Advisory Group on Endocrine Disrupters Testing and Assessment (EDTA AG) - Virtual Meeting, May 26, 2021

RETINOID SIGNALING IN SKELETAL DEVELOPMENT:

SCOPING THE SYSTEM FOR PREDICTIVE TOXICOLOGY

Thomas B. Knudsen, PhD
Developmental Systems Biologist, US EPA

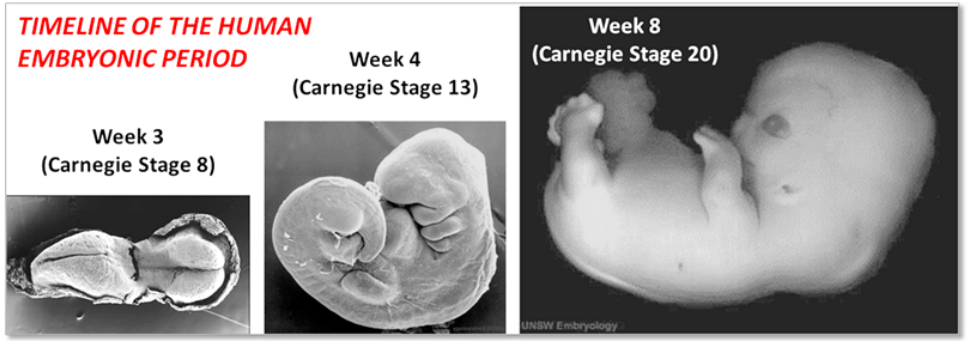
DISCLAIMER: The views expressed are those of the presenters and do not necessarily reflect Agency policy.

OECD
BETTER POLICIES FOR BETTER LIVES

Evaluating prenatal developmental toxicity

"The first trimester is the most crucial to your baby's development. During this period, your baby's body structure and organ systems develop."

www.ucsfhealth.org

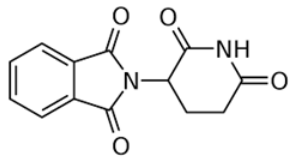


peak sensitivity (3rd – 8th wk)

Embryonic Period | **Fetal Period** | **Fetal Period**

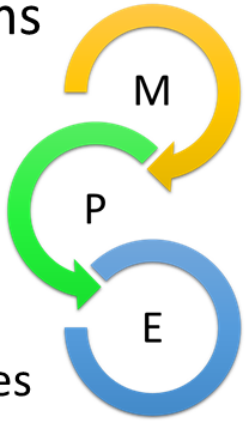
T1 | T2 | T3

OECD TG 414
OPPTS 870.3700



Adverse Birth Outcomes (CDC)
- neonatal mortality (1-2%)
- malformations (3-4% live births)
- premature births (10%)
- low birth weight (11%)
- functional deficits (17% children)

Complex Systems
- gene networks
- multiscale
- autopoiesis
- canalization
- temporality
- state trajectories
- and more …

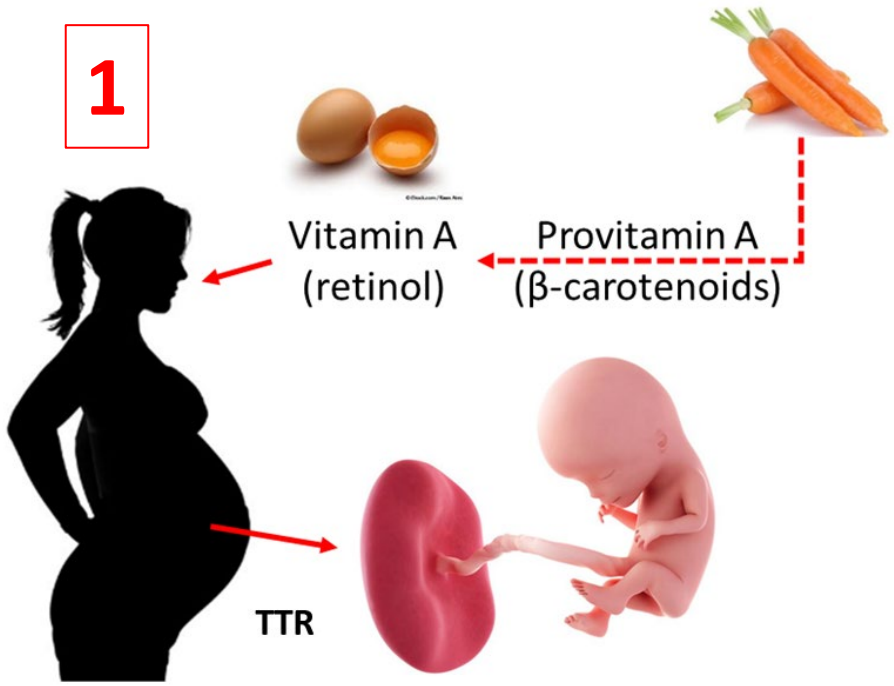


1. Key messages from DRP 4.97 (Annex-B)

- The retinoid pathway has parallels with endocrine signaling (EATS), but its many unique functions are essential to morphogenesis, growth and differentiation of the embryo.
- ATRA signaling collaborates with some of the most powerful morphogenetic signals known to the embryo (FGF, BMP, SHH, WNT, …).
- Threshold ATRA responses are transduced by intracellular transport, liganding of nuclear RAR/RXR receptors bound to RARE genomic sequences, and recruitment of NCoA.
- ATRA-dependent gene expression is a molecular determinant of early growth, patterning and differentiation of the fetal skeleton (facial, vertebral, appendicular).

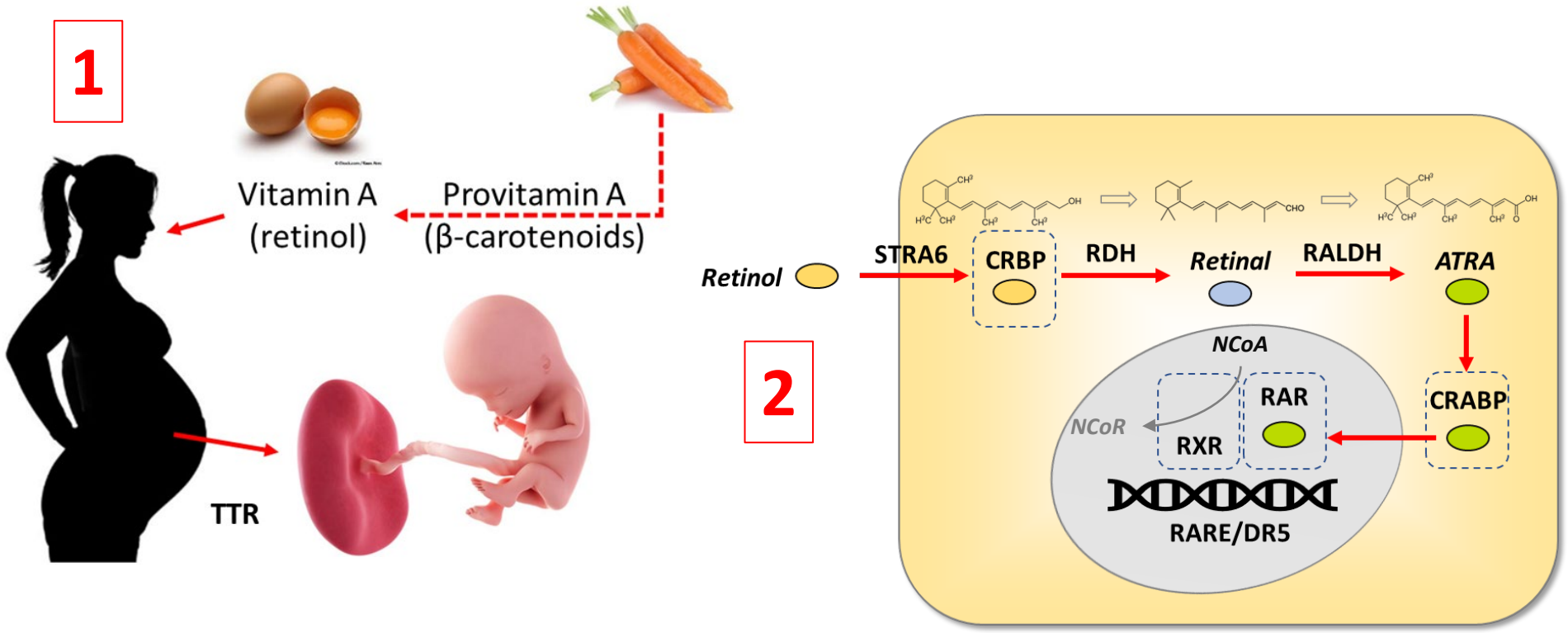
KEY POINT: Although it is clear that disruption of endogenous retinoid signaling has adverse consequences to skeletal development, from a broad DevTox regulatory context it is not clear which (if any) apical outcomes are attributable exclusively to retinoid-related mechanism(s).

The retinoid pathway and morphogenetic signaling



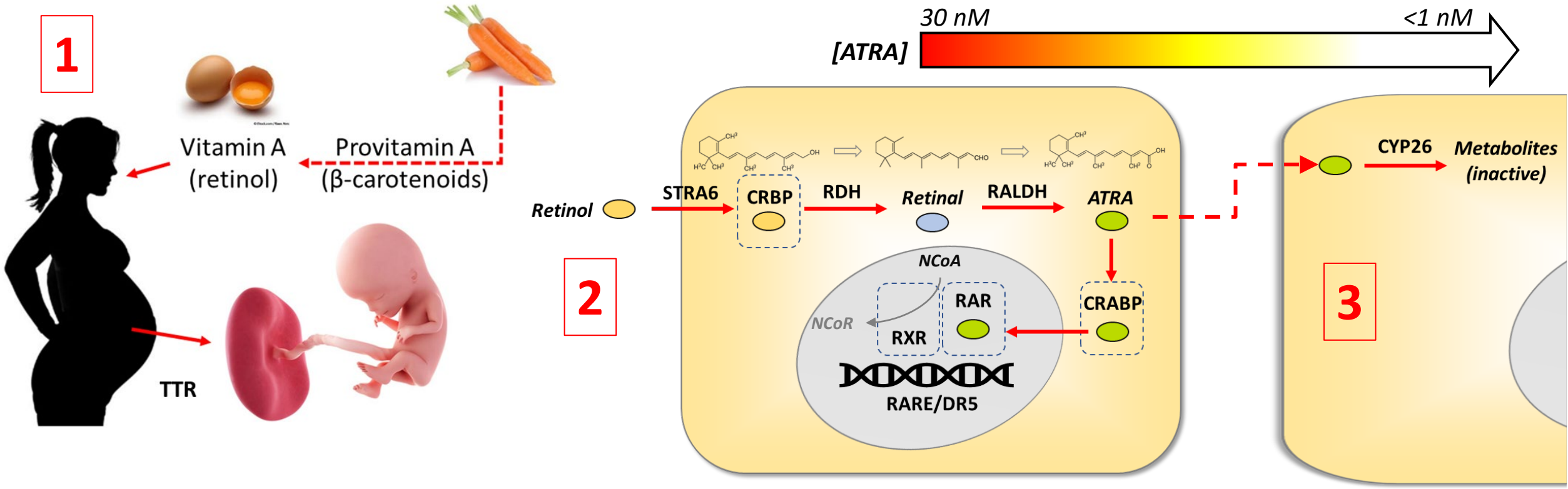
1. Retinol (vitamin A) is ingested by the mother and stored in her liver; it circulates to target tissues bound to serum transporters such as transthyretin (TTR).

The retinoid pathway and morphogenetic signaling



2. In the embryo, retinol is enzymatically converted to all-trans retinoic acid (ATRA). This capacity is acquired during gastrulation and continues through organogenesis.

The retinoid pathway and morphogenetic signaling



3. ATRA is fashioned into biochemical gradients by the cell-specific expression of enzymes for its biosynthesis (RDH10, RALDH2) and breakdown (CYP26a/b/c).

The retinoid pathway and morphogenetic signaling

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Retinoid signaling in skeletal development: Scoping the system for predictive toxicology

Thomas B. Knudsen[a,*], Jocylin D. Pierro[a], Nancy C. Baker[b]

ARTICLE INFO

ABSTRACT

Preface

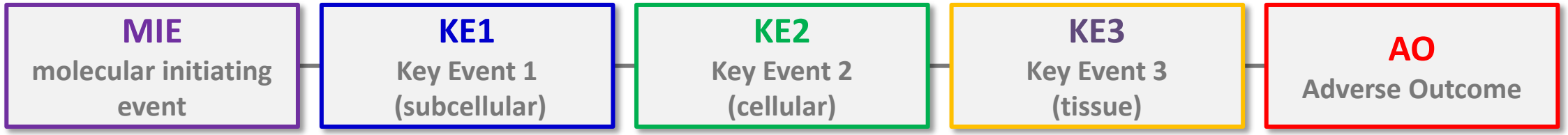
Dosimetric	Conc.	Indication	Reference
baseline ATRA (5 somite zebrafish embryo)	< 1 nM	non-morphogenetic	(Shimozono, Iimura et al. 2013)
maternal serum (animal study)	1.7 nM	non-teratogenic	(Daston, Beyer et al. 2014)
devTOX^{qp} assay (pluripotent hESC)	3.0 nM	teratogenic threshold	(Zurlinden, Saili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

4

4. It is not clear which (if any) apical skeletal outcomes in chemically-induced 'retinoid embryopathy' are attributable exclusively to retinoid-related mechanism(s).

Knudsen et al., Reprod Toxicol (2021) – special issue devoted to retinoid signaling (Guest Editor: H Håkansson).

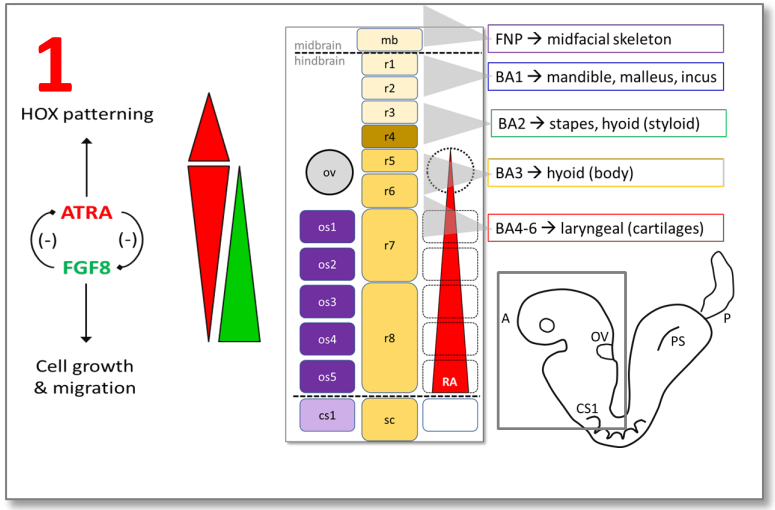
2. Postulated AOPs and status of development

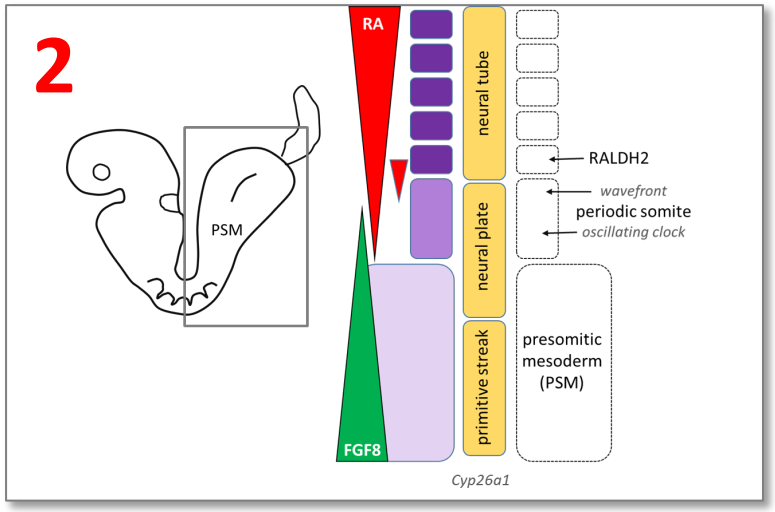


REGION	*MIE*	*KE1*	*KE2*	*KE3*	*KE4*	*KE5*	*AO*
Anterior Neural Tube	Inhibition of CYP26A1 enzymatic activity	Local increase in endogenous ATRA levels	Hyperactivation of the RAR/RXR heterodimer	Repression of *Fgf8* limits FGF8 signaling	Mis-specification of CNC cell fate and behavior	Maxillary arch dysplasia 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

Postulated AOPs for each skeletal domain based on literature for dietary, genetic or chemical disruption of endogenous ATRA signaling during embryogenesis.

Domain-specific AOPs (provisional examples)





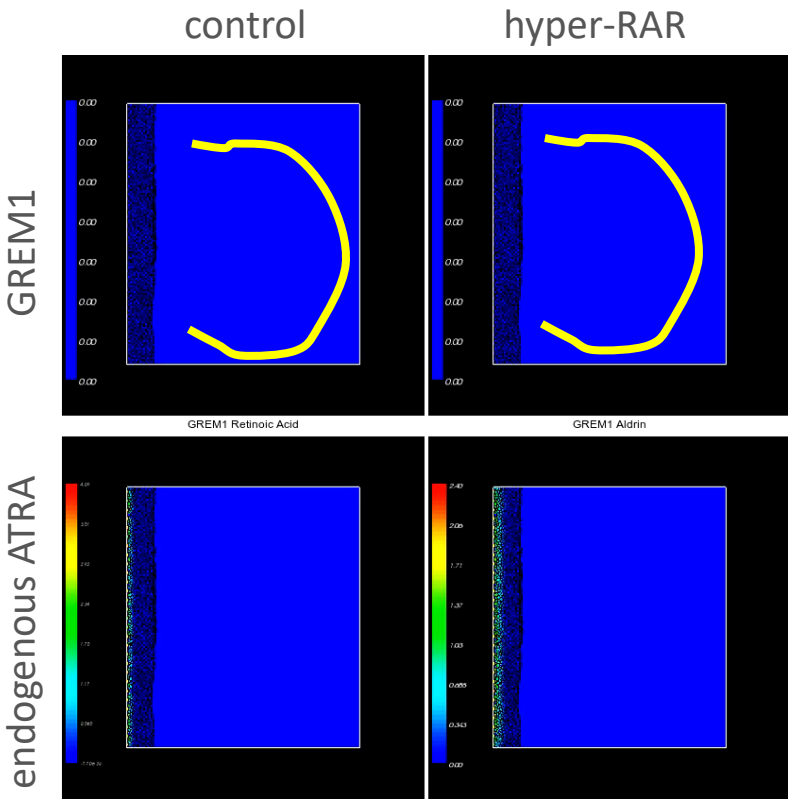
1. Facial skeleton: positional information of premigratory neural crest cells destined for branchial arches (5- to 11 somite stage).

2. Vertebral skeleton: size, registration, and specification of somites giving rise to individual vertebrae/ribs (0- to 36 somite stage).

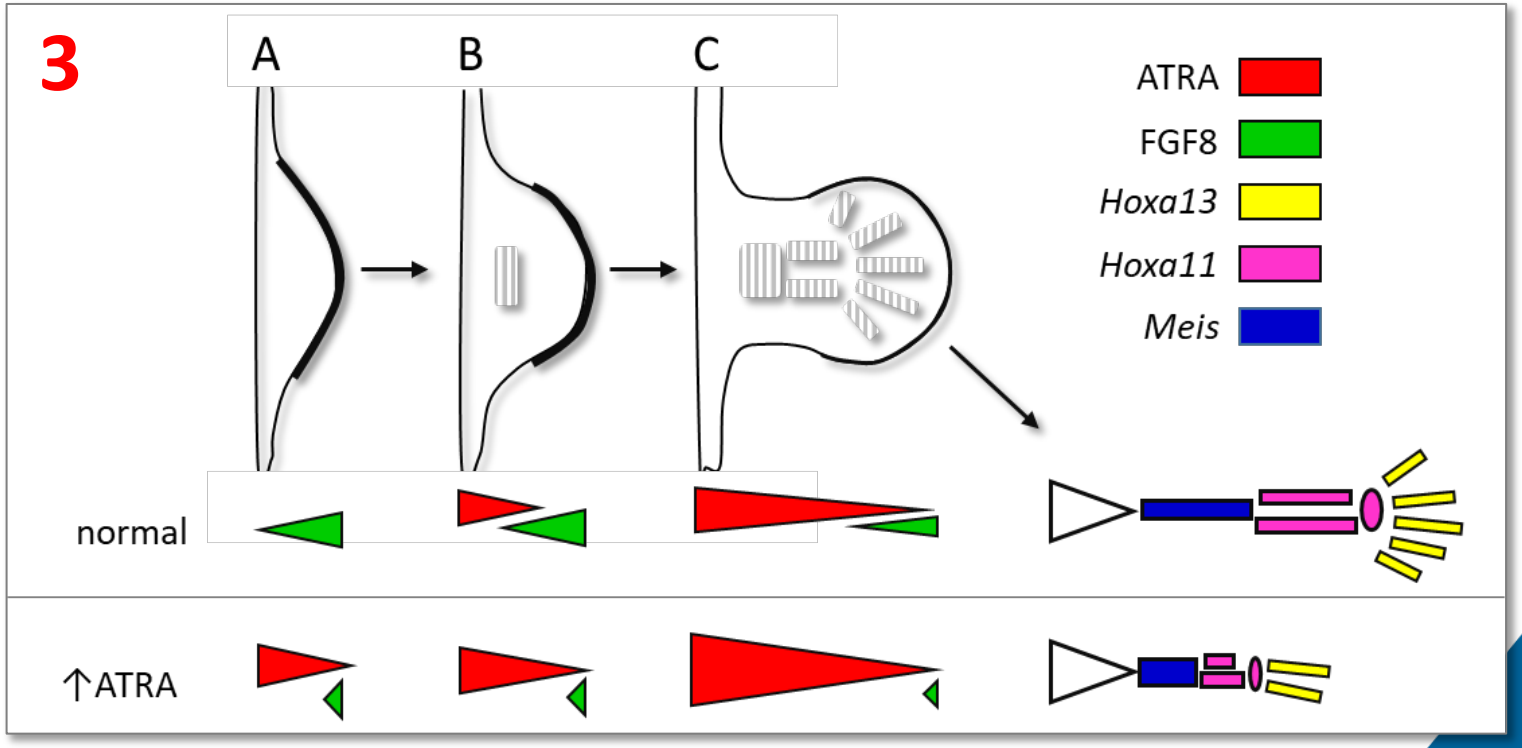
3. Appendicular skeleton: limb-bud initiation, outgrowth, patterning, and differentiation (12- to 36+ somite stage).

Example: phocomelia due to hyperactivity of RAR

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

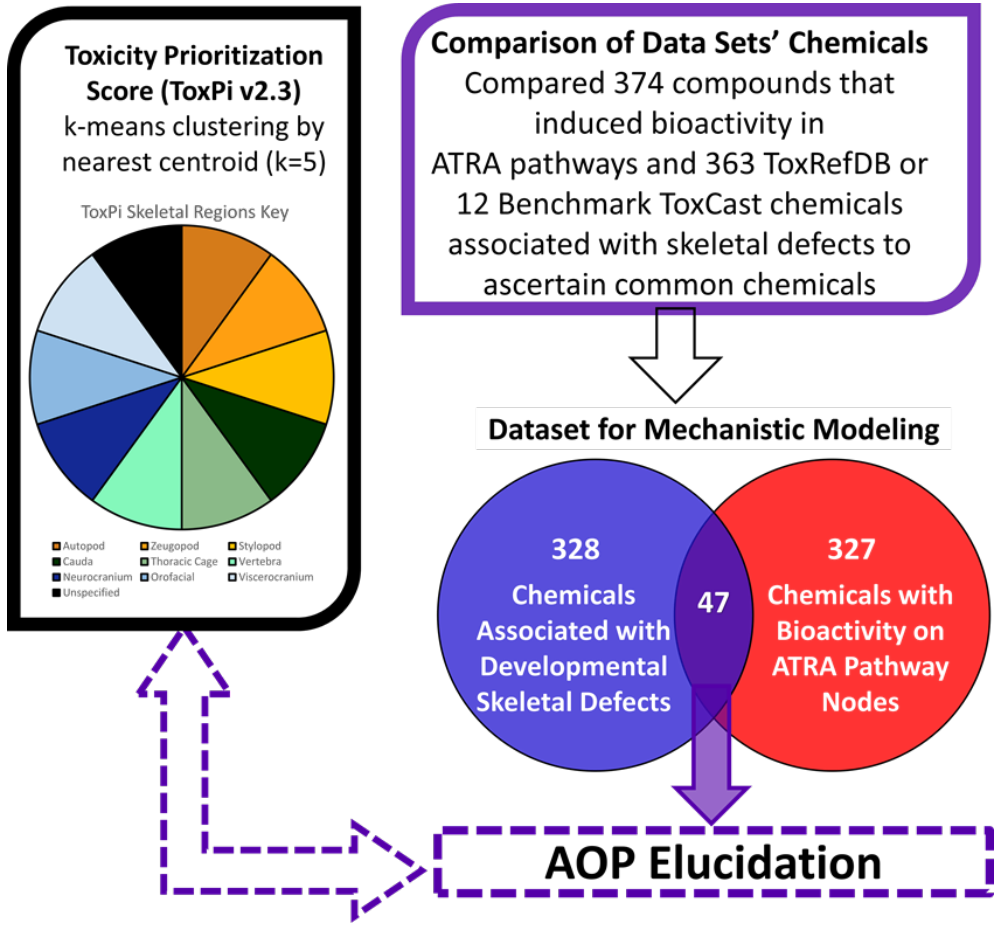


Systems model, work in progress



Based on Uzkudunet al. (2015) Mol Syst Biol

Broadening the landscape



- 2946 developmental studies in EPA's ToxRefDB with an adverse skeletal outcome (328 chemicals x 57,198 features).
- ToxPi slices annotated per chemical by composite and potency (mg/kg dosage) across these anatomical domains:

Cranium
- viscerocranium (oral, face)
- neurocranium (base, vault)

Axial skeleton
- vertebra (cervical, thoracic, lumbar)
- thoracic cage (ribs, sternum)
- cauda (tail)

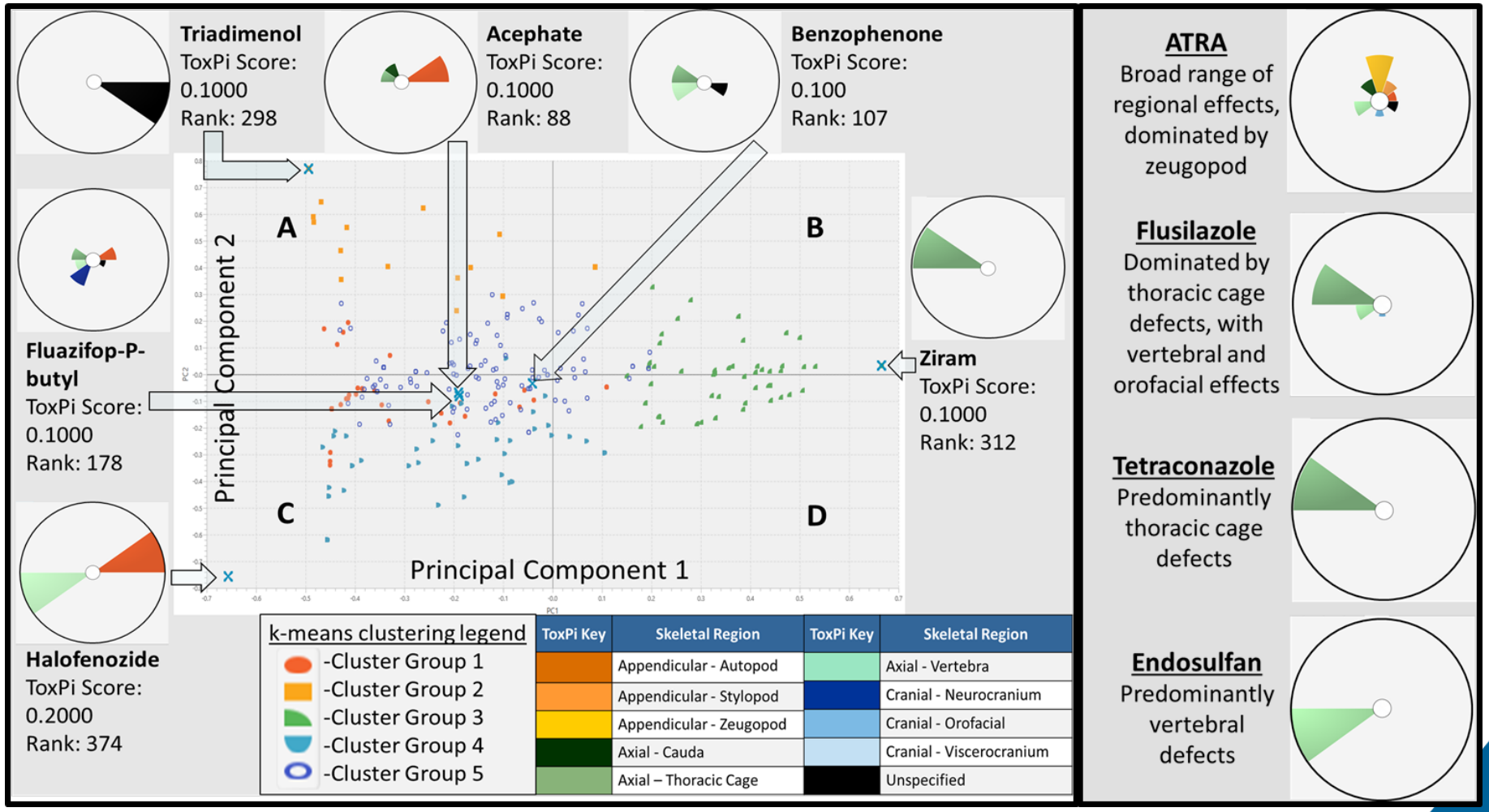
Appendicular skeleton
- stylopod, zeugopod, autopod

Other - insufficient detail for mapping

Jocylin Pierro, EPA/CCTE

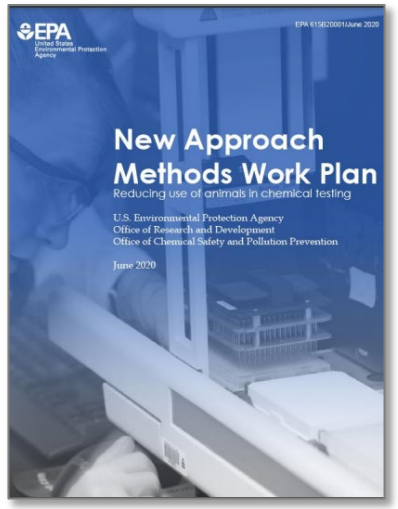
Chemicals (375) clustered by ToxPi phenotype (k=5)

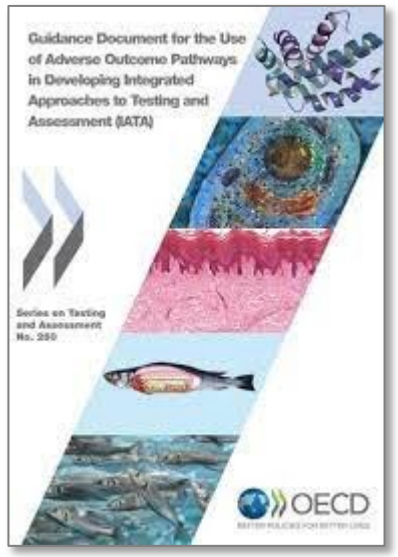
- **Phenotyping:** *are defects of the fetal skeleton consistent with AOPs linked to ATRA pathway?*
- **Chemotyping:** *are distinct structural features shown by chemicals in each phenotype cluster?*



Work in progress, Jocylin Pierro, EPA/CCTE

3. Candidate assays/endpoints and value for standardization





- New Approach Methods (NAMs): *in vitro* data and *in silico* models for hazard identification with low reliance on animal testing.
- An important consideration is building NAMs for early lifestages, reflecting the best knowledge of human developmental biology.
- Integrated Approaches to Testing and Assessment (IATA): integrating information with data generated with new testing strategies.
- The ATRA system provides an excellent opportunity to establish reliability of NAMs and defining IATA case studies.

ToxCast class distribution for retinoid target assays

1. Retinoids (ATRA, retinol, bexarotene).

2. Triazoles (CYP inhibition – CYP1A1 as a surrogate to CYP26a/b/c).

3. persistent organic pollutants (selective RAR activation).

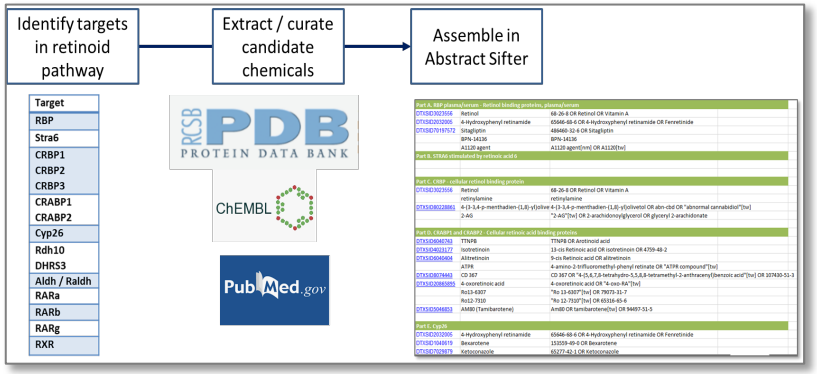
4. tert-butyl and organotin compounds (selective RXR activation)

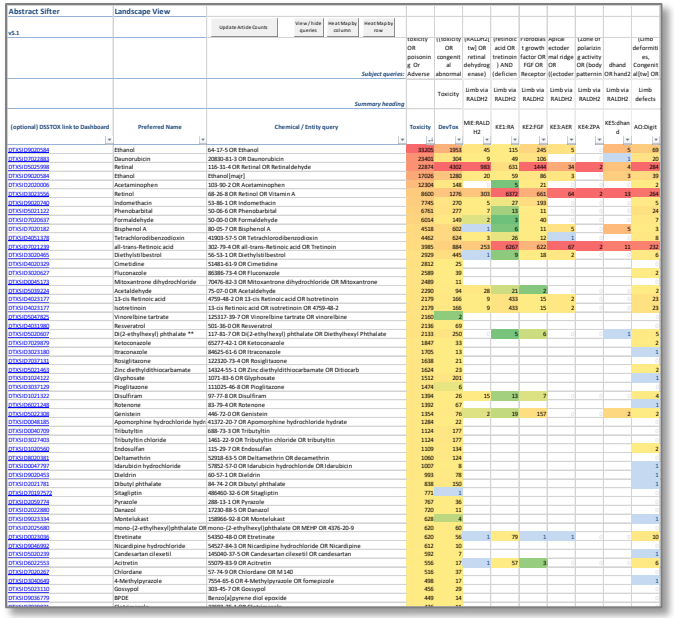
Other: mitochondrial disrupters, anticonvulsants (VPA), flame retardants (PBDEs).

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	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	2	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	3	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	4	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

Baker et al. (2018); Knudsen et al. (2021)

Reference chemicals for the ATRA system

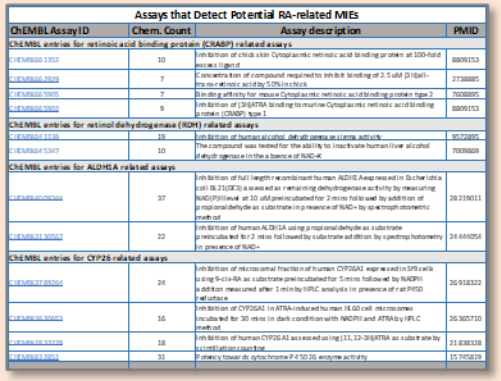




Baker et al. (manuscript in preparation)

- **Assemble candidate chemical list for each target:**
  - search databases (Protein Data Bank, ChEMBL, ToxCast/Tox21, …);
  - advanced literature mining to define bioactivity at each target.
- **For each candidate chemical:**
  - mine literature for MIEs, key events, and adverse outcomes;
  - e.g., skeletal defects, CNS defects, cardiovascular defects, ...
- **Compile top candidate chemicals for each target:**
  - detailed analysis of the Tox21 qHTS 10K dataset;
  - consult with domain experts.

Direct assays for modeling the ATRA pathway



Assays that Detect Potential RA-related MIEs

ChEMBL Assay ID	Chem. Count	Assay description	PMID
ChEMBL entries for retinoic acid binding protein (CRABP) related assays			
CHEMBL666 1353	10	Inhibition of chick skin Cytoplasmic retinoic acid binding protein at 100-fold excess ligand	8809153
CHEMBL666 2928	7	Concentration of compound required to inhibit binding of 2.5 uM [3 H]all-trans-retinoic acid by 5.0% in chick	2738895
CHEMBL666 5905	7	Binding affinity for mouse Cytoplasmic retinoic acid binding protein type 2	7638895
CHEMBL666 5902	9	Inhibition of [3H]ATRA binding to murine Cytoplasmic retinoic acid binding protein (CRABP) type 1	8809153
ChEMBL entries for retinol dehydrogenase (RDH) related assays			
CHEMBL666 1338	19	Inhibition of human alcohol dehydrogenase 4 kcmp activity	9572895
CHEMBL666 5367	10	The compound was tested for the ability to inactivate human liver alcohol dehydrogenase in the absence of NAD+K	7009869
ChEMBL entries for ALDH1A related assays			
CHEMBL6005362	37	Inhibition of full length recombinant human ALDH1 A expressed in Escherichia coli BL21(DE3) assessed as remaining dehydrogenase activity by measuring NAD(P)H level at 10 uM preincubated for 2 mins followed by addition of propionaldehyde as substrate in presence of NAD+ by spectrophotometric method	28.219011
CHEMBL21 30567	22	Inhibition of human ALDH1A using propionaldehyde as substrate preincubated for 2 mins followed by substrate addition by spectrophotometry in presence of NAD+	24.444054
ChEMBL entries for CYP26 related assays			
CHEMBL27 89266	24	Inhibition of microsomal fraction of human CYP26A1 expressed in Sf9 cells using 9-cis-RA as substrate preincubated for 5 mins followed by NADPH addition measured after 1 min by HPLC analysis in presence of rat P450 reductase	26.918322
CHEMBL36 30653	16	Inhibition of CYP26A1 in ATRA-induced human HL60 cell microsomes incubated for 30 mins in dark condition with NADPH and ATRA by HPLC method	26.365710
CHEMBL18 33238	18	Inhibition of human CYP26A1 assessed using [11, 12-3H]ATRA as substrate by scintillation counting	21.838328
CHEMBL23 2851	31	Potency towards cytochrome P-450 26 enzyme activity	15.795819

ChEMBL has limited data on 12 upstream assays (ATRA metabolism) for drug-like compounds

ToxCast has target-specific data on 11 assays for <2K Ph-I/II chemicals.

Tox21 has 2 assays on the integrated retinol pathway (LOPAC and Tox21 10K chem) libraries.

ToxCast/Tox21 Assay ID	Chem. Active	Assay description	PMID
ToxCast assays for CYP1A1			
NVS_ADME_hCYP1A1*	72	Using a type of enzyme reporter, loss-of-signal activity can be used to understand changes in the enzymatic activity as they relate to the gene CYP1A1	
NVS_ADME_rCYP1A1*	6	Using a type of enzyme reporter, loss-of-signal activity can be used to understand changes in the enzymatic activity as they relate to the gene Cyp1a1	
ToxCast assays for RARalpha, RARbeta, RARgamma			
ATG_DR5_CIS_up*	56	Measures of mRNA for gain-of-signal activity can be used to understand the reporter gene at the transcription factor-level as they relate to the gene RARA and RARB and RARG	
ATG_RARa_TRANS_up*	80	Using a type of inducible reporter, measures of mRNA for gain-of-signal activity can be used to understand the reporter gene at the transcription factor-level as they relate to the gene RARA	
ATG_RARb_TRANS_up*	26	Measures of mRNA for gain-of-signal activity can be used to understand the reporter gene at the transcription factor-level as they relate to the gene RARB	
ATG_RARg_TRANS_up*	63	Measures of mRNA for gain-of-signal activity can be used to understand the reporter gene at the transcription factor-level as they relate to the gene RARG	
NVS_NR_hRARa_Antagonist*	41	Using a type of binding reporter, loss-of-signal activity can be used to understand changes in the binding as they relate to the gene RARA	
NVS_NR_hRARa_Agonist*	42	Using a type of binding reporter, loss-of-signal activity can be used to understand changes in the binding as they relate to the gene RARA	
Tox21 assays for reporter assays RARalpha			
TOX21_RAR_LUC_Agonist*	305	Using a type of inducible reporter, gain-of-signal activity can be used to understand changes in the reporter gene as they relate to the gene RARA	
TOX21_RAR_LUC_Antagonist*	985	Using a type of inducible reporter, loss-of-signal activity can be used to understand changes in the reporter gene as they relate to the gene RARA	
ToxCast assays for RXRalpha, RXRbeta, and RXRgamma			
ATG_RXRa_TRANS_up*	19	Measures of mRNA for gain-of-signal activity can be used to understand the reporter gene at the transcription factor-level as they relate to the gene RXRA	

ATRA processes that can be modeled

Pregnancy
- exposure (diet, pharma, chemicals)
- hepatic mobilization (retinol)
- transplacental kinetics (RBP)

Microphysiology
- bioactivation (RDH, RALDH2)
- molecular transporter (Stra6)
- metabolism (CYP26a/b/c)

Cell signaling
- endogenous ATRA gradients
- ATRA kinematics (retinoid dosimetry)
- nuclear delivery (CRABP)

Nuclear signaling
- RAR/RXR liganding
- NCOR – NCOA switching
- DR5 mediated transactivation

4. Connections with other on-going projects at OECD and elsewhere

- Knowledgebase (skeletal development)
- AOP-WIKI (limb defects)
- HTS-based signatures (ToxPi classifier)
- HTS data analysis (ToxCast/Tox21/ChEMBL)
- Pregnancy IVIVE models (targeted case studies)
- Performance-based prediction (ATRA pathway in Devtox)
- Morphoregulatory simulation (Limb ABM)

1. CPP 13 Predictive Toxicology of the Retinoid Signaling Pathway.

US EPA, CCTE
Nancy Baker, Leidos
Richard Judson
Thomas Knudsen
Jocylin Pierro
Ann Richard
Laura Taylor

NCTR/FDA
Annie Lumen

NTP/NIEHS
Nicole Kleinstreuer,

NIH/NCATS
Srilatha Sakamuru
Menghang Xia

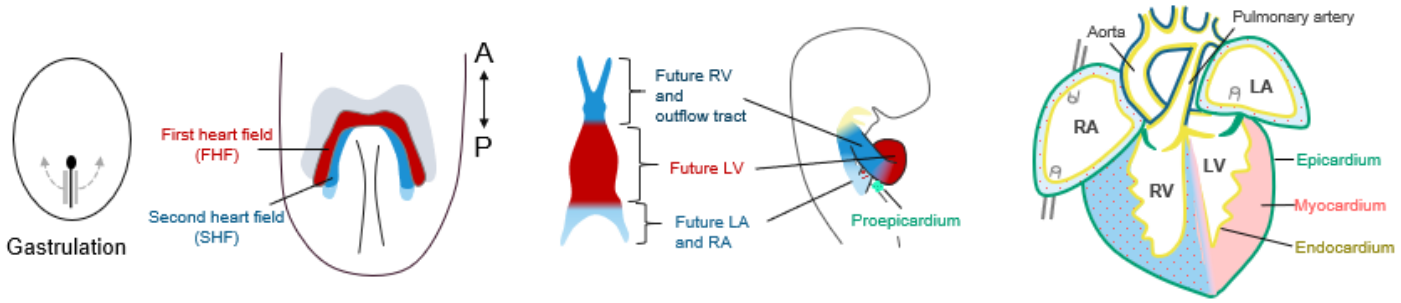
OECD
Patience Browne

Aim: mine HTS bioactivity profiles for retinoid transporters, metabolism, receptors, and responsive pathways that can be formally integrated with embryological knowledge on the embryo-fetal skeletal system as a potential IATA case study.

4. Connections with other on-going projects at OECD and elsewhere

2. Retinoid Signaling and Cardiovascular Development.

	Cardiac Precursors	Cardiac Crescent	Linear Heart Tube	Cardiac Looping	4-Chambered Heart
Mouse:	6.5	E7.5	E8.0	E8.5	E14.5
Human:	CS6	CS7	CS8	CS8	CS20



Roles for Retinoic Acid Signaling	• Restricting heart field size • Patterning the SHF	• Addition of SHF cells to growing heart tube	• Left-right asymmetry • Outflow tract development	• Myocardial expansion • Formation of fibroblast and vascular smooth muscle cell populations

US EPA

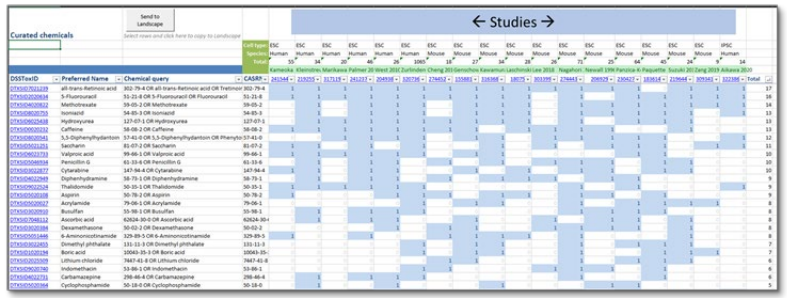
Nancy Baker
Rachel Brunner, OPP
Sid Hunter
Thomas Knudsen
Jocylin Pierro
John Rogers
Other experts (TBD)

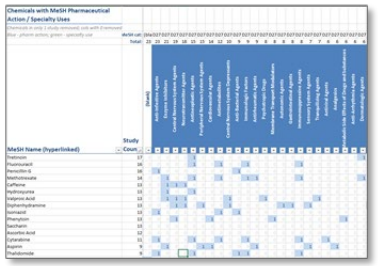
Aim: comprehensive review of the scientific literature on endogenous ATRA signaling in the regulation of cardiovascular (CV) development, functioning and health focusing on the heart and great vessels. To be a new annex in DRP 4.79.

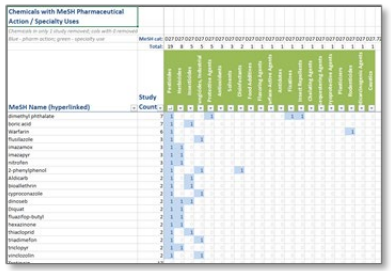
4. Connections with other on-going projects at OECD and elsewhere

3. DRP on Stem Cell Assays for Developmental Toxicity (EST).

- **1,250 chemicals:** 18 studies tested ≥ 10 chems, 174 studies 1-9 chems.
- **Most overlap:** ATRA (17 studies), and the most potent of 1065 ToxCast chemicals.







EST team

Nancy Baker - Leidos
George Daston – Procter & Gamble Co.
Burkhard Flick – BASF (Berlin)
Michio Fujiwara – Astellas Pharma Inc. (Japan)
Thomas Knudsen – USEPA
Hajime Kojima – NIHS/JaCVAM (Japan)
Aldert Piersma – RIVM (Netherlands)
Horst Spielmann – Berlin (retired)
Noriyuki Suzuki – Sumitomo Chemical Co. (Japan)
Katya Tsaioun – Johns Hopkins University

Aim: systematic scoping review on pluripotent stem cell assay modalities and applications for predictive developmental toxicity: chemical and biological domains, readout endpoints, standardized protocols, reproducibility and predictive performance.

Outlook

- Harmonized protocol(s) assessing the retinoid pathway would be useful for developmental toxicity hazard prediction in AOPs leading to fetal skeletal defects.

- Translatability will depend on the integration of *in vitro* data with embryological knowledge (*in vivo*) using multifaceted computational tools, approaches, and models.

- Due to complexity of the retinoid pathway and related networks, computer modeling will be useful for quantitative simulation for animal-free hazard evaluation.