Expert Group on Retinoid Pathway Signaling
Advisory Group on Endocrine Disrupters Testing and Assessment (EDTA), Test Guidelines Program
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Retinoid Signaling in Skeletal Development:
Scoping the System for Predictive Toxicology

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

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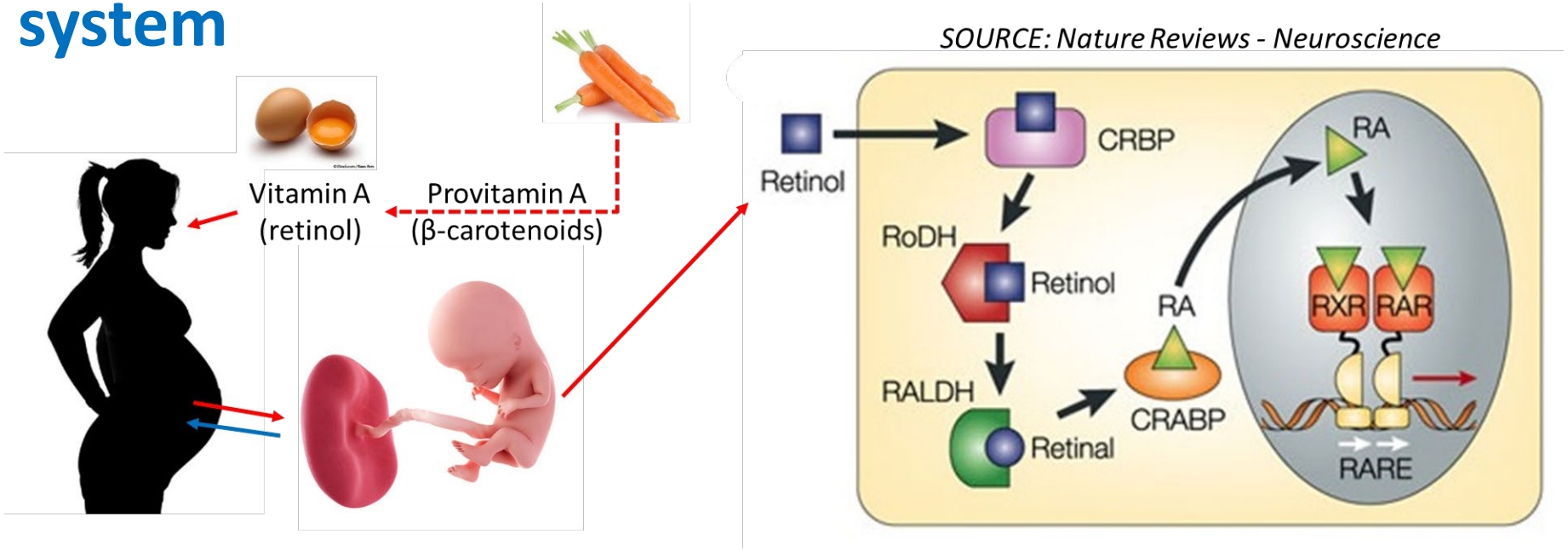
Scope and relevance of this annex

- Retinoids influence development: reflected in the ancestry and conservation of genes for retinoid signaling, from gastropods to humans.
- Two major themes relevant to state of science: (1) role of endogenous retinoids, their receptors, and cellular effects; (2) chemical disruption during pregnancy.
- A common theme between developmental processes and toxicities is the regulation, homeostasis, and physiology of retinoic acid (biologically active form of vitamin A).
- RA signaling collaborates with patterning the embryo: altered skeletal morphology is a common outcome following genetic or chemical perturbation.
- Case study approach: chemicals that interact with retinoid pathway targets (*in vitro* data) and quantitative models (*in silico*) for adverse skeletal phenotypes (*in vivo*).

Retinoid system



- Vitamin A (retinol), unlike many vitamins, can be stored in several tissues (eg, liver, RPE) and mobilized as needed; however, unlike endocrine hormones the active ligand (atRA) is not produced at a specific gland.

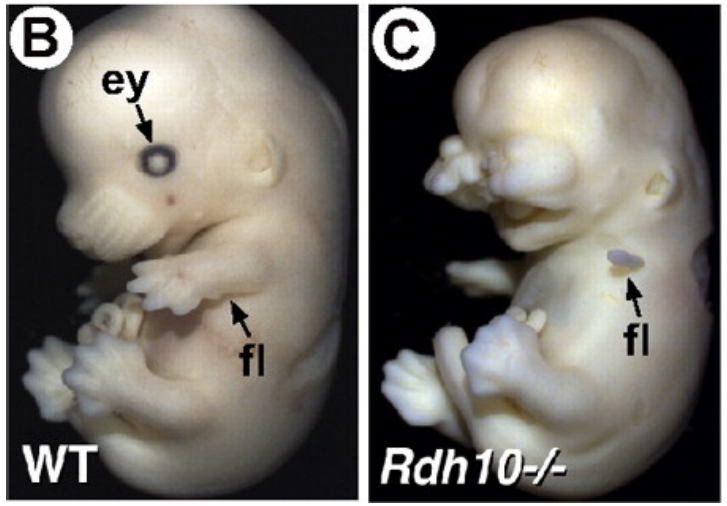
- Maternal vitamin A (retinol) conveys via retinol-binding protein, freely crosses the placenta to enter the embryonic circulation, and is locally activated to retinoic acid (atRA) and binds its nuclear receptors (RARs);

- RAR/RXR heterodimers bind cognate ‘RARE’ elements having a direct repeat (DR) hexanucleotide spaced by five nucleotides (DR5); lesser spacing converts RXR specificity to other nuclear receptors.

RA signaling

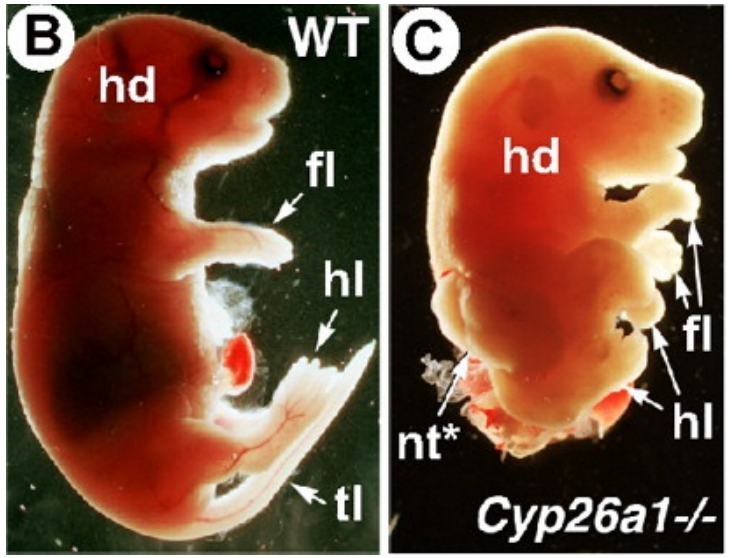
- RA was the ‘first morphogen’ characterized in vertebrate embryos [Thaller and Eichle, 1987].
- Control of RA production (RDH/RALDH2) and degradation (CYP26 a/b/c) is critical for proper skeletal development;
- over 500 RA-responsive genes regulate many biological processes at the cellular, tissue and organ levels;
- form positive and negative feedback loops with key morpho-regulatory signals (SHH, FGF, WNT, TGFbeta, RTKs, …);
- WOCBP on retinoid therapy (eg, Accutane for acne) must be wary of the ‘Fetal Retinoid Syndrome’.

Suboptimal atRA production



SOURCE: Rhinn et al. (2011) PNAS

Suboptimal atRA breakdown



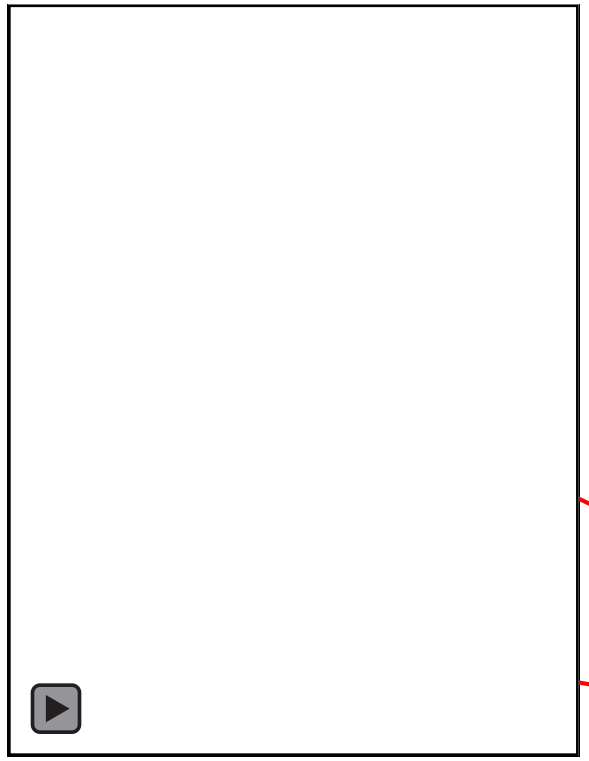
SOURCE: Rhinn and Dolle (2012)

Skeletal development

- The fetal skeleton (>200 bones) 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 [5,6].

- Annex reviewed concepts in retinoid signaling that are generally distinct for three skeletal fields, investigating normal regulation by endogenous RA and abnormalities induced by exogenous retinoids:

https://embryology.med.unsw.edu.au/embryology



craniofacial skeleton
(*neurocranium, viscerocranium*)

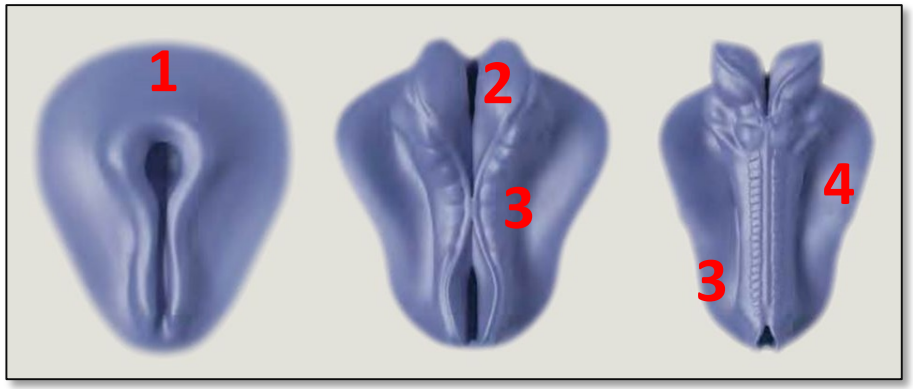
axial skeleton
(*ribs, sternum, vertebral column*)

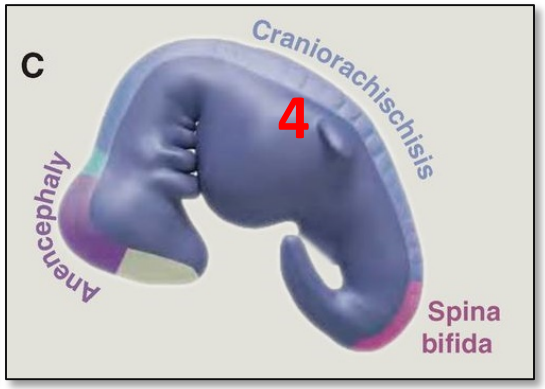
appendicular skeleton
(*upper, lower limbs*)

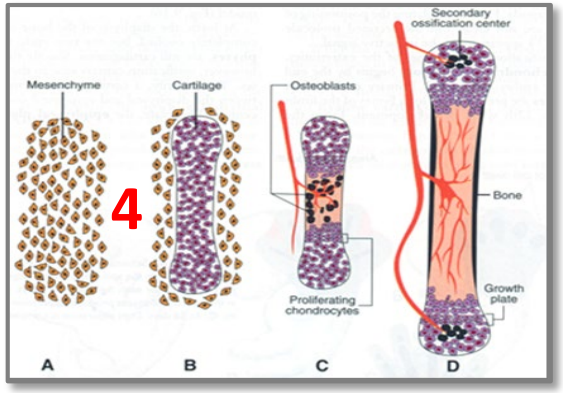
sciencephoto.com

Stage-specific morphogenesis of retinoid-sensitive structures

1. **Presomitic**: appearance of notochord and interaction with floor plate of the neural tube patterns the primary body axis (neuraxis) that subsequently elongates during neurulation.







2. **Craniofacial:** retinoid effects closely linked to patterning cranial neural crest cells (CNCs) prior to their emigration from the anterior neural tube (hindbrain).

3. **Axial:** vertebral column segmentation has its origin in somitogenesis - somites spur cells (sclerotome) that migrate medially around the neural tube and elsewhere to form ribs and sternum.

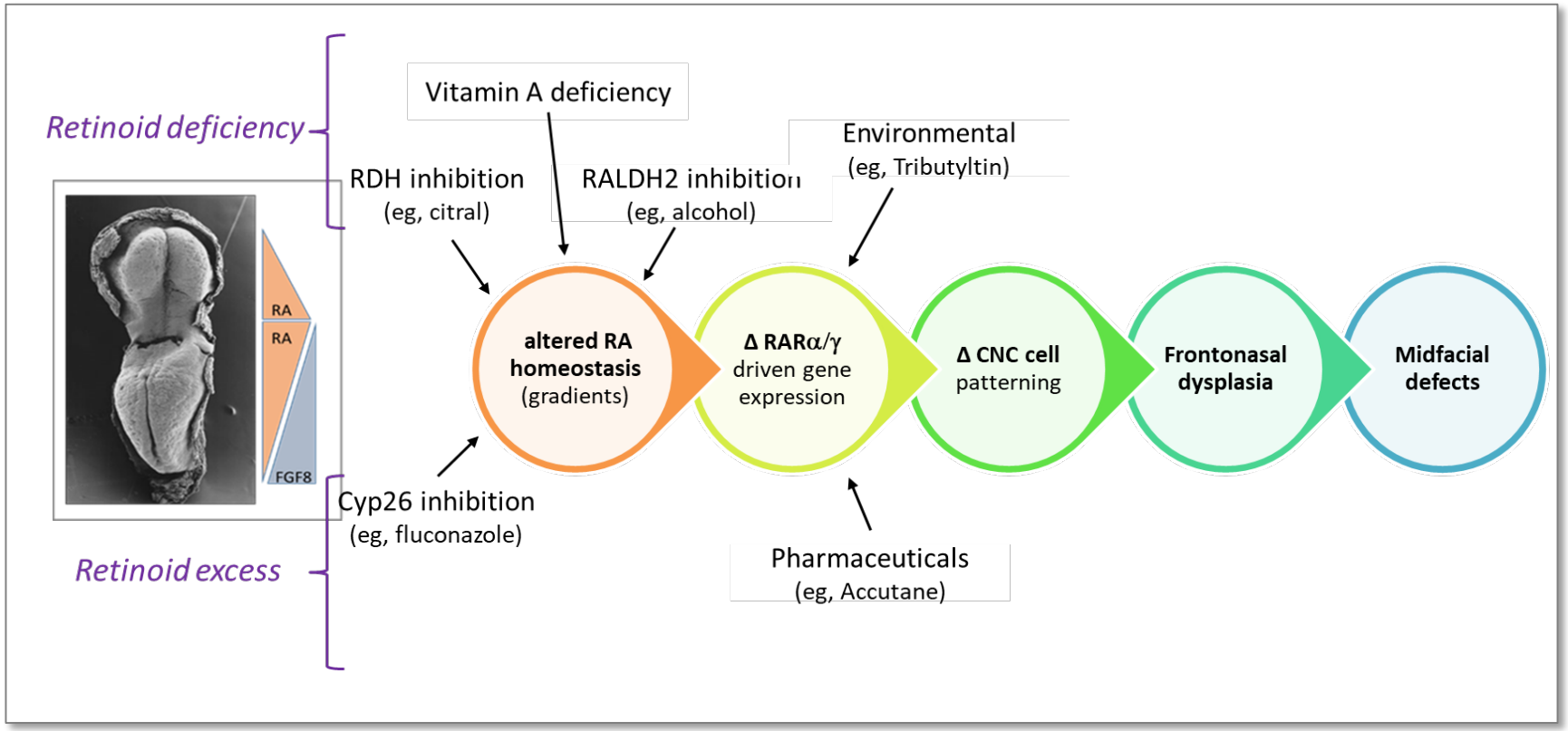
4. **Appendicular:** important considerations for limb-bud prepatterning and subsequent differentiation (chondrogenesis, osteogenesis, vascularization).

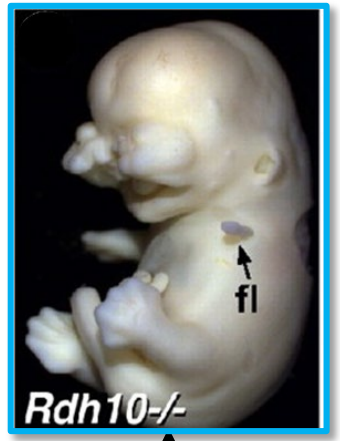
Generalized AOP framework

- End-goal is a performance-based model of the retinoid system for predictive toxicology that addresses the regulation, homeostasis, and biological activity of the retinoid signaling pathway.
- An AOP framework is necessary to organize the *in vitro* data and *in silico* models and help identify relevant analytical tools, statistical relationships and insight into the biological domain.
- Despite a wealth of information on retinoid signaling pathways during development, only two literature reports refer to an AOP for the retinoid system [Tonk et al. 2015, Baker et al. 2018].
- Searching the AOPWiki [https://aopwiki.org/] for 'retinoic acid' or 'retinoid' returned key events for only 5 AOPs (#ID = 43, 297, 7, 37, 38).
- Some information available for profiling the retinoid system *in vitro* (ToxCast/Tox21) and building performance-based models to predict skeletal dysmorphogenesis *in vivo*.

This Annex reviews literature on retinoid signaling pathways since 1970s as an initial conceptualization of an AOP framework for developmental toxicity of skeletal system.

Craniofacial AOP: Abstract Sifter query '*cranial development and (embryo or fetus)*' (August 2019) picked up 3747 records (1978-2019) with relevant MeSH headings; 158 contained '*retino**' for this review.





Adverse Outcome
retinoids primarily affect midface and branchial structures

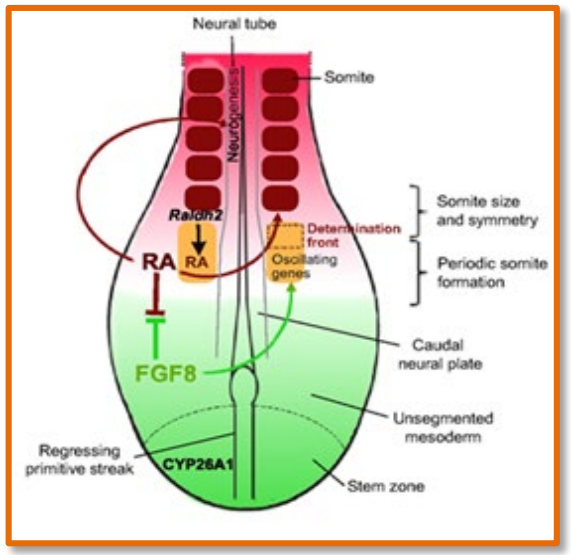
Molecular (MIE)
disruption of RA synthesis/breakdown → during gastrulation

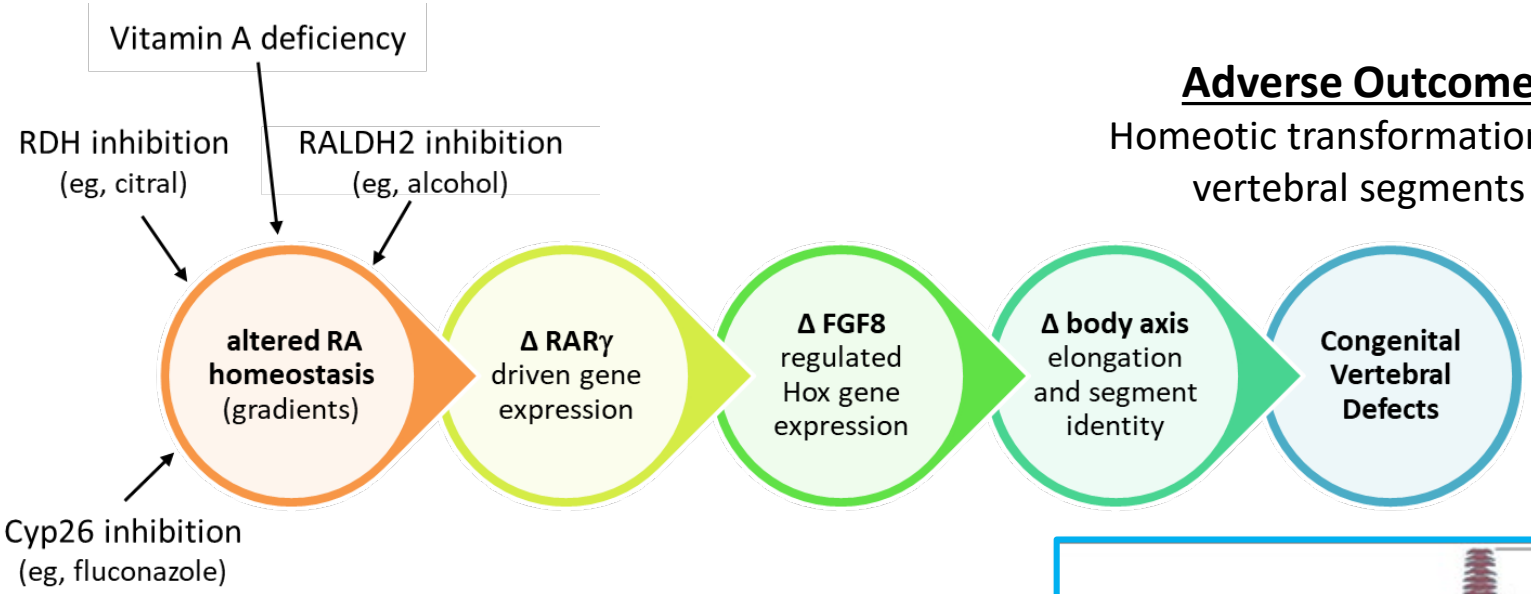
Neuroepithelium
RAR-driven gene expression in crosstalk with BMP, WNT, and FGF signaling

Cranial Neural Crest
misprogramming of migratory CNC functions during gastrulation

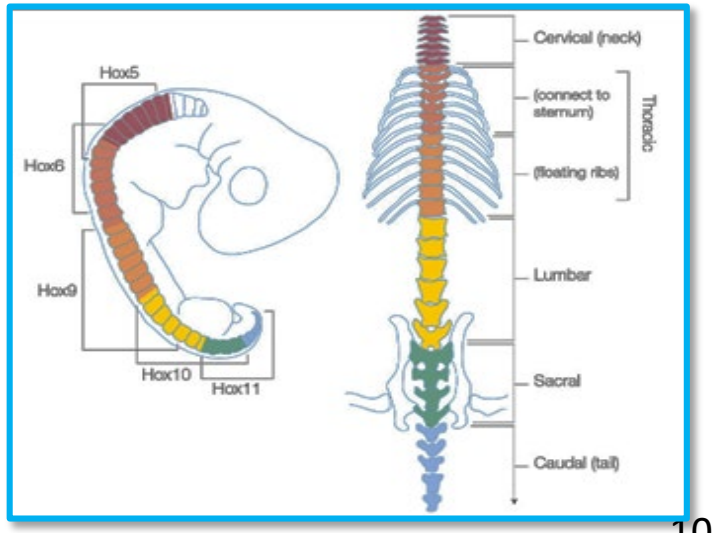
Dysmorphogenesis
altered CNC fate and behaviors (ectopic migration, ↑ apoptosis)

Axial AOP 1: Abstract Sifter query '*cranial development and (embryo or fetus)*' picked up 1679 records (1989-2019) with relevant MeSH headings; 80 contained at least one use of text '*retino**' for this review.



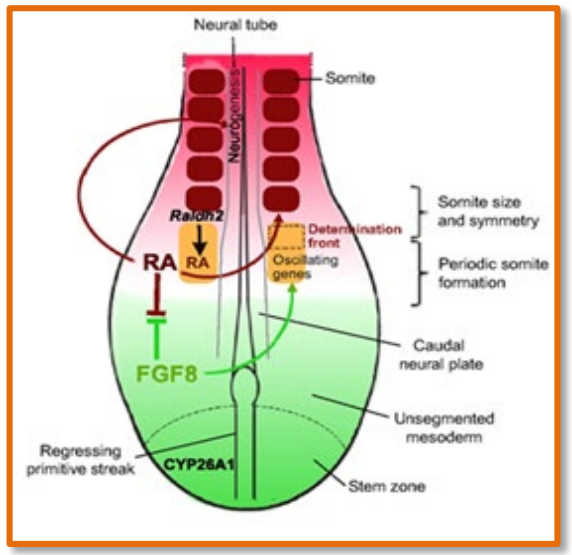


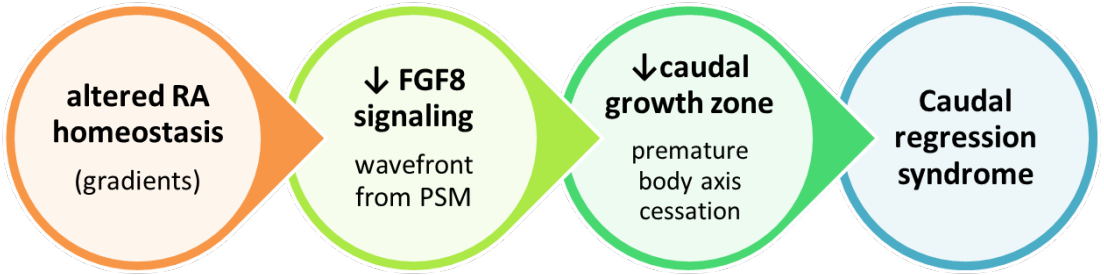
Adverse Outcome
Homeotic transformations of vertebral segments

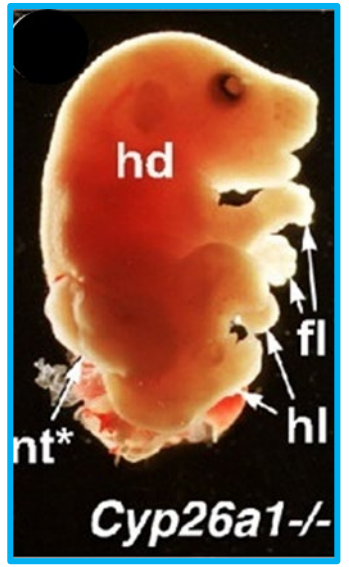


- retinoid signaling posteriorizes the neuraxis (gastrulation) → somites (neurulation)
- positional information encoded during gastrulation but decoded during somitogenesis
- result of complex interactions involving oscillatory activity of Notch and WNT signaling
- mutually antagonistic gradients of FGF8 and RA
- FGF8 wavefront (caudally) opposed by RA signaling (RALDH2 in newly formed somites)
- FGF8 and RA influence pattern of homeobox gene expression (HOX code)
- crosstalk with molecular clock genes determine periodicity.

Axial AOP 2: Abstract Sifter query '*cranial development and (embryo or fetus)*' picked up 1679 records (1989-2019) with relevant MeSH headings; 80 contained at least one use of text '*retino**' for this review.



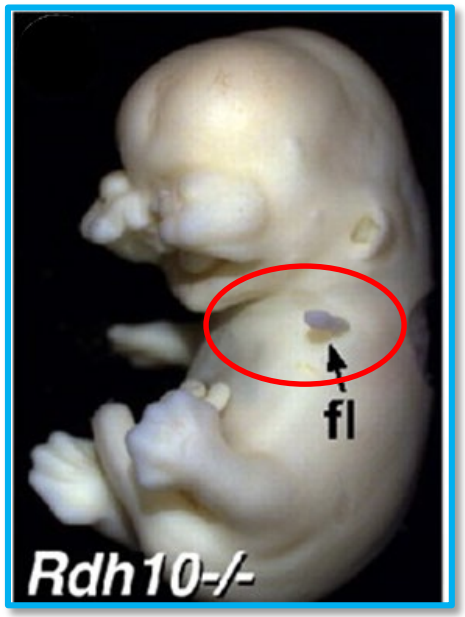


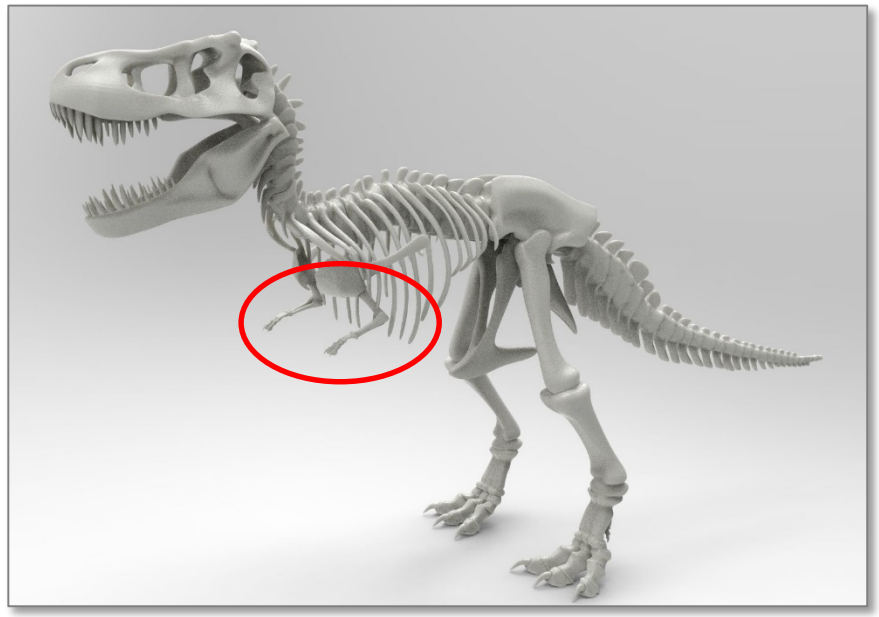


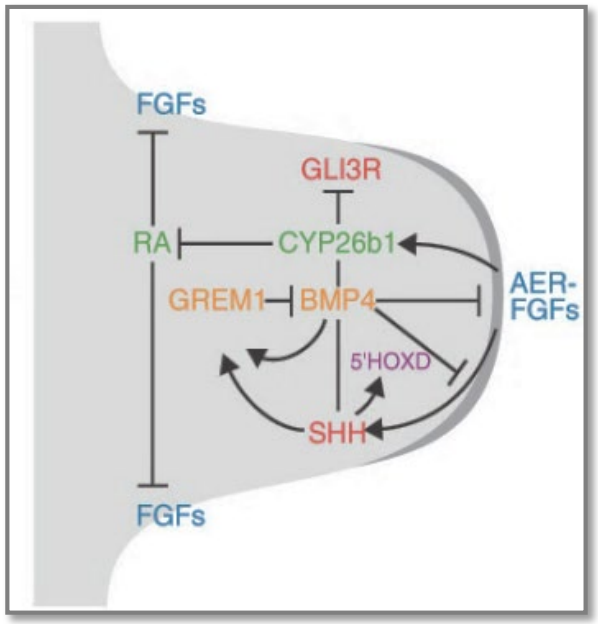
- rostral to caudal progression of somitogenesis during extension of the neuraxis
- draws from proliferating cells in presomitic mesoderm (PSM) of tailbud in response to FGF8
- FGF8 expression maintained in PSM (caudally) by WNT signaling
- anterior extent of FGF8 expression limited by RA (RALDH2) from newly formed somite
- caudal growth zone maintained by RA clearance (Cyp26a1)
- premature cessation of body axis elongation
- crosstalk with molecular clock genes determine periodicity in 'clock-and-wavefront' model.

Adverse Outcome
disruption of posterior axial elongation

Appendicular AOP: Abstract Sifter query '*limb development and (embryo or fetus)*' picked up 4470 records (1973-2019) with relevant MeSH headings; 299 contained at least one use of text '*retino**' for this review.

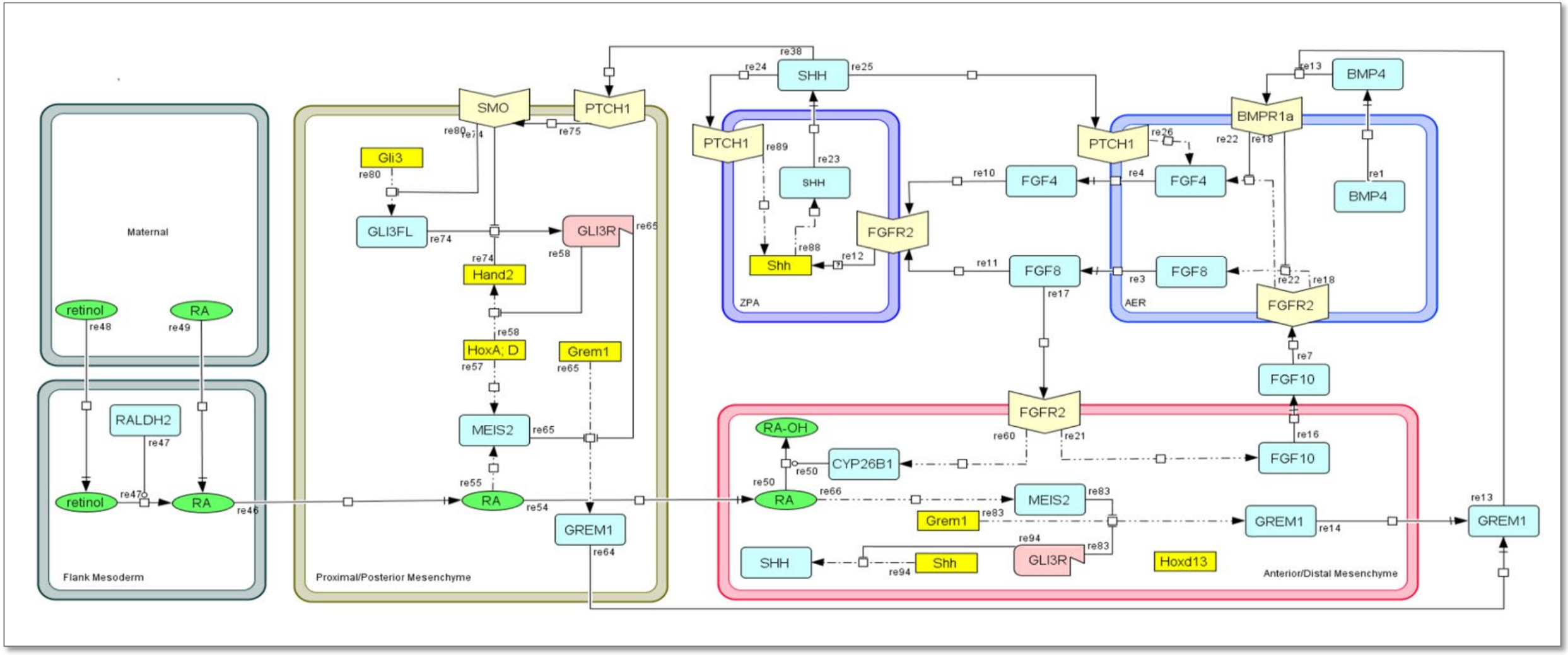






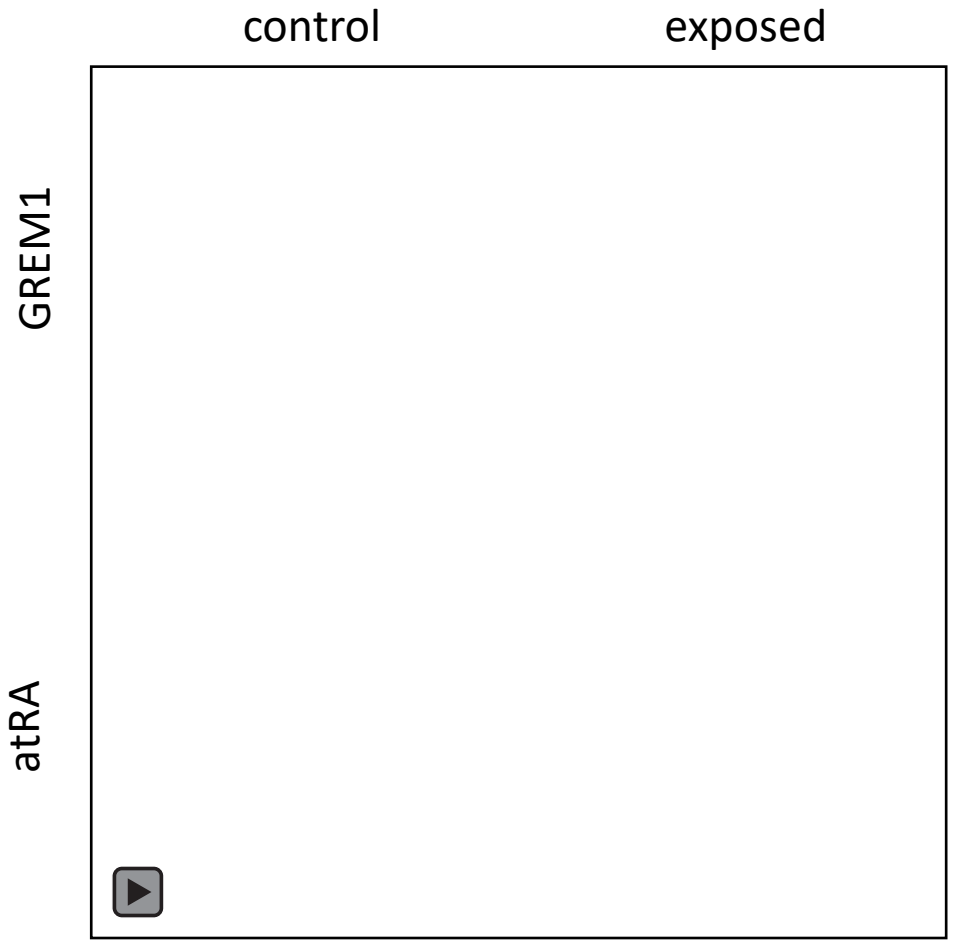
- a complete retinoid system exists in the limbs and somites during critical stages of appendicular patterning
- *Rdh-null* mouse embryos show stunted forelimbs and apparently normal hindlimbs reminiscent of *Tyrannosaurus rex*
- RA from somites enters limb-bud proximally and is degraded by CYP26B1 distally
- RA (through RARα/γ) proximalizes the limb-bud and facilitates distal anterior-posterior patterning via Hox genes
- RARs regulate the transition of precartilage anlagen to chondroblast differentiation
- retinoid teratogenesis on limb may be linked to RARβ hyperactivity and vascular disruption.

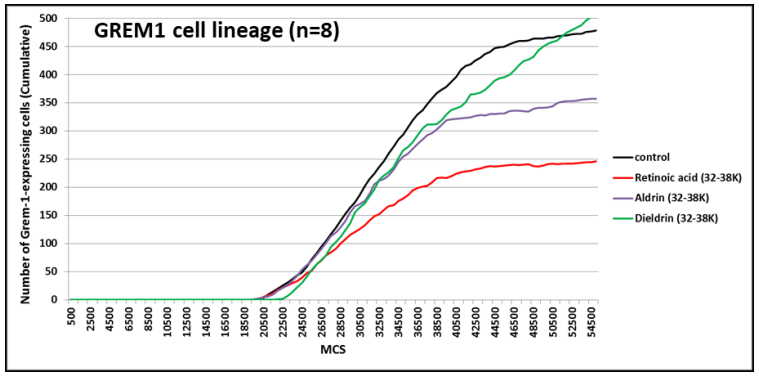
Control system: *more complex and dynamical than conveyed by an AOP network*

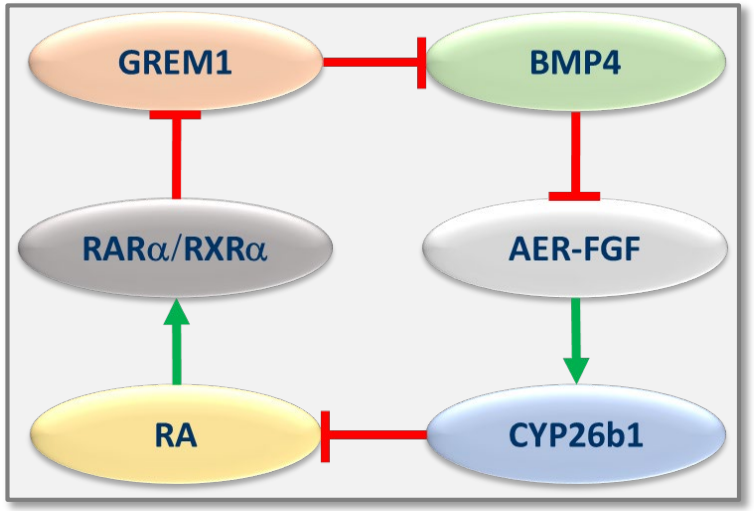


SOURCE: B Ahir (CCTE), CellDesigner

Computer simulation







Model suggests premature shutdown of GRM1-BMP4 loop is a key event terminating FGF-dependent limb-bud outgrowth associated with excessive RA signaling

Skeletal endpoints (developmental)

Toxicity Reference Database (ToxRefDB)
http://www.epa.gov/ncct/toxrefdb/
(Open access)

↓

Currently consists of 1,159 chemical across 6727 animal studies (subchronic, chronic, prenatal development, and multigenerational reproductive studies)

↓

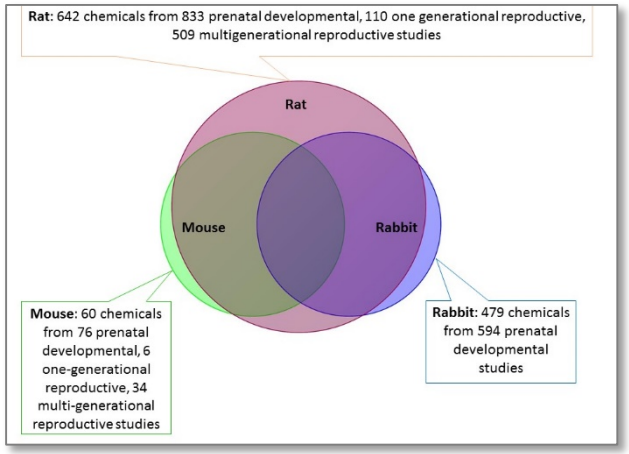
FILTER: Study types associated with prenatal development/ developmental and reproductive
(Returns 896 chemicals across 2747 studies)

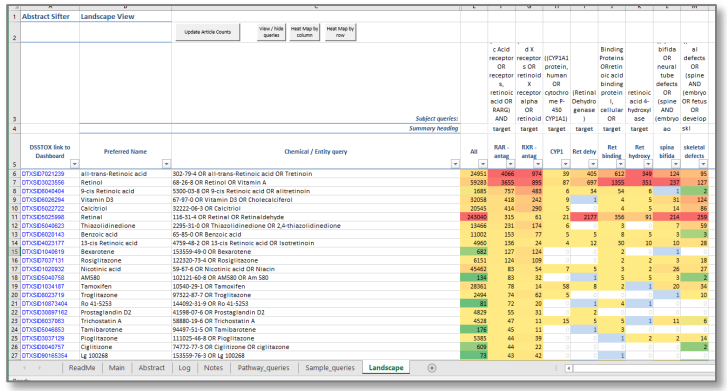
↓

POSITIVES: Identify Studies with skeletal developmental defects (Appendicular, Axial, and Cranial)
(returns 369 chemicals (41% of 896) across 2592 studies)

↓

Limit to Chemicals with limb developmental defects
(returns 44 chemicals (4% of 896) across 310 studies)

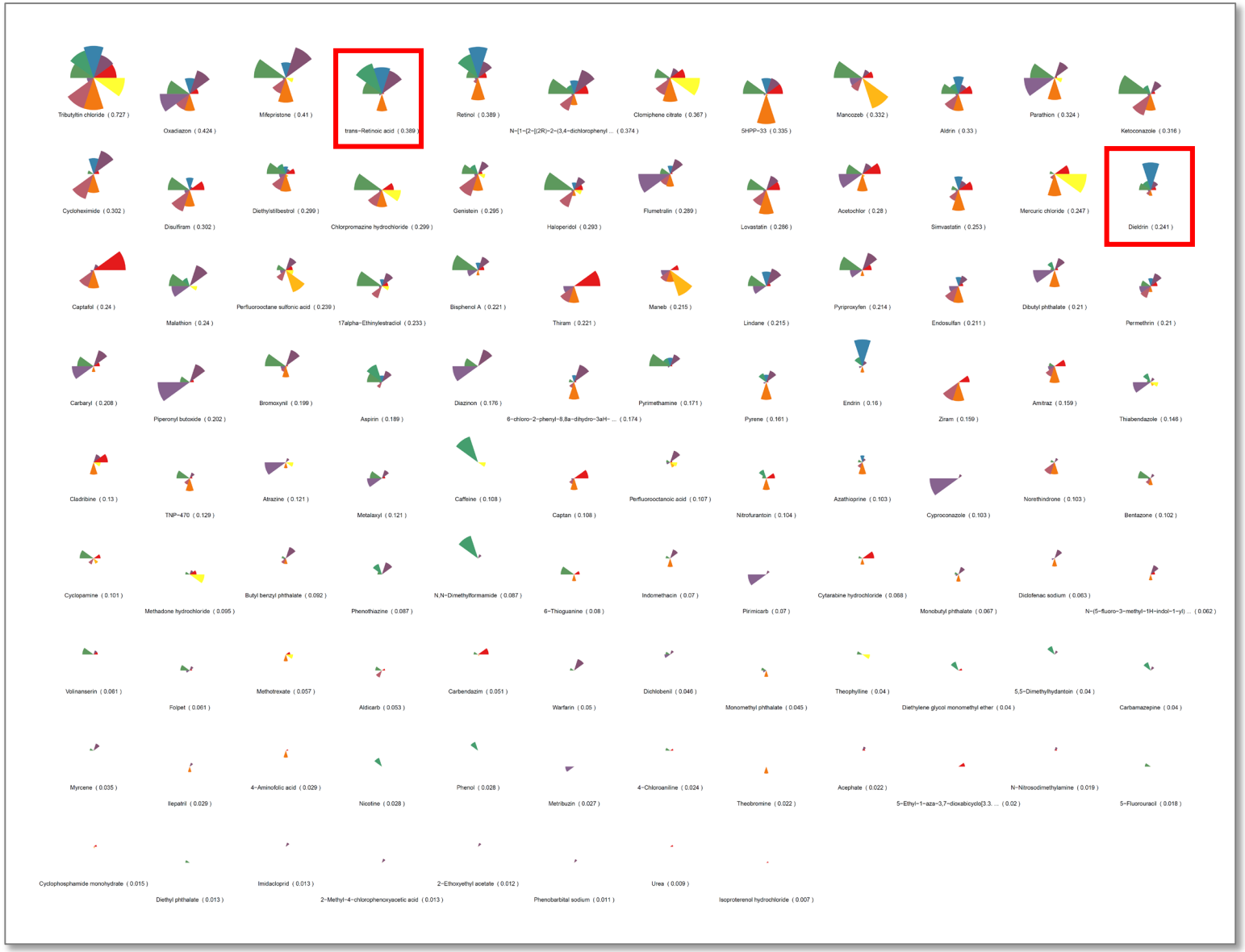


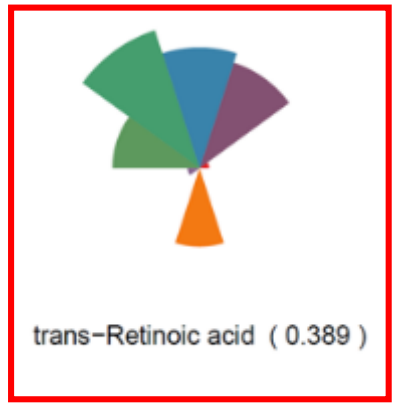


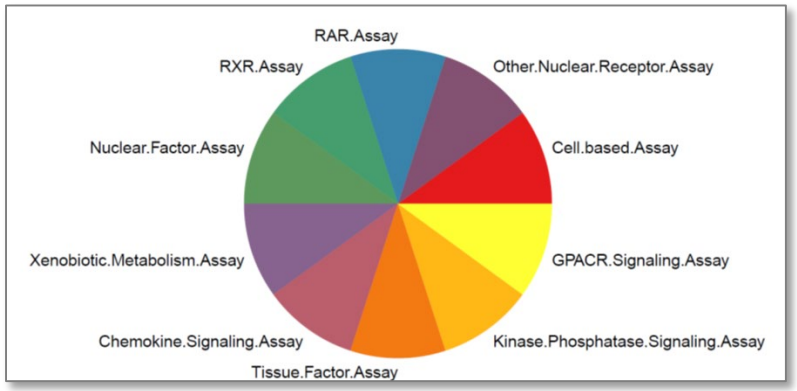
Literature supplement = 104 ToxCast chemicals associated with limb defects

SOURCE: Ahir et al., manuscript

ToxPi ranking: univariate features correlating statistically with skeletal (limb) defects

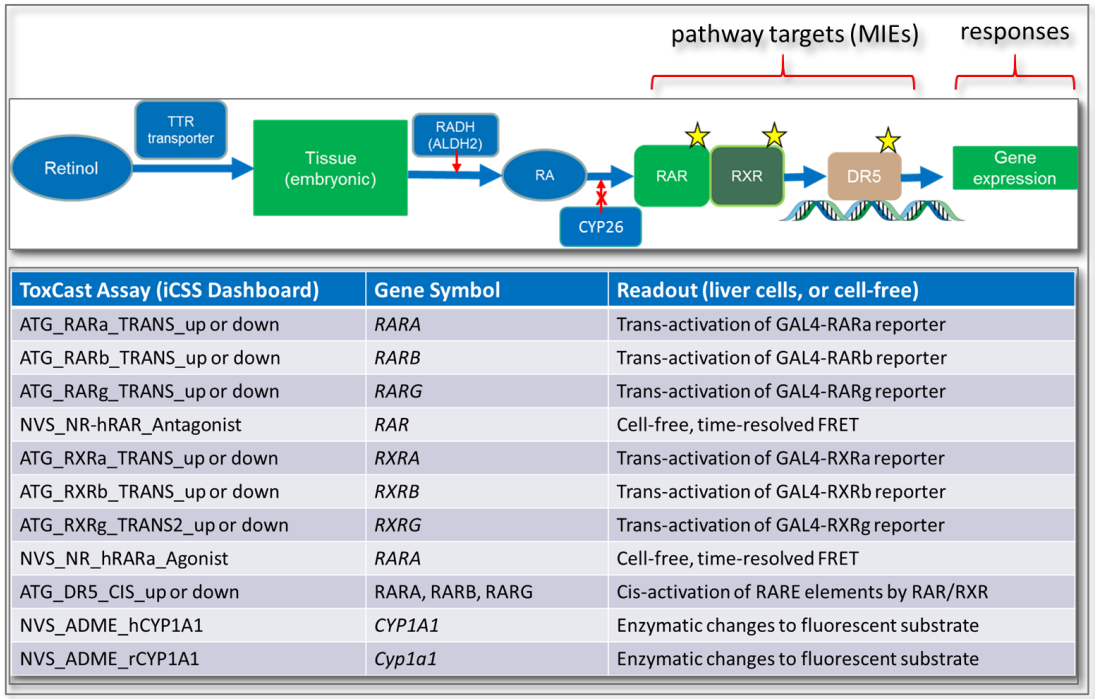






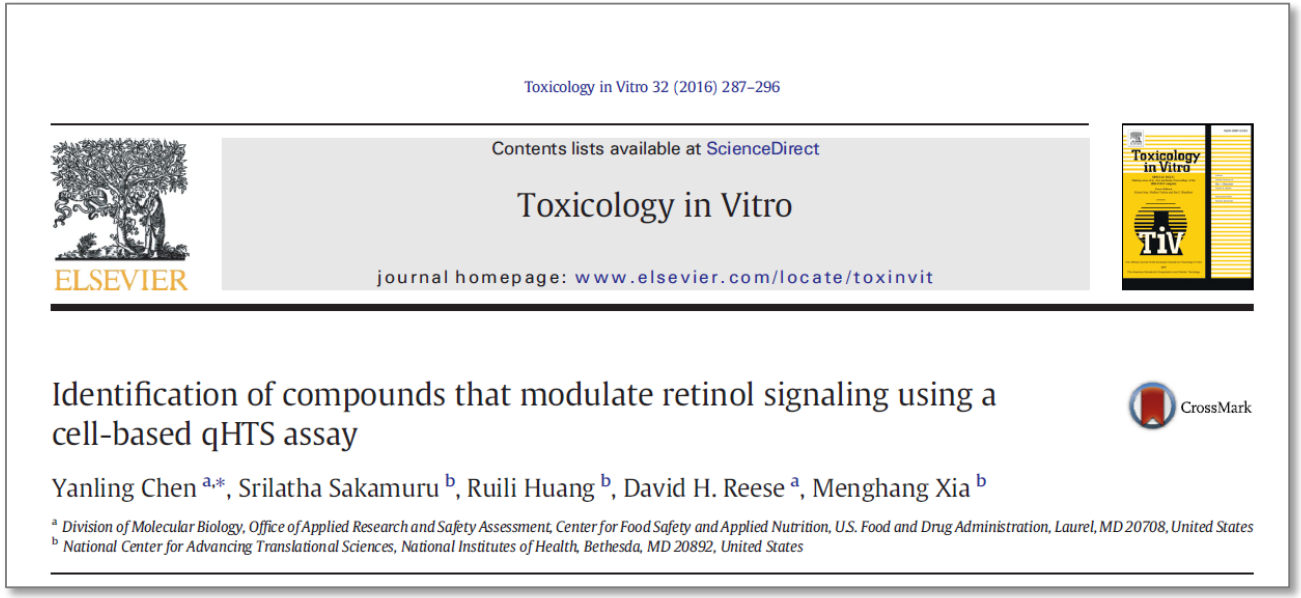
104 chemicals hit ~90 assays, with **RAR-RXR-DR5** well represented

HTS and qHTS



ToxCast Assay (iCSS Dashboard)	Gene Symbol	Readout (liver cells, or cell-free)
ATG_RARa_TRANS_up or down	*RARA*	Trans-activation of GAL4-RARa reporter
ATG_RARb_TRANS_up or down	*RARB*	Trans-activation of GAL4-RARb reporter
ATG_RARg_TRANS_up or down	*RARG*	Trans-activation of GAL4-RARg reporter
NVS_NR-hRAR_Antagonist	*RAR*	Cell-free, time-resolved FRET
ATG_RXRa_TRANS_up or down	*RXRA*	Trans-activation of GAL4-RXRa reporter
ATG_RXRb_TRANS_up or down	*RXRB*	Trans-activation of GAL4-RXRb reporter
ATG_RXRg_TRANS2_up or down	*RXRG*	Trans-activation of GAL4-RXRg reporter
NVS_NR_hRARa_Agonist	*RARA*	Cell-free, time-resolved FRET
ATG_DR5_CIS_up or down	RARA, RARB, RARG	Cis-activation of RARE elements by RAR/RXR
NVS_ADME_hCYP1A1	*CYP1A1*	Enzymatic changes to fluorescent substrate
NVS_ADME_rCYP1A1	*Cyp1a1*	Enzymatic changes to fluorescent substrate

- ToxCast has a number of ligand-binding and reporter assays for retinoid coverage.
- Obvious omissions for RDH/RALDH2 and CYP26 a/b/c (surrogate Cyp1a1 biochemical assay).


Toxicology in Vitro 32 (2016) 287–296
Contents lists available at ScienceDirect
Toxicology in Vitro
journal homepage: www.elsevier.com/locate/toxinvit
ELSEVIER

Identification of compounds that modulate retinol signaling using a cell-based qHTS assay

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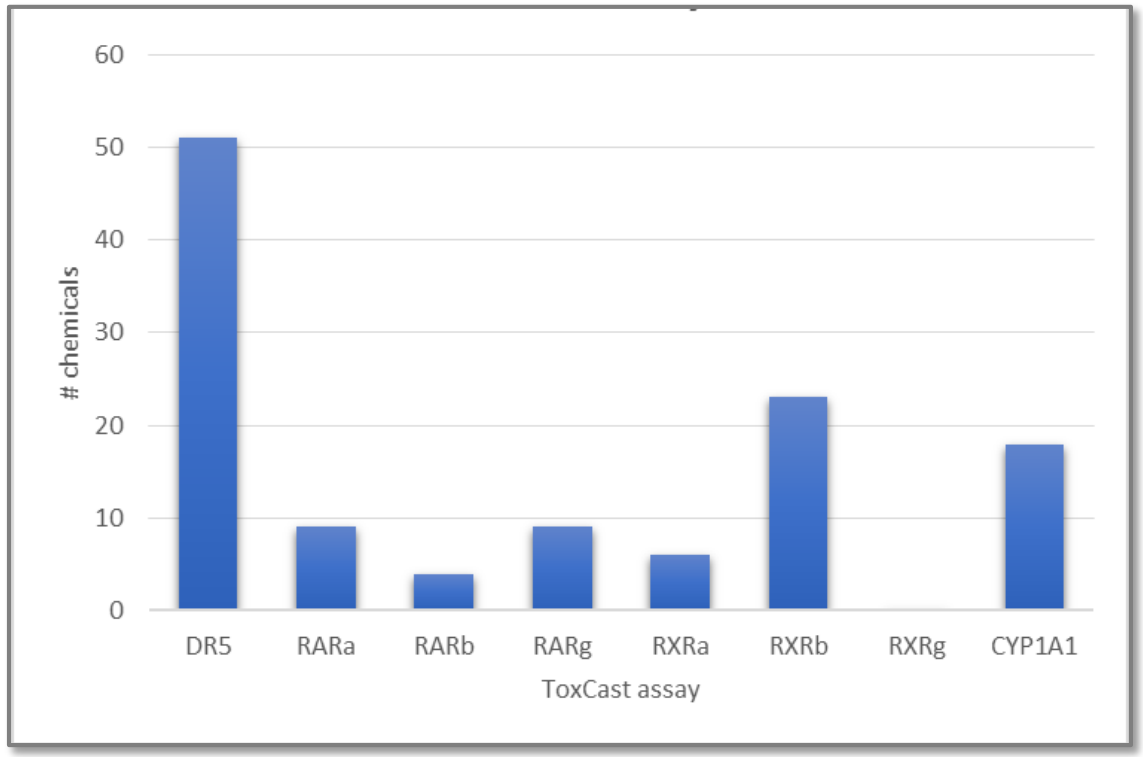
[b] National Center for Advancing Translational Sciences, National Institutes of Health, Bethesda, MD 20892, United States

- NCATS established the C3RL4 reporter gene cell line which contains a functional retinoid signaling pathway;
- firefly luciferase reporter gene (Luc) engineered under control of RARE;
- screened a library of 1280 pharmacologically active compounds in both agonist and antagonist modes;
- have run the Tox21 library (some of that data in Wei et al. 2019, in press).

ToxCast HTS profile



Chemicals ranked by potency	ATG_DR5_CIS_up	ATG_RARa_TRANS_up	ATG_RARb_TRANS_up	ATG_RARg_TRANS_up	ATG_RXRa_TRANS_up	ATG_RXRb_TRANS_up	NVS_ADME_hCYP1A1	NVS_ADME_rCYP1A1	NVS_NR_hRAR_Antagonist	NVS_NR_hRARa_Agonist
trans-Retinoic acid	0.0063	0.0004			0.0003	1.0400	1.3200			0.9430
Retinol	0.1470	0.0690		0.2080	1.5400	0.4730				
Tributyltin benzoate	0.0227				0.0053	0.0364				
Tributyltin methacrylate	0.0055				0.1470	0.0250				
Tributyltin chloride	0.0028				0.1760	0.0777				
Tetrabutyltin	0.2790				0.7410	0.0333				
2,4,6-Tris(tert-butyl)phenol	1.8300				0.4770	0.1850				
Pyraclostrobin	0.5420	0.7790					1.6800			
Dieldrin	0.5790	0.7700		1.6800						
Endrin	0.8060		1.6100	1.7000						
SR271425	0.8340					0.7600				
Triflumizole	0.2980	1.4500								
2,6-Di-tert-butyl-4-methoxyphenol		1.0700				0.7000				
Coumaphos	1.6000						0.2740			
CP-532623	0.5030	1.5000								
Imazalil		0.9080					1.4100			
Prochloraz							0.4130	1.9300		
Endosulfan I	1.8300	1.3800								

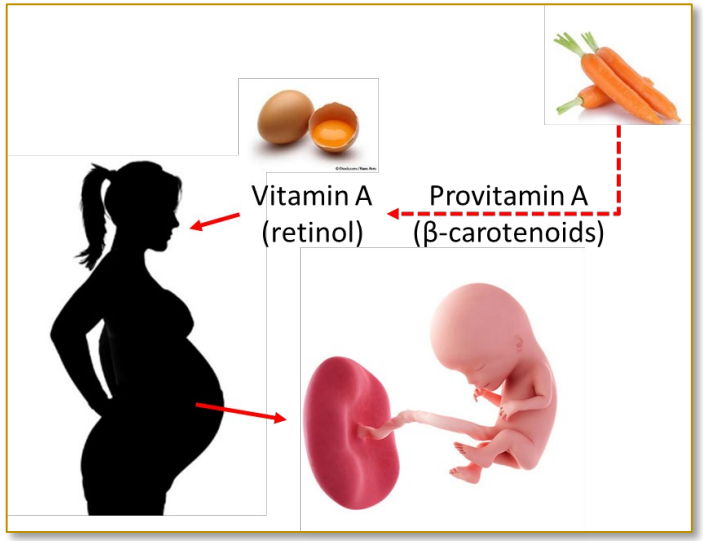
- 97 of 1858 chemicals (5.2%) registered an AC50 $\leq$ 2 µM in one or more relevant assays
- some persistent organic pollutants preferentially activated RARs (eg, aldrin, dieldrin, endrin, endosulfan)
- some tert-butyl compounds and organotins preferentially activated RXRs (eg, butylphenol, tributyltin)
- in addition to above, some mitochondrial disrupters displayed activity on DR5 (e.g., strobins, rotenone)
- others: retinoids (atRA, retinol), anticonvulsants (VPA), triazoles (fluconazole), flame retardants (PBDEs), …

SOURCE: Baker et al. 2018, Birth Def Res

Pregnancy dosimetry

- To learn the HTTK model, case study was applied to 7 retinoid analogs to determine real-world exposure scenario for respective T.I.s reported in the iPSC DevTox^{qP} assay by Palmer et al. 2017.

- Such analysis underway for the Tox21_VPA & Tox21_NTP iPSC series, linking the T.I. concentration to maternal dosimetry reflective of exposure during the critical developmental toxicity window.



Retinoid analogs	DevTox potential (TI) in µM [Palmer et al. 2017]	HTTK predicted external dose (mg/kg/day)
Retinol	191.536	4.05E+01
Etretinate	1.694	9.59E-02
13-cis-RA	0.065	6.34E-03
9-cis-RA	0.036	3.51E-03
ATRA	0.019	2.20E-03
TTNPB	0.062	4.31E-04
Acitretin	ND*	-

Lumen A, Chang X, Xia M, Zurlinden TJ, Knudsen TB, and Kleinstreuer NC. In Vitro Stem-Cell Based Developmental Toxicity Testing and In Vivo Dose Extrapolation (abstract in preparation for SOT 2020).

Summary: *scoping the system for predictive toxicology toward developing guideline testing strategies or IATA/Defined Approaches …*

- Although skeletal defects are common outcomes in experimental and clinical retinoid perturbation, the sensitive biology is on early patterning of the body plan.
- HTS profiling (ToxCast) identifies aspects of the RA pathway in statistical correlation models associating *in vitro* bioactivity with skeletal defects.
- Mechanistic data and information for chemicals that interact with retinoid pathway targets can be framed into various AOPs.
- Computational intelligence may help isolate human-relevant inputs and downstream events during uncontrolled RA signaling invoked with chemical exposure.