11TH WORLD CONGRESS ON ALTERNATIVES AND ANIMAL USE IN THE LIFE SCIENCES

Organising frameworks: AOPs, MOAs and KCs –
Mutually Informative, Not Mutually Exclusive
S104, Monday, August 30

3Rs in transition From development to application
VIRTUAL CONGRESS - AUGUST 2021

Modeling the Retinoid System in Biology and Toxicology

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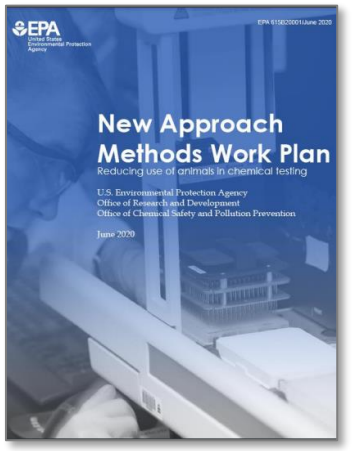
New Approach Methods (NAMs)



- NAMs refer to *in vitro* data and *in silico* models for toxicological assessment with low reliance on animal testing (accelerating data acquisition with fewer resources).
- Healthy pregnancy is an important consideration, that data generated with new testing strategies can be used for developmental hazard evaluation.
- Computational (*in silico*) models that integrate *in vitro* data with knowledge of embryology and development can fill regulatory data gaps for more mechanistic assessments.
- The molecular and cellular determinants of retinoid signaling provide a case study of mutually informative organizing frameworks for NAM-based developmental hazard evaluation.

Can *in vitro* and *in silico* mechanistic tests identify chemicals that perturb development through the retinoid signaling pathway, rather than *in vivo* animal testing?

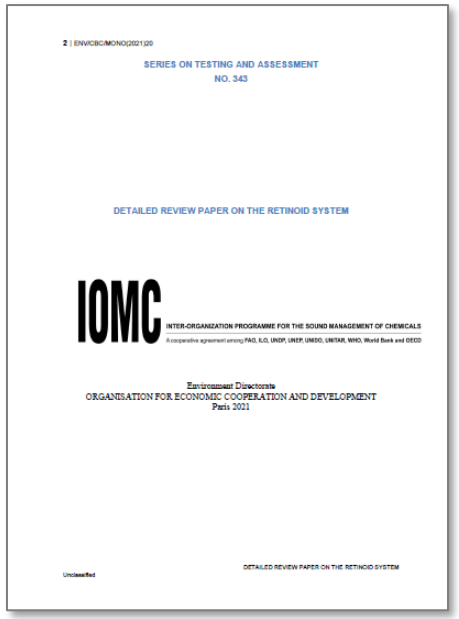
Regulatory interest in the retinoid signaling pathway

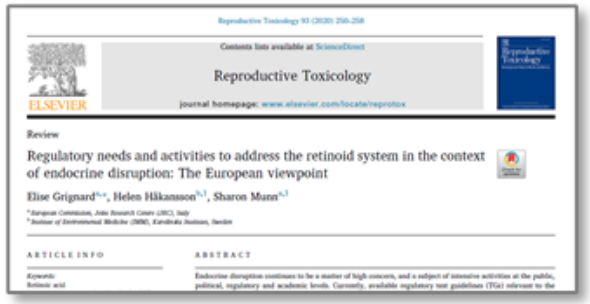
2012: DRP 178 of OECD Test Guidelines Programme highlighted a critical need for harmonized regulations on this system for toxicity screening and evaluation.

2015: OECD EDTA Advisory Group initiated DRP Project 4.97 to review the retinoid signaling pathway across diverse organ systems.

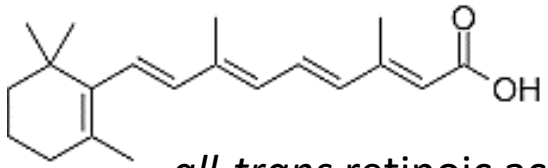
2018: DRP 4.97b narrowed to four areas: Overview, Reproductive System (Annex A), Skeletal Patterning (Annex B), and CNS Development (Annex C).

2021: individual publications in *Reproductive Toxicology* (H Hakannson); final report published in *OECD Testing and Assessment Series* No. 343 (P Browne).

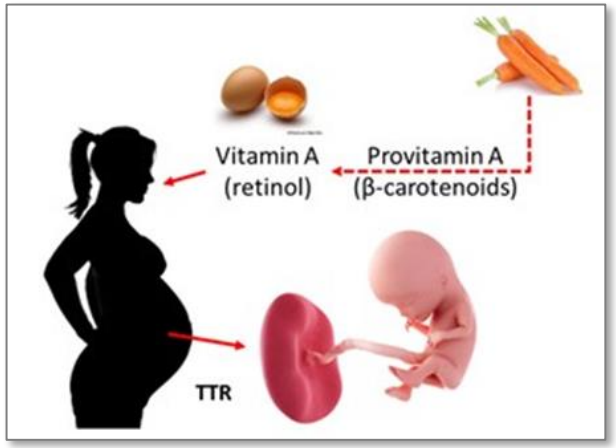


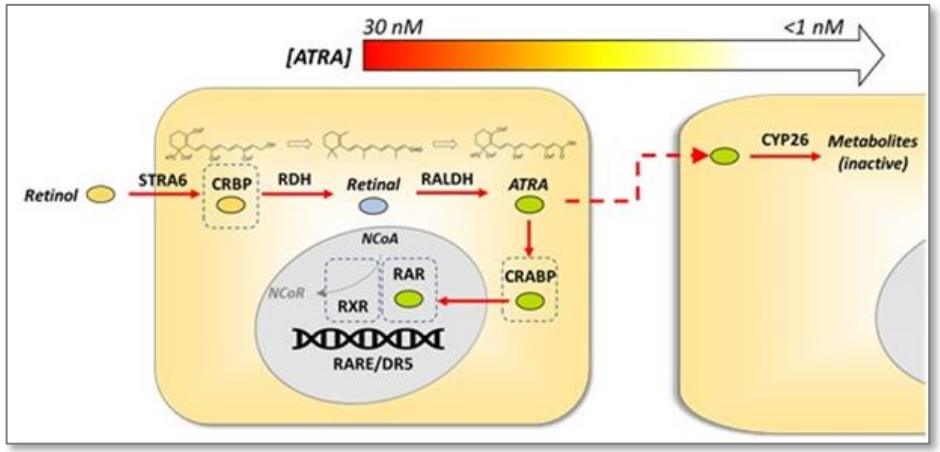


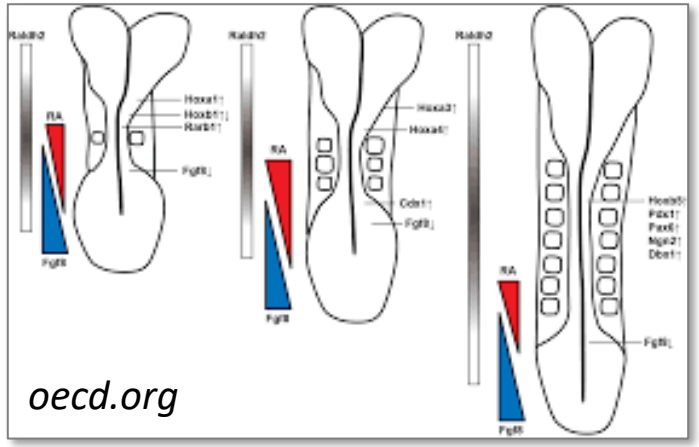
Retinoid signaling pathway



all-trans retinoic acid (ATRA)







- ATRA is locally regulated by a complex network of enzymes, molecular transporters, and nuclear receptors (RARs) determined by cell-specific expression.
- ATRA gradients collaborate with some of the most powerful morphogenetic signals that shape embryonic growth and development (e.g., FGF, BMP, SHH, WNT, …).
- Local regulation of ATRA homeostasis and its disruption may be captured in diverse AOP frameworks linking molecular initiating events (MIEs) to adverse developmental outcomes.

ATRA thresholds

Regional ATRA concentration thresholds reported in different studies on morphogenesis, differentiation, and pregnancy.

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)

SOURCE: Knudsen et al. (2021), Reprod Toxicol

Information retrieval: *biological targets*

Retinoid Pathway Targets
Retinol Binding Proteins (plasma and cellular transporters)
Molecular transporters for retinol uptake (STRA6, STRA8)
Retinol Dehydrogenase (RDH10)
Retinaldehyde Dehydrogenase (RALDH2)
Cellular Retinoic Acid Binding Proteins (CRABP-I, CRABP-II)
Retinoic Acid Receptors (RARs) alpha, beta, and gamma
Retinoid X Receptors (RXRs) alpha, beta, and gamma
Nuclear Coactivators (NCOAs) and Corepressors (NCORs)
Cytochrome P450 family 26 (CYP26A, CYP26B, CYP26C)

Assemble candidate chemical list for each target:

- ChEMBL has data on 12 metabolic assays for drug-like compounds;
- Bioactivity from high-throughput screening (HTS);
- ToxCast has HTS data on 11 downstream assays testing ~2K chemicals;
- Tox21 has HTS data on an intact retinol signaling pathway for ~10K chemicals;
- Potential disruption of ATRA signaling identified for 213 compounds;
- Literature mining (AbstractSifter v5.7) → 5903 related publications;
- Compile information for pMIEs, key events, and adverse outcomes.

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

potency

Retinoids

Triazoles (CYP)

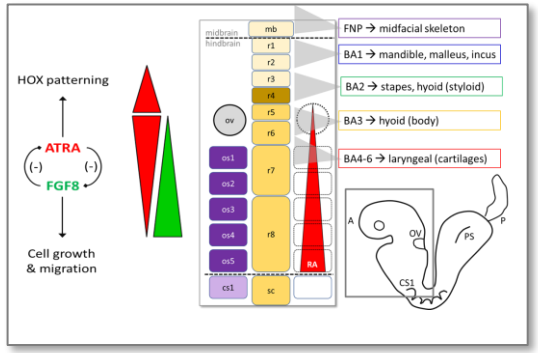
Organochlorines (RAR)

Organotins (RXR)

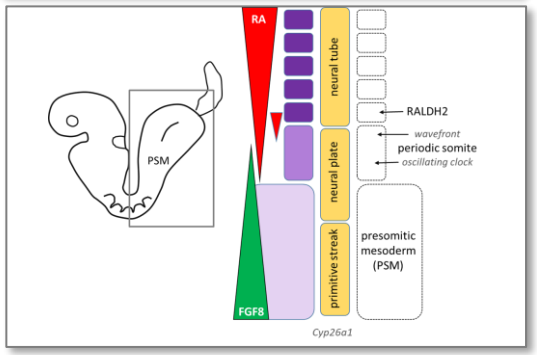
HTS data exists for modeling downstream RAR/RXR responses but less available for ATRA metabolism (eg, RDH10, RALDH2, CYP26a/b/c) and molecular transporters (eg, STRA6, CRABP-I, CRABP-II).

SOURCE: Baker et al. (2018); Knudsen et al. (2021); Baker et al. (manuscript under technical review)

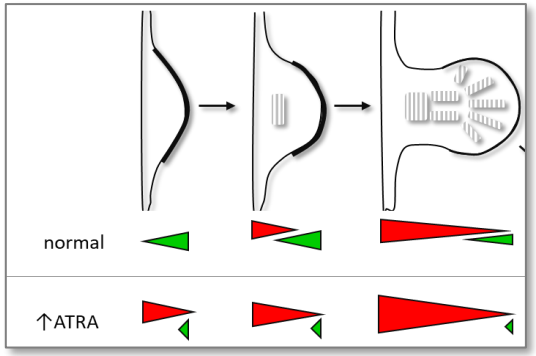
Regional domains for ATRA-dependent skeletal patterning



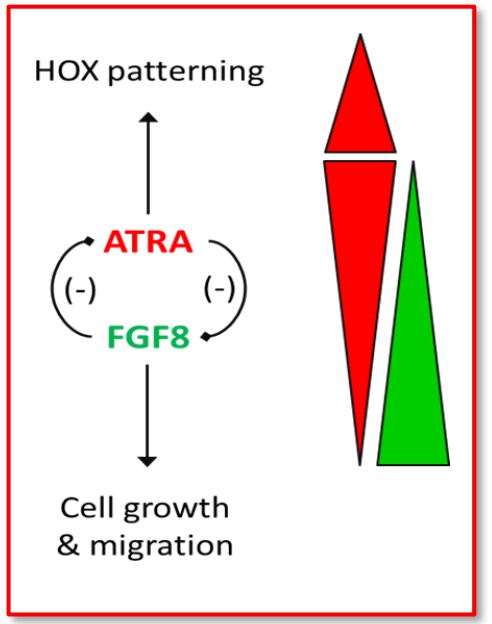
Facial skeleton: positional information of premigratory neural crest cells destined for branchial arches (5- to 11 somite stage).



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



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



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

Putative AOPs for ATRA-dependent skeletal embryopathy

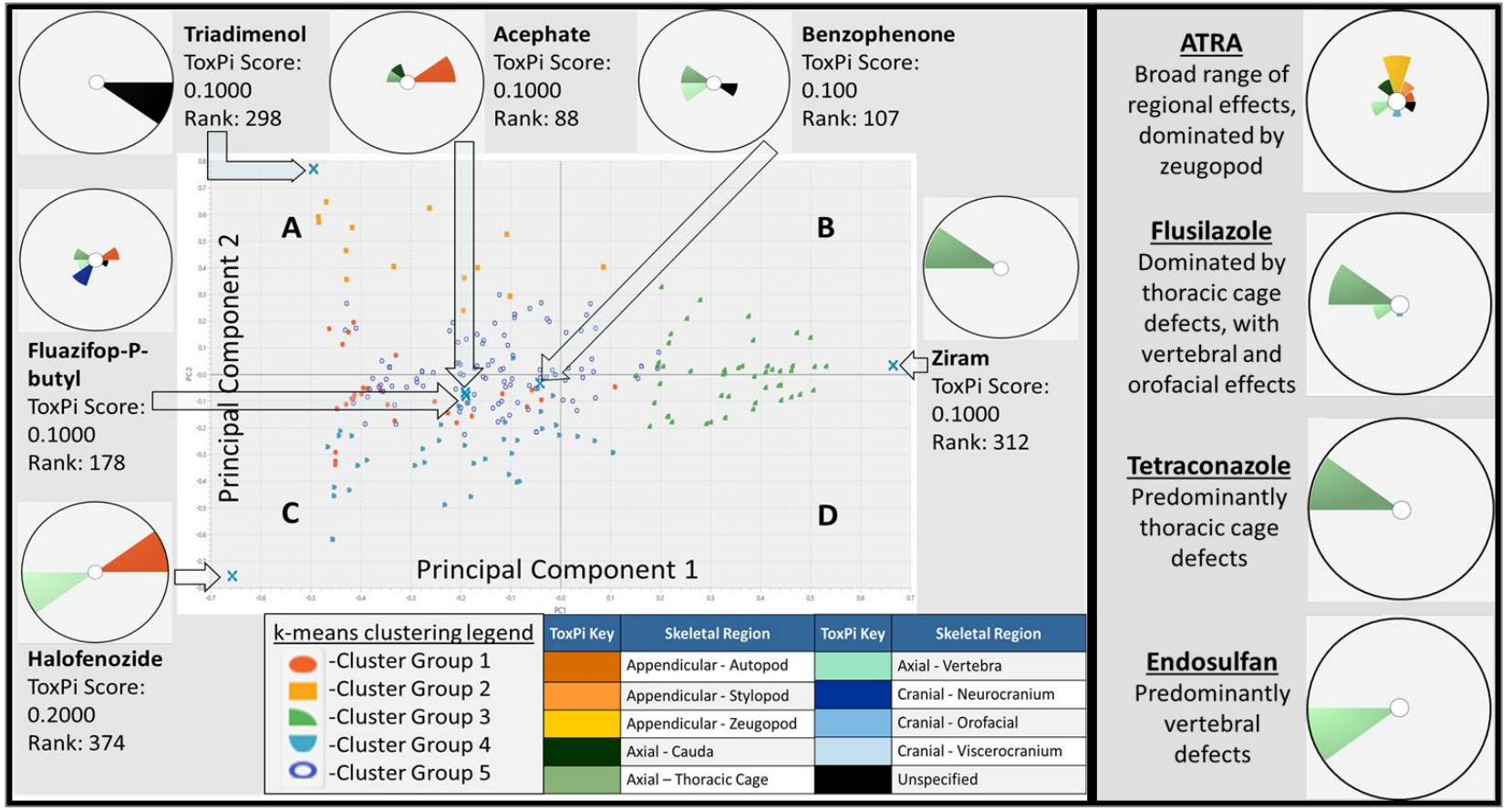
REGION	*MIE*	*KE1*	*KE2*	*KE3*	*KE4*	*KE5*	*AO*
Anterior Neural Tube	Inhibition of CYP26A1 enzymatic activity	Local increase in endogenous ATRA levels	Hyperactivation of the RAR/RXR heterodimer	Repression of *Fgf8* limits FGF8 signaling	Mis-specification of CNC cell fate and behavior	Maxillary arch dysplasia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

Although it is not clear which AOPs may be attributable exclusively to a retinoid-related mechanism, harmonized protocols assessing retinoid signaling can aid developmental hazard prediction.

Knudsen et al., Reprod Toxicol (2021)

ToxRefDB chemicals (370) clustered by ToxPi phenotype

- Culled fetal effects data from 2,946 ToxRefDB studies;
- 57,198 skeletal defects across rodent/nonrodent studies;
- clustered chemicals (k=5) by phenotypic domains (ToxPi).



Are fetal skeletal defects consistent with AOPs linked to ATRA pathway?

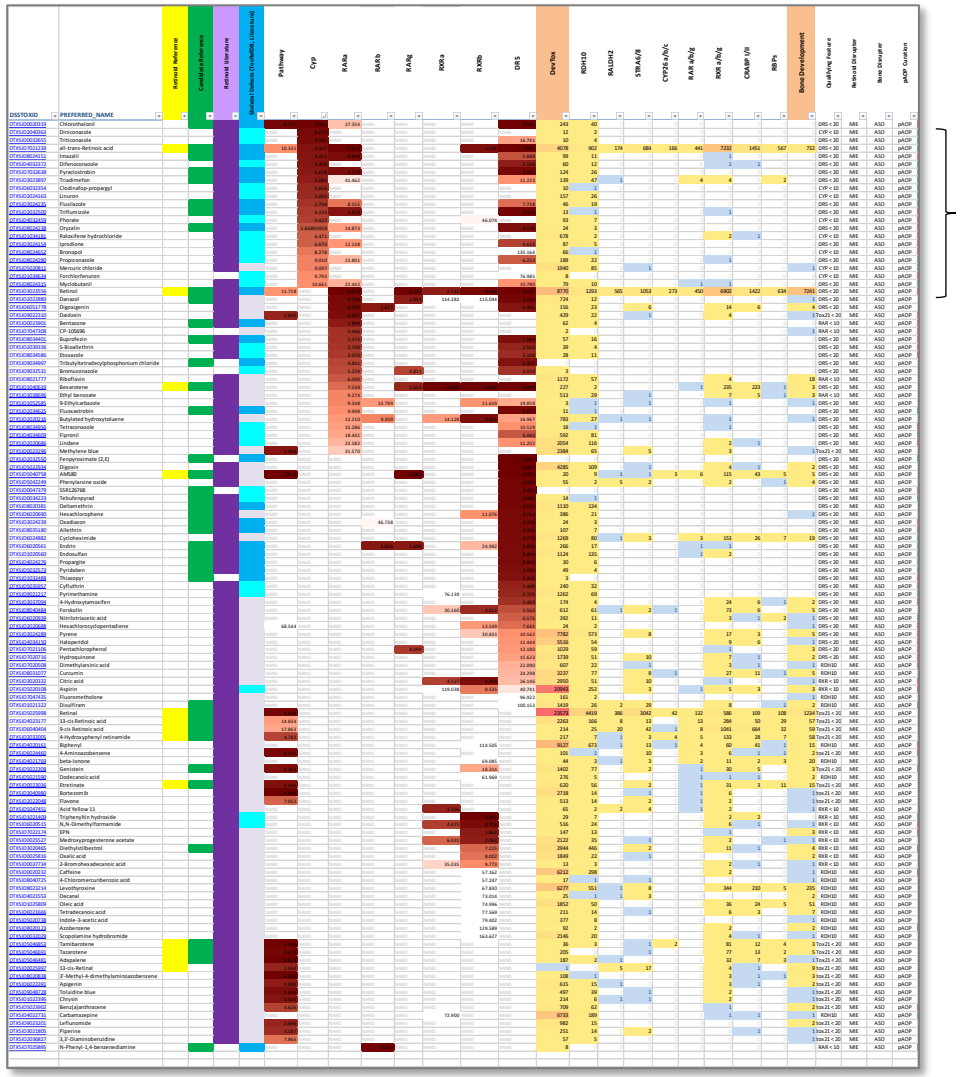
SOURCE: Jocylin Pierro, manuscript in preparation

Mapping potential MIEs to ASOs in the ATRA pathway

Chemicals (n=117)

In vitro biactivity (k=8 assays)

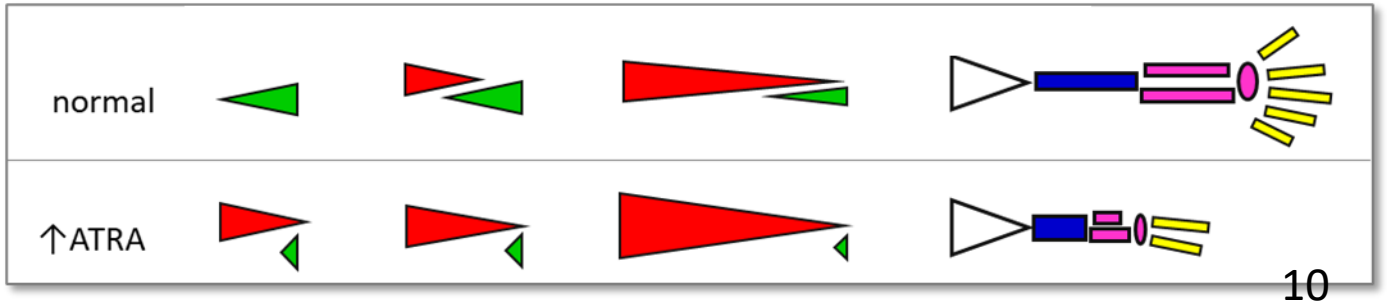
Literature (k=8 assays)



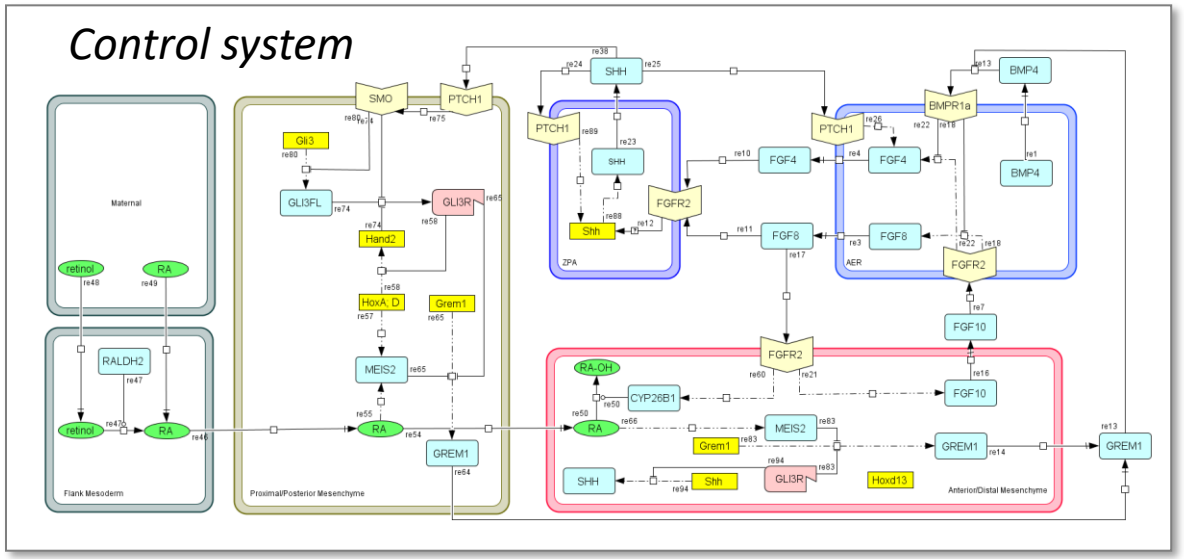
PREFERRED_NAME	Retinoid Reference	Candidate Reference	Retinoid Literature	Skeletal Defects (ToxRefDB, Literature)	Pathway	Cyp	RARa	RARb	RARg	RXRa	RXRb	DR5	DevTox	RDH10
Chlorothalonil					0.472	0.18192162	27.354	NAND	NAND	NAND	NAND	0.185	243	40
all-trans-Retinoic acid					10.335	1.317	0.126	NAND	NAND	NAND	1.036	0.006	4078	902
Imazalil					NAND	1.413	0.908	NAND	NAND	NAND	NAND	5.888	99	11
Pyraclostrobin					NAND	1.678	0.779	NAND	NAND	NAND	NAND	0.542	124	26
Triadimefon					NAND	2.085	41.462	NAND	NAND	NAND	NAND	11.223	139	47
Flusilazole					NAND	3.704	8.155	NAND	NAND	NAND	NAND	7.718	46	19
Triflumizole					NAND	4.134	1.453	NAND	NAND	NAND	NAND	0.298	13	1
Oryzalin					NAND	5.86895958	14.873	NAND	NAND	NAND	NAND	1.116	24	3
Retinol					11.718	NAND	0.076	NAND	0.227	2.142	0.464	0.197	8770	1293

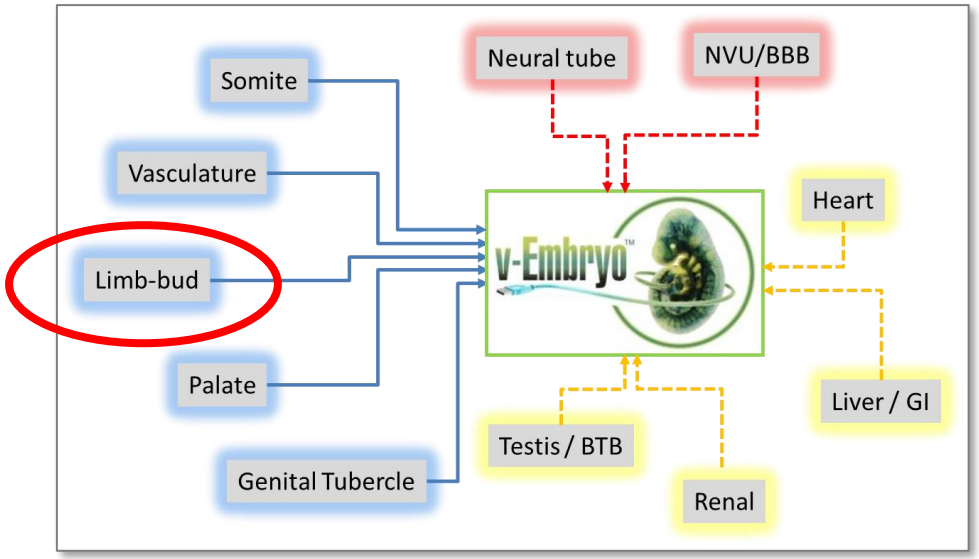
Limb-Bud Mesoderm	Hyperactivation of the RAR/RXR heterodimer	Underextension FGF8 signaling from the AER	Dysregulation of *Meis1/2* and *Hox* gene expression	Proximalization of the limb-bud mesenchyme	Mis-specification of precartilage blastema	Malformed cartilaginous bone rudiment	Phocomelia

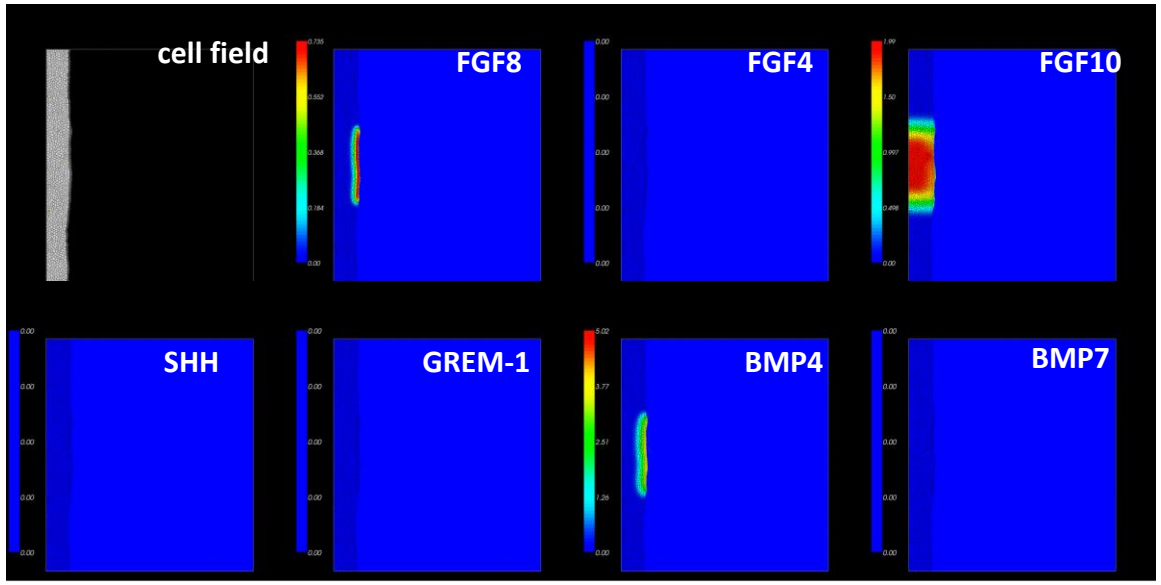
Example: phocomelia can result when a 30-somite embryo experiences ATRA overload.

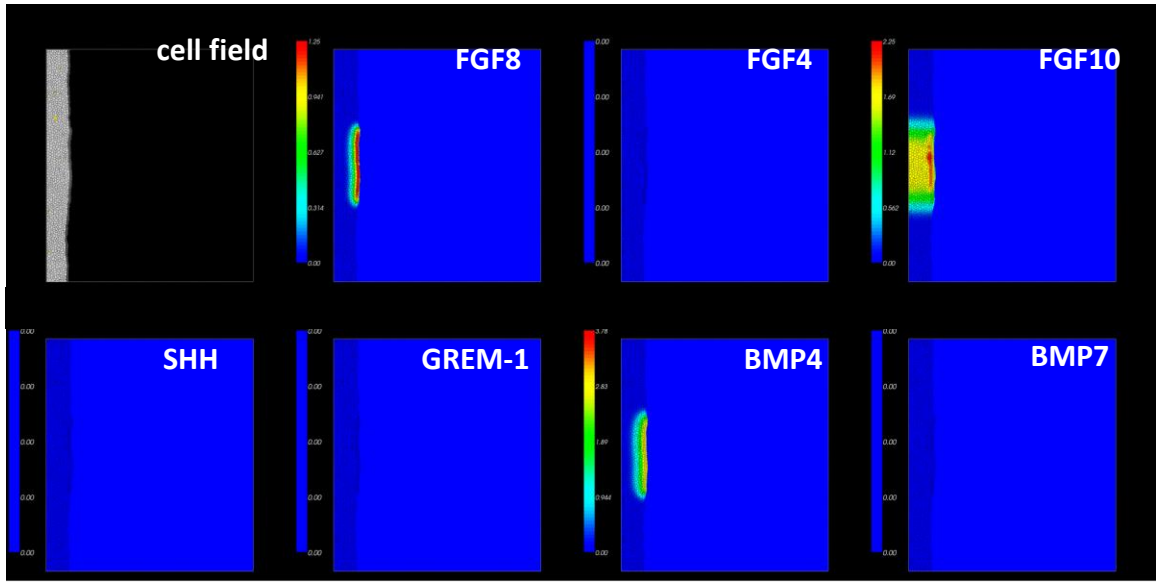


Putting an AOP in motion: *loss of SHH signaling impairs limb-bud outgrowth*









EPA/ORD/CCTE control system built with CellDesigner and simulated with CompuCell3D.org

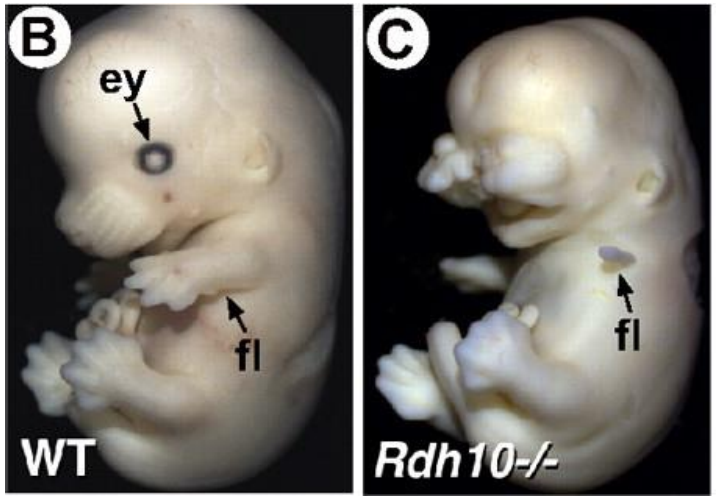
Example: *mode-of-action for ATRA-mediated limb-bud truncation*

Limb-bud initiation requires ATRA signaling but morphogenetic patterning is sensitive to ATRA overload

Several ToxCast compounds inhibit CYP26-like enzymatic activity and weakly activate RAR signaling *in vitro.*

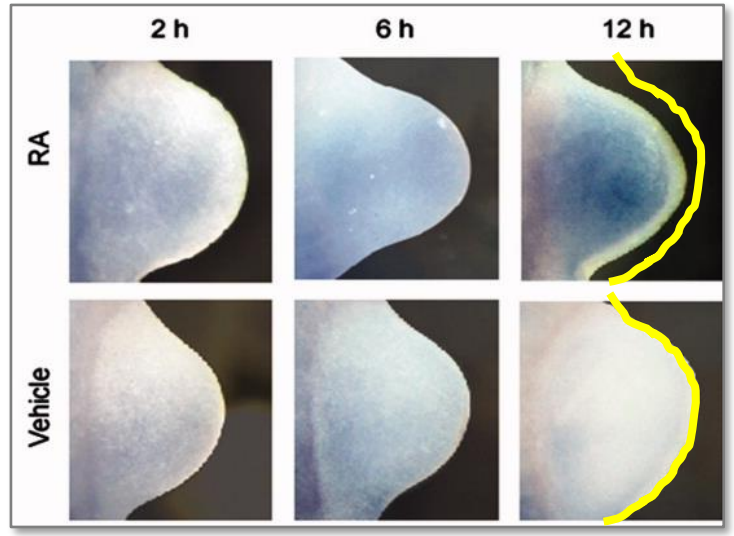
ATRA deficiency

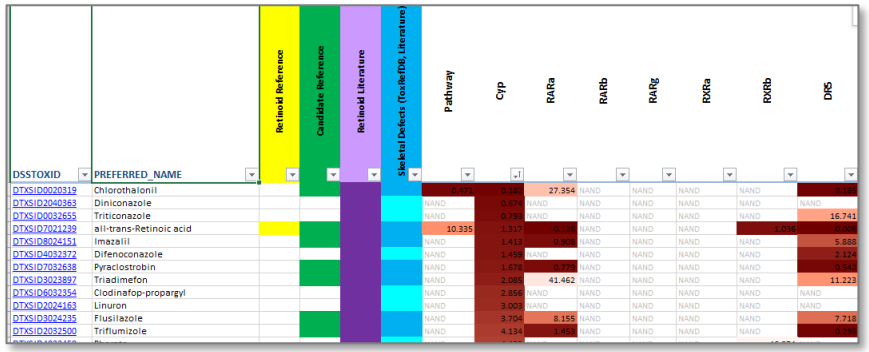
Rhinn et al. (2011), PNAS



ATRA overload

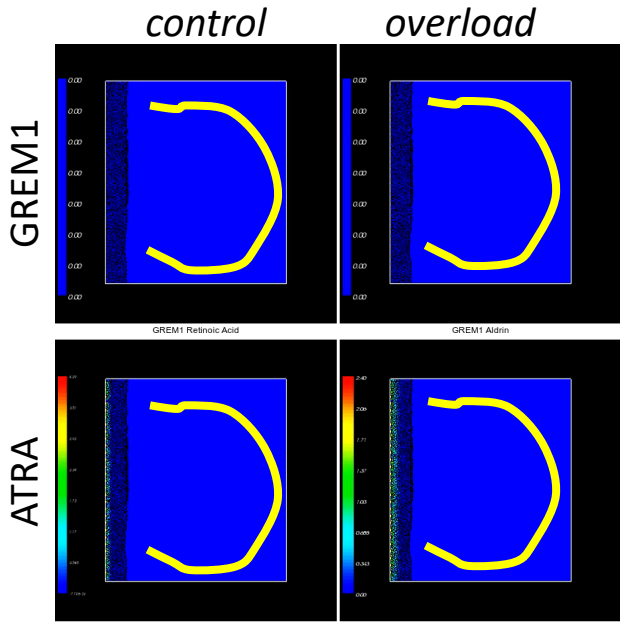
Lee et al. (2010), BDRA





DSSTOXID	PREFERRED_NAME	Retinoid Reference	Candidate Reference	Retinoid Literature	Skeletal Defects (ToxRefDB, Literature)	Pathway	Cyp	RARa	RARb	RARg	RXRa	RXRb	DHS
DTXSID0020319	Chlorothalonil					0.472	0.117	27.354	NAND	NAND	NAND	NAND	0.148
DTXSID2040363	Diniconazole					NAND	0.671	NAND	NAND	NAND	NAND	NAND	NAND
DTXSID0032655	Triticonazole					NAND	0.795	NAND	NAND	NAND	NAND	NAND	16.741
DTXSID7021239	all-trans-Retinoic acid					10.335	1.317	0.126	NAND	NAND	NAND	1.036	0.008
DTXSID8024151	Imazalil					NAND	1.413	0.909	NAND	NAND	NAND	NAND	5.888
DTXSID4032372	Difenoconazole					NAND	1.451	NAND	NAND	NAND	NAND	NAND	2.124
DTXSID7032638	Pyraclostrobin					NAND	1.678	0.771	NAND	NAND	NAND	NAND	0.544
DTXSID3023897	Triadimefon					NAND	2.083	41.462	NAND	NAND	NAND	NAND	11.223
DTXSID6032354	Clodinafop-propargyl					NAND	2.856	NAND	NAND	NAND	NAND	NAND	NAND
DTXSID2024163	Linuron					NAND	3.003	NAND	NAND	NAND	NAND	NAND	NAND
DTXSID3024235	Flusilazole					NAND	3.704	8.155	NAND	NAND	NAND	NAND	7.718
DTXSID2032500	Triflumizole					NAND	4.134	3.451	NAND	NAND	NAND	NAND	0.054

Simulating an ATRA overload *in silico:* 'cybermorph' foreshadows deficiencies of distal elements.



Predictive Toxicology of the Retinoid Signaling Pathway

Goal: mine HTS bioactivity profiles for the retinoid system and build relevant AOPs based on embryological knowledge for fetal (skeletal) development.

- 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. Formalize an Adverse Outcome Pathway (AOP) framework for the retinoid system.
2. Map HTS data from relevant assays in ToxCast/Tox21 profiles to the AOP framework.
3. Build and test computational models for quantitative disruption of ATRA signaling.

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

Tox21 contact
Nisha Sipes, CCTE

NTP/NIEHS
Nicole Kleinstreuer

NIH/NCATS
Srilatha Sakamuru
Menghang Xia

NCTR/FDA
Annie Lumen

OECD
Patience Browne