*Pharmaceutical and BioScience Society (PBSS)*
*Reproductive and Juvenile Toxicity Workshop (virtual)*
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# Translatability of Cell-Based and *In Silico* Models of Developmental Toxicity

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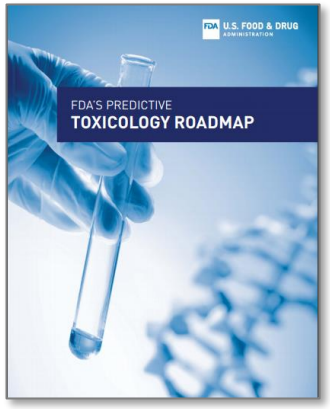
*DISCLAIMER: The views expressed are those of the presenters and do not reflect Agency policy.*

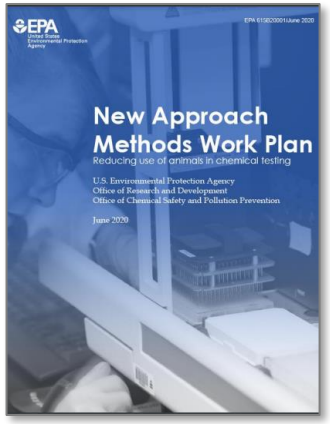
# Summary

- High-throughput screening (HTS) assays with pluripotent stem cells (PSCs) offer a promising alternative to pregnant animals for assessing developmental toxicity.
- Their unique properties help them to be excellent models of the developing embryo during stages when the basic body plan is ‘decoded’ from the genomic blueprint.
- Many studies have shown predictive value for developmental toxicity; however, descaling the human embryo to hPSCs can be blind to systems reconstitution.
- Engineered microsystems and integrative computational models that recapitulate the full complementation of an embryo will be needed for translatability.

# Regulatory drivers

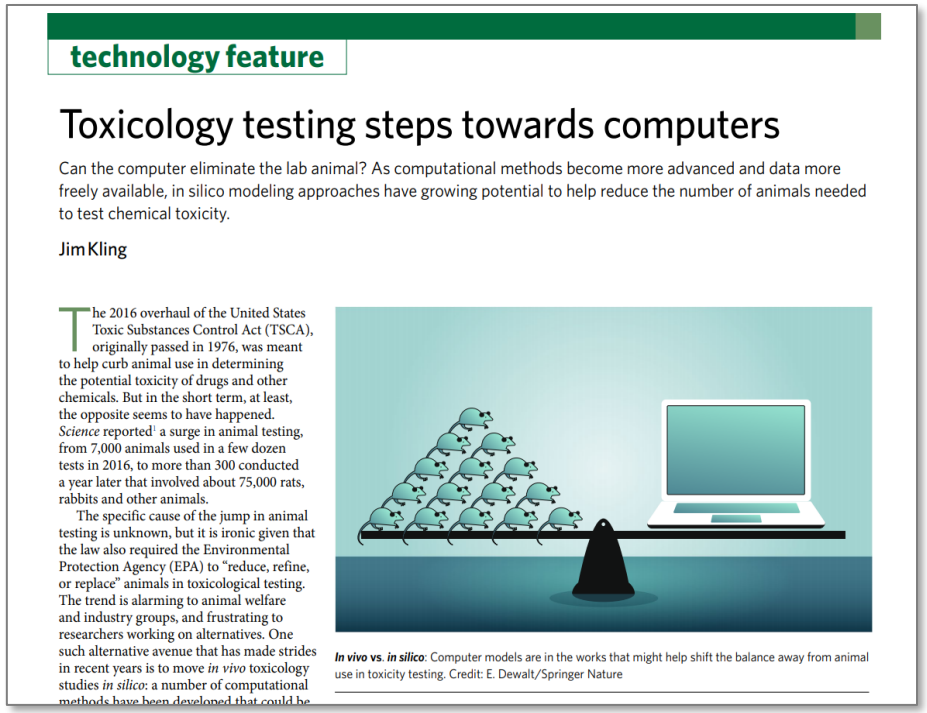



**FDA's Predictive Toxicology Roadmap** - created to identify the toxicology areas that could benefit from improved predictivity as well as promising new technologies that could potentially meet these needs and support animal 3Rs (Replacement, Reduction, and Refinement).
**https://www.fda.gov**




**EPA's New Approach Methods Work Plan** - created to prioritize agency efforts and resources toward activities that aim to reduce the use of animal testing while continuing to protect human health and the environment (NAMs).
**https://www.epa.gov**

# Can the computer replace lab animal testing?



technology feature

## Toxicology testing steps towards computers

Can the computer eliminate the lab animal? As computational methods become more advanced and data more freely available, in silico modeling approaches have growing potential to help reduce the number of animals needed to test chemical toxicity.

Jim Kling

The 2016 overhaul of the United States Toxic Substances Control Act (TSCA), originally passed in 1976, was meant to help curb animal use in determining the potential toxicity of drugs and other chemicals. But in the short term, at least, the opposite seems to have happened. *Science* reported[1] a surge in animal testing, from 7,000 animals used in a few dozen tests in 2016, to more than 300 conducted a year later that involved about 75,000 rats, rabbits and other animals.

The specific cause of the jump in animal testing is unknown, but it is ironic given that the law also required the Environmental Protection Agency (EPA) to "reduce, refine, or replace" animals in toxicological testing. The trend is alarming to animal welfare and industry groups, and frustrating to researchers working on alternatives. One such alternative avenue that has made strides in recent years is to move *in vivo* toxicology studies *in silico*: a number of computational methods have been developed that could be



*Kling (2019) Nature Lab Animal 48: 40-42*

- **mapping the (chemical) world:** structural alerts based on black-box read-across [Structure-Activity Relationships].
  - *Luechtefeld et al. (2018) Toxicol Sci 165: 198-212.*
- **opening the black box:** data-driven machine learning using high-throughput bioactivity datasets [ToxCast/Tox21].
  - *Ciallella and Zhu (2019) Chem Res Toxicol 32: 536-547.*
- **a step further:** engineered microsystems (*in vitro*) and dynamic simulation (*in silico*) [Virtual Tissue Models].
  - *Knudsen et al. (2021) Toxicol Sci 180: 198-211.*

**DART translation:** *in vitro* assays and *in silico* models that reflect embryo-fetal development and human pregnancy will be important for NAM-based evaluation of developmental hazard potential.

# Pluripotent stem cell (PSC) assays

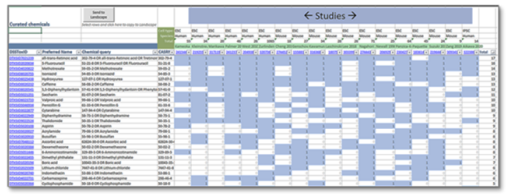
An active area of investigation and one of the most promising *in vitro* alternatives to pregnant animal testing for assessing developmental hazard potential; novel features:

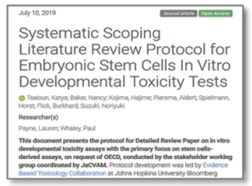
- **Self-renewal:** cells replicate themselves indefinitely when cultured under appropriate growth factor conditions.
- **Pluripotency:** cells have the potential to form most of the different cell types comprising the embryo-fetus.
- **Autopoiesis:** capacity to self-organize into rudimentary tissues and more complex organoid structures.

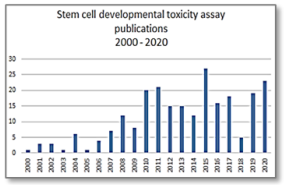
Established PSC lines can recapitulate **some** of the biology driving embryogenesis during the period covered by guideline prenatal studies (e.g., OECD TG 414, OPPTS 870.3700).

# PSC assays in developmental toxicity

**Detailed Review Paper (DRP) on EST platforms**






*Abstract Sifter, SWIFT, MeSH terms, Chemicals Dashboard, …*

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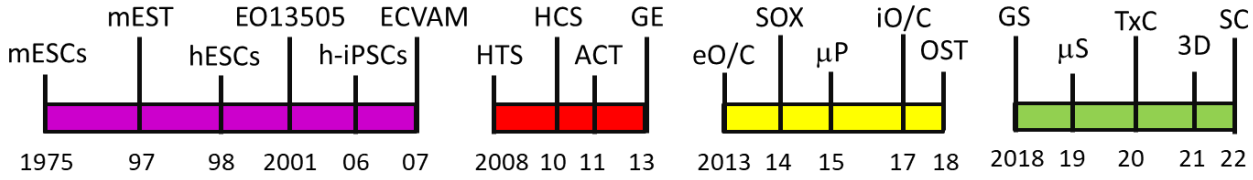



**1997-07 traditional mEST [accuracy ~80%]**
**2008-13 improve mEST sensitivity and scalability [accuracy 72-83%]**
**2013-18 targeted biomarker readouts in hPSCs [accuracy 77-87+%]**
**2018- hPSC biomimetics and throughput [accuracy 77-87+%]**

- **Strategy:** search for studies that used PSCs to classify developmental toxicants:
  - chemical and biological domains
  - standardized protocols, biomarkers, readouts
  - reproducibility and performance.

- **Corpus:** 1,533 PubMed records *(circa 1991-2021):*
  - reduced to 192 papers by AI and manual curation
  - 18 papers tested ≥ 10 compounds (primary)
  - 174 papers tested 1-9 (evidentiary support).

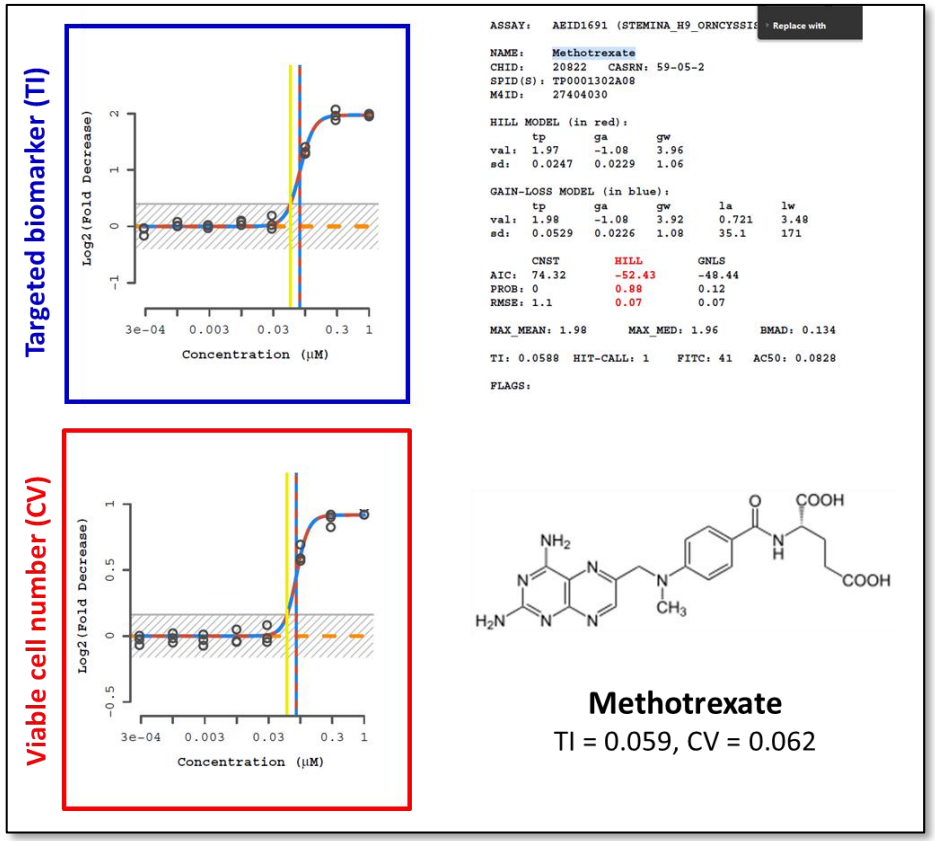
- **1,250 annotated chemicals:**
  - accuracies 72-87% for well-curated compounds
  - most commonly represented: ATRA, 5-FU, MTX.

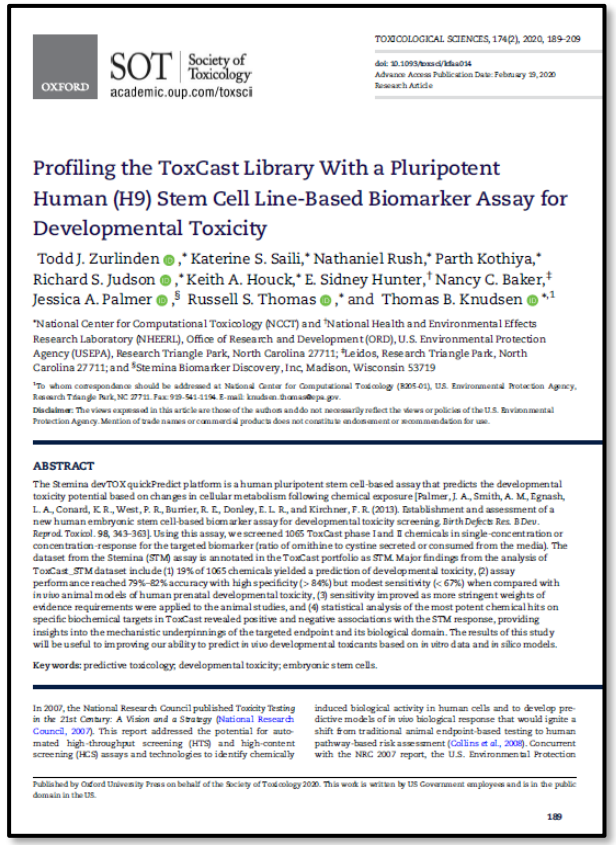
*Piersma et al., manuscript in final preparation*

# **ToxCast_STM:** *devTOX$^{qP}$ assay contracted from Stemina Biomarker Discovery*

- 1065 ToxCast Ph I/II chemicals at single-conc. or multi-conc.;
- Data tcpl-pipelined into ToxCast database (now >1125 assays);
- Raw and pipelined data in EPA's CompTox Chemicals Dashboard.
- Bioactivity concentration predicting DevTox potential.

https://comptox.epa.gov/dashboard



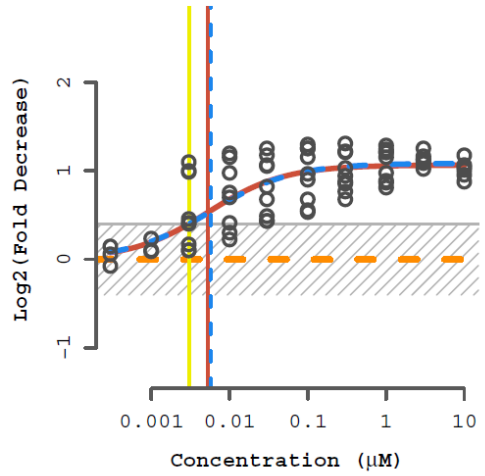




19.2% positivity rate indicative of teratogenic potential

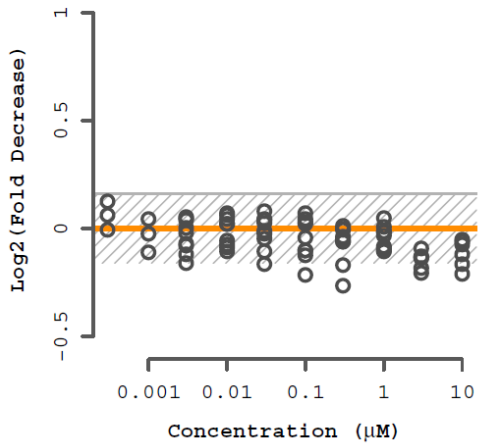
*Zurlinden et al. (2020) Toxicol Sci*

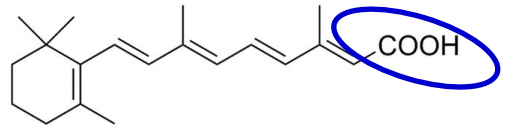
# **Example:** *vitamin-A and its morphogenetic metabolite (all-trans Retinoic acid)*

**Targeted biomarker (TI)**




**Viable cell number (CV)**



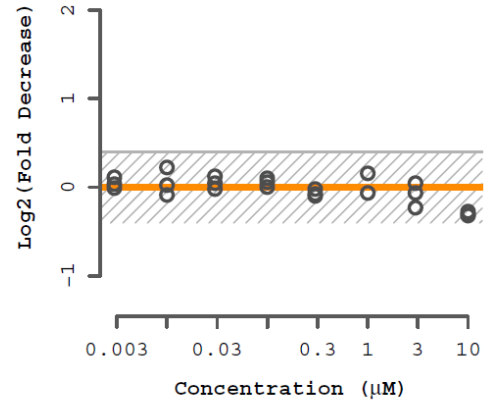



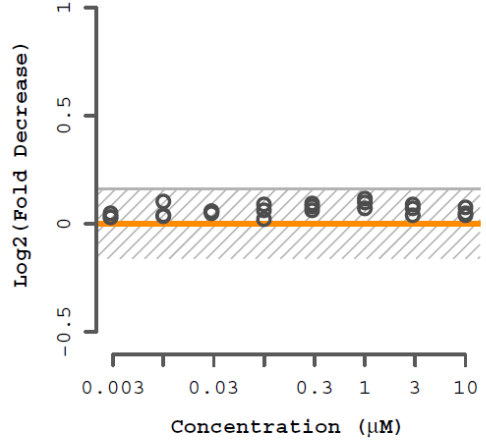

**all trans Retinoic acid (ATRA)**

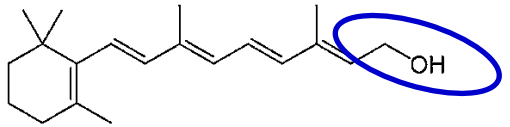
TI = 0.003 μM, CV = NA

dLEL rat = 2.5 mg/kg/day

dLEL rabbit = 0.5 mg/kg/day



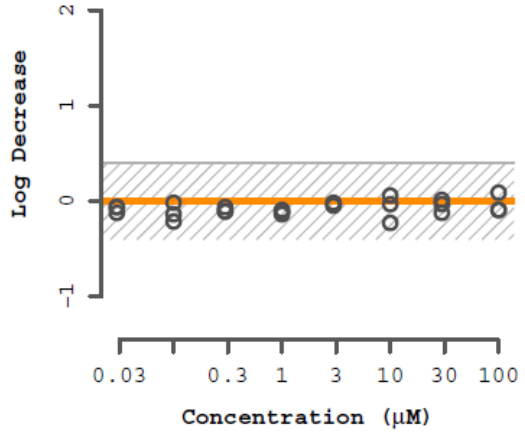

**Retinol (vitamin-A)**

TI = NA, CV = NA

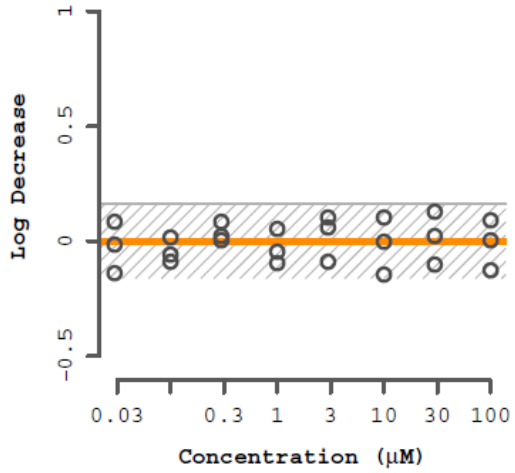
(True Negative)

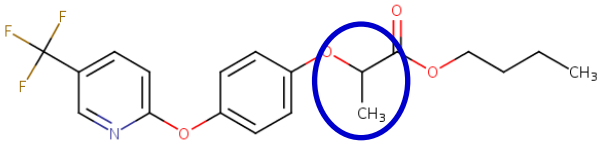
# **Example:** *R-enantiomer (Fluazifop-P-butyl) is the active herbicide*

**Targeted biomarker (TI)**




**Viable cell number (CV)**



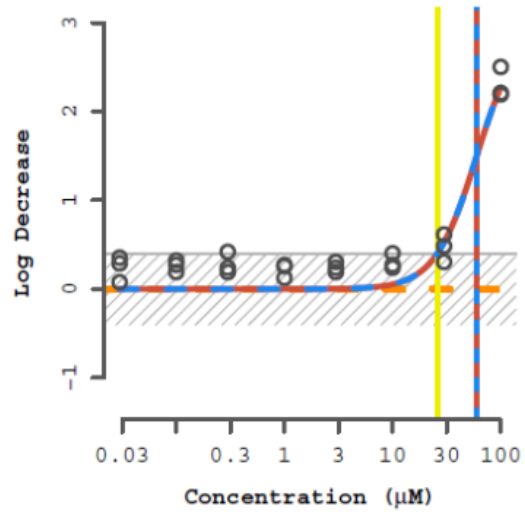



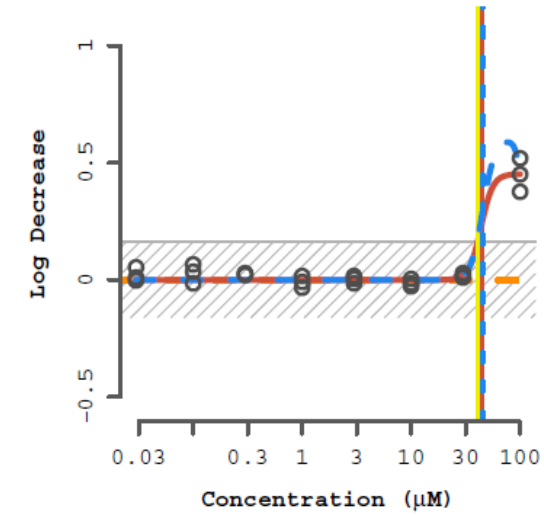

**Fluazifop butyl**

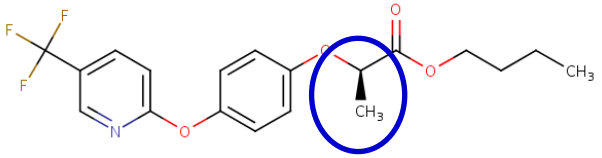
TI = not active, CV = no effect

dLEL rat = 10 mg/kg/day (< mLEL)

dLEL rabbit = 90 mg/kg/day (mLEL)




**Fluazifop-P-butyl**

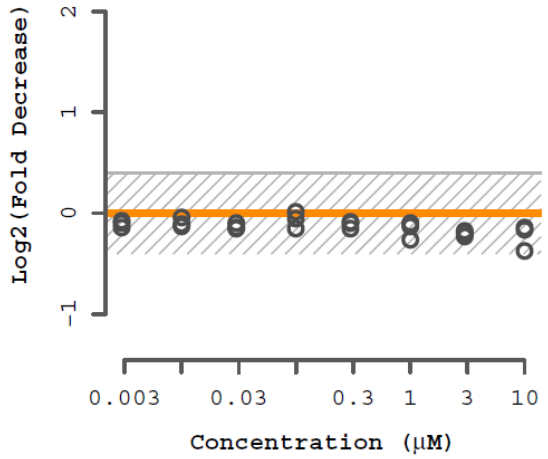
TI = 26 µM, CV = 40.8 µM

dLEL rat = 5 mg/kg/day (< mLEL)

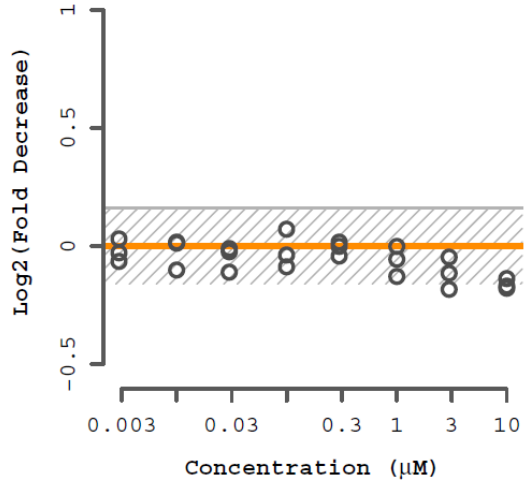
dLEL rabbit = 50 mg/kg/day (mLEL)

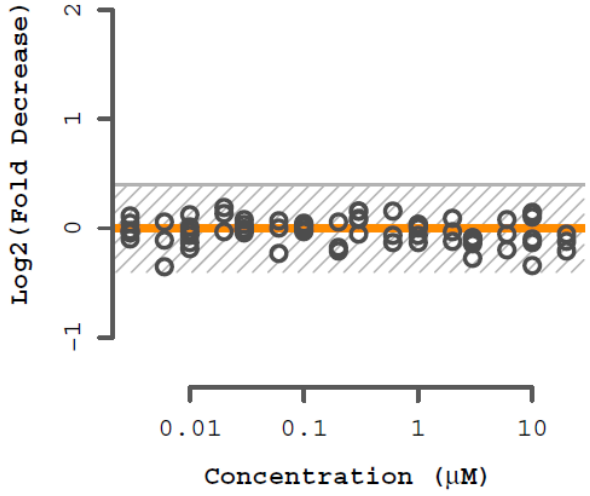
# **Example:** *false negatives (not detected in ToxCast_STM)*

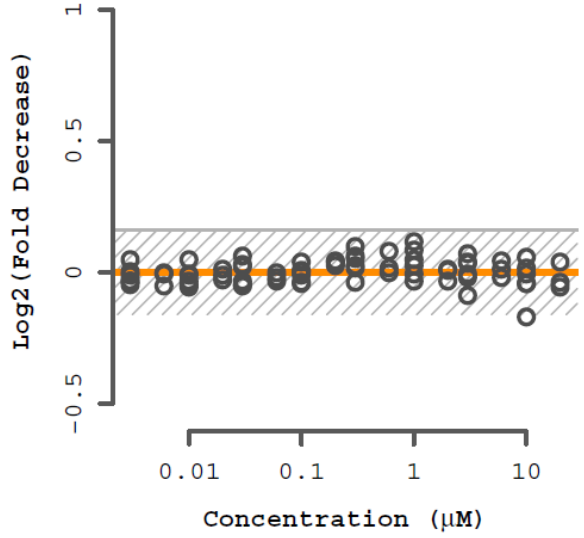
**Targeted biomarker (TI)**

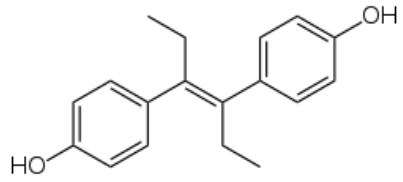



**Viable cell number (CV)**



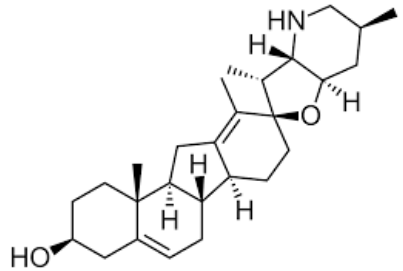




**Diethylstilbestrol (DES)**

TI = NA, CV = NA

dLEL rat = 0.03 mg/kg/day (= mLEL)

(no rabbit data in ToxRefDB)

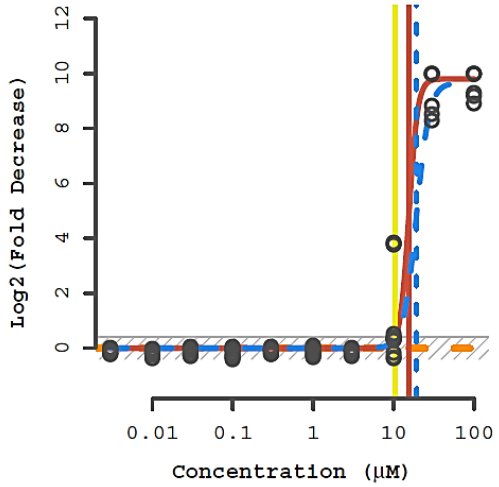



**Cyclopamine**

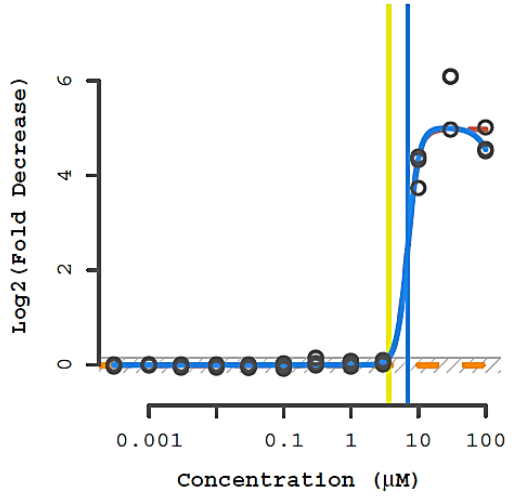
TI = NA, CV = NA

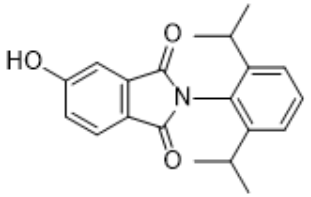
# **Example:** *pharmacological angiogenesis inhibitors*

**Targeted biomarker (TI)**




**Viable cell number (CV)**



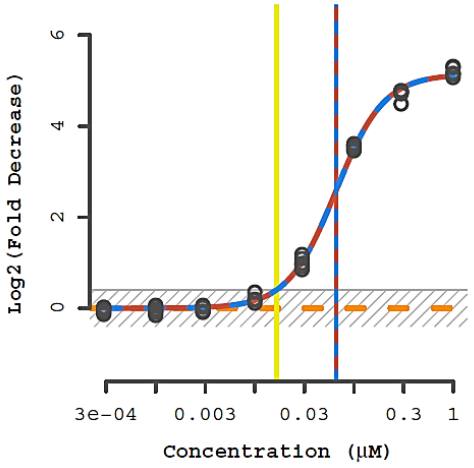



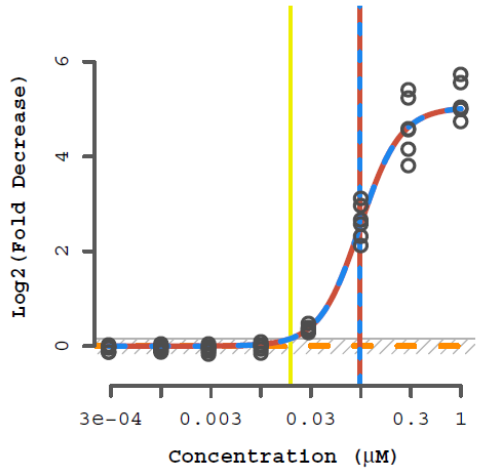

*synthetic thalidomide analog*

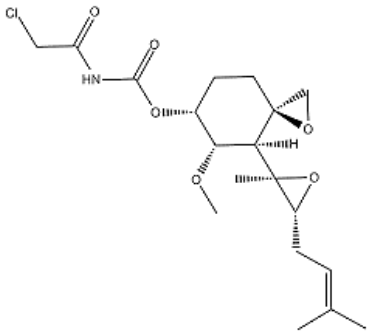
**5HPP-33**

TI = 10.5, CV = 16.4

(no rat or rabbit data)




*synthetic fumagillin analog*

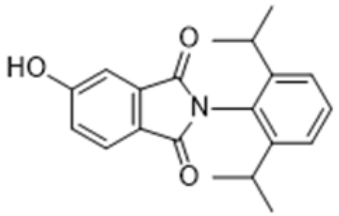
**TNP-470**

TI = 0.017, CV = 0.020

(no rat or rabbit data)

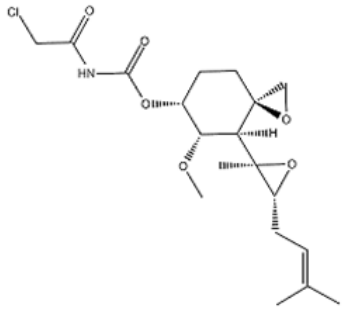
# **Quantitative prediction:** *checking forward predictivity of the hPSC readout*

Colleagues at Dow Chemical, led by Ed Carney, tested T.I. predictions for two structurally diverse potential vascular disrupters (pVDCs) in rat whole embryo culture (WEC):




**5HPP-33:** *synthetic thalidomide analog*

- T.I. predicted by hESC 10.5 µM
- AC50 observed in WEC 21.2 µM (embryo viability)



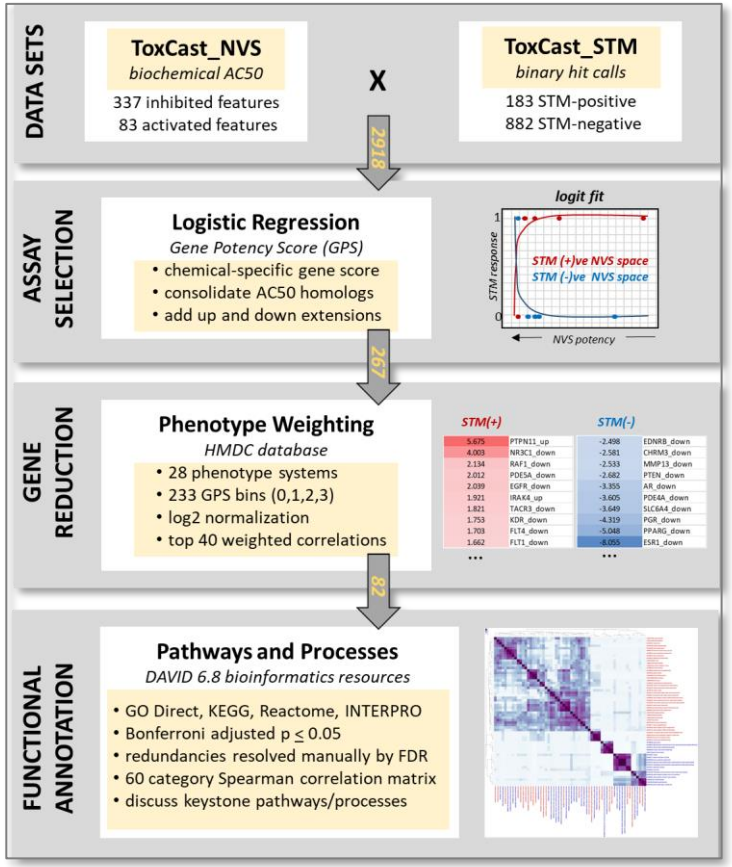

**TNP-470:** *synthetic fumagillin analog*

- T.I. predicted by hESC 0.02 µM
- AC50 observed in WEC 0.04 µM (dysmorphogenesis)

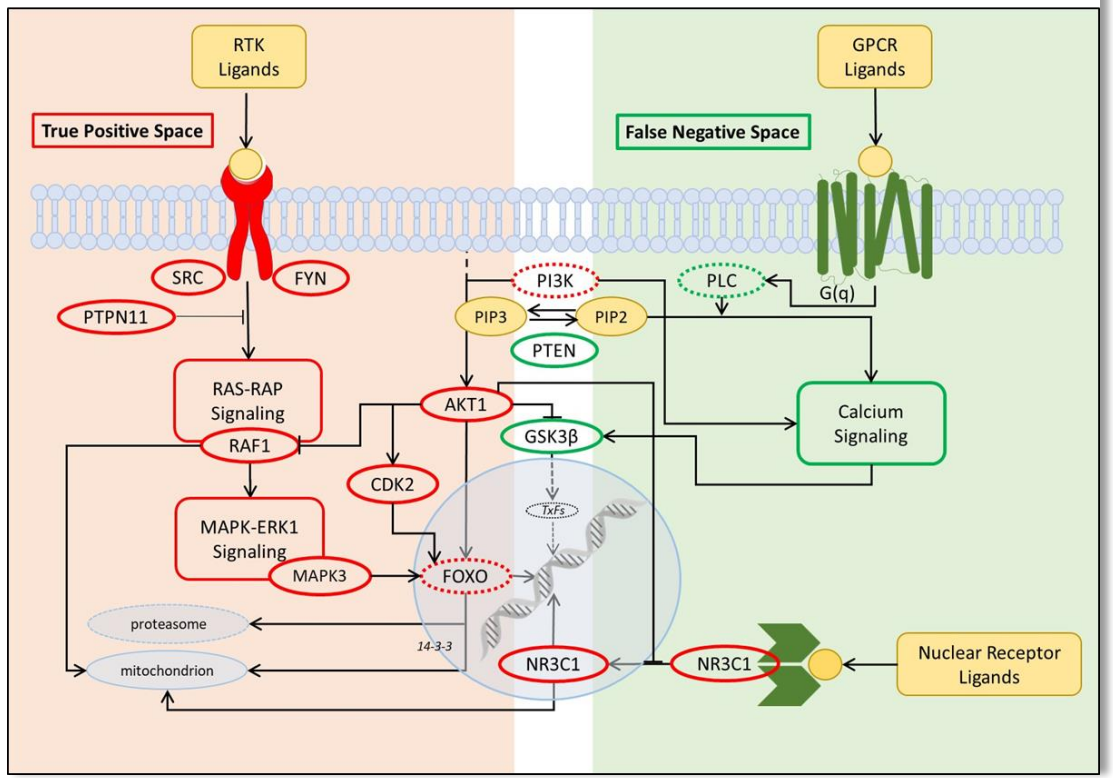
*Ellis-Hutchings et al. (2017) Reprod Toxicol*

# What human relevant pathways are detected or missed?

*Workflow to mine hPSC bioactivity against 337 cell-free biochemical assays (ToxCast_NVS)*




| Annotation System | Keystone Pathway / Process | # MIEs | Class |
|---|---|---|---|
| GOTERM_BP_DIRECT | GO:0014066~regulation of phosphatidylinositol 3-kinase signaling | 6 | TP |
| KEGG_PATHWAY | hsa04068:FoxO signaling pathway | 8 | TP |
| KEGG_PATHWAY | hsa04510:Focal adhesion | 13 | TP |
| GOTERM_BP_DIRECT | GO:0007200~phospholipase C-activating G-protein coupled receptor signaling pathway | 10 | FN |
| INTERPRO | IPR001723:Steroid hormone receptor | 7 | FN |
| GOTERM_MF_DIRECT | GO:0005496~steroid binding | 5 | FN |




**Sensitive Domain** **Insensitive Domain**

*Zurlinden et al. (2020) Toxicol Sci*

# Performance check for hPSC-based classification of DevTox

- Qualification on 42 well-curated reference compounds often used to validate alternative DevTox platforms[1].
- Balanced Accuracy (BAC) = 82% (0.65 sensitivity, 1.00 specificity) for these reference chemicals.
- Metrics are consistent with the original pharma-trained model [Palmer et al. 2013].

Many PSC studies have been validated with a limited set of data-rich chemicals, inflating predictive accuracy >80%.

ATRA was most potent across 1065 compounds tested.

| CASRN | Chemical | CV (µM) | TI (µM | | |
|---|---|---|---|---|---|
| 302-79-4 | all-trans-Retinoic acid | NA | 2.00 | | |
| 69-74-9 | Cytarabine hydrochloride | 0.083 | 0.05 | | |
| 59-05-2 | Methotrexate | 0.062 | 0.05 | | |
| 147-24-0 | Diphenhydramine hydr | 3.76 | 0.58 | | |
| 50-35-1 | Thalidomide | NA | 1.2 | | |
| 51-21-8 | 5-Fluorouracil | 1.45 | 2.02 | D | TP |
| 298-46-4 | Carbamazepine | NA | 2.29 | C | TP |
| 55-98-1 | Busulfan | 4.91 | 2.31 | D | TP |
| 13292-46-1 | Rifampicin | NA | 2.46 | C | TP |
| 19774-82-4 | Amiodarone hydrochlor | NA | 5.1 | D | TP |
| 75330-75-5 | Lovastatin | NA | 5.1 | X | TP |
| 3056-17-5 | Stavudine | NA | 32.5 | C | TP |
| 2392-39-4 | Dexamethasone sodiur | 21.8 | 37.7 | C | TP |
| 53-86-1 | Indomethacin | 44.1 | 72.7 | D | TP |
| 127-07-1 | Hydroxyurea | 237 | 74.9 | D | TP |
| 99-66-1 | Valproic acid | 271 | 155 | D | TP |
| 4376-20-9 | MEHP | NA | 167 | D | TP |
| 57-41-0 | 5,5-Diphenylhydantoin | NA | NA | D | FN |
| 51-52-5 | 6-Propyl-2-thiouracil | NA | NA | D | FN |
| 10043-35-3 | Boric acid | NA | NA | NTP | FN |
| 4449-51-8 | Cyclopamine | NA | NA | D | FN |
| 6055-19-2 | Cyclophosphamide mor | NA* | NA | D | FN |
| 56-53-1 | Diethylstilbestrol | NA | NA | X | FN |
| 107-21-1 | Ethylene glycol | NA | NA | NTP | FN |
| 57-30-7 | Phenobarbitol sodium | NA* | NA | D | FN |
| 81-81-2 | Warfarin | NA | NA | X | FN |
| 69-72-7 | Salicylic acid | 1795 | 513 | C | TN |
| 103-90-2 | Acetaminophen | NA* | NA | B | TN |
| 79-06-1 | Acrylamide | NA | NA | NTP | TN |
| 50-78-2 | Aspirin | NA* | NA | C | TN |
| 80-05-7 | Bisphenol A | 39.4 | NA | NTP | TN |
| 94-26-8 | Butylparaben | NA | NA | GRAS | TN |
| 58-08-2 | Caffeine | NA | NA | B | TN |
| 464-49-3 | D-Camphor | NA | NA | C | TN |
| 131-11-3 | Dimethyl phthalate | NA | NA | NTP | TN |
| 59-30-3 | Folic acid | NA | NA | A | TN |
| 54-85-3 | Isoniazid | NA* | NA | C | TN |
| 57-55-6 | 1,2-Propylene glycol | 327552 | 246664 | NTP | TN |
| 68-26-8 | Retinol | NA | NA | A | TN |
| 81-07-2 | Saccharin | NA | NA | A | TN |
| 134-03-2 | Sodium L-ascorbate | NA* | NA | A | TN |
| 599-79-1 | Sulfasalazine | NA* | NA | B | TN |

True Positive

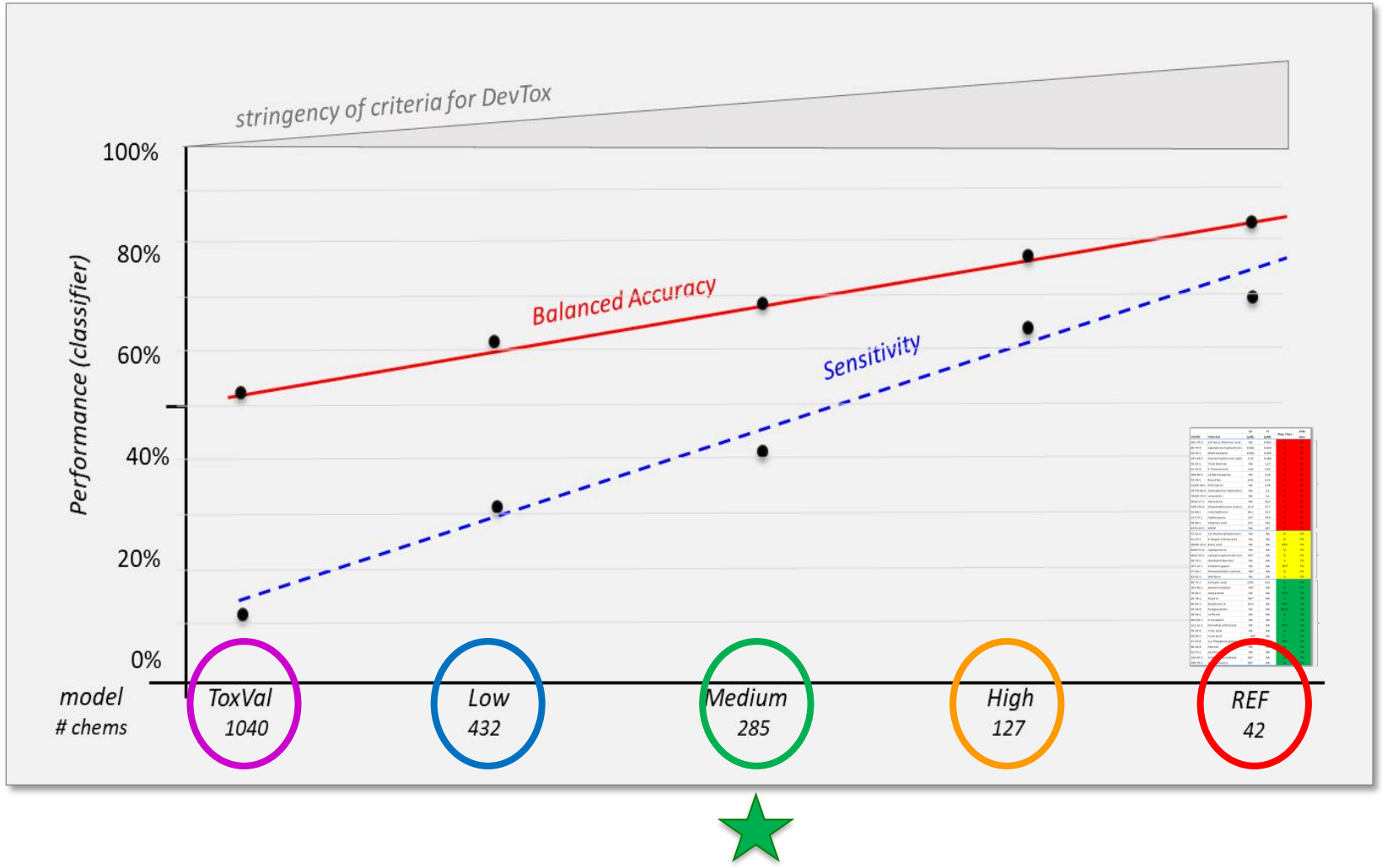
False Negative

True Negative

*Zurlinden et al. (2020), Toxicol Sci*

[1] Genschow et al. 2002; West et al. 2010; Daston et al. 2014; Augustine-Rauch et al. 2016; Wise et al. 2016

# Chemical landscape: *hPSC biomarker (in vitro) and ToxRefDB (in vivo)*



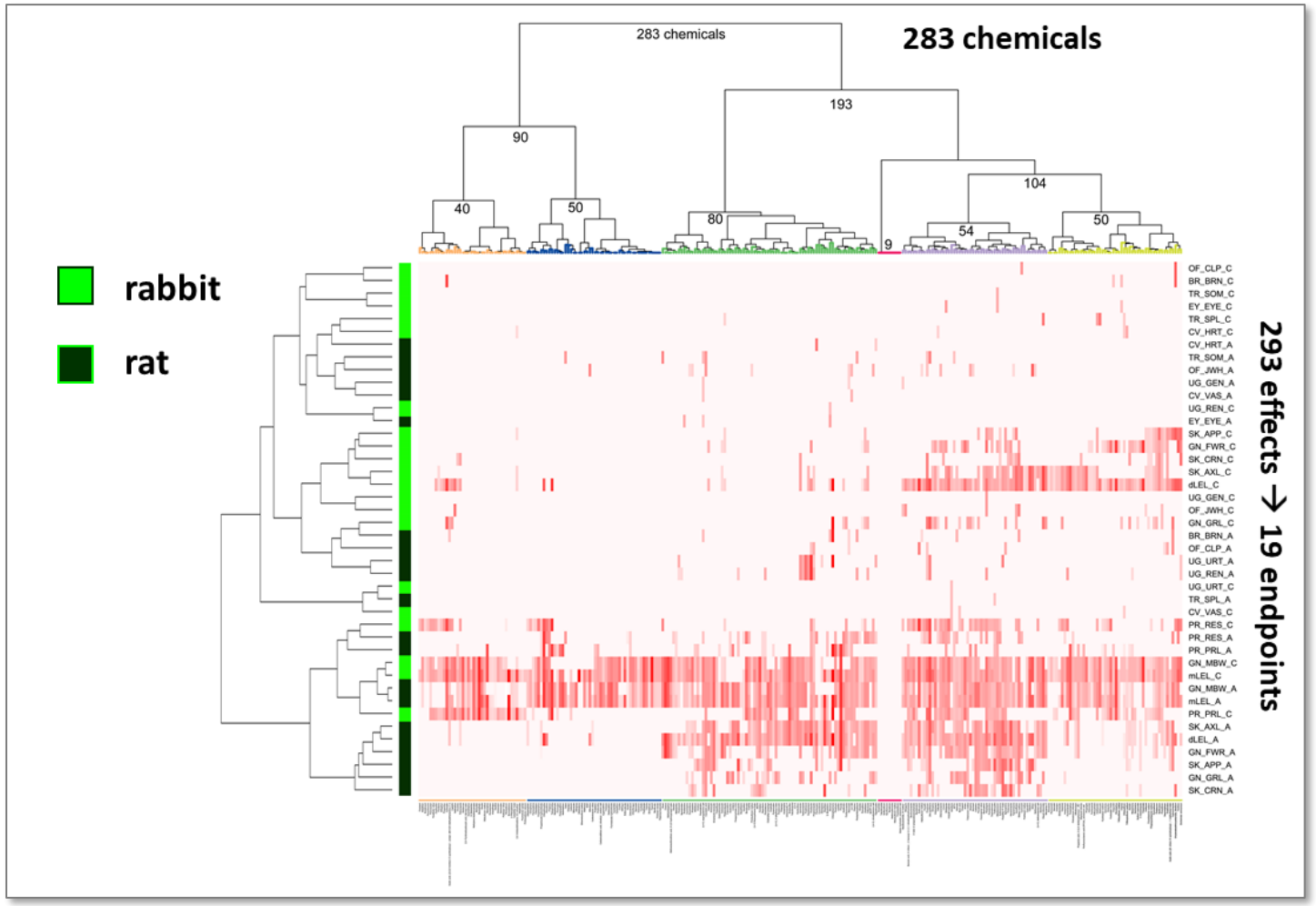

***Scaling Criteria (ToxRefDB)***

- **BM-42 reference**
- **concordant, rat AND rabbit**
- **dLEL < mLEL, rat OR rabbit**
- **dLEL ≤ 200 mg/kg/day**
- **LEL for any study type**

Although hPSC positivity rate (19.2%) was similar to concordant rat-rabbit studies (18.7%), only a subset was detected by both platforms.

*Zurlinden et al. (2020) Toxcol Sci*

# Prenatal developmental toxicity outcomes in ToxRefDB

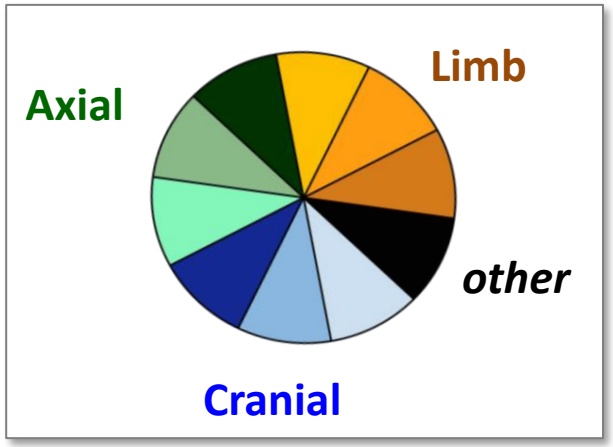


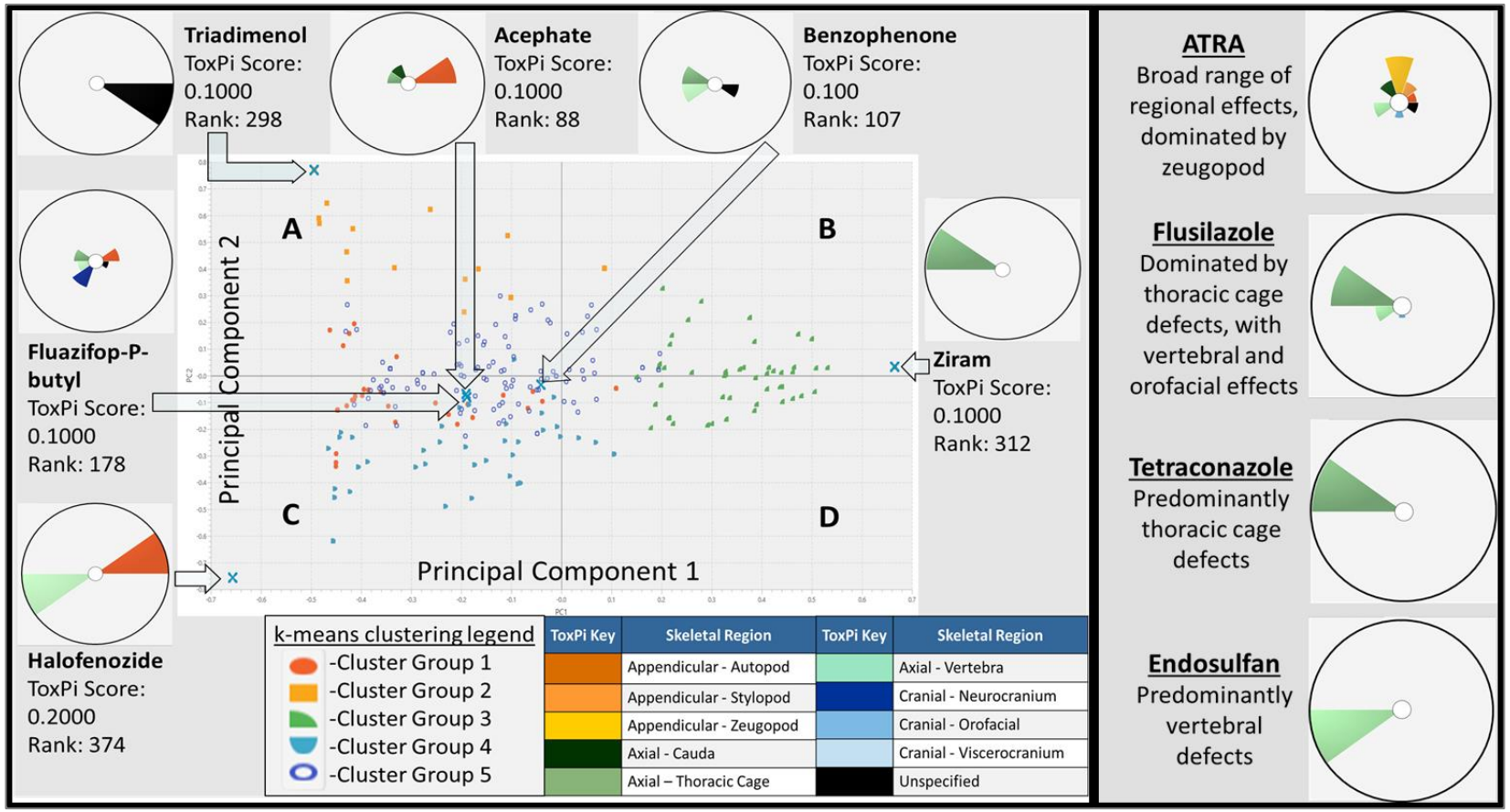

- Hierarchical clustering of 283 chemicals (*columns*) by 19 adverse outcomes (*rows*) observed in guideline rat and rabbit studies [*circa 2009*].
- Skeletal defects are among the most common adverse fetal outcomes in rat and rabbit studies.

*SOURCE: Knudsen et al. (2009) Reprod Toxicol*

# ToxRefDB chemicals (370) clustered by regional 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).



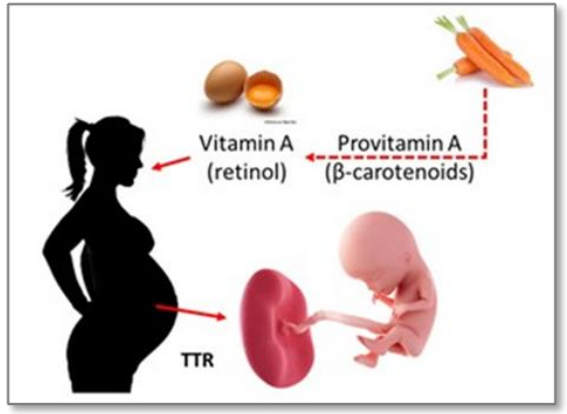



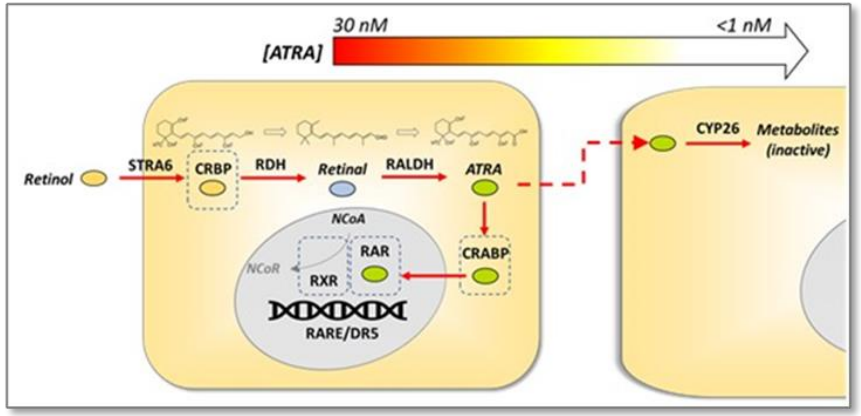

Are fetal skeletal phenotypes consistent with AOPs linked to disruption of the ATRA signaling pathway?

*SOURCE: Jocylin Pierro, work in progress*

# Case Study: *all-trans* retinoic acid (ATRA) pathway

The central role of ATRA in patterning and homeostasis of the skeleton makes it a logical choice for case studies in computational toxicology.

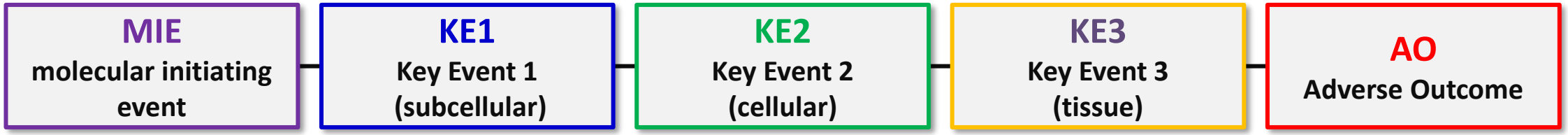



- ATRA gradients locally generated by cell-specific expression of enzymes, molecular transporters, and nuclear receptors (RARs) collaborate with powerful morphogenetic signals (e.g., FGF, BMP, SHH, WNT, …).

- Local regulation of ATRA homeostasis and its disruption may be captured in diverse Adverse Outcome Pathway (AOP) frameworks linking molecular initiating events (MIEs) to developmental phenotypes.

# Adverse Outcome Pathways (AOPs)




***Some AOPs for skeletal embryopathy linked to ATRA disruption***

| *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 |

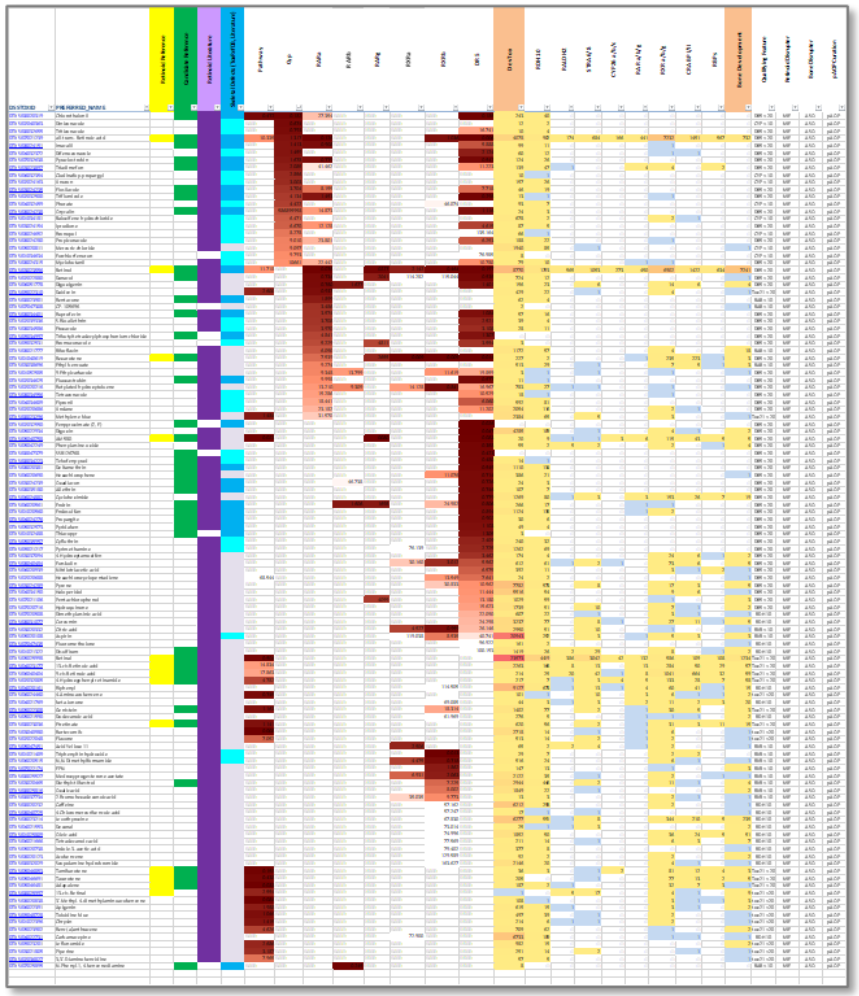
*Knudsen et al., Reprod Toxicol (2021)*

# *In vitro* profiling

| 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) |

Data available on 117 chemicals to model skeletal embryopathies linked to disruption of ATRA metabolism and signaling.

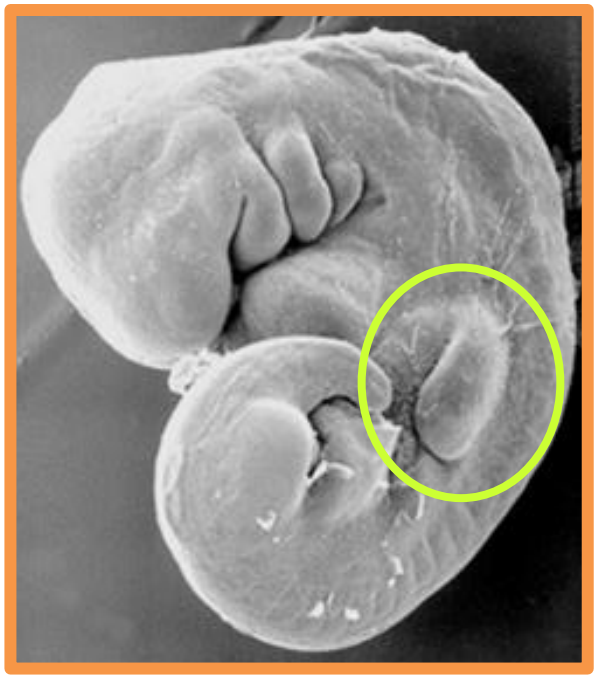
***Chemicals (n=117)*** ***In vitro biactivity (k=8 assays)*** ***Literature (k=8 assays)***



- ChEMBL data on 12 metabolic assays for drug-like compounds;
- ToxCast HTS data on 11 reporter assays for ~2K chemicals;
- Tox21 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.

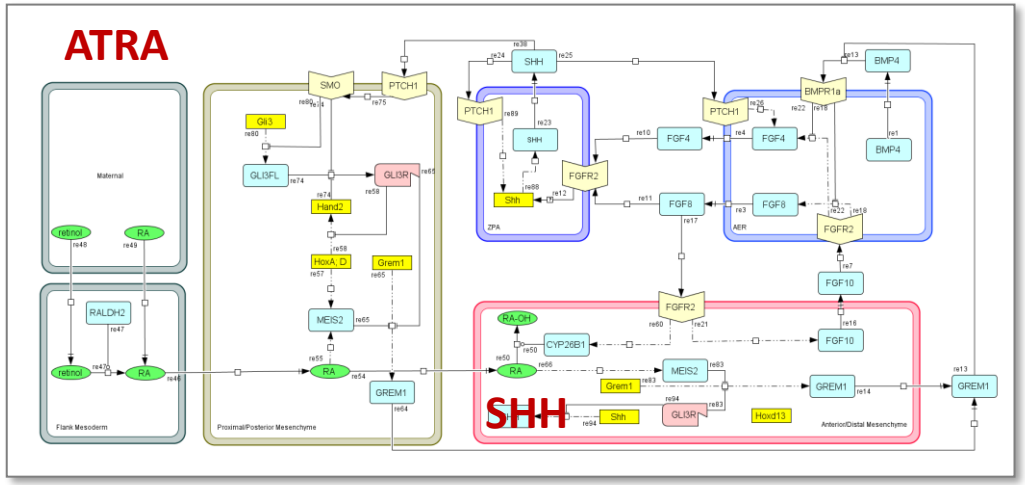
*Baker et al. (manuscript under technical review)*

# **Cybermorphs:** cellular ‘agents’ in a self-organizing system executed computationally into a spatially-perturbed morphology reconstructing effects of an ATRA overload.

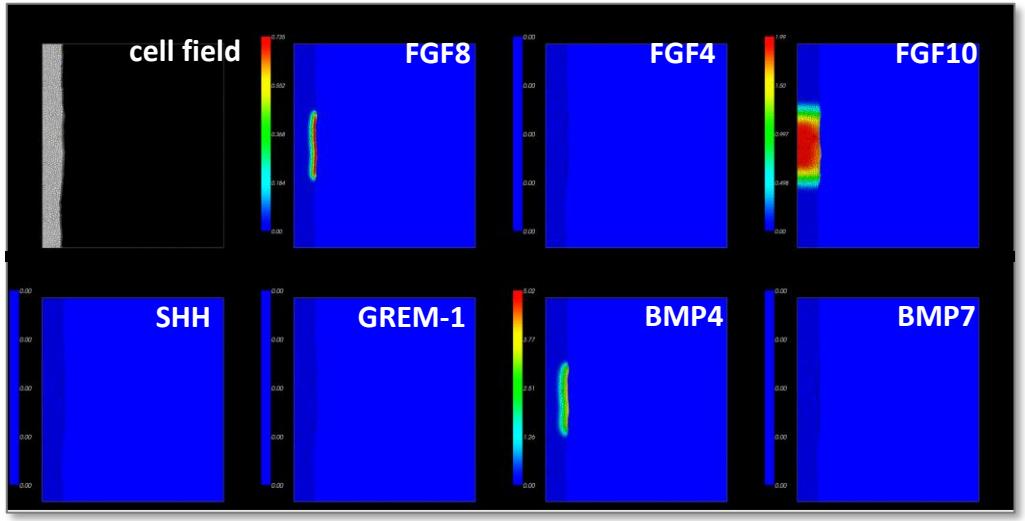


Early limb development
(~4-weeks gestation)

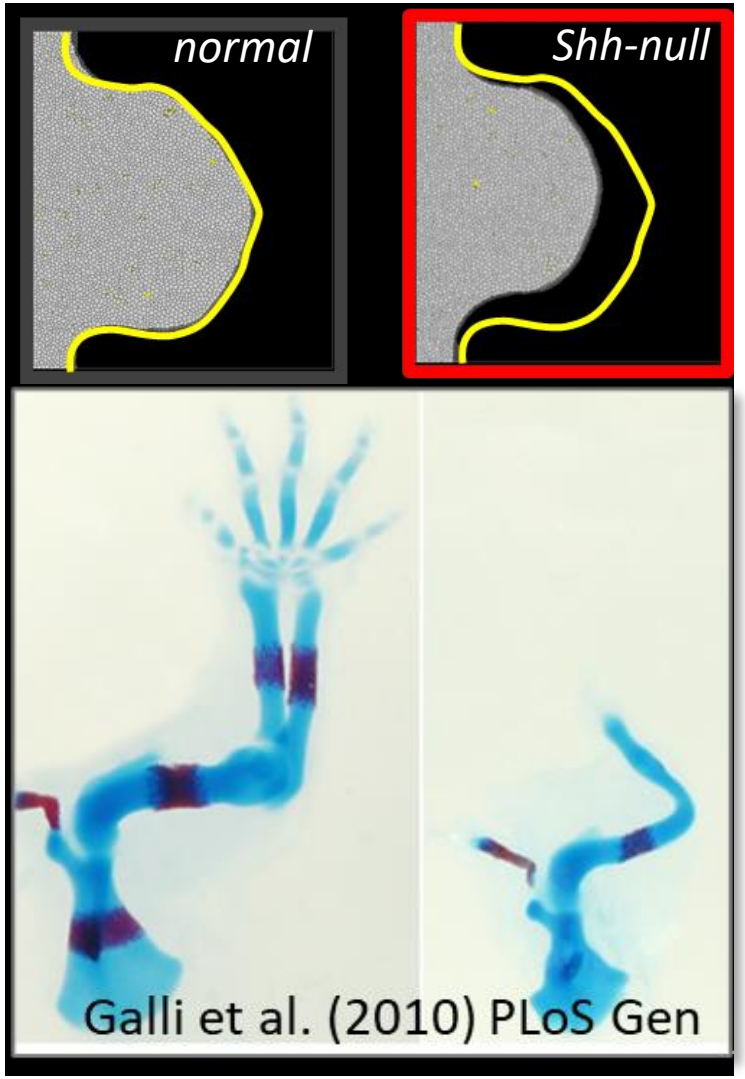
*Control system driving polarized outgrowth*



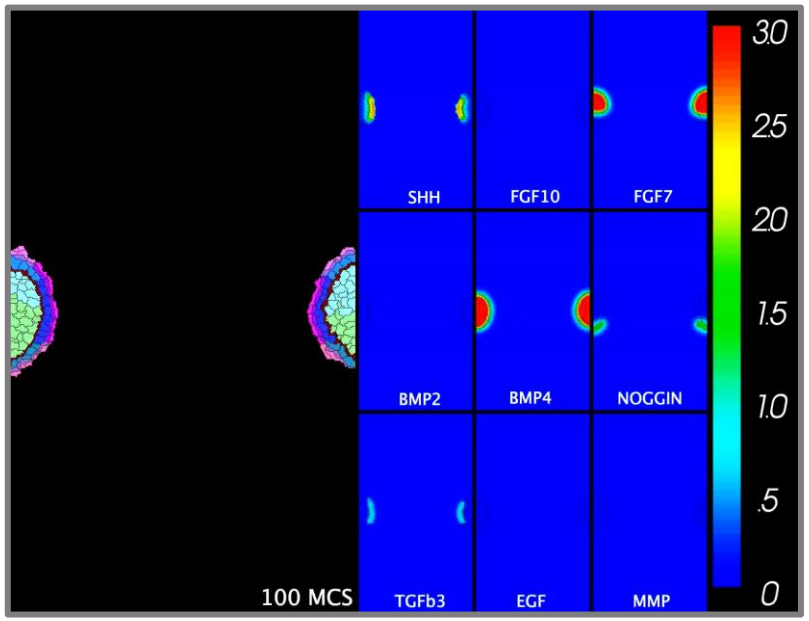

*Cell agent-based model (compucell3d.org)*




*Hacking the biology*

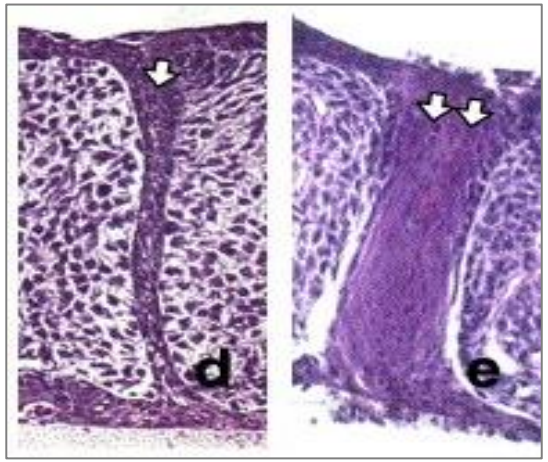


# **Smart models:** specifying the right biology enables real outcomes to self-organize.

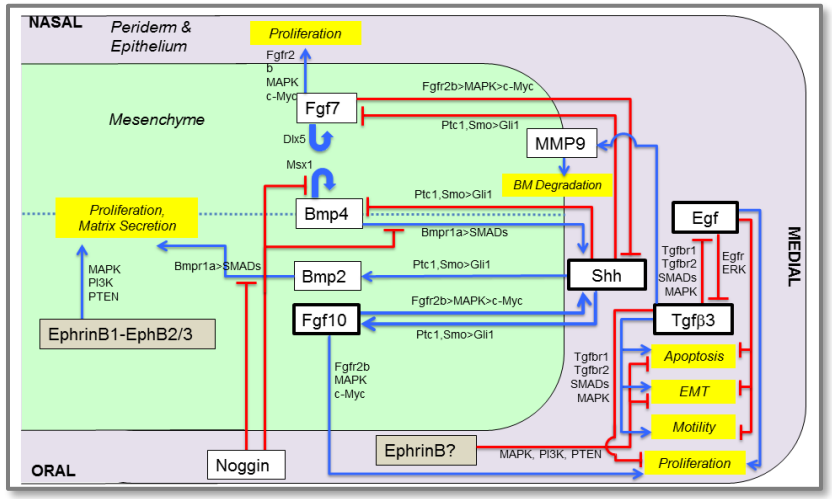



Reviewer Comment: "*Crucial mechanisms occurring during palate fusion, especially opposing palatal shelf adhesion, are not considered in the model. … Even in those strains in which palatal shelves adhere partially, I have never seen a MES as the one shown in Fig. 5.*"

Our Response: TGF-b3 knockout palates *in vitro (Dudas et al. 2004).*

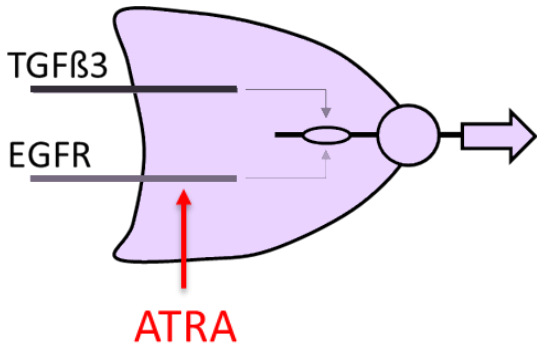


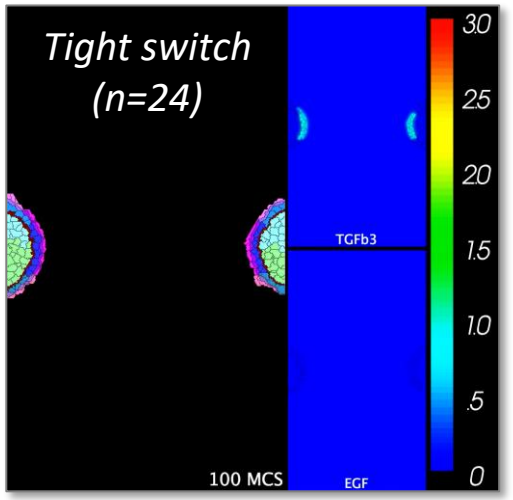


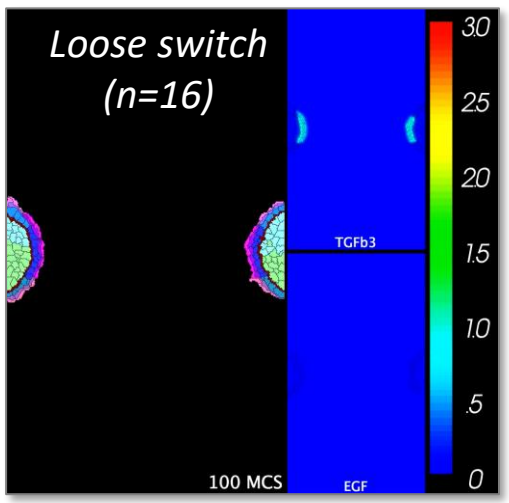



*Hutson et al. (2017) Chem Res Toxicol*

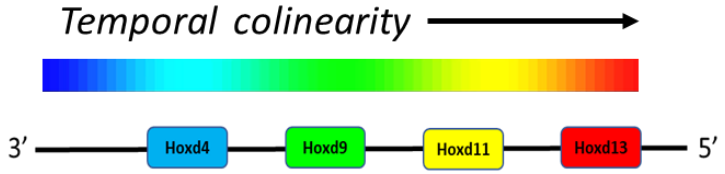
*Bistable MES Switch*

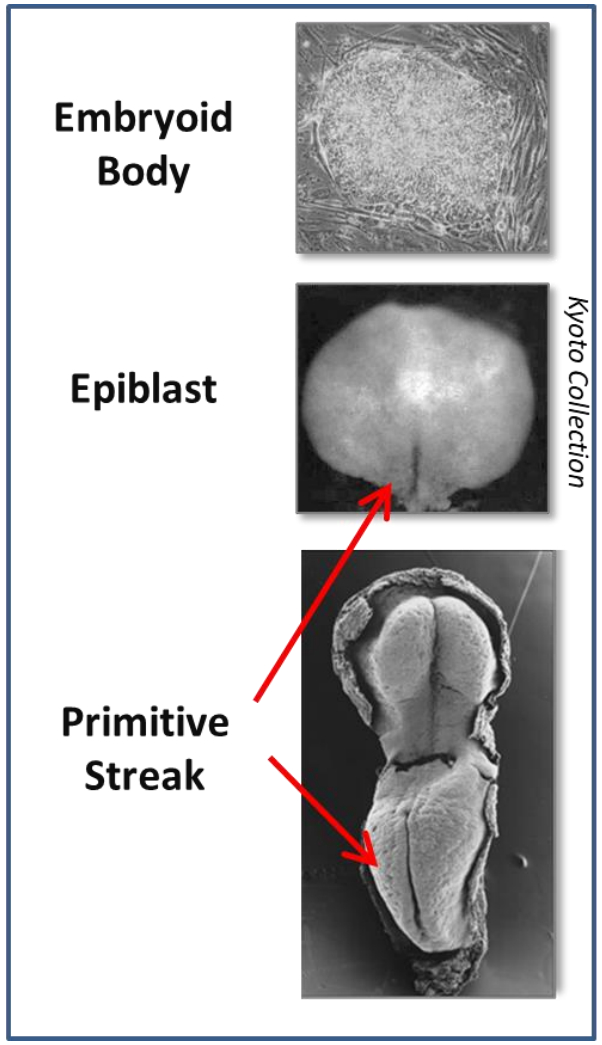


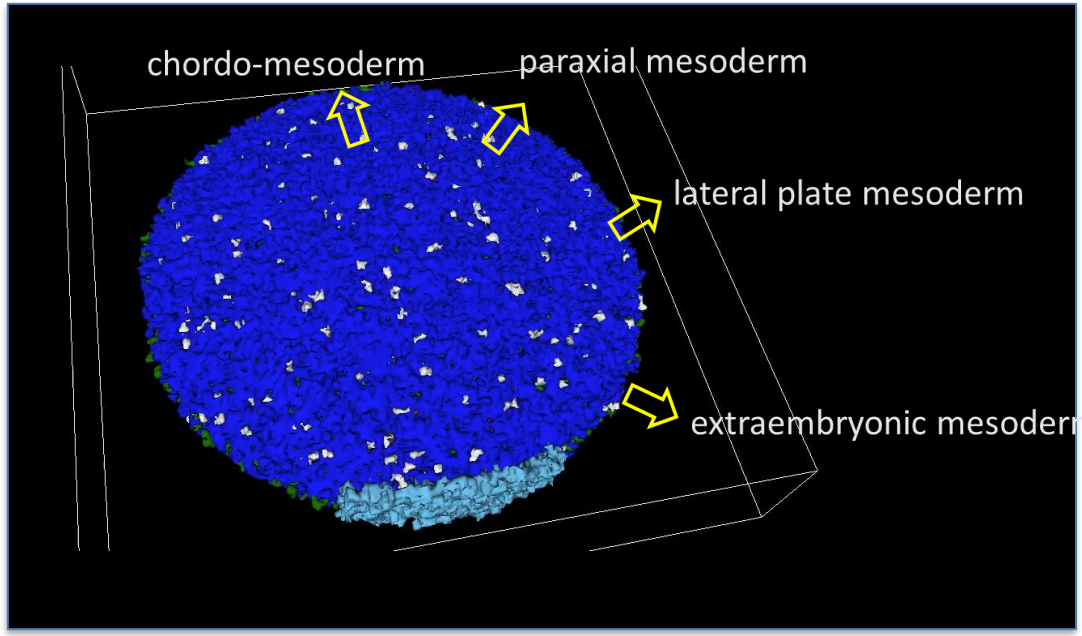



# **Patterning:** *computable emergence of regional mesoderm*




- Cultured hPSCs most closely represent the 'epiblast' of an early embryo during gastrulation (3rd week human), the hallmark of which is the primitive streak (PS).
- Cell migration through the PS is an early determinant of endomesodermal fate for '*decoding the genomic blueprint of the fetal body plan*'.




*Agent-Based Model of the epiblast built in CompuCell3D.org*

- Input parameters: dynamic signals (eg, FGF2), autonomous HOX clock.
- Stochastic determinants: cell position, timing of migration through PS.
- Emergent property: computable cell numbers for anatomical destiny.
- Editable features: kinematics of ATRA signaling, rate of HOX clock.

# Interactive Cinematic Scientific Visualization (iCSV)

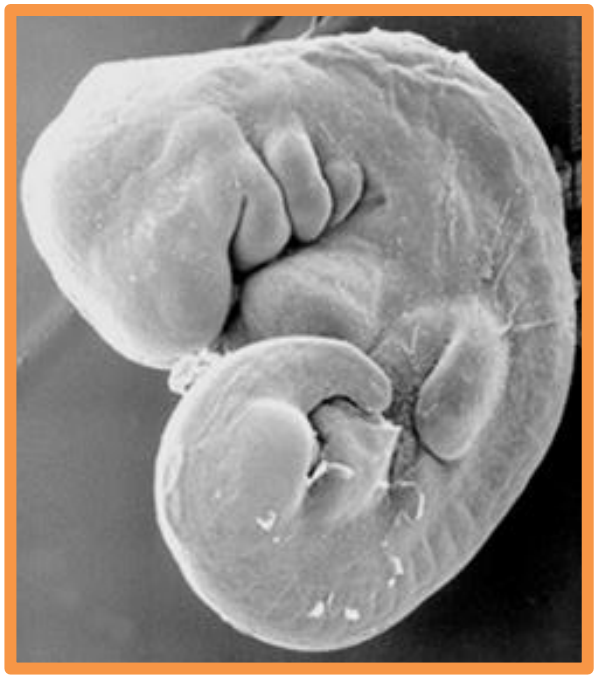
Presents scientific data in a way that is understandable and aesthetically pleasing, putting dynamical systems into motion (e.g., award-winning documentaries in planetary science).

*Same data displayed by traditional and CSV … capturing one theory on 'Birth of the Earth'*


*[video provided by KM Borkiewicz, National Center for Supercomputing Applications (NCAS), Univ. Illinois]*

Photorealistic animation can put AOPs into motion in time and space for simple, direct, and impactful translation of complex scientific data to a variety of audiences.

# Morphing NAMs data across levels of biological organization



- Vast collections of bioactivity data from *in vitro* chemical profiling are now in hand (https://comptox.epa.gov/dashboard).
- These complex datasets provide a new resource to examine key cellular and molecular determinants of developmental toxicity.
- However, virtual reconstitution of a self-organizing system from unidimensional data (embryogeny) remains a challenge.

A fully computable synthetic embryo ('synbryo') may be a distant goal, but cinematic representation of time-evolved scientific data is perhaps a new frontier.

# Acknowledgements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