DRP section of male reproduction organised similarly to that of the female with a review of the role of retinoids on germ cell meiosis, testis development, function and health of male reproductive tissues, and the impact of chemicals that affect

male reproduction via the retinoid system. The timing of germ cell meiosis in the testis is very different than in that of the ovary. Within the seminiferous tubule, RA produced by Sertoli cells from ROH plays a role in differentiation of spermatogonia, meiosis, spermiation (in rodents) and expression in the epithelium of the seminiferous tubule during specific stages of sperm maturation. The RA degrading enzyme CYP26B1, expressed in the peritubular cells of the testis, constitutes a barrier against extra-tubular/circulating RA levels.

406. The role of RA in germ cell meiosis based on experimental results in male rodents are similarly difficult to resolve. Impaired RA signalling in transgenic and rodent VAD experimental models are associated with undifferentiated spermatogonia and arrested spermatogenesis/spermiation, effects which can be reversed with retinoid supplementation. VAD males are infertile, and data indicate that presence of RA in the fetal testis initiates (premature) germ cell meiosis. However, delayed germ cell meiosis does occur in transgenic mice lacking RALDH necessary for RA synthesis. Again as in the female, RA may be facultative rather than required or differences may occurred due to the experimental model evaluated. Several male reproductive impairments (e.g. testicular dysgenesis syndrome; testicular cancer) are associated with undifferentiated spermatogonia, and cryptorchidism is observed in VAD rats. Several chemicals are known to have adverse effects on male reproduction and alter retinoid synthesis/signalling, however, for most of these, the link between male reproductive effects and retinoids has not been demonstrated. There is a strong indication that there are somatic effects of RA (e.g. the ovotesits has granulosa-like cells in the gonad) and there may be utility in examining RA interactions with other signalling molecules.

407. Two “provisional” draft AOPs for retinoid effects on the male reproductive system were proposed: inhibition of Cyp26b1 in the fetal rodent testis leading to impaired male fertility, and inhibition of RALDH or induction of Cyp26b1 in the adult rodent testis leading to impaired male fertility. Humans have lower fertility than mice, so decreased sperm counts are much more relevant predictors of impaired fertility. For example, it was pointed out that spermatogenesis can be reduced by 95% in rats without altering fertility. The physiology of spermatogenesis (e.g., the length of time in each stage and total time of spermatogenesis) also differs substantially between rodents and humans and this should be considered in AOP development.

408. The group discussed the role of retinoids on male reproduction. While there have been a number of recent publications on the topic, there are no definitive answers. The group discussed the aims of the OECD project, which is first to determine if current *in vivo* test guidelines may miss adverse effects of chemicals acting through the retinoid signalling pathway, and if so, which endpoints/assays could be added, and what *in vitro* assays are available to evaluate targets. It was pointed out that many simple organisms (e.g. yeast) have functional meiosis without retinoids, so the evolutionary advantage of retinoid involvement in the process is not known. Rather than a direct effect, disrupted retinoid signalling may perhaps affect male reproduction due to an indirect and poorly understood effect such as disruption of the blood-testis barrier. Other comments included that “impaired fertility” as an adverse outcome (AO) in the draft AOPs may be too general and it may be more useful to focus on more specific effects.

D.3.3. Scoping exercise for assays/endpoints.

409. Charlotte Nilsson (Sweden) gave a presentation on the initial scoping effort to identify assays and endpoints for identifying chemicals that effect male and female reproduction via the retinoid system. The targets highlighted for in silico and *in vitro* assay development are activity assays for key enzymes responsible for maintaining RA levels (RALDH and CYP26), induction assays for Stra8 (a meiosis-inducing protein regulated by RA), and binding/transactivation assays for RAR and RXR that mediate RA signalling.

410. Sweden highlighted some existing and future in silico methods that might be useful for identifying chemicals that interact in the retinoid pathway. For level 1 of the OECD Conceptual Framework, there are currently QSARs and computational docking models to identify CYP26A1 and CYP26B1 inhibitors and various QSAR models for binding/activation of other nuclear receptors and CYP enzymes. The

recommendation from the DRP is that future models focus on RALDH and CYP26 inhibition and RAR/RXR binding.

411. The group noted that these in silico approaches could be used as a prioritisation method and agreed with the recommendation for future development. The US noted that the existing computational docking models (for RAR) depend on the predicting binding site of the protein, but in reality, a variety of other proteins interact with the receptor and the shape can be quite dynamic. Useful information can be obtained, but there is a question of the specificity of chemicals predicted to interact with the receptor using this type of approach. There is also some information available from industry, from the cheminformatics evaluation (discussed below), and from evaluations of RXR species conservation. It was noted that CYP26 is an interesting target for development as the enzyme is thought to be specific for the retinoid system.

412. For levels 2 and 3 of the OECD Conceptual Framework, there are currently *in vitro* assays available for RAR/RXR transactivation and CYP26 isomer expression/activation. Potential targets for assay development include CYP26 and RALDH transcription induction and protein inhibition, and Stra8 transcription induction. The section reviewed *ex vivo* rodent and human foetal gonad models but acknowledged that, due to potential variability in tissues obtained from different individuals, these models may be more suitable for research purposes than regulatory test guideline development.

413. The expert group supported the suggestions to focus *in vitro* assays development to on: CYP26 and RALDH (mRNA induction, protein), Stra8 (mRNA induction), and RAR and RXR (receptor binding, transactivation, mRNA induction). The group also suggested considering other existing assays that may be informative. For example retinoid perturbation of gonadal somatic cells may affect steroid synthesis and other CYPs. More sophisticated models like the organ-on-a-chip models for male and female reproduction developed by Teresa Woodruff (Northwestern University) could potentially be useful in the future for evaluating chemicals that are known to affect retinoid signalling.

414. An evaluation of potential assays and endpoints at Conceptual Framework levels 4 and 5 include gonadal histopathology and sperm parameters, which are already included in several OECD test guidelines. Other suggested potential parameters included ovarian histopathology of pups (TG 421/422), serum anti-Mullerian hormone (AMH) as a proxy for ovarian follicle reserve, and tissue retinoid levels. Denmark mentioned that the FREIA project (Horizon 2020) will be evaluating AMH and other markers of female reproductive performance and the work may provide an indication of the feasibility of the endpoint. However, there was a question of how AMH or another proxy would be interpreted as adversity (e.g. what level of change).

415. Retinoid levels have been measured in liver and other tissues following published protocols so it is possible, but depending on the experimental model it could, depending on tissue of interest, be challenging to get enough sample/sufficient levels for quantification. In addition, the analysis requires that samples are protected from natural light and there is still some controversy regarding the biological activity of specific retinoid metabolites. WHO publishes human levels associated with maternal vitamin A deficiency/hypervitaminosis, but serum levels are likely to be much more stable than liver. It was suggested that a biomarker, if identified, could be used as a proxy of RA levels in tissues, and that a biomarker may be easier to measure (especially in foetal and pup tissues of rodents). Denmark pointed out that changes in hepatic levels may be difficult to associate with decreased reproductive function, while severe decreases in oocyte number is clearly adverse.

416. Adding ovarian histopathology to an existing screening test guideline must consider a) the number of animals at each dose level (i.e., statistical power of the endpoint), 2) relevant timing of exposure, 3) an understanding of temporal gene expression. While histopathology of the pup ovary obtained in TG 421/422 is possible, measuring the endpoint would require time, and the power of the endpoint may be limited given the group size, to conclude on a negative result. However a positive result would give valuable information. The group also noted that associating histopathological changes of the ovary with RA-linked adversity may not be straight-forward as it is an endpoint that is not RA-specific. However, it is still important as an

indicator of an overall adversity, which may be caused by retinoid disruption. Coupling histopathology with *in vitro* data (e.g. an alert that a chemical is active in an *in vitro* retinoid assay) would strengthen the link. A quantitative evaluation of how much perturbation is needed to have an adverse effect is also missing. For example, Grey *et al.* (2016) quantitatively evaluated the level of testosterone suppression required to see an effect (i.e. "the biologically relevant reduction"), though this was also noted to have a temporal component and may be affect by other factors such as route of exposure which also must be determined for retinoids.

417. Currently, there are no good systems biology models of retinoids. As more AOPs are developed, adverse effects could be linked to changes in e.g. RA synthesizing and metabolising enzymes. To date, good reference chemicals to look at the entire retinoid signalling pathway have not been identified. Further, the specificity of chemicals that have been identified for interaction with the retinoid pathway is questionable, though this may not be critically important from a regulatory perspective. The scoping effort highlighted the difficulty associating adverse reproductive outcomes with disrupted retinoid signalling due to the extensive crosstalk between various pathways involved in reproduction and also noted that chemicals affecting reproduction specifically via retinoid signalling disruption have not been identified. With regard to reproductive endpoints that could be added to *in vivo* test guidelines, the EG agreed that there are no known *in vivo* endpoints that are specific to the retinoid pathway.

D.4. Abstract sifter tool/information science for retinoid pathway

418. Nancy Baker (Leidos, contractor to US EPA) provided an overview of two approaches for identifying candidate reference chemicals and supporting literature for the retinoid signalling pathway. In the first example provided, candidate chemicals were identified from 12 ToxCast high throughput *in vitro* assays relevant to the retinoid signalling pathway. The second example used information from US EPA Lit DB, a database of MeSH terms from PubMed articles, to identify chemicals with retinoid targets and annotated with the MeSH terms "agonist", "antagonist" or "inhibitor". This information is then exported to excel and includes the number of articles associated for each chemical/target pair and is hyperlinked to the MeSH term and a second sheet is hyperlinked to the PubMed citation. The information is then managed in AbstractSifter, a publically available tool developed at US EPA that builds an Excel sheet front end linked to PubMed citations that allows retrieval, sorting, searching, tagging, and export from the tool. AbstractSifter is intended for simple queries that are likely to return large numbers of hits. It is not a systematic literature review but can be very helpful for conducting literature searches around AOPs or KEs, though the search is limited to terms in the title and abstract of the publication. A unique query was created for this project "AbstractSifter_RetinoidPathway.xlsm" (Appendix 2) and shared with the authors of each organ system and the Expert Group. A demonstration of the AbstractSifter for the retinoid pathway was provided and the group was given an opportunity to explore the tool and prepared worksheet on their own. The tool is available through US EPA's Chemistry Dashboard and is supported by training (https://www.youtube.com/watch?v=NTS-BUJcfO4&feature=youtu.be).

419. The experts commented that not all relevant articles are included in PubMed. Although the public version of AbstractSifter is limited to PubMed, other literature databases have been tapped in newer version 'AbstractSifter-Plus' under development (not in the public domain). Because PubMed has the strongest controlled vocabulary, other literature databases would need a different tool.

D.5. Skeletal/craniofacial development.

420. Tom Knudsen (US EPA) presented an overview of the annex on retinoid effects on skeletal and craniofacial development. The draft annex was developed under a tight timeline and started with a literature review approach (using AbstractSifter) to evaluate what is known about the role of retinoids in normal biology and consequences of perturbed retinoid signalling, organizing information in a "generalised"

AOP framework. Information from 12 retinoid-relevant ligand-binding and reporter gene assays included in ToxCast suite and results from ~1300 chemicals screened in a quantitative high throughput screening (HTS) assay (Chen *et al.* 2016) were used as a starting point. The absence of HTS assays for the key enzymes involved in RA synthesis (RDH/RALDH2) and degradation (CYP26) were noted. Results indicated compounds with known MOAs (e.g. triazoles, organochlorine pesticides, organotins) had anticipated *in vitro* assay profiles.

421. A search for publications on retinoid effects on skeletal development brings up more than 20,000 papers. There are a lot of publications on skeletal homeostasis/differentiation (e.g. osteoporosis), however, those were excluded from this annex due to the complex cross-talk between retinoids and oestrogens. The process of RA production/degradation is critical for maintaining spatial gradients responsible for body patterning of the facial skeleton, vertebral column and limbs. In test guidelines which evaluate skeletal endpoints, missing, malformed, and retarded ossification of bones in the foetal skeleton are among the most commonly affected endpoints. It was acknowledged and reflected in several comments that an understanding of the normal biology may be different from the understanding of retinoids as teratogens.

422. Retinoid signaling largely impacts early development processes (gastrulation, early organogenesis). For cranial, axial, and appendicular skeleton there are different cellular targets. In order to make sense of this complexity, a generalised AOP framework is helpful for organising data, and though there are two in the AOP wiki, a search of the wiki indicated only five key events and thus illustrates gaps that need to be filled. There was some discussion by the expert group as to whether retinoid signalling is amenable to AOP development, as developmental biology is very complex and AOPs are inherently reductionist, thus it is unlikely that "true" AOPs may be developed. The experts agreed that at this stage, there is utility in grouping events in a generalised pragmatic approach using "AOP frameworks". This approach could be organised by windows of susceptibility (and linked to the OECD TGs that include those time points), as perturbations at different times may results in different effects.

423. The group discussed several tools that are or may be under development, beyond those described in GD 150 and GD 178. The experts mentioned computer models of developmental biology to understand phenotypes (US), QSARs (Denmark), *in vitro* RDH and CYP26 assays (US), and potential work from the EU ToxRisk and Horizon 2020 projects that may be informative, as well as other biomarkers or surrogates. There was also recognition that dosimetry is very important and though reverse toxicokinetics can be used to extrapolate *in vitro* levels to the exposure predicted in the foetus, there is uncertainty in extrapolated values, especially with regard to metabolism.

D.6. Central Nervous system

424. Joshua Robinson (University of California, San Francisco) presented an overview of the annex on retinoid effects on the CNS in humans and rodents using a case study approach. Retinoids play a role in several critical processes including anterior/posterior brain patterning, neurogenesis, and formation of the hindbrain and blood-brain barrier. Epidemiological data indicate variable results regarding teratogenicity of excess retinoid exposure during pregnancy. Experimental animal studies indicate a variety of adverse developmental CNS outcomes due to excess or deficiency that vary based on dose, timing, and duration of exposure and genetic background.

425. As with other organ systems, *in vivo* evaluation of retinoid effects on CNS development must take into account metabolising enzymes and carrier proteins critical to how RA exerts effects. Various enzymes and pattern of expression in CNS are both regionally and temporally variable. Results of studies conducted in mouse knock-out models indicate there is some redundancy. For example, effects are observed in double knock-out RAR/RXR models, but not when a single receptor is inactivated. Genetic deletion of Cyp26a1 is associated with severe hindbrain and caudal defects; whereas Cyp26c1 knockouts are generally phenotypically normal, and double knockouts Cyp26a1/Cyp26c1 display severe defects in the

hindbrain/caudal regions as well as forebrain, midbrain and branchial arches. As with other systems, the temporal window is also an important consideration. For example, exposure of Swiss mice to a single IP injection of RA on GD 7 causes adverse CNS effects, the severity increases if exposure is on GD 8 (during neural tube closure), however, minimal effects are observed with exposures on GD 9.

426. Several *in vitro* (but not always non-animal) model systems for evaluating retinoid effects on CNS development were discussed including rodent whole embryo culture (WEC), embryonic stem cells (ESC), specific cell lines, primary rodent cell lines, and zebrafish. There are various strengths and limitations of each, though it was specifically noted that several ESC model systems use vitamin A/retinoic acid to promote neuronal differentiation, so testing retinoids in this system may be more difficult to interpret. Similarities in temporal expression patterns of genes associated with neurogenesis between rat WEC models versus rat and/or human embryogenesis are observed, supporting relevance of the model system. Similar gene expression patterns were also observed in comparisons between human ESC neural differentiation models and human embryos undergoing early CNS development. Despite the heterogeneity of ESC models developed in different labs, gene expression patterns for neurodifferentiation systems among five models were similar. Differentiated hESCs that form neural rosettes are a model for neural tube development. Exposure to a range of RA concentrations over 3 orders of magnitude indicated effects in a concentration-dependent manner that associated with morphological changes. While specific aspects of neural development differ between zebrafish and mammals, neurobehavioral studies can be conducted in zebrafish and not in WEC models, which are a limited-time exposure spanning neurulation and early organogenesis.

427. The group discussed the annex and comments. It was pointed out that for an endogenous signal like RA, deciding where to focus resources in AOP and assay development should take into account the sensitivity and severity of effects. The example provided was reduced fertility may not be an important effect if chemical exposure causes anencephaly. There was also discussion as to whether RA regulates CNS development, in which case one would expect to see an early effect, or is a modulator of other pathways, in which case one would expect to see a range of RA concentrations before effects are detected. Further, substantial CNS malformations are unlikely to be reversible. Some experts suggested that endpoints related to structural variants and/or (neuro)behaviour may be utilized due to higher sensitivity, but more difficult to observe (than malformations, for example). Toxicokinetics may provide a way to measure more subtle or difficult to measure endpoints.

428. The group also noted that for the model systems discussed, zebrafish are high throughput while WEC is not. Also, hESC and other cell models may not be fully representative of CNS development *in vivo*. To note, gene expression is an element to potentially connect *in vitro* and *in vivo* models. It was also noted that the early efforts are intended to be approaches for hazard identification which may/not be relevant to human risk. Assays developed expressly to measure effects of perturbation of retinoid signalling may not be well covered in the DNT battery under development which is focused on neurogenic processes but not patterning. CNS dysmorphogenesis is not necessarily part of the ENT battery, however, there may be subtle effects on CNS patterning that do not show up as overt malformations in standard animal studies (e.g. congenital deafness).

D.7. Overall recommendations from Expert Group

429. Following the presentations on each draft section of the DRP, the expert group discussed general recommendations and next steps.

D.7.1. Possible sources of additional information

430. The Expert Group was asked to identify other potential sources of information that were not discussed. It was noted that the European Food Safety Authority (EFSA) has a great deal of information

on triazole effects on cleft palate which may be useful to consider. Similarly, there is a great deal of information on vitamin A related blindness, which could be summarised in its own annex or included as part of CNS. The fish embryo assay and early development in the frog include eye and heart development endpoints and may be informative. From research on retinoid effects on cardiovascular development, there is some information on the role of retinoids on barrier functions which could be included in each relevant section (e.g. blood-brain barrier, blood-testis barrier, etc.). There is also information on the role of retinoids for valve and septal systems of heart. It was discussed that, when possible, information on susceptible windows for the organ systems captured in this report should be included. Experts were asked to send relevant references to be included in the updated section on retinoid biology.

D.7.1.a. In silico

431. A pilot study at US EPA/NCCT chemotyped chemicals (based on information including phys-chem properties, reactivity, ADME, use categories, and bioactivity) that seem to be able to interact with retinoid receptors (unpublished). Using an in silico pathway modelling and bioinformatics approach, there are datasets that can be used to model, though these may be early research tools to help understand biology and not directly applicable for regulatory purposes.

D.7.1.b. In vitro assays:

432. It was pointed out that in contrast to perturbations in endocrine (EAT) signalling which cause neotonous defects (i.e. the slowing of development), retinoids are involved in patterning of the embryo, and thus, it may be helpful to focus on the types of effects unique to retinoids and not covered by other MoAs considered in regulatory toxicology (e.g. effects on meiosis and expression of homeobox genes).

433. There are currently available *in vitro* assays that measure some retinoid pathway targets, though many are emerging science and would benefit from a critical evaluation of the strengths and weaknesses. Comments on the draft DRP noted that some researchers have had difficulty reproducing HTS results, though it was noted that this was a limited effort and did not necessarily follow the same protocols used in the HTS assays. Results from the HTS analyses indicate chemicals with known modes of action indicated the results made logical sense. More work may be need for technical readiness of the ToxCast assays, but based on results, these seem to be good target candidates for development. Biodetection Systems (BDS Netherlands) has developed RAR and RXR CALUX assays as part of the EDC-MixRisk project, and these may also be candidates for additional development.

434. While CYP26 is considered to be specific for retinoids, other CYP interactions (e.g. steroidogenesis, CYP1B1) could be evaluated as proxies, as a CYP26 inhibition assay is not currently available. There may be CYP1A1 assays under development (e.g. Japan, ISO, H2020) that may be useful, as well. There are commercially available ADH/RDH assays that may be informative though not highly specific, but an RALDH2 assay, which is rumoured to be under development from Tox21, would be high priority because it is responsible for spatial regulation of RA within tissues. The group generally agreed that CYP26 and RALDH assays were high priorities for development. The expression profile of CYP26 and other enzymes (e.g., DHRS3) may also be highly informative as a first step for understanding biology.

435. There are existing cell-based assays (e.g. ESC, development of cranial neural crest, somatic development) that could be straightforward and more easily interpreted for measuring retinoid effects. It would be helpful to identify chemicals for downstream effects, as well. One challenge of this approach may be teasing out cross-talk with other pathways, and identifying endpoints that are specific to retinoid pathways (e.g. cleft palate; phocomelia endpoints which are somewhat diagnostic for retinoid effects).

436. For some general endpoints such as cell migration, measuring these in WEC systems and adding toxicogenomics can add a high degree of specificity for the endpoint affected. However, in evaluation of the effects of anti-androgens on the testis, a transcriptomic signal may not be detected or may be detected in other tissues. At this stage, it may be difficult to know what to look for.

D.7.1.c. In vivo:

437. The group noted that histopathology from pups is of high technical relevance (and Denmark has been measuring the endpoint for years), however, the number of animals examined needs to be taken into account and if there is no observed histopathology, one cannot be sure there is no effect of the chemical. It was also again noted that ovarian histopathology is not an RA-specific endpoint. Some retinoid signalling pathways are similar to thyroid signalling (RXR-crosstalk) and could be considered with thyroid parameters (e.g. tested in the thyroid *in vitro* battery under development in the JRC) to be mutually informative. A zebrafish model is included in the 17 candidate assays for thyroid disruption, and it may be useful to test retinoids in that assay.

438. An approach could be adopted similar to that used to investigate Zika virus, where (in that case, human paediatric) neuroimaging showed differences in affected individuals and *in vitro* organoids were used to identify Zika targets. In this case, histopathology of individuals treated with reference chemicals known to affect retinoid signalling could identify exposed individuals, and *in vitro* assays could help resolve specific markers. Several experts pointed out adverse outcomes affected by a number of pathways may not help identify chemicals that are specific to retinoid signalling but are still helpful from a regulatory perspective. Using an AOP framework will help to understand the KERs and gaps in biological understanding. In the interim, a retinoid AOP may be helpful for screening.

439. Denmark noted that the discussion thus far has focused on human health and ecotoxicology assays should also be taken into account, echoing earlier comments from the UK. Somatic cells in the gonad, feminisation, and reproductive disorders may be may be useful endpoints to consider in ecotoxicology assays. Several chemicals (e.g. benomyl) that interfere with retinoid signalling and have effects on the eye in mammals and zebrafish but may be missed in standard endpoint measurements (or with some animal models, e.g. white mice that lack a pigmented retina). These chemicals have similar impairments on hearing, and potentially affect the lateral line system in fish. It was also noted that Patrick Allard (UCLA) has a *C. elegans* model for evaluating retinoid effects.

440. The group discussed Josephine Bowles's transgenic mouse model for examining Stra8 expression but noted the need for heterozygotes, and thus a limited number of chemicals can be screening. The model may help to provide foundational work for *in vitro* assay development.

D.7.2. Next steps

441. The UK commented that retinoids are common cosmetics ingredients and animal testing is prohibited. For this reason there is an urgent regulatory need for development of *in vitro* assays. The group discussed a possible future IATA for retinoids.

442. For the preliminary AOPs discussed, the group pointed out there were a lot of commonalties in the early steps. General screens could be developed to look at the early parts of the AOPs (MIEs and KEs) and cell-based screens could be developed. It would be helpful to have target cells of interest, for example coelomic cells of the gonad are not included in any MOA. A recommendation was that the work to summarise the biology on retinoids is anchored in 1) AOPs intended to guide the (pre)screening of chemicals, and 3) *in vivo* function effects to determine what is currently covered in regulatory test guidelines. Nancy Baker will continue to work to identify candidate reference chemicals for retinoid effects on each of the organ systems discussed and an update will be provided.

443. The group concluded that more research is needed to fill data gaps.

444. Revised draft sections will be sent to the secretariat Q4 2019/Q1 2020, at which time the project will no longer have support from the authors of each section. The sections led by Sweden that provide an overview of retinoid biology and effects of retinoid signalling on the male and female reproductive systems will also be published in early 2020 as a Nordic Report.

445. An updated draft DRP will be consolidated and shared with the EDTA prior to the April 2020 meeting for additional input and feedback on regulatory considerations. The draft DRP will be circulated for a second WNT commenting round later in 2020 with the aim of having a document for approval in April 2021.