Future of Reproductive and Developmental Toxicity Testing: computational and organoids

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American College of Toxicology and Teratology Society

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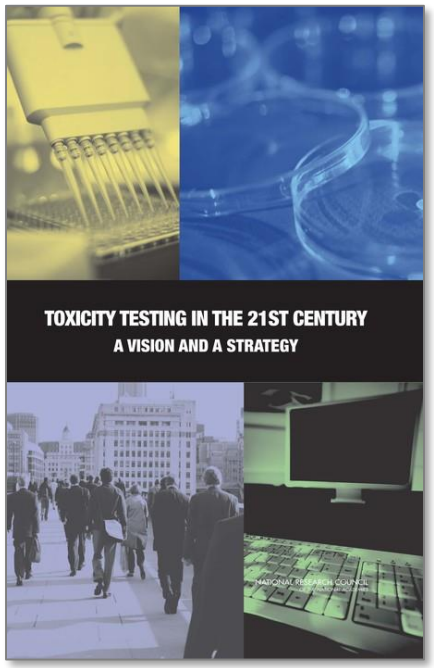
Paradigm for ‘predictive toxicology’

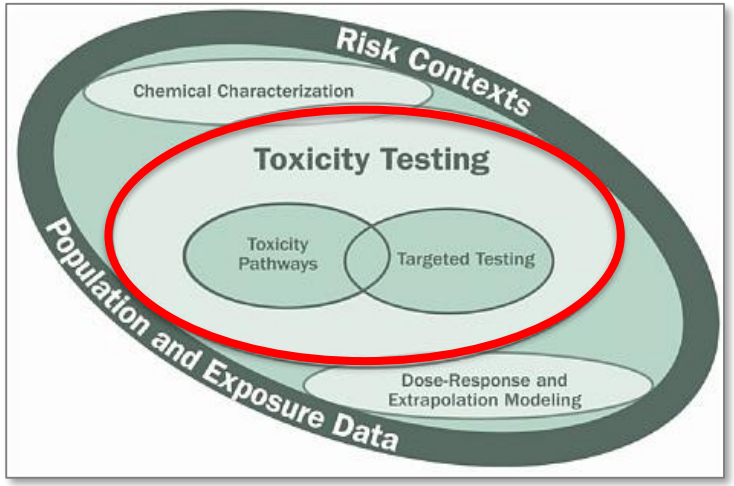


- Mechanistic understanding of biology is becoming increasingly dependent on ‘big data’: by 2025 the volume of genomics data is projected to exceed that from astronomy, YouTube and Twitter combined.

- *Toxicity Testing in the Twenty-first Century* [National Academy of Sciences, 2007] flips testing from data-poor observation in animal studies (*in vivo*) to data-rich evaluation associated with pathway-level profiling (*in vitro, in silico*).

- Emphasis on new approach methods (NAMs): HTS/HCS data, human cells and cell lines, chemical-biological interaction(s), computational and organotypic models, concentration-response and extrapolation based on exposure models.

- An integrated approach to toxicity assessment (eg, IATAs) brings it all together in the ‘animal-free zone’ where possible and targeted testing where necessary, assimilating hazard-exposure information into pathway-based risk assessment.



https://www.nap.edu/catalog/11970/toxicity-testing-in-the-21st-century-a-vision-and-a-strategy

Key Points

"It's tough to make predictions, especially about the future"
-Yogi Berra

- Automated HTS assays enable rapid chemical screening to help '*decode the toxicological blueprint of active substances that interact with living systems*' [Sturla et al. 2014].
- Reducing a self-organizing biological system to simpler assays for chemical profiling disrupts the spatial and temporal dynamics that render it adaptive in the first place.
- Vast HTS data now in hand, the need arises for organotypic culture models (*in vitro*) and computer (*in silico*) systems that can rebuild this complexity.
- Focus of this lecture is on predicting the potential for human developmental and reproductive toxicity (DART) testing with less reliance on vertebrate animal testing.

"Molecular biology took Humpty Dumpty apart …
mathematical modeling is required to put him back together again."

– Schnell et al. (2007) Amer Scientist

Outline: computational and organoid approaches

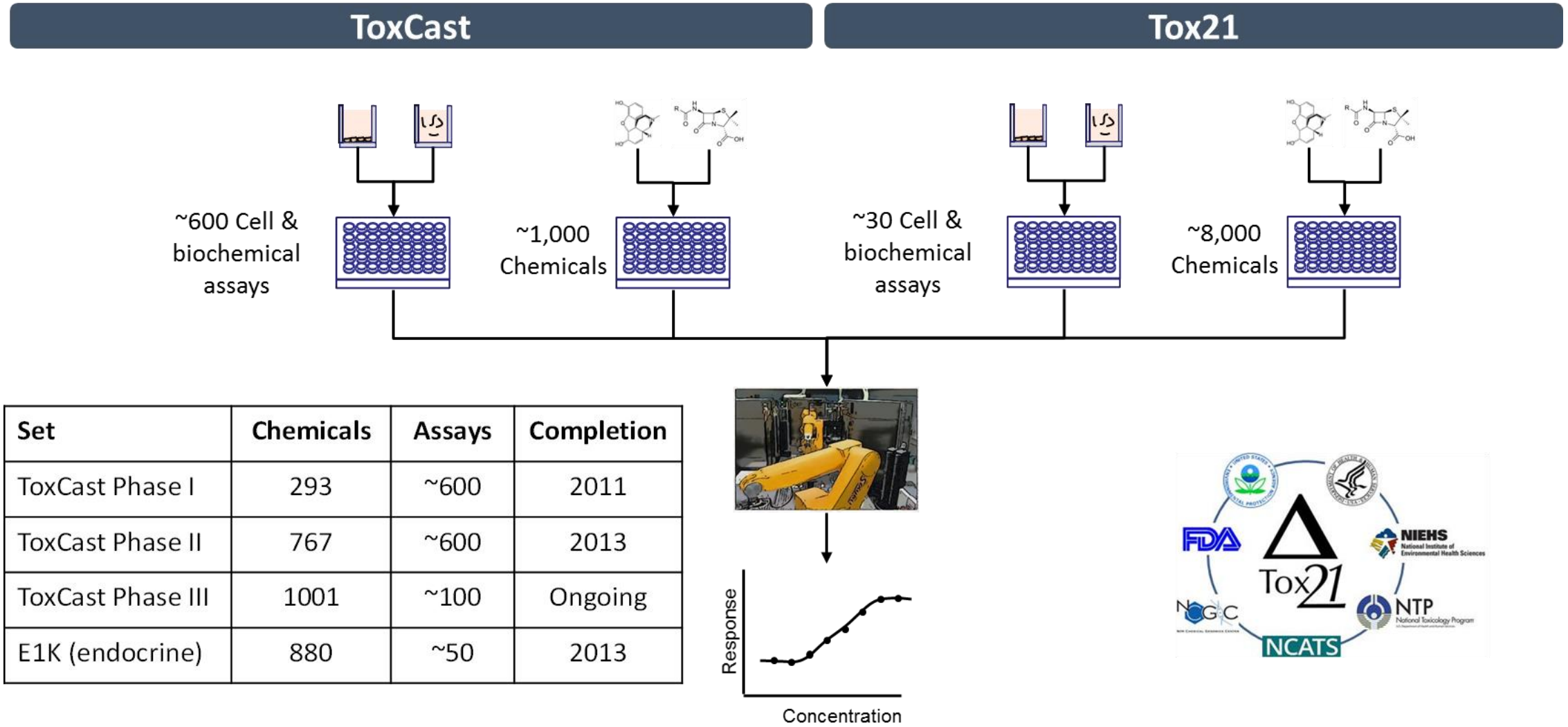
1. Profiling the ToxCast library with a pluripotent human (H9) embryonic stem cell assay.

2. AOP-based ontologies for developmental toxicity: case study on developmental vascular toxicity.

3. Virtual Tissue Models (VTM): computer simulation and biomimetic systems.

January 23, 2006

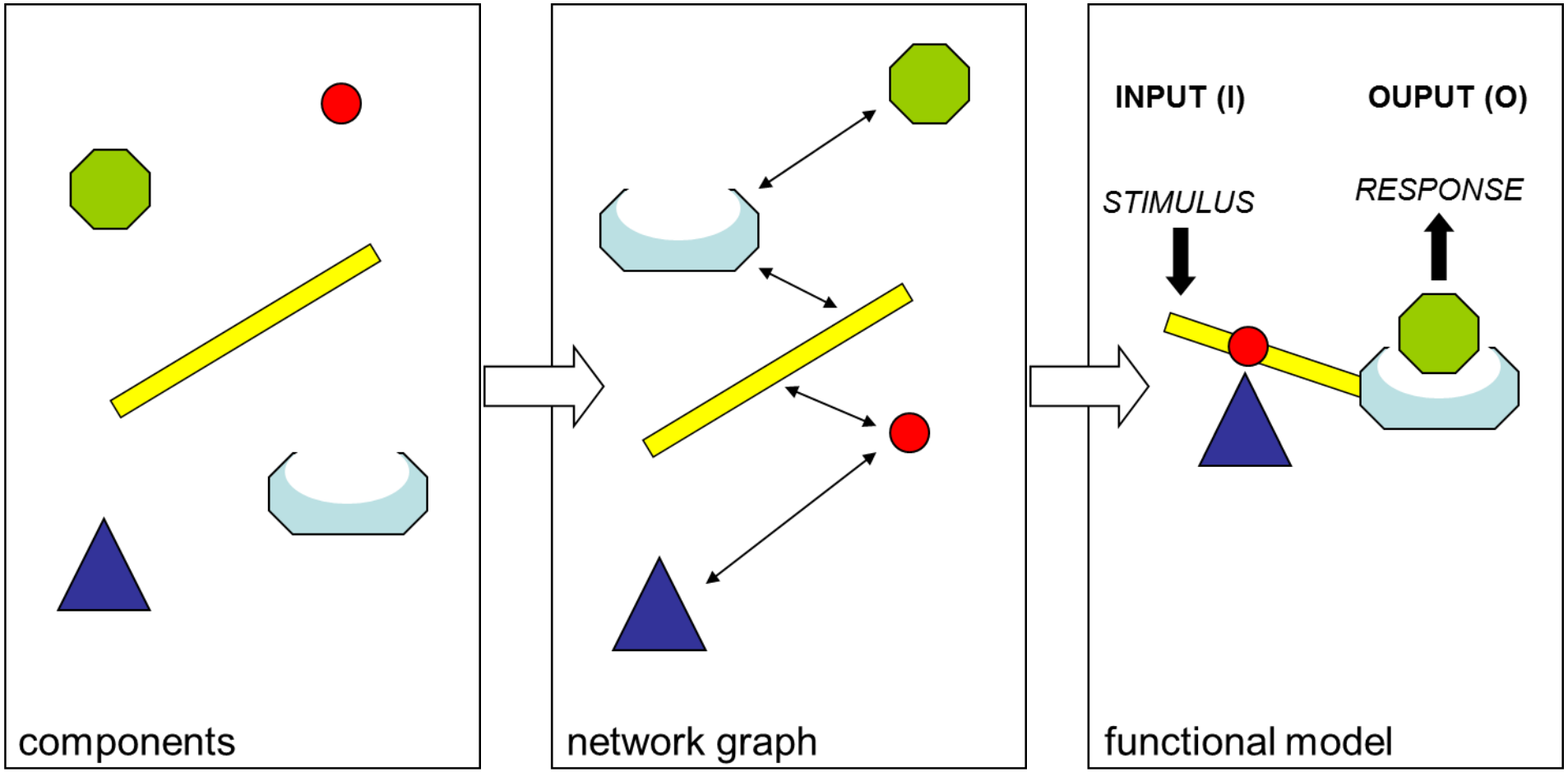
Shifting toxicology to pathway-based approaches



Set	Chemicals	Assays	Completion
ToxCast Phase I	293	~600	2011
ToxCast Phase II	767	~600	2013
ToxCast Phase III	1001	~100	Ongoing
E1K (endocrine)	880	~50	2013

https://www.epa.gov/chemical-research/toxicology-testing-21st-century-tox21

Why systems models are needed …

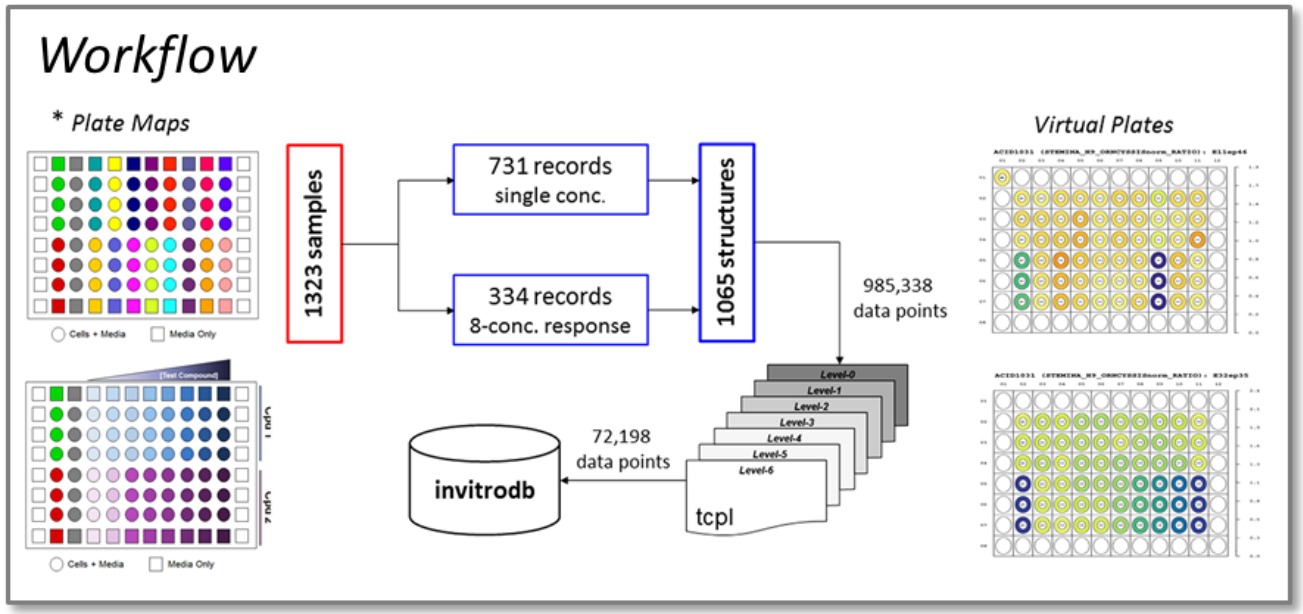


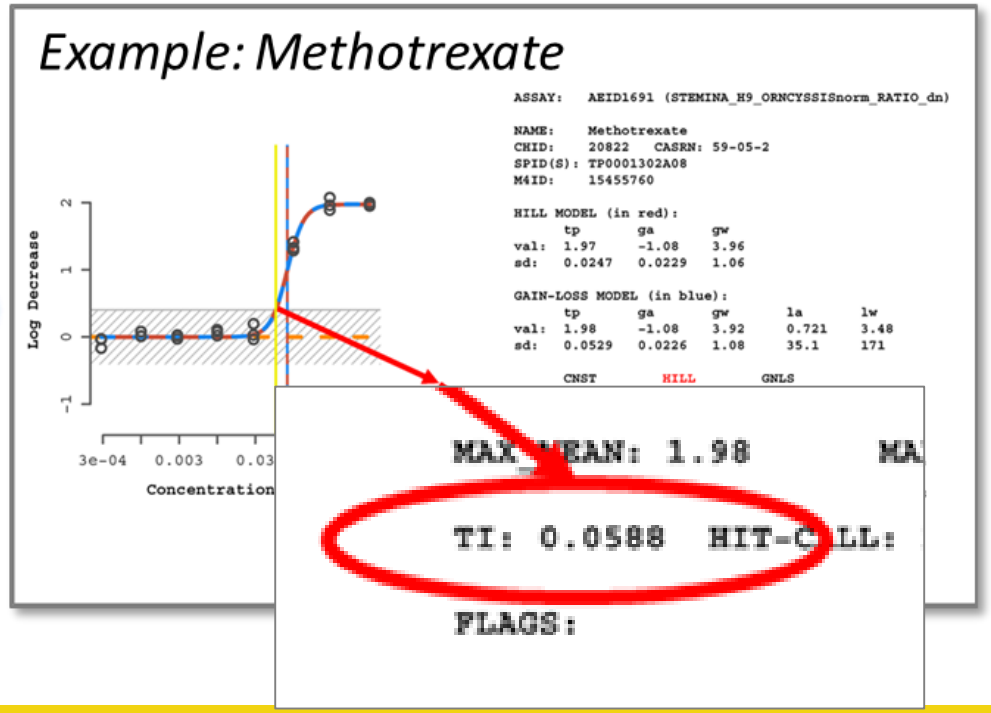
Profiling the ToxCast library with a pluripotent human (H9) embryonic stem cell assay

Objective: increase the diversity and relevance of assays in ToxCast that can be used to profile chemicals for potential adverse effects on human embryonic development.

ToxCast_STM assay

- devTOX^{qP} assay from Stemina Biomarker Discovery
- pluripotent H9 stem cells exposed → secretome of 3rd day analyzed by metabolomics
- critical drop of ornithine:cystine ratio is the targeted biomarker
- pharma test set yields 77% accuracy (0.57 sensitivity, 1.00 specificity) [Palmer et al. 2013]
- **Key point:** 183 of 1065 (17%) ToxCast chemicals tested positive in this screen



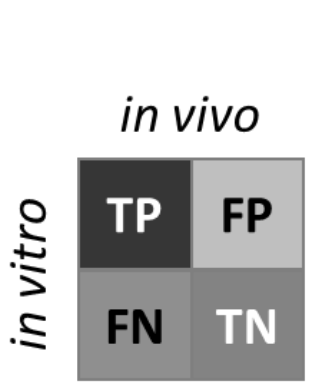


SOURCE: Zurlinden et al. (NCCT manuscript in clearance)

Performance anchored to ToxRefDB

Key point: balanced accuracy improves with evidence for DevTox

		Stringency Filter Applied to DevTox Anchor		
Condition[2]	*Base*	*Low*	*Medium*	*High*
TP	85	60	35	19
FP	14	37	23	9
FN	217	127	51	11
TN	116	208	176	88
n	432	432	285	127
sensitivity	0.281	0.321	0.407	0.633
specificity	0.892	0.849	0.884	0.907
PPV	0.859	0.619	0.603	0.679
NPV	0.348	0.621	0.775	0.889
ACC	46.5%	62.0%	74.0%	84.3%
MCC	0.190	0.202	0.332	0.554
	any dLEL rat OR rabbit	SOME evidence rat OR rabbit	CLEAR evidence rat OR rabbit	CLEAR evidence rat AND rabbit



STM versus rat WEC

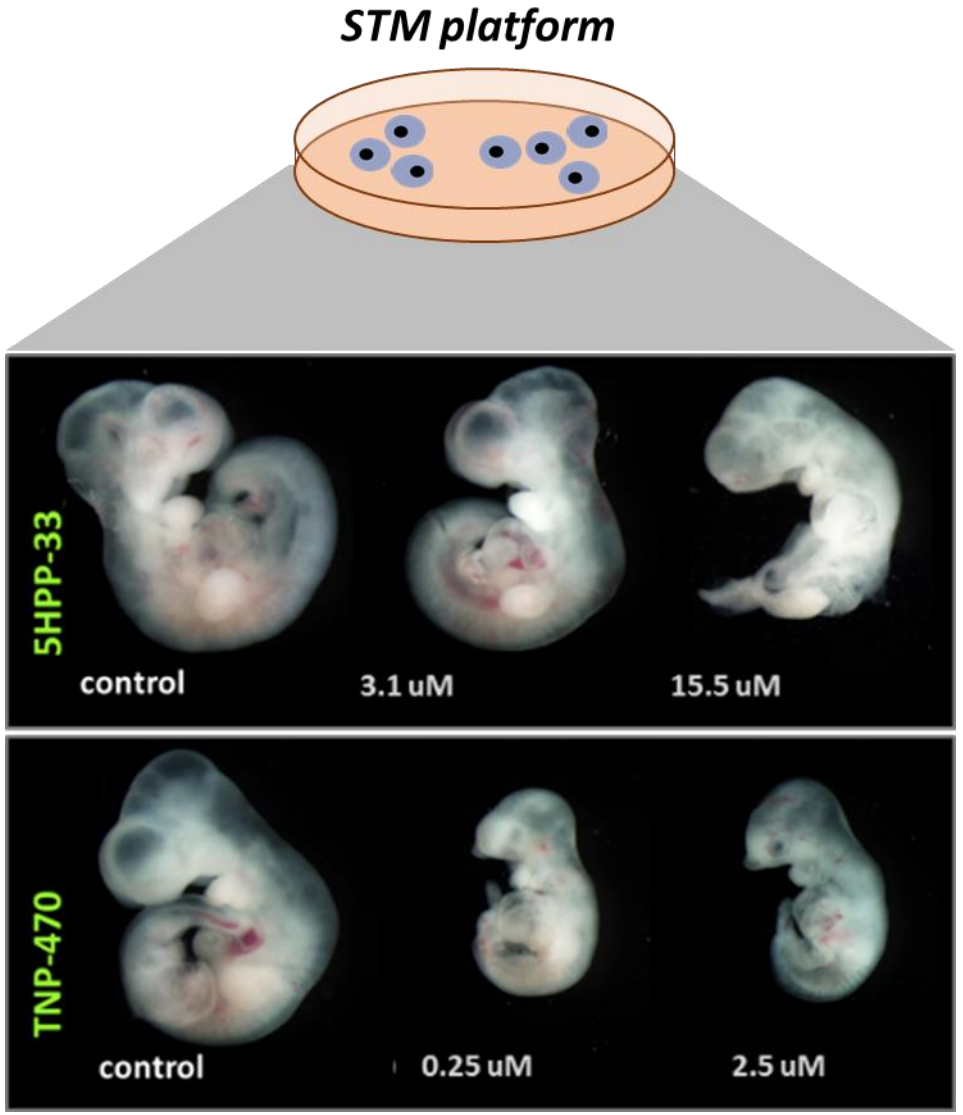
5HPP-33: *synthetic thalidomide analog*

- T.I. predicted 9.5 µM
- AC50 observed 21.2 µM (embryo viability)

TNP-470: *synthetic fumagillin analog*

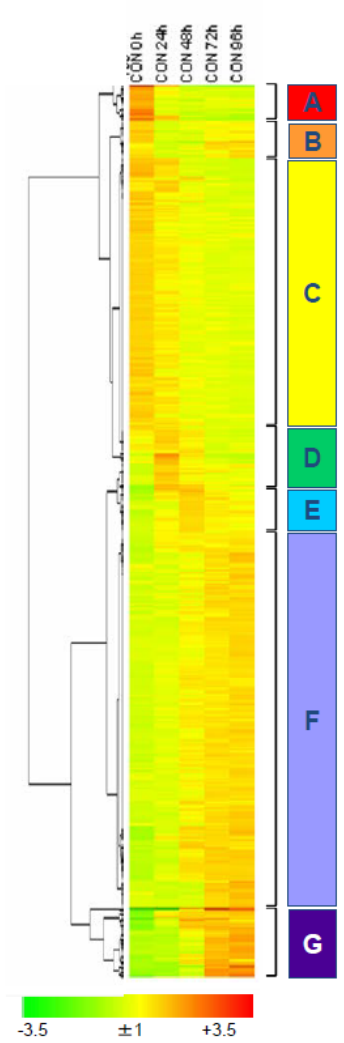
- T.I. predicted 0.01 µM
- AC50 observed 0.04 µM (dysmorphogenesis)

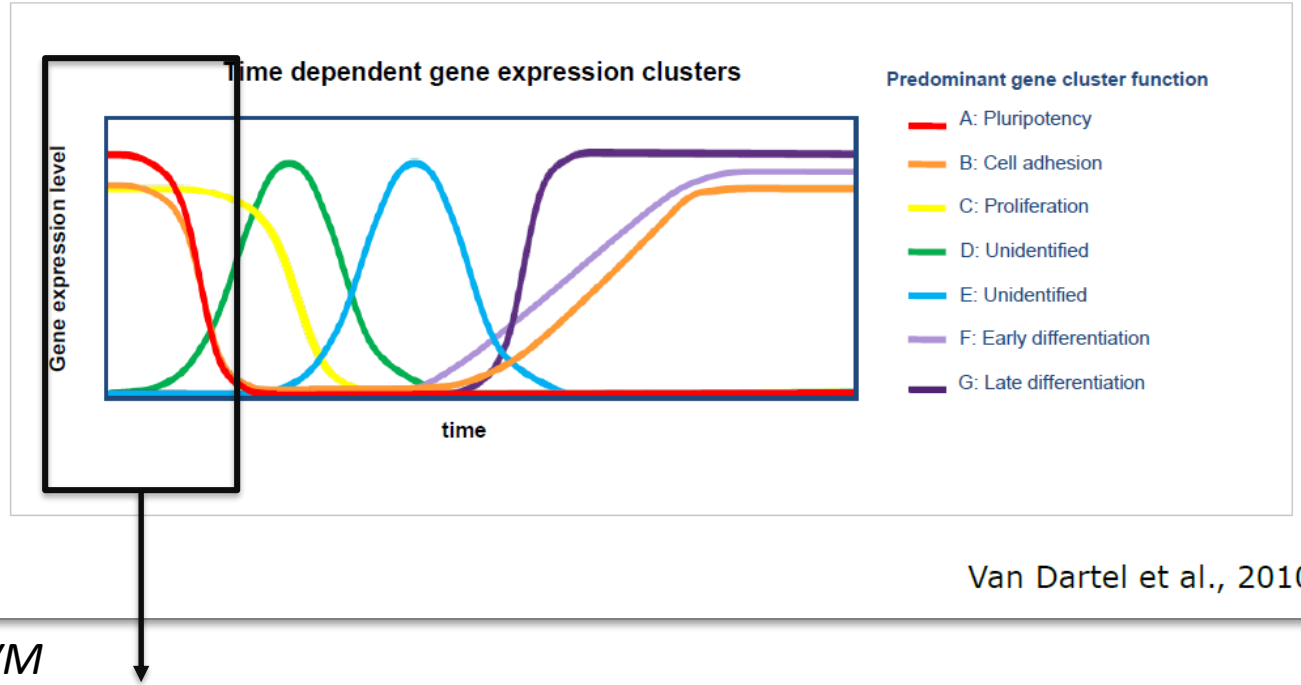
Key point: exposure-based potential for DevTox predicted by STM assay (quantitative prediction).



SOURCE: Ellis-Hutchings et al. (2017) Reprod Toxicol

EST differentiation – related gene expression

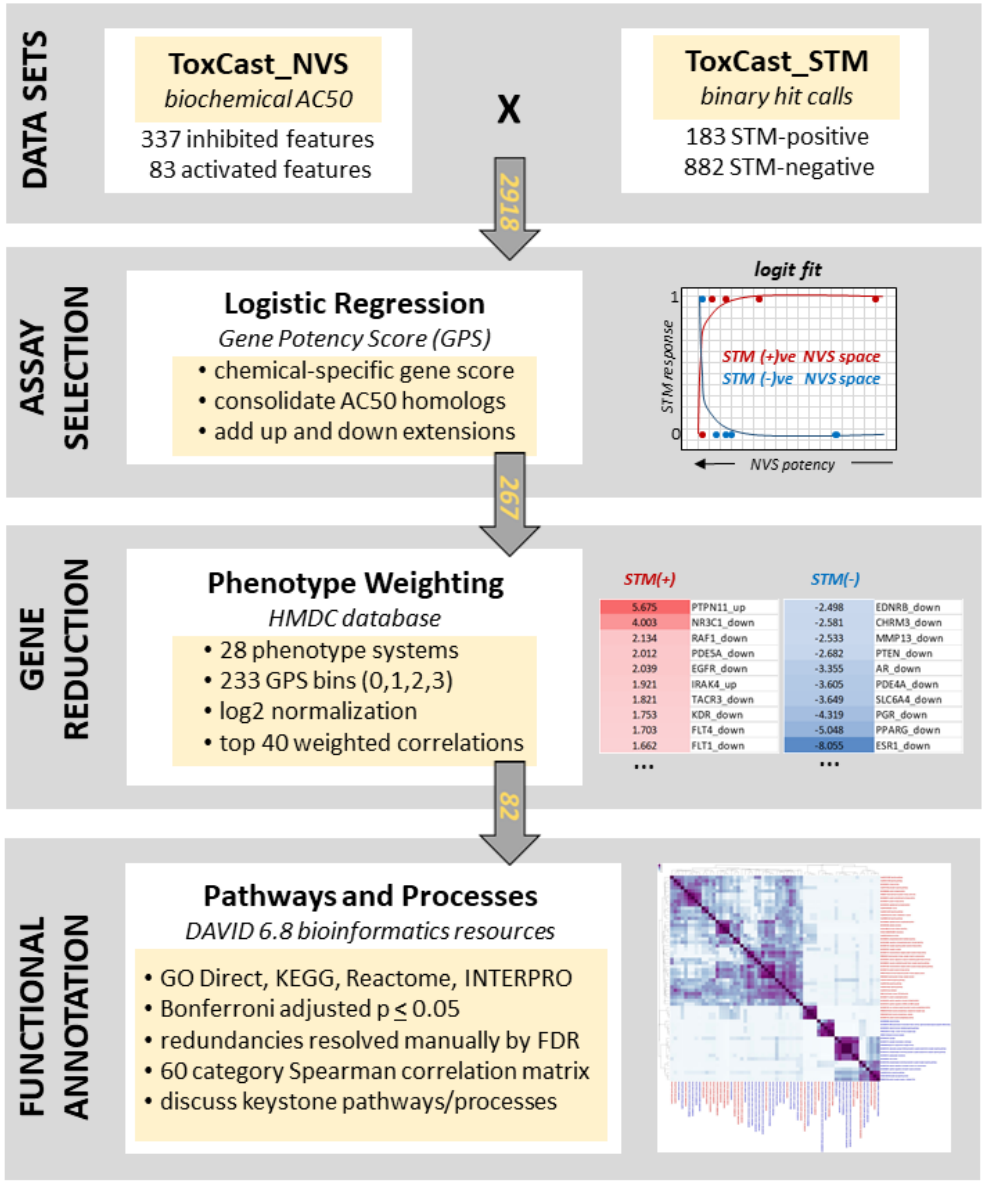




Van Dartel et al., 2010

SLIDE SOURCE: Aldert Piersma, RIVM

phase of the differentiation trajectory addressed by ToxCast_STM assay



SOURCE: Zurlinden et al. (NCCT manuscript in clearance)

Keystone Pathways

(predicted)

- Mining STM response against biochemical pathways constructed from ToxCast_NVS.
- What we can and cannot say about the applicability domain of the STM response:

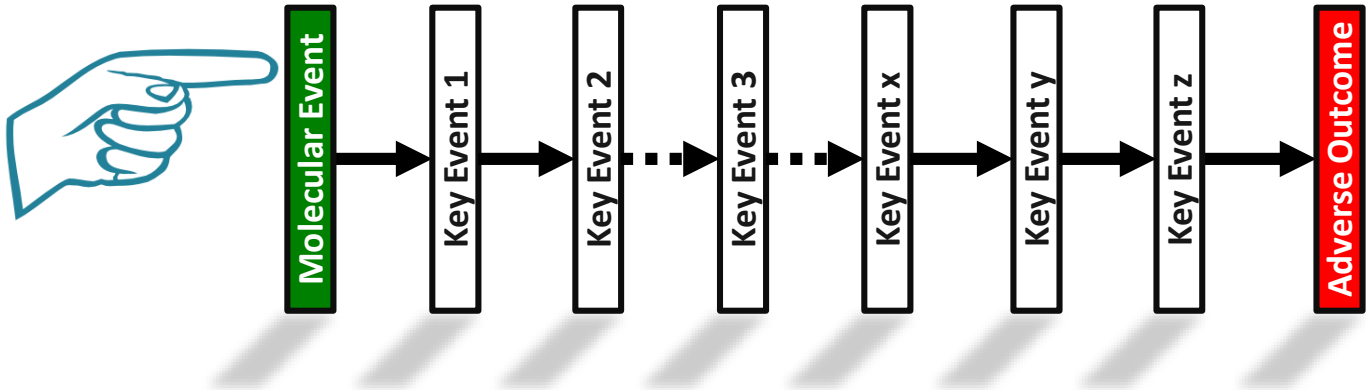
  - sensitive domain: regulation of PI3K signaling, FoxO signaling pathway, and focal adhesion pathway.

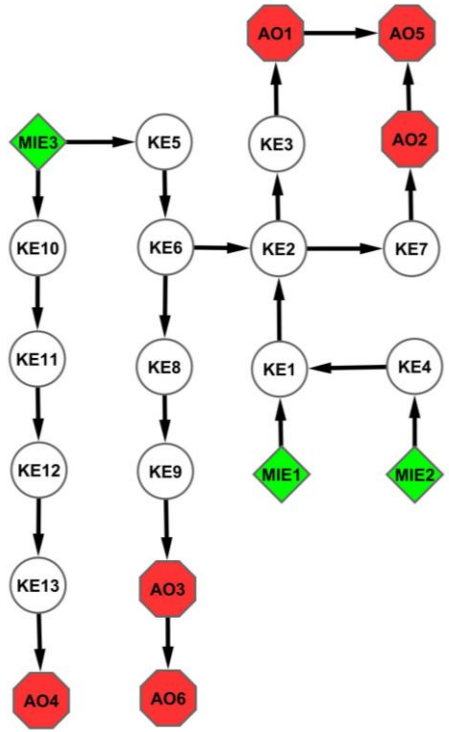
  - insensitive domain: GPCR signaling through G(q) and steroid hormone mediated signaling pathways.

AOP-based ontologies for developmental toxicity:
case study on developmental vascular toxicity

Objective: formalize a mechanistic framework for developmental toxicity that can be used to quantitatively link adverse outcomes with MIEs and KERs in the angiogenic cycle.

AOP Core Principles





1. ***AOPs are not chemical-specific*** (based on biological motifs of failure)
2. ***AOPs are modular*** (individual relationships based on weight of evidence)
3. ***Individual AOPs are a pragmatic simplification*** (linearized sequence of biology)
4. ***AOP networks are the functional unit of prediction*** (in most cases)
5. ***AOPs are living documents*** (evolve as knowledge grows)

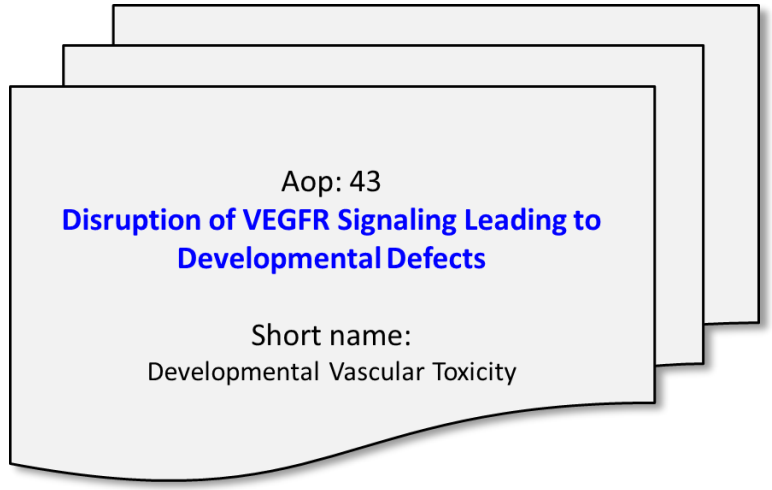
SOURCE: Villeneuve et al. (2014) Toxicol Sci

Vascular Development

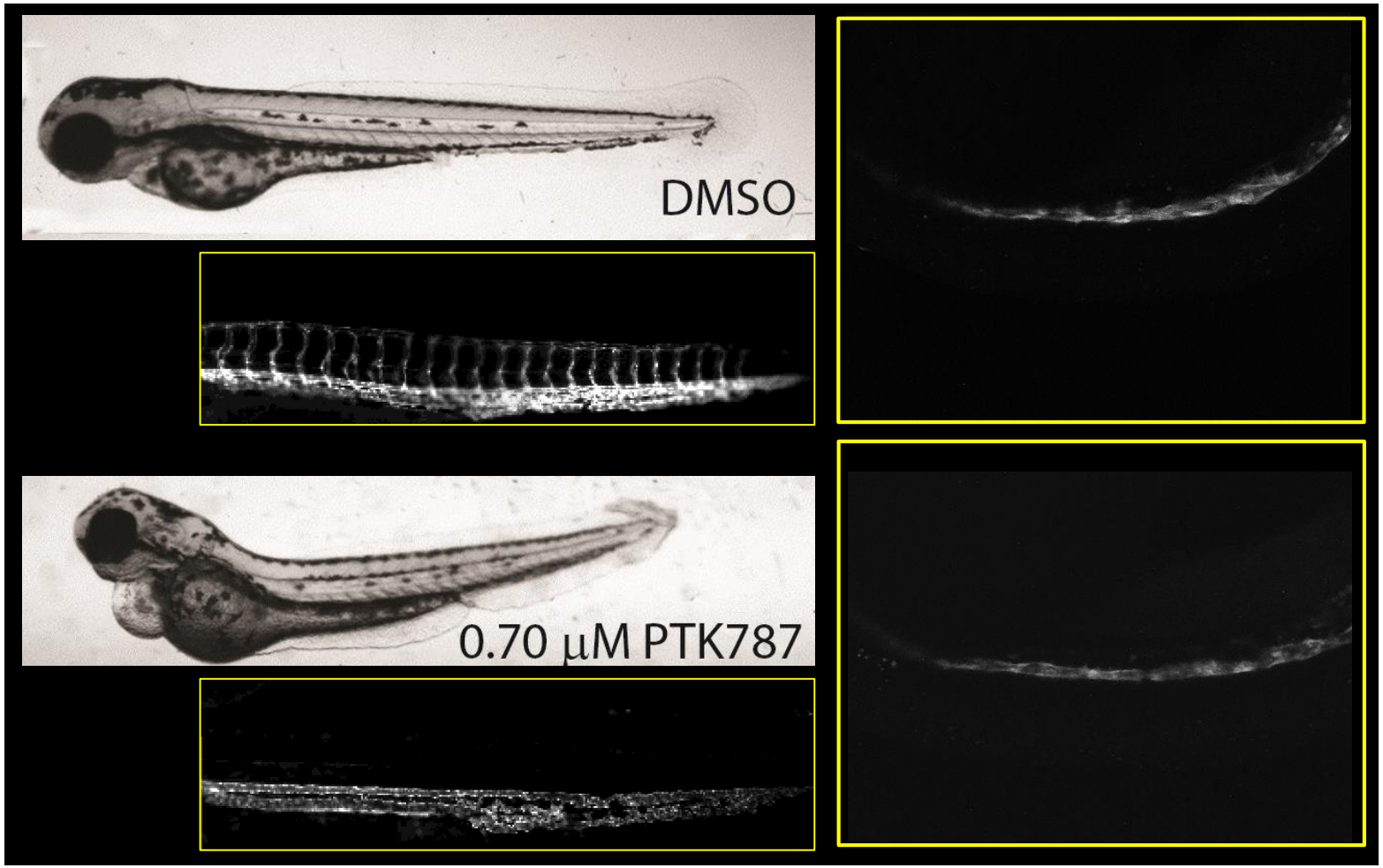
- Blood vessel development is essential to embryogenesis (cardiovascular is first functioning organ system across *Vertebrate* species).

- Vascular insufficiency is tied to many disease processes (stroke, diabetes, preeclampsia, neonatal respiratory distress, osteoporosis, teratogenesis, …).

- Aop43: one of 28 AOPs included in the OECD work plan with status ‘open for citation & comment’ [**https://aopwiki.org/aops/43**].



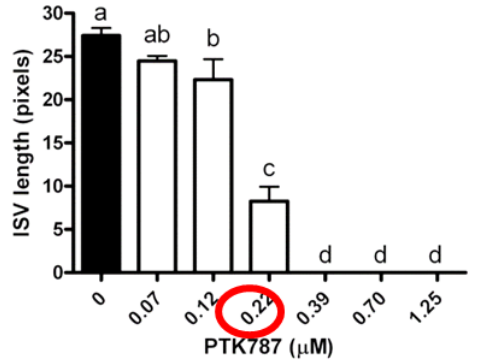
VEGFR2 inhibition (PTK787)



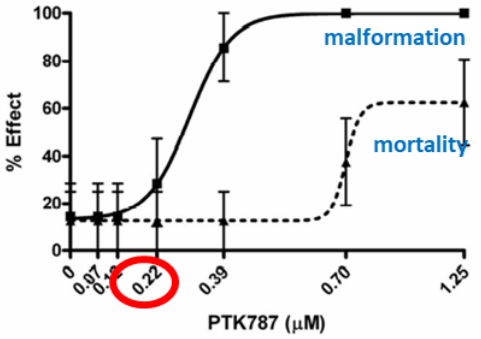
video of angiogenesis

SOURCE: Tal et al. (2014) Reprod Toxicol

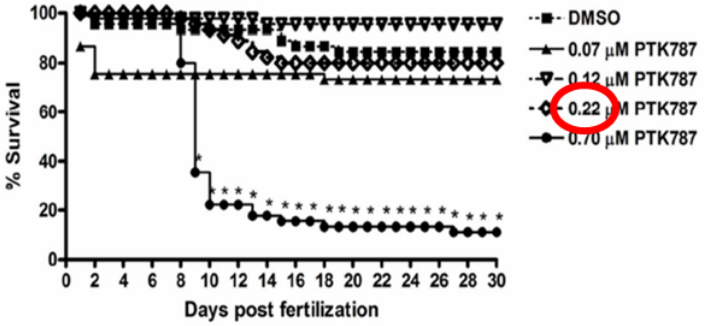
ISV length (72 hpf)



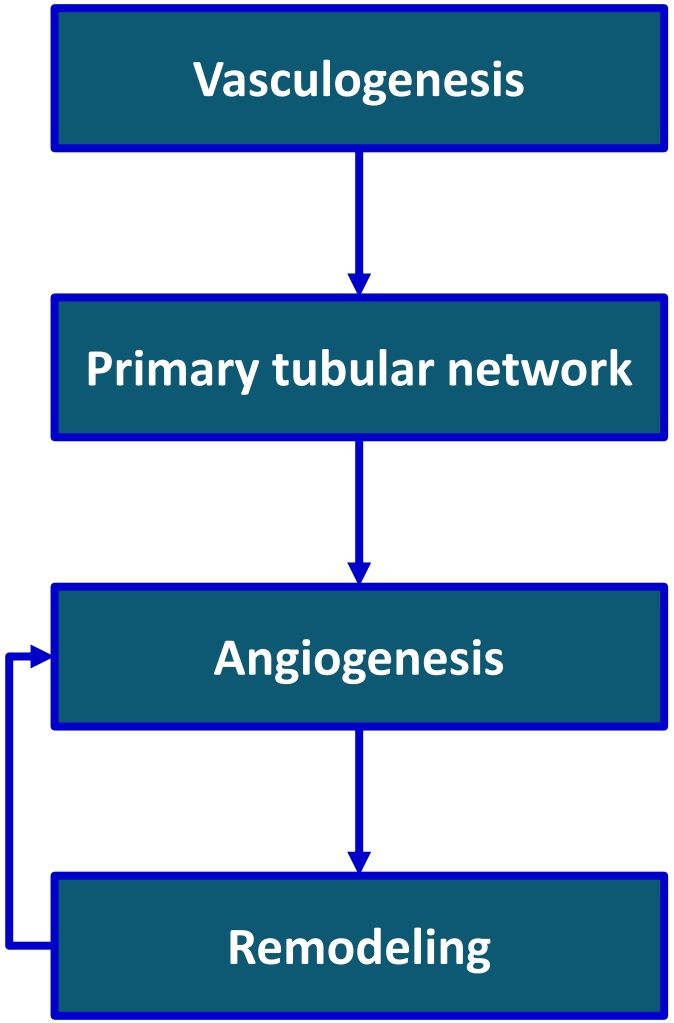
Terata (120 hpf)

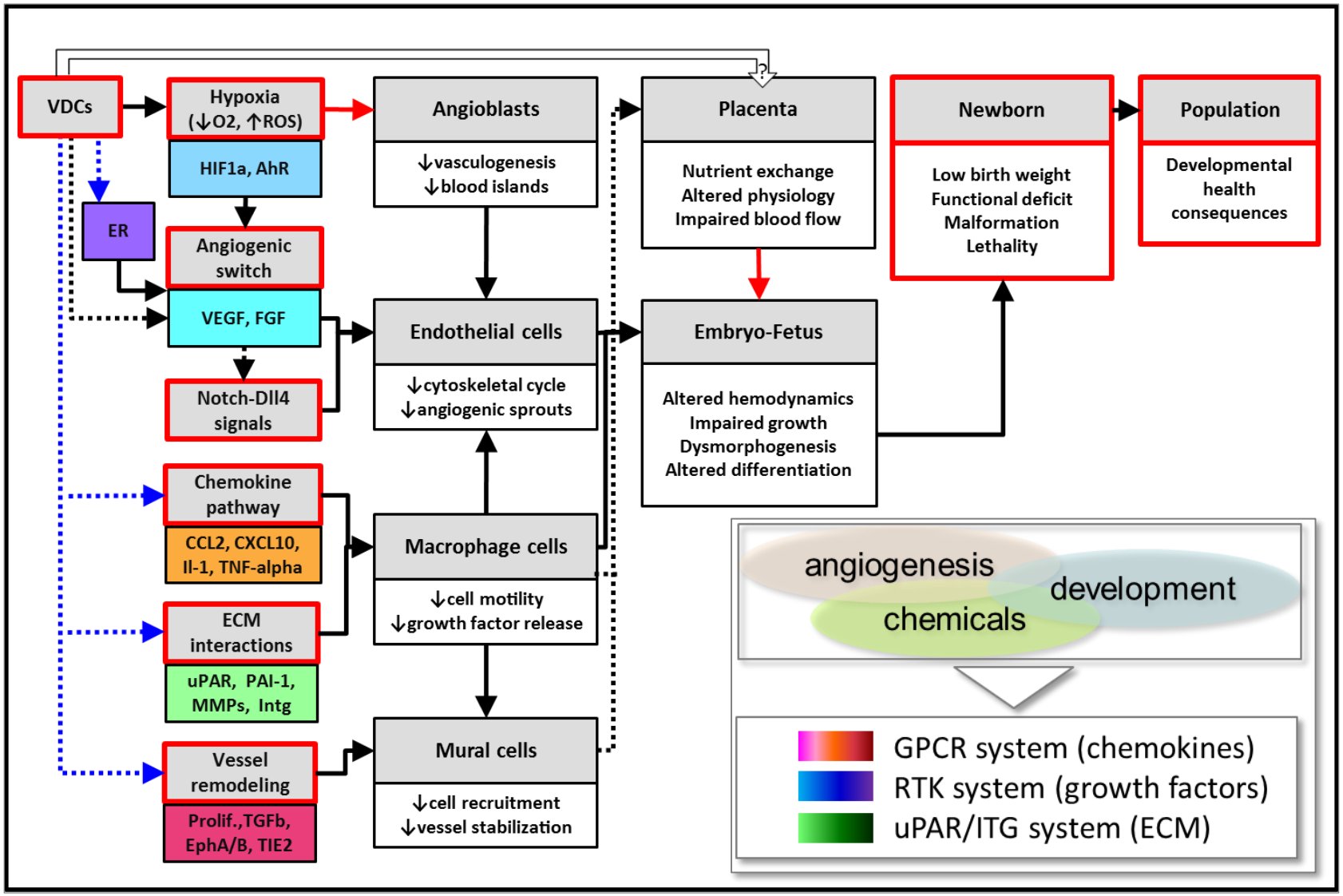


Lifespan (10 dpf)



Aop43 framework

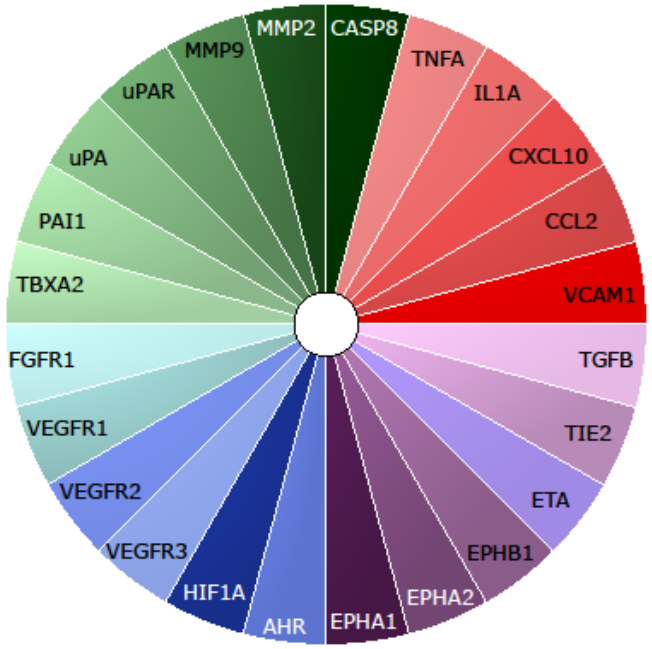




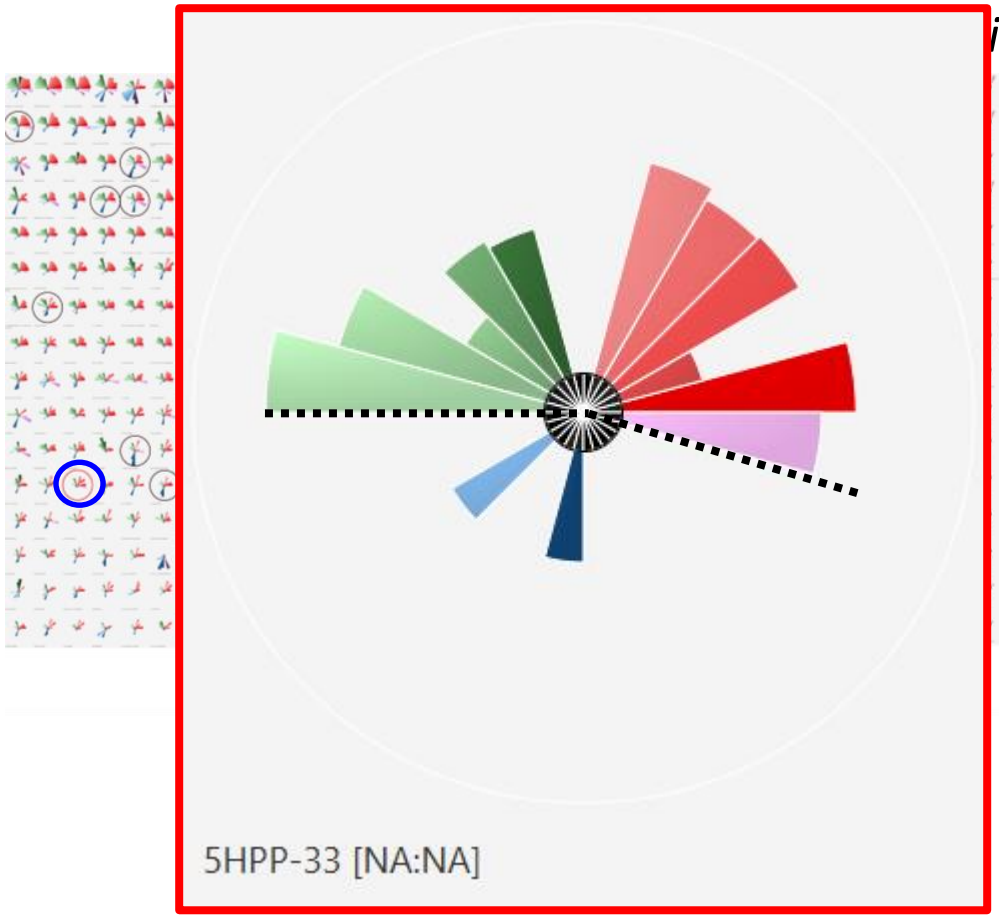
SOURCE: Knudsen and Kleinstreuer (2011) Birth Defects Res

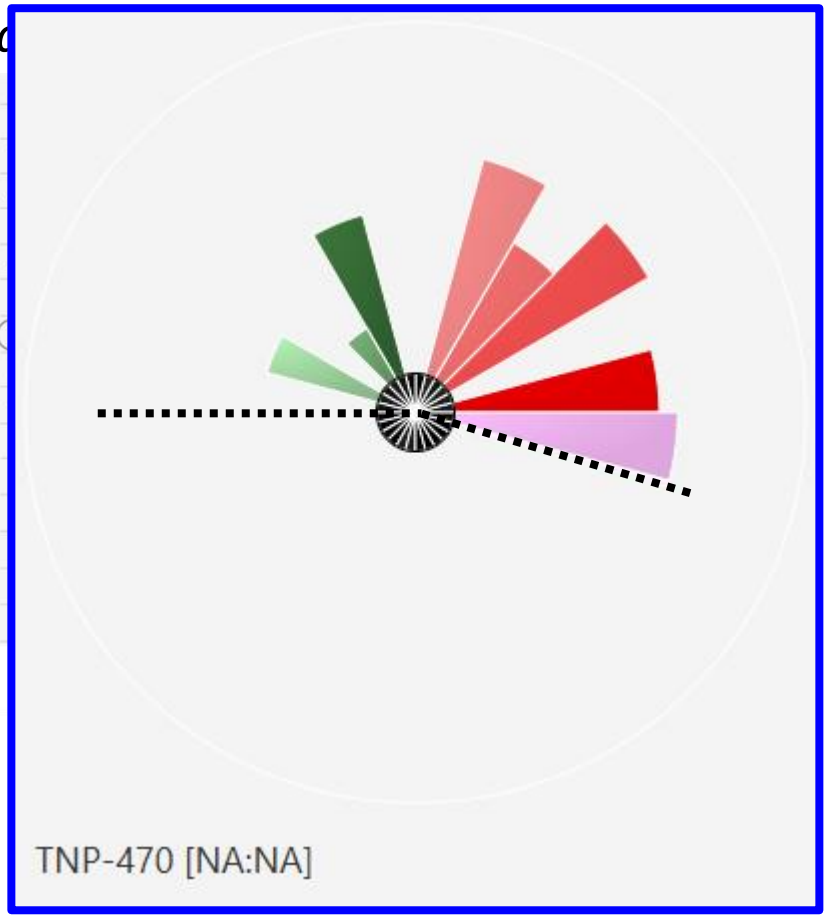
AOP-based ranking: predicted vascular disrupting chemicals (pVDCs)

24 ToxCast target assays
(pVDC ToxPi)

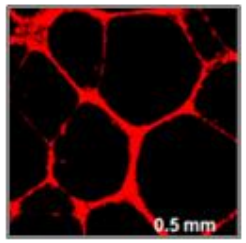


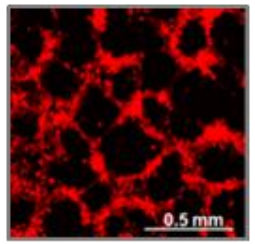
1058 ToxCast chemicals ranked by pVDC ToxPi

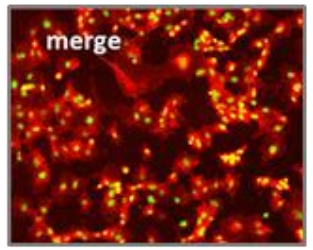


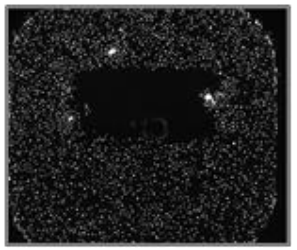
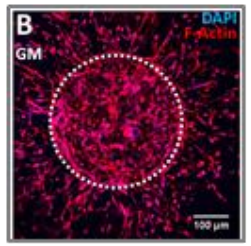


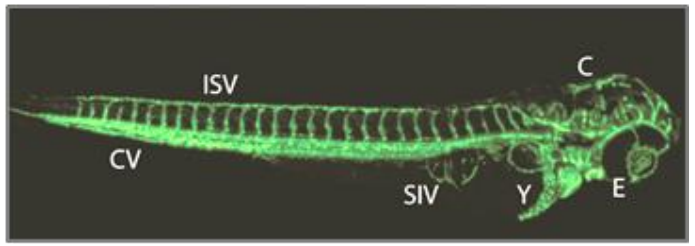
SOURCE: Kate Saili, NCCT



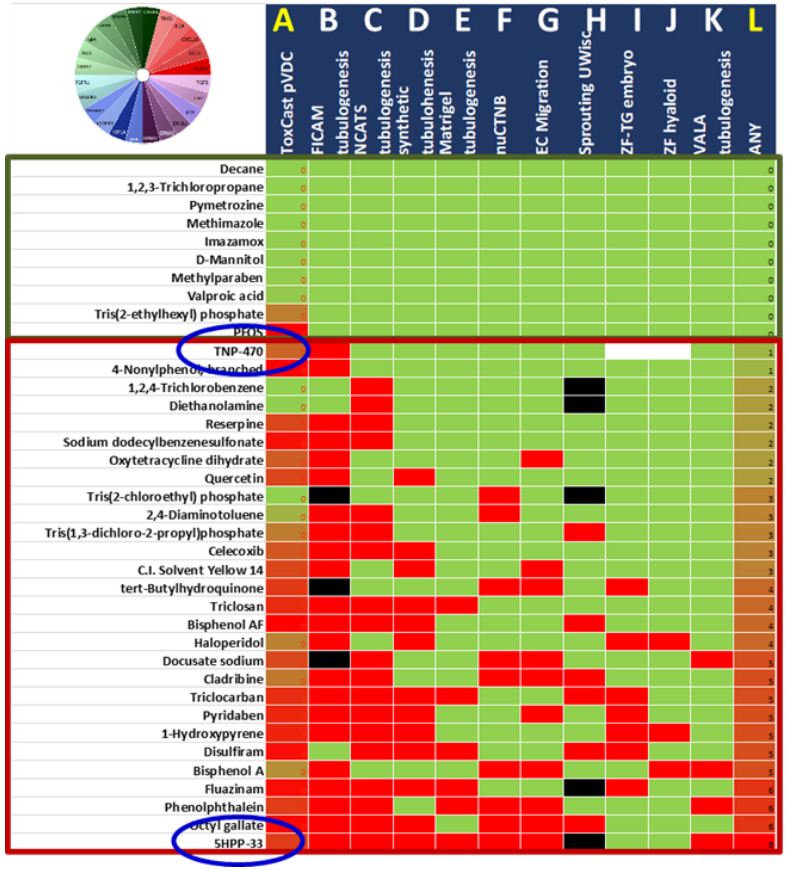






- inactive
- active
- cytotoxic
- no data



- A pVDC ToxPi
- B HUVEC tubulogenesis (FICAM)
- C HUVEC tubulogenesis (NCATS)
- D tubulogenesis in synthetic matrices (HMAPS)
- E tubulogenesis in Matrigel (HMAPS)
- F nuCTNB biomarker (VALA)
- G endothelial cell migration (VALA)
- H iPSC endothelial sprouting (HMAPS)
- I ISV reporter zebrafish (NHEERL)
- J reporter zebrafish (UDUBLIN)
- K HUVEC tubulogenesis (VALA)
- L ANY (B to K)

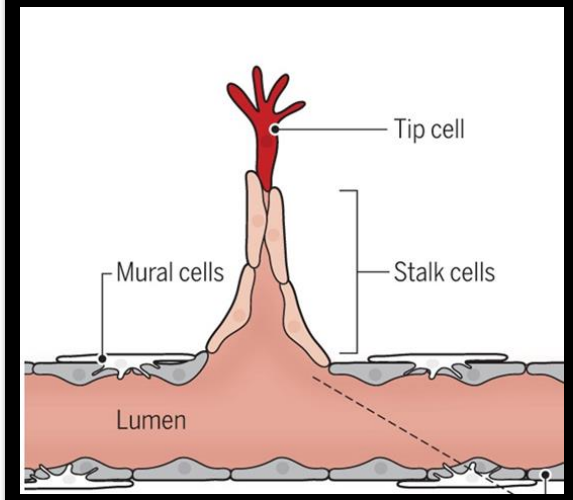
sensitivity 0.89, specificity 0.80
balanced accuracy 87% (PPV 93%, NPV 73%)

SOURCE: Saili et al. (submitted)

Virtual Tissue Models (VTM):
computer simulation and biomimetic systems

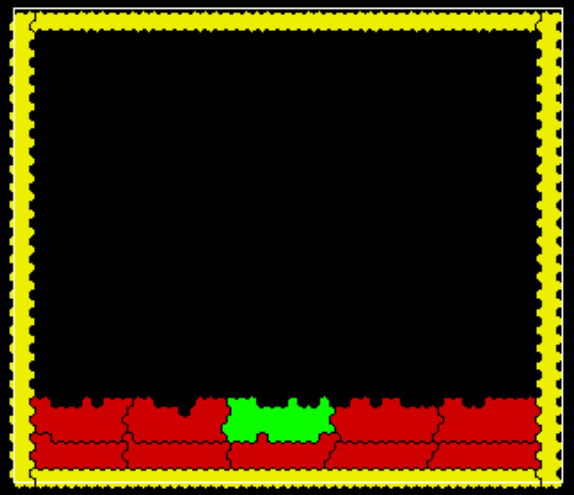
Objective: build and test computer models of complex tissues that advance critical phenomena (specificity, canalization, plasticity) for quantitative prediction for virtual screening and *in silico* testing.

Computer simulation: cell agent-based models (cABMs)



Li and Carmeliet (2018) Science

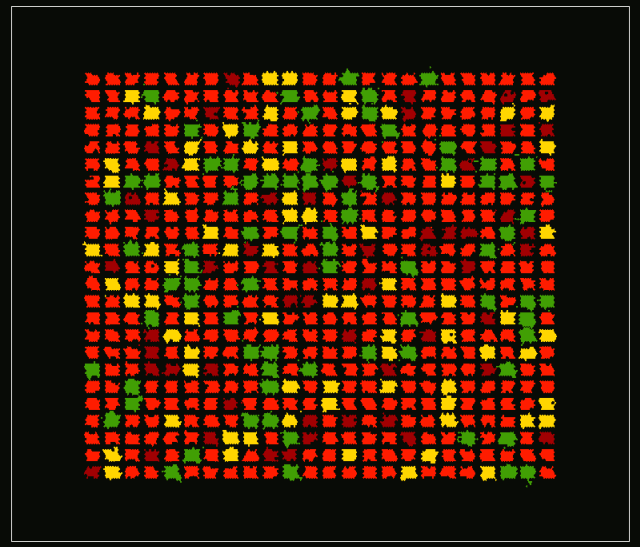
VEGF corridors



Nicole Kleinstreuer

VEGF165
MMPs
VEGF121
sFlit1
TIE2
CXCL10
CCL2

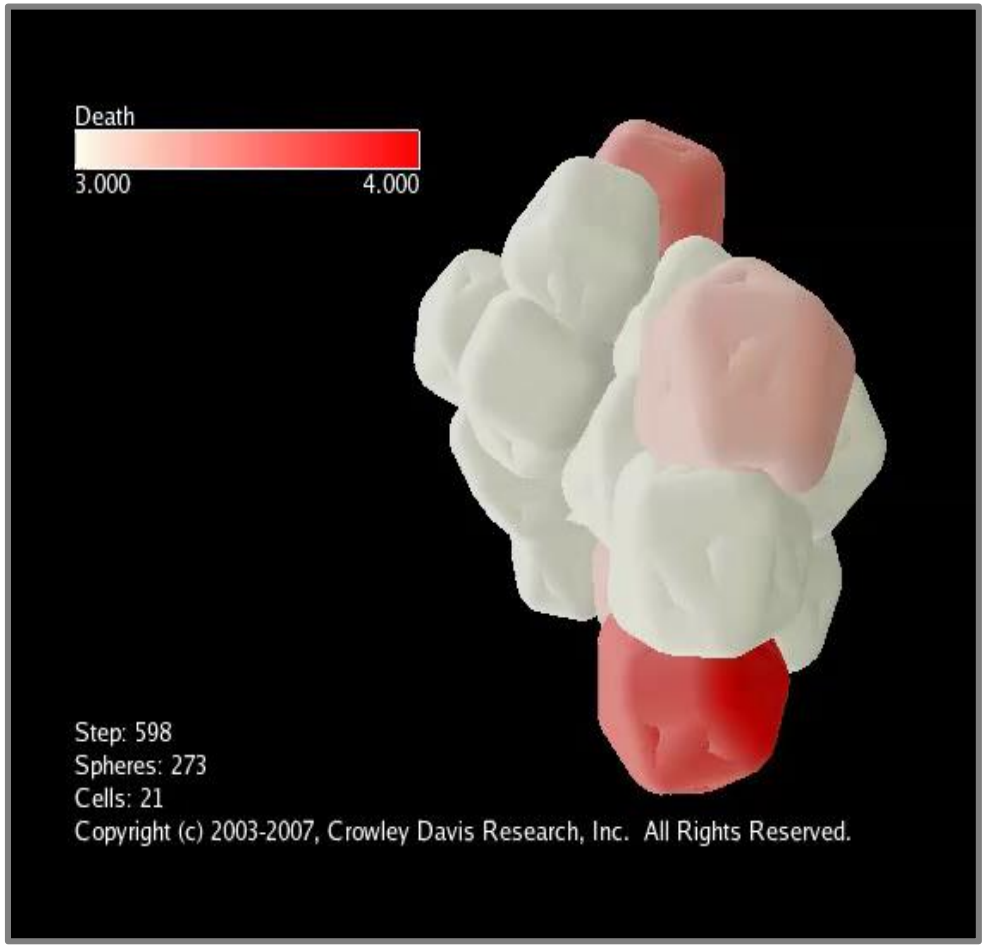
Network assembly



Kleinstreuer et al. (2013) PLoS Comp Biol

SOFTWARE: www.compucell3d.org
BioComplexity Institute, Indiana U

Anatomical homeostasis in a self-regulating ‘Virtual Embryo’



SOURCE: Andersen, Newman and Otter (2006) Am. Assoc. Artif. Intel.

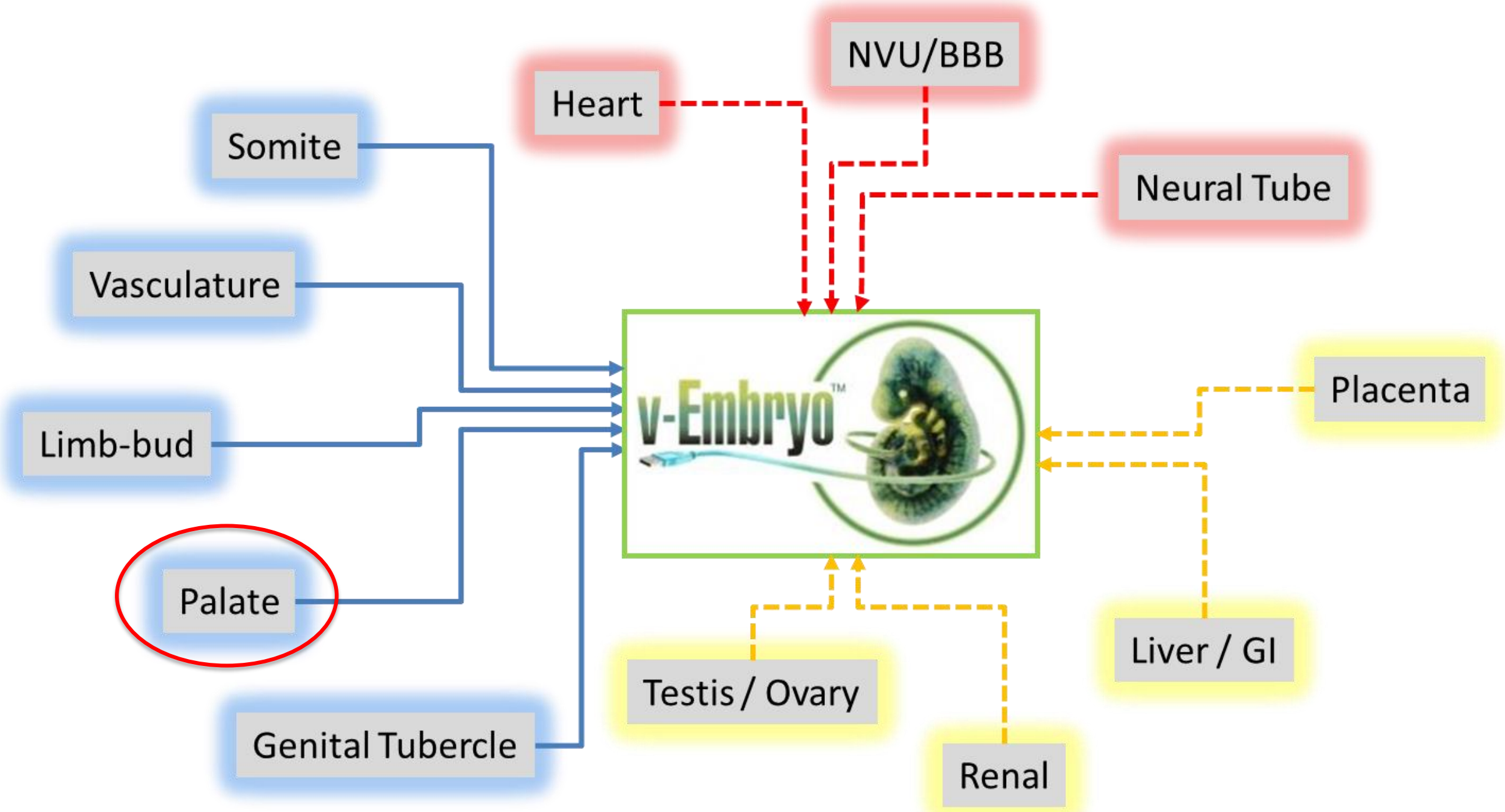
cABMs in predictive DART

Approach: build and test self-organizing morphogenetic systems *in silico* using an open-source modeling environment (CompuCell3d.org).

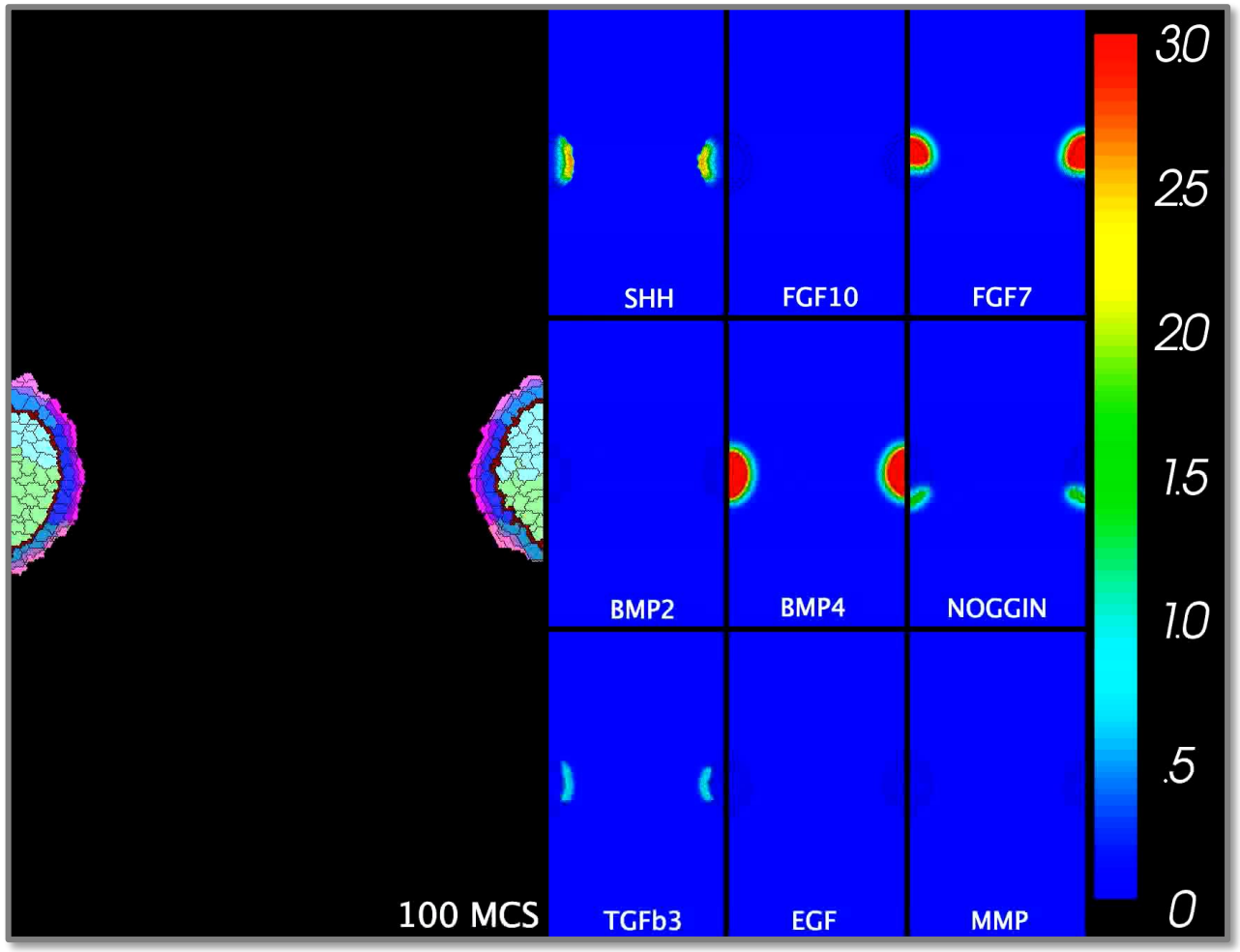
Input: A.I. cast into mathematically-defined cells (agents), synthetic gene circuits, and viscoelastic properties to emulate developmental progression (embryogeny).

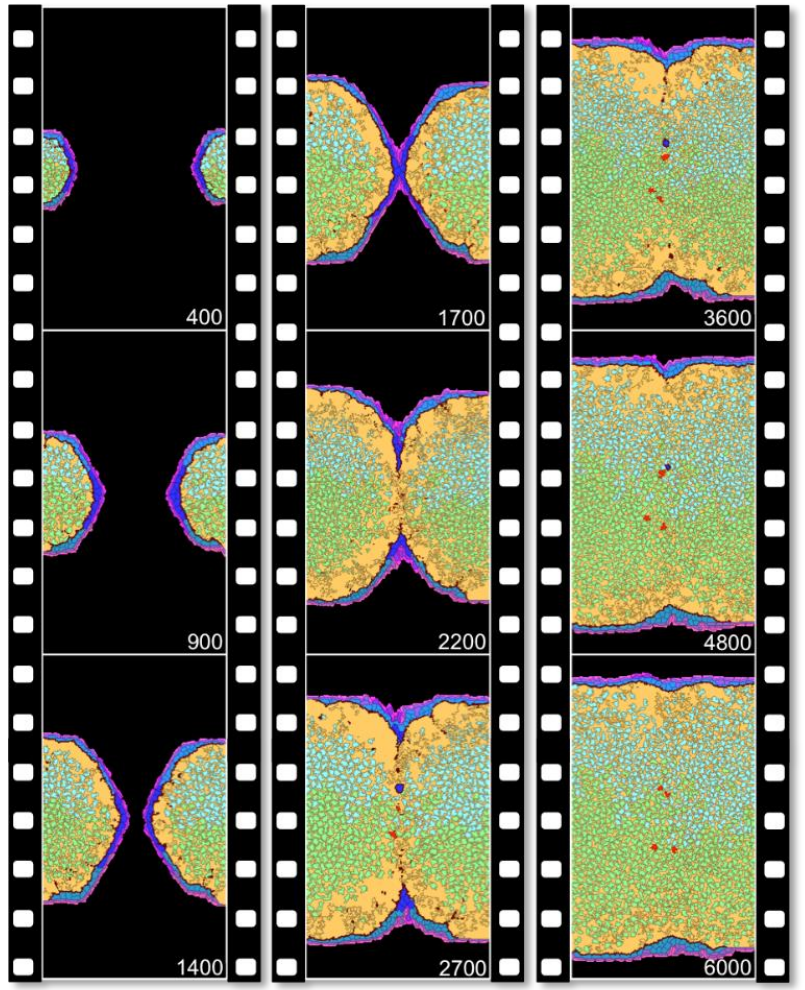
Emergence: simulation resolves into normal or perturbed phenotypes reading *in vitro* data input from specific ToxCast assays (cybermorphs).

Output: probabilistic rendering of where, when and how a developmental defect might occur (critical phenomena).


NVU/BBB
Heart
Somite
Neural Tube
Vasculature
v-Embryo™
Placenta
Limb-bud
Palate
Liver / GI
Testis / Ovary
Genital Tubercle
Renal

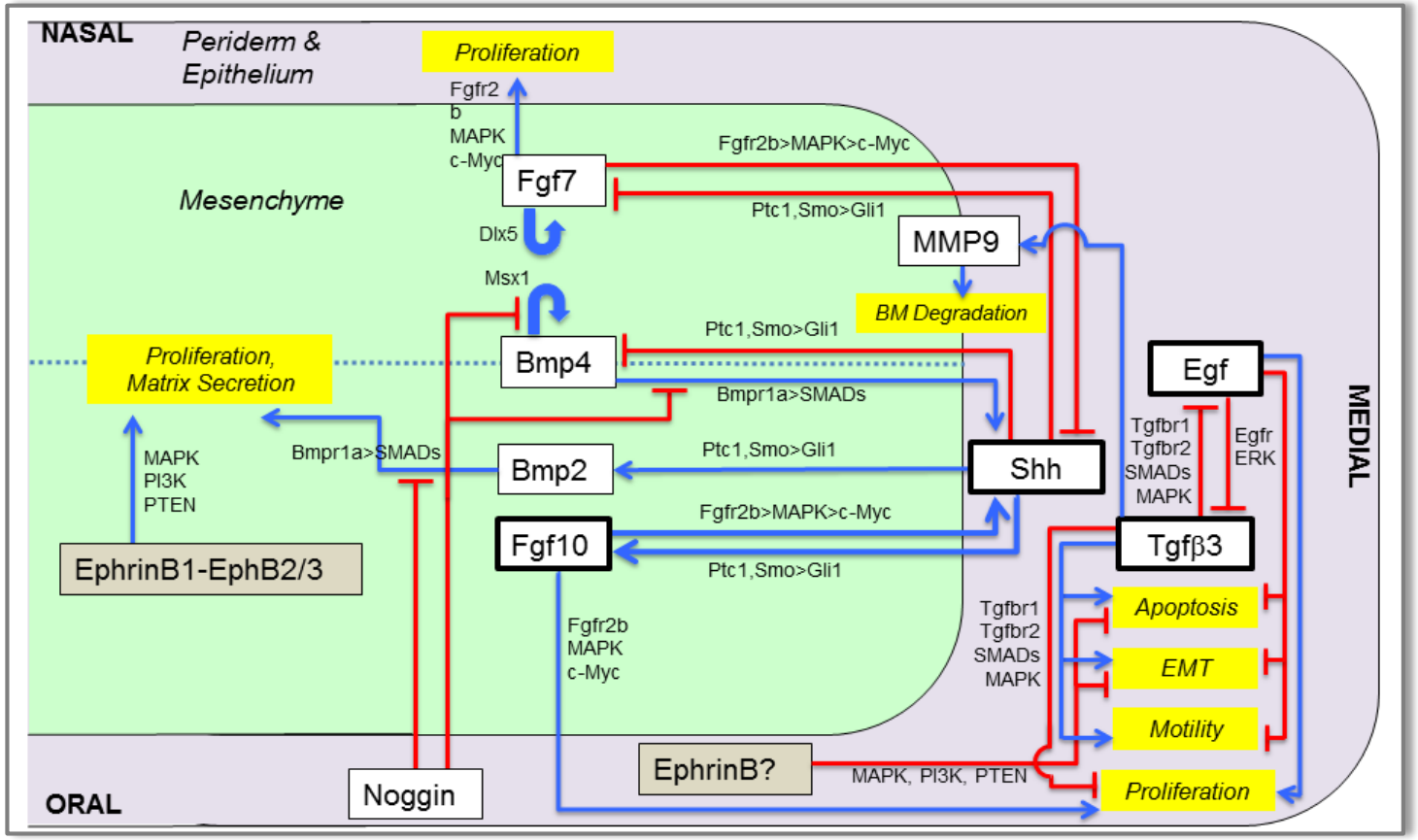
Palatal fusion: epithelial seam breakdown and mesenchymal confluence

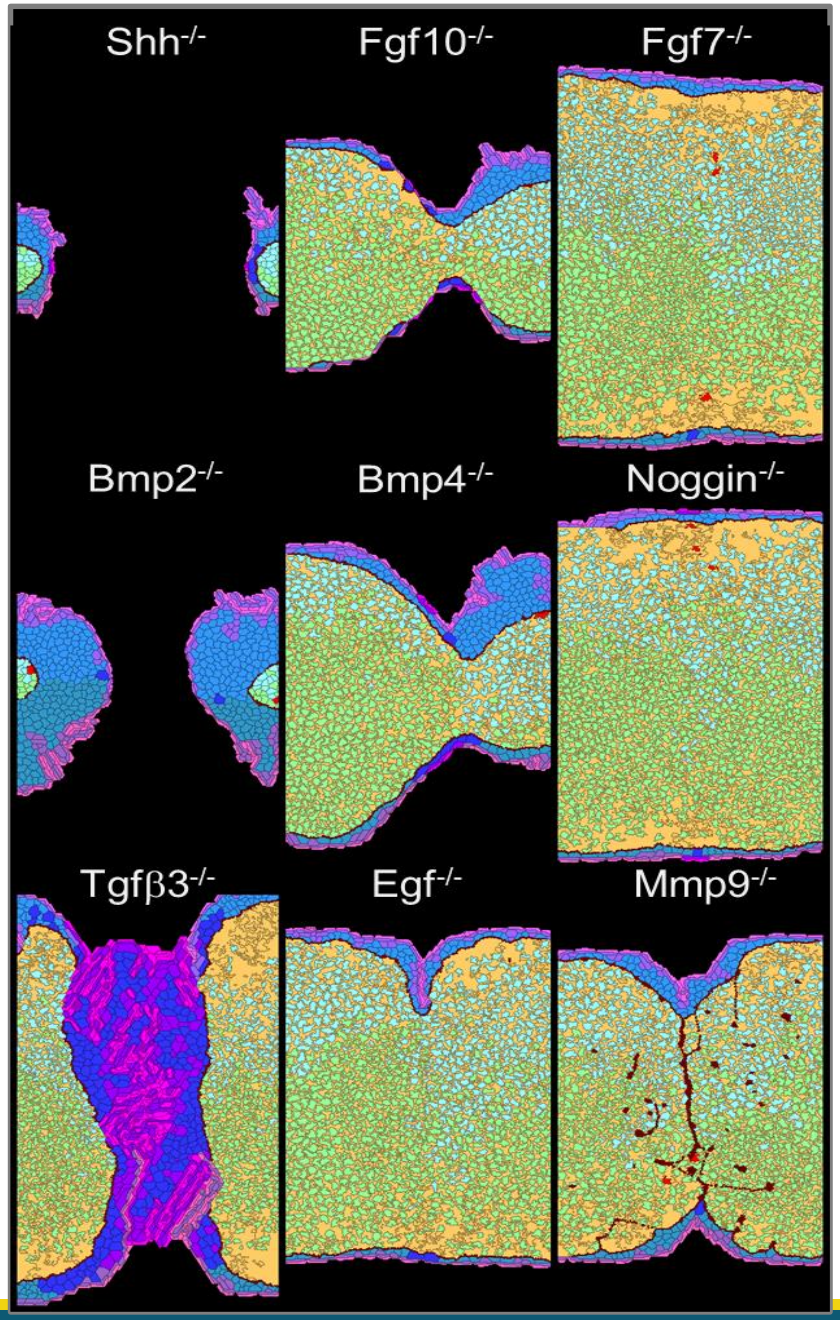




SOURCE: Hutson et al. (2017) Chem Res Toxicol

Hacking the control network



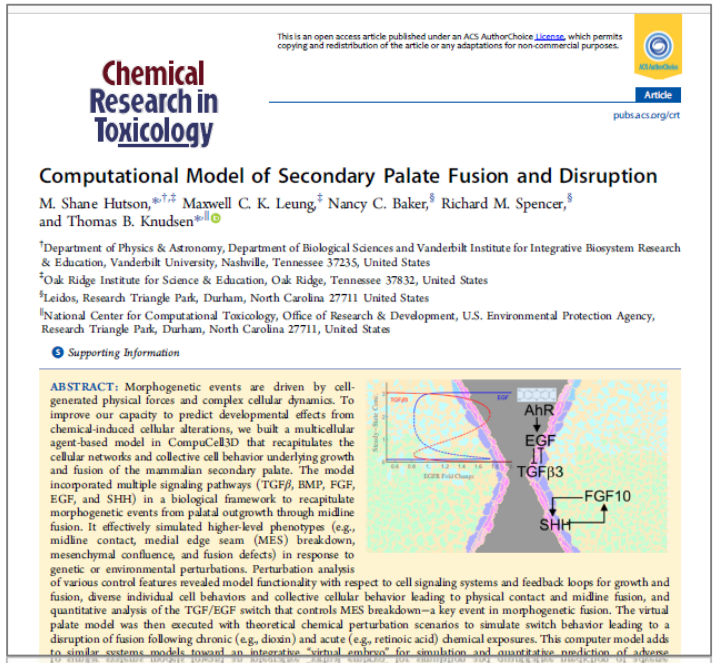


A.I. = synthetic cell signaling networks

Cybermorphs = simulated loss of function

SOURCE: Hutson et al. (2017) Chem Res Toxicol

Smart model …



Chemical Research in Toxicology

Article

pubs.acs.org/crt

Computational Model of Secondary Palate Fusion and Disruption

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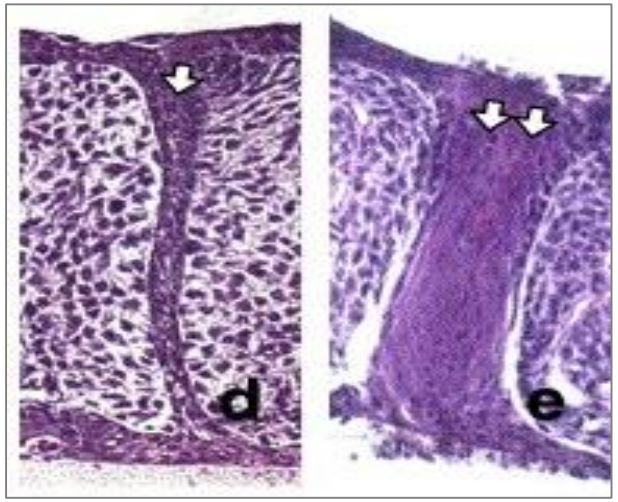
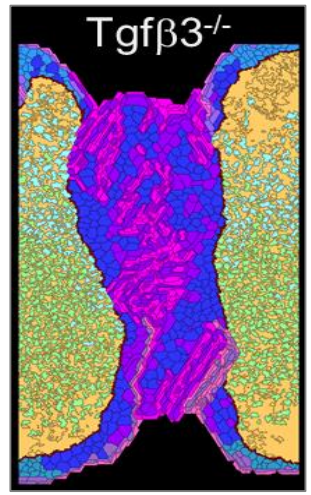
Supporting Information

ABSTRACT: Morphogenetic events are driven by cell-generated physical forces and complex cellular dynamics. To improve our capacity to predict developmental effects from chemical-induced cellular alterations, we built a multicellular agent-based model in CompuCell3D that recapitulates the cellular networks and collective cell behavior underlying growth and fusion of the mammalian secondary palate. The model incorporated multiple signaling pathways (TGFβ, BMP, FGF, EGF, and SHH) in a biological framework to recapitulate morphogenetic events from palatal outgrowth through midline fusion. It effectively simulated higher-level phenotypes (e.g., midline contact, medial edge seam (MES) breakdown, mesenchymal confluence, and fusion defects) in response to genetic or environmental perturbations. Perturbation analysis of various control features revealed model functionality with respect to cell signaling systems and feedback loops for growth and fusion, diverse individual cell behaviors and collective cellular behavior leading to physical contact and midline fusion, and quantitative analysis of the TGF/EGF switch that controls MES breakdown—a key event in morphogenetic fusion. The virtual palate model was then executed with theoretical chemical perturbation scenarios to simulate switch behavior leading to a disruption of fusion following chronic (e.g., dioxin) and acute (e.g., retinoic acid) chemical exposures. This computer model adds to similar systems models toward an integrative "virtual embryo" for simulation and quantitative prediction of adverse

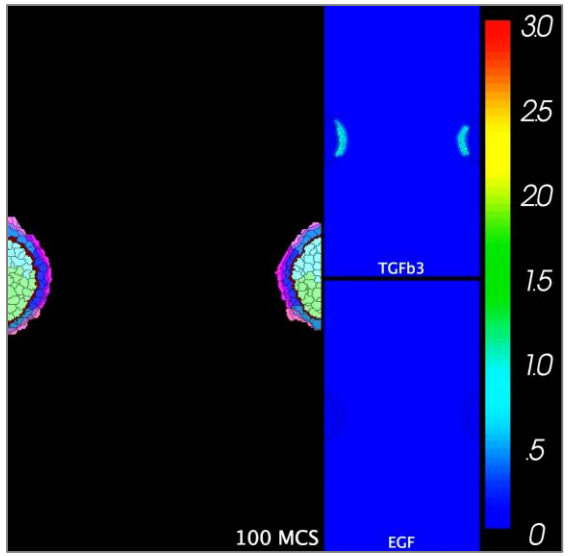
Reviewer Comment: *"Crucial mechanisms occurring during palate fusion, especially opposing palatal shelf adhesion, are not considered in the model. In fact, the main reason why Tgf-b3 KO mice have cleft palate is a failure of opposing MEE adhesion, leading to separation of palatal shelves after their initial contact. Even in those strains in which palatal shelves adhere partially, I have never seen a MES as the one shown in Fig. 5."*

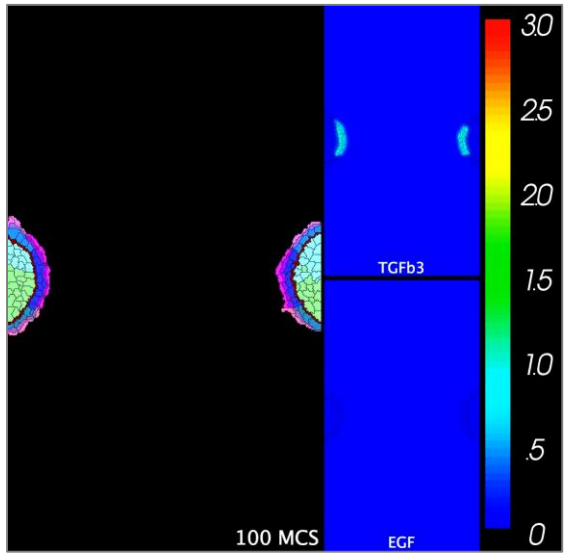
Our Response: TGF-b3 knockout mouse palates transduced with ALK vectors *in vitro. (from Dudas et al. 2004).*

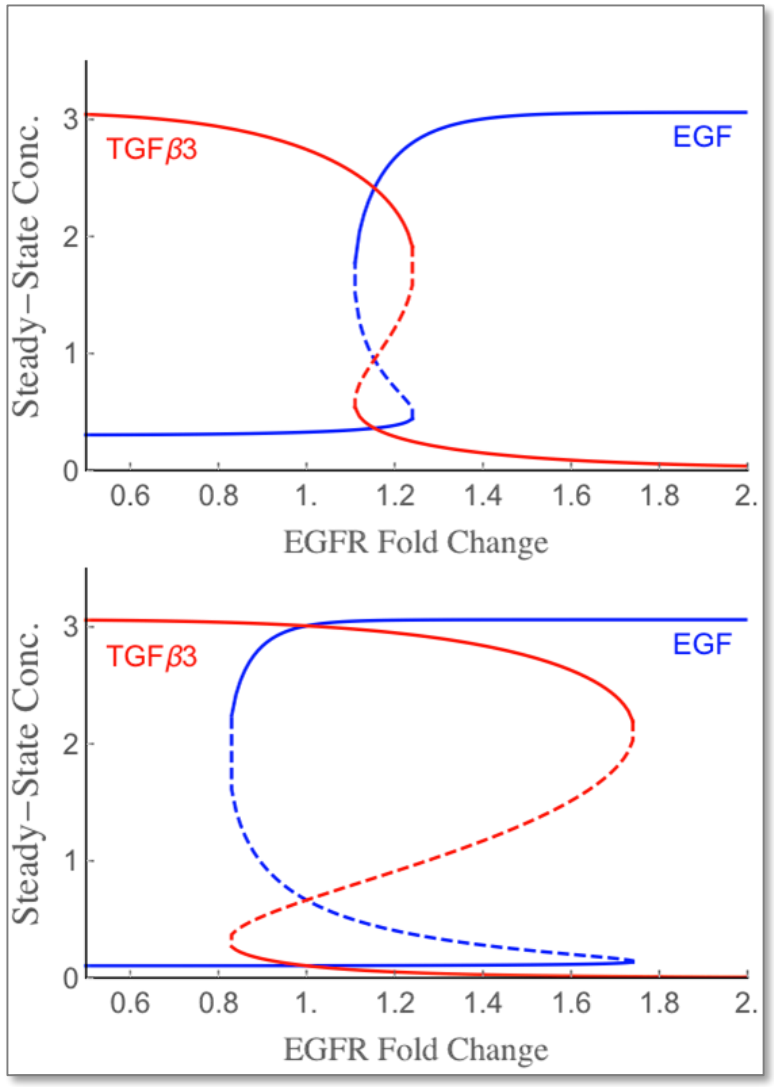


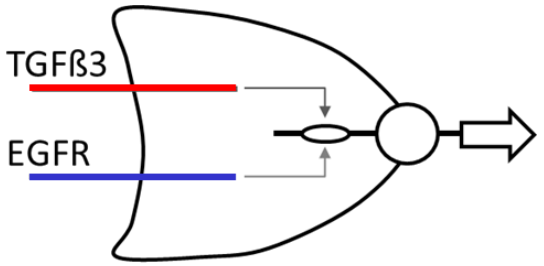


Messin' with the switch: *two scenarios for bistable switch dynamics*









1. Narrow hysteresis:
less resilient but reversible

2. Broad hysteresis:
more resilient but irreversible

ToxCast dataset

(-) ToxRefDB_DEV

(+) ToxRefDB_DEV

ChemicalName	FR_up(/	GFR_up(	1_dow	b1_dow	ToxRef(lo
Methylene bis(thiocyanate)	1.14	2.13	5.93	4.26	NEG
Zoxamide	14.22	1.85	17.37	9.69	NEG
2-(Thiocyanomethylthio)benzothiazole	2.28	1.54	6.48	7.21	NEG
Diphenylamine	32.71	1.49	5.95	1.63	NEG
Azamethiphos	0.89	1.81	1000.00	1000.00	NEG
Bromacil	20.50	1.57	1000.00	1000.00	NEG
Forchlorfenuron	0.02	1.53	1000.00	1000.00	NEG
Methyl isothiocyanate	4.60	1.44	1000.00	1000.00	NEG
Diuron	16.51	1.44	1000.00	1000.00	NEG
Rotenone	0.82	1.42	1000.00	1000.00	NEG
Captan	4.59	2.57	7.15	7.25	POS
Triflumizole	32.71	2.48	19.88	19.88	POS
Butachlor	32.71	2.47	17.85	17.85	POS
Captafol	1.02	2.20	3.76	3.25	POS
Thiram	4.45	1.96	6.95	5.38	POS
Raloxifene hydrochloride	12.40	1.91	15.94	10.94	POS
Fluazinam	2.39	1.61	2.48	4.84	POS
Carbaryl	0.07	1.55	1000.00	1000.00	POS
Linuron	10.91	1.46	1000.00	1000.00	POS
Maneb	0.01	1.46	1000.00	1000.00	POS
Bendiocarb	8.75	1.43	1000.00	1000.00	POS
Fipronil	1.18	1.43	1000.00	1000.00	POS
Propoxur	1.67	1.43	1000.00	1000.00	POS
TNP-470	7.78	1.57	3.97	3.61	x
1-(2,3,8,8-Tetramethyl-1,2,3,4,5,6,7,8-octal	8.33	2.10	9.74	1.88	x
Trimethylolpropane triacrylate	2.02	1.80	5.17	1.41	x
Diiodomethyl 4-methylphenyl sulfone	3.15	1.77	3.74	17.68	x
1,2-Benzisothiazolin-3-one	8.22	1.74	11.91	14.70	x
Tralopyril	18.30	1.68	0.87	1.08	x
Bis(trichloromethyl)sulfone	1.95	1.61	4.49	5.74	x
N,N,N-Trimethyloctadecan-1-aminium chl	2.22	1.56	1.77	1.45	x
beta-Nitrostyrene	7.12	1.52	2.01	2.34	x
4,5-Dichloro-3H-1,2-dithiol-3-one	2.71	1.47	6.42	6.56	x
Tri-o-cresyl phosphate	8.95	1.45	9.54	1.56	x
Isobornyl methacrylate	13.66	1.44	21.86	1.97	x
SAR102779	0.05	1.43	12.95	14.97	x
PharmaGSID_48511	12.19	1.37	11.22	17.33	x
Perfluoroundecanoic acid	6.81	1.35	4.76	5.04	x
FR167356	17.65	2.06	1000.00	1000.00	x
Monobutyl phthalate	0.01	1.35	1000.00	1000.00	x
Niclosamide	0.58	2.14	1000.00	1000.00	x
Tripropylene glycol diacrylate	26.52	2.09	1000.00	1000.00	x
CP-457920	3.50	1.92	1000.00	1000.00	x
Trimethylolpropane trimethacrylate	32.85	1.81	1000.00	1000.00	x
alpha-Terpinyl acetate	39.18	1.64	1000.00	1000.00	x
3-(4-tert-Butylphenyl)-2-methylpropanal	35.26	1.62	1000.00	1000.00	x
1,4-Dinitrobenzene	2.95	1.54	1000.00	1000.00	x
SB281832	34.72	1.54	1000.00	1000.00	x
2-(Morpholin-4-yldithio)-1,3-benzothiazol	5.61	1.52	1000.00	1000.00	x
Tolclofos-methyl	7.71	1.49	1000.00	1000.00	x
1,1':3',1''-Terphenyl	11.98	1.38	1000.00	1000.00	x
Estrone	0.03	1.35	1000.00	1000.00	x

	↑EGFR	↓TGFβ1		ToxRefDB		
	μM effect in vitro	AC50	top	AC50	top	DevTox
Captan		4.59	2.57	7.15	7.25	POS
Triflumizole		32.71	2.48	19.88	19.88	POS
Butachlor		32.71	2.47	17.85	17.85	POS
Captafol		1.02	2.20	3.76	3.25	POS
Thiram		4.45	1.96	6.95	5.38	POS
Raloxifene hydrochloride		12.40	1.91	15.94	10.94	POS
Fluazinam		2.39	1.61	2.48	4.84	POS

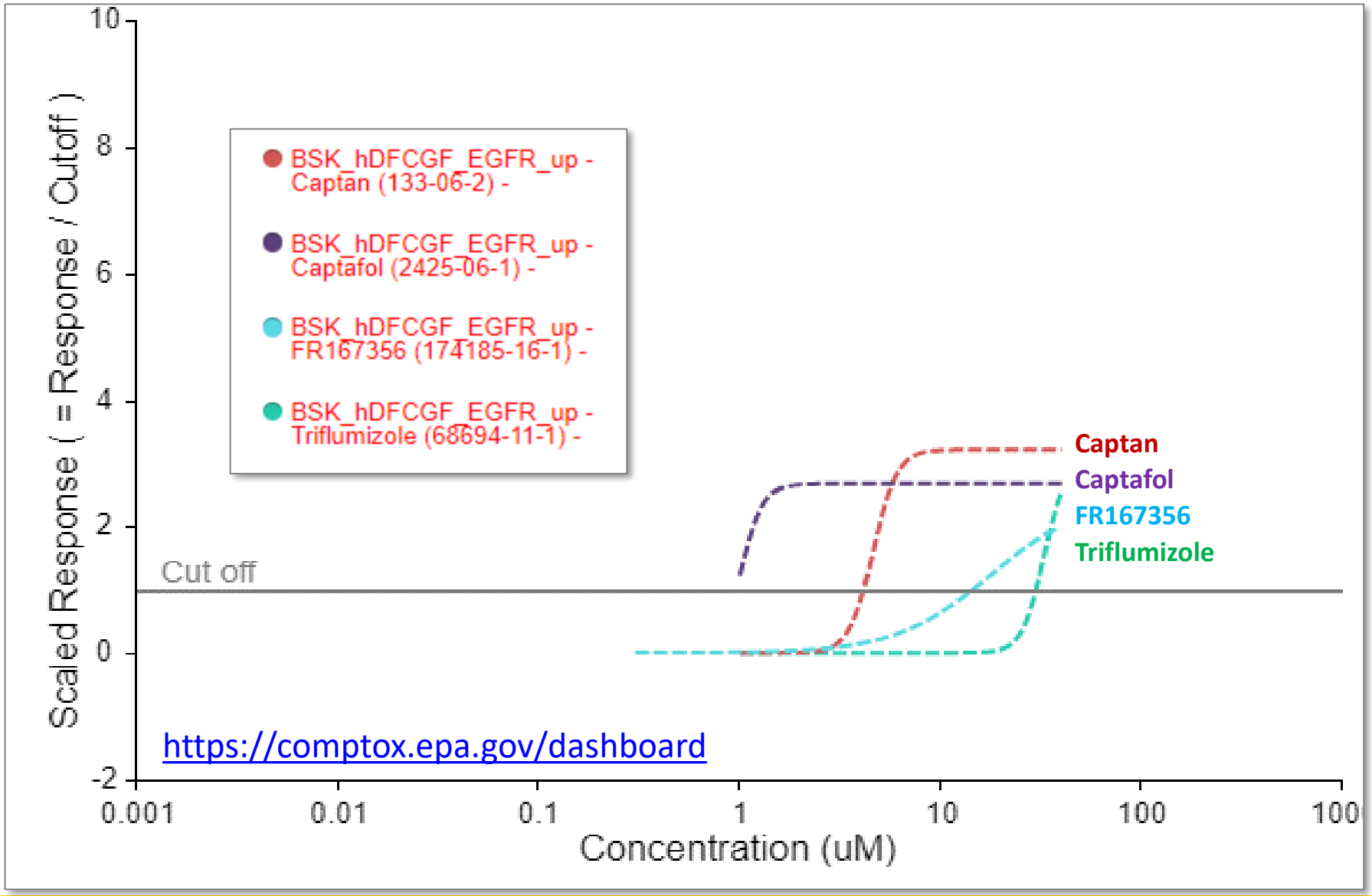
- *54 chemicals ↑EGFR density*
- *some also ↓TGF-beta signaling*

negative for developmental toxicity in ToxRefDB

positive for developmental toxicity in ToxRefDB

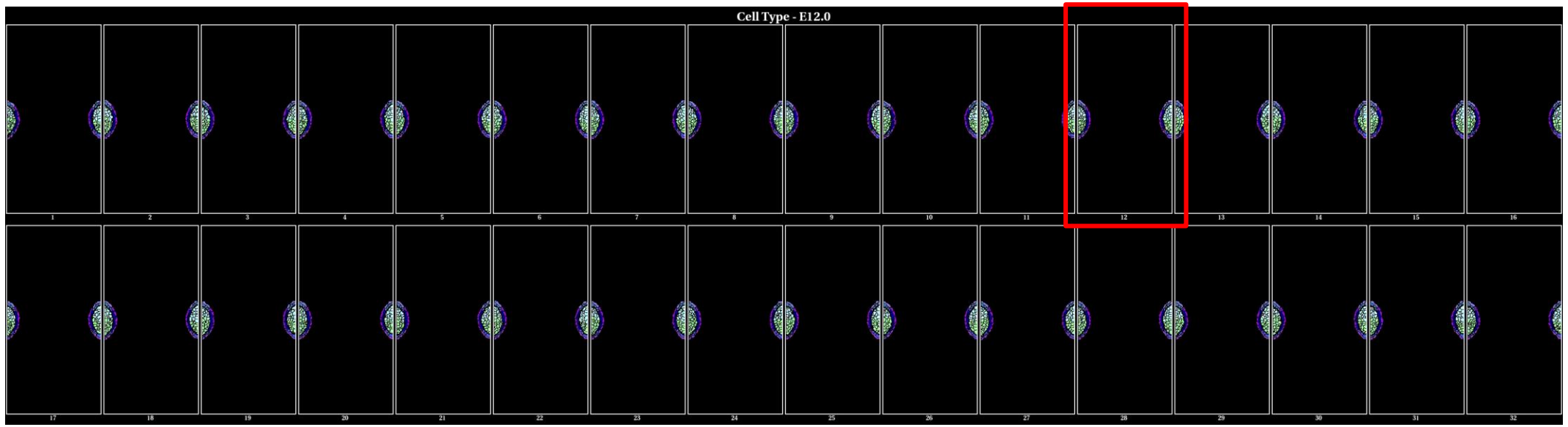
no developmental toxicity data in ToxRefDB

EGFR signaling: ↑ immunoreactivity relative to DMSO

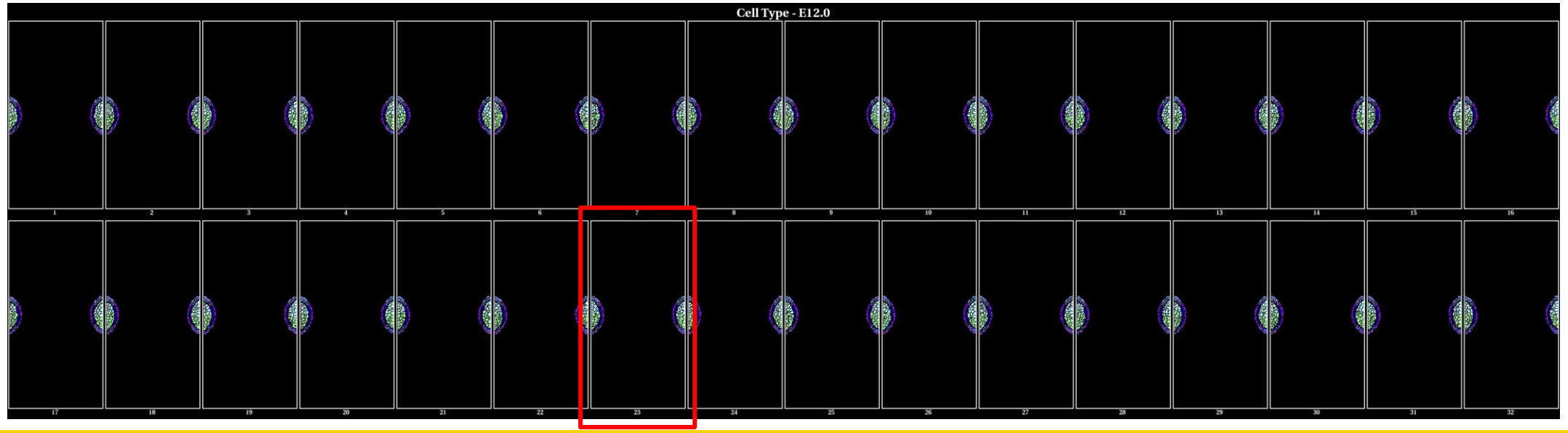


In silico dose-response: ↑*EGFR conc. response in topological context*

Captan

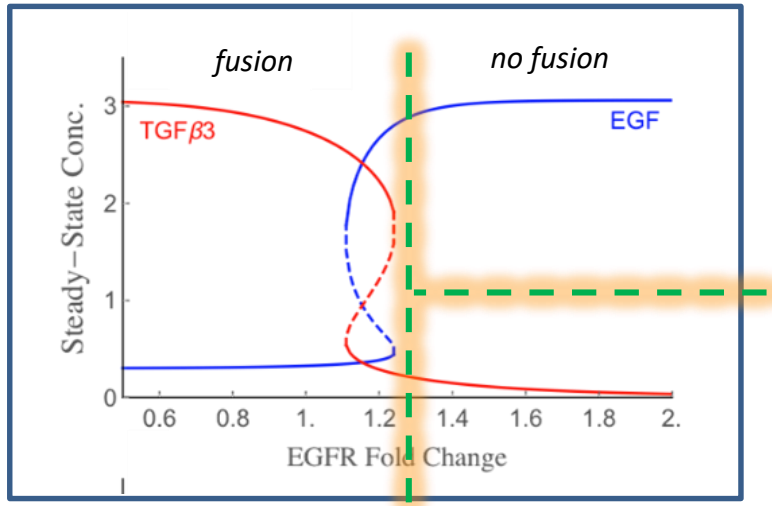


FR167356



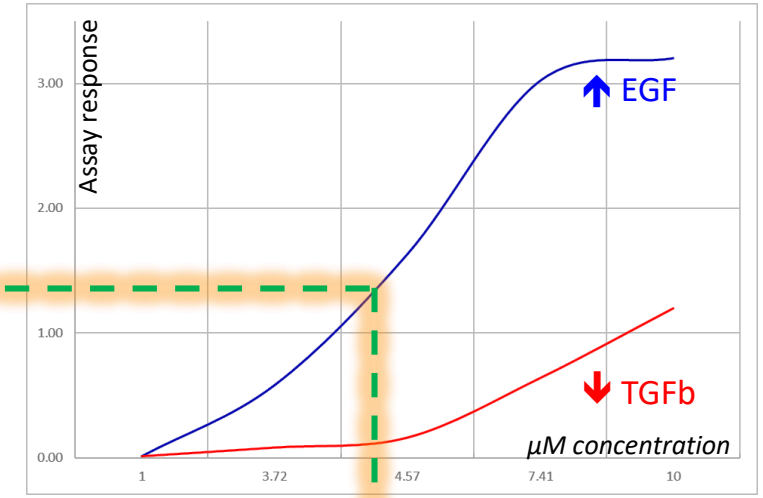
Predictive model: critical phenomenon

INPUT: switch dynamics



tipping point predicted by computational dynamics (hysteresis switch)

Captan in ToxCast



OUTPUT: tipping point mapped to concentration response (4 µM)

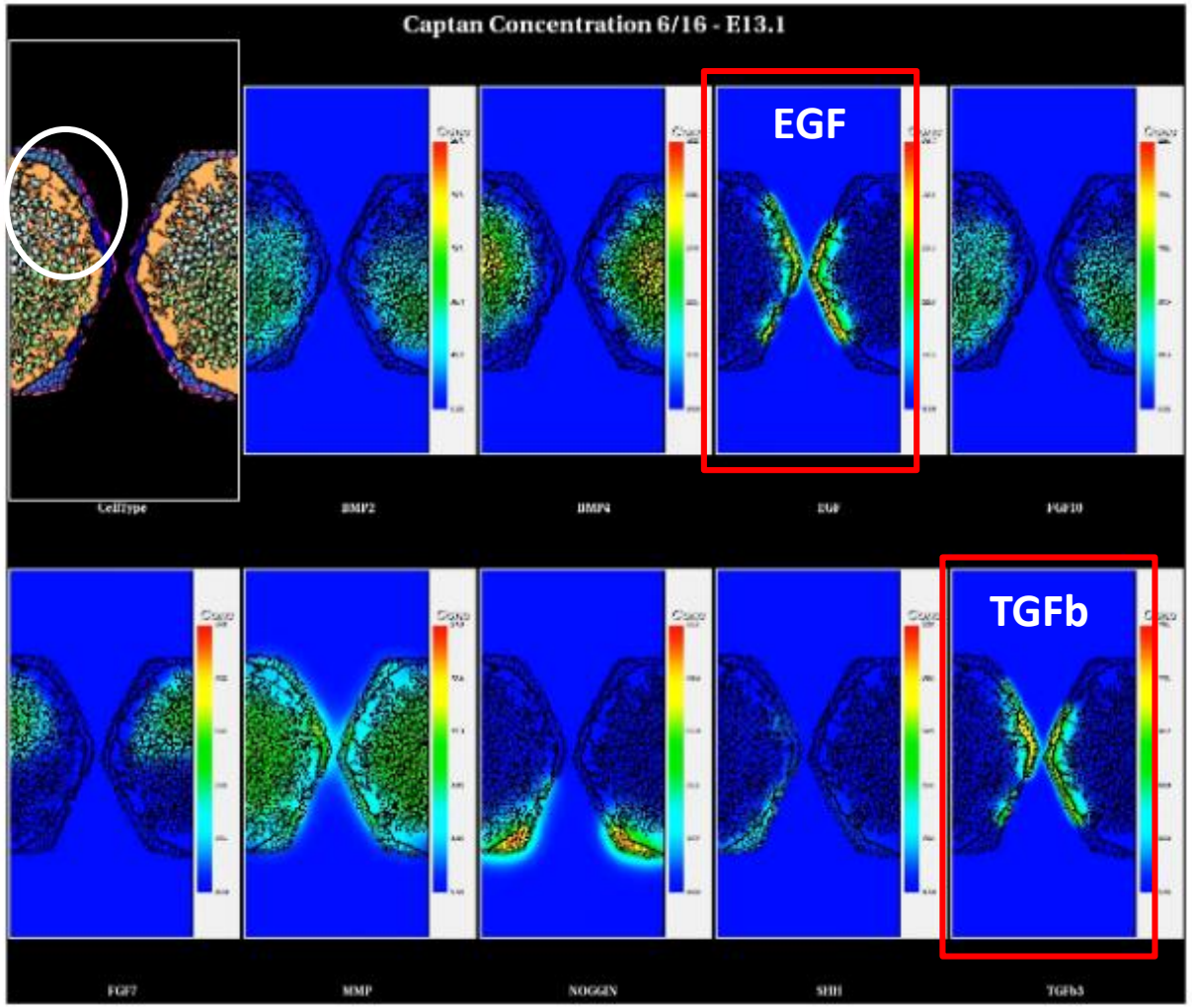
Captan in ToxRefDB
NOAEL = 10 mg/kg/day
LOAEL = 30 mg/kg/day

human HTTK model
2.39 mg/kg/day would achieve a steady state of 4 µM in fetal plasma

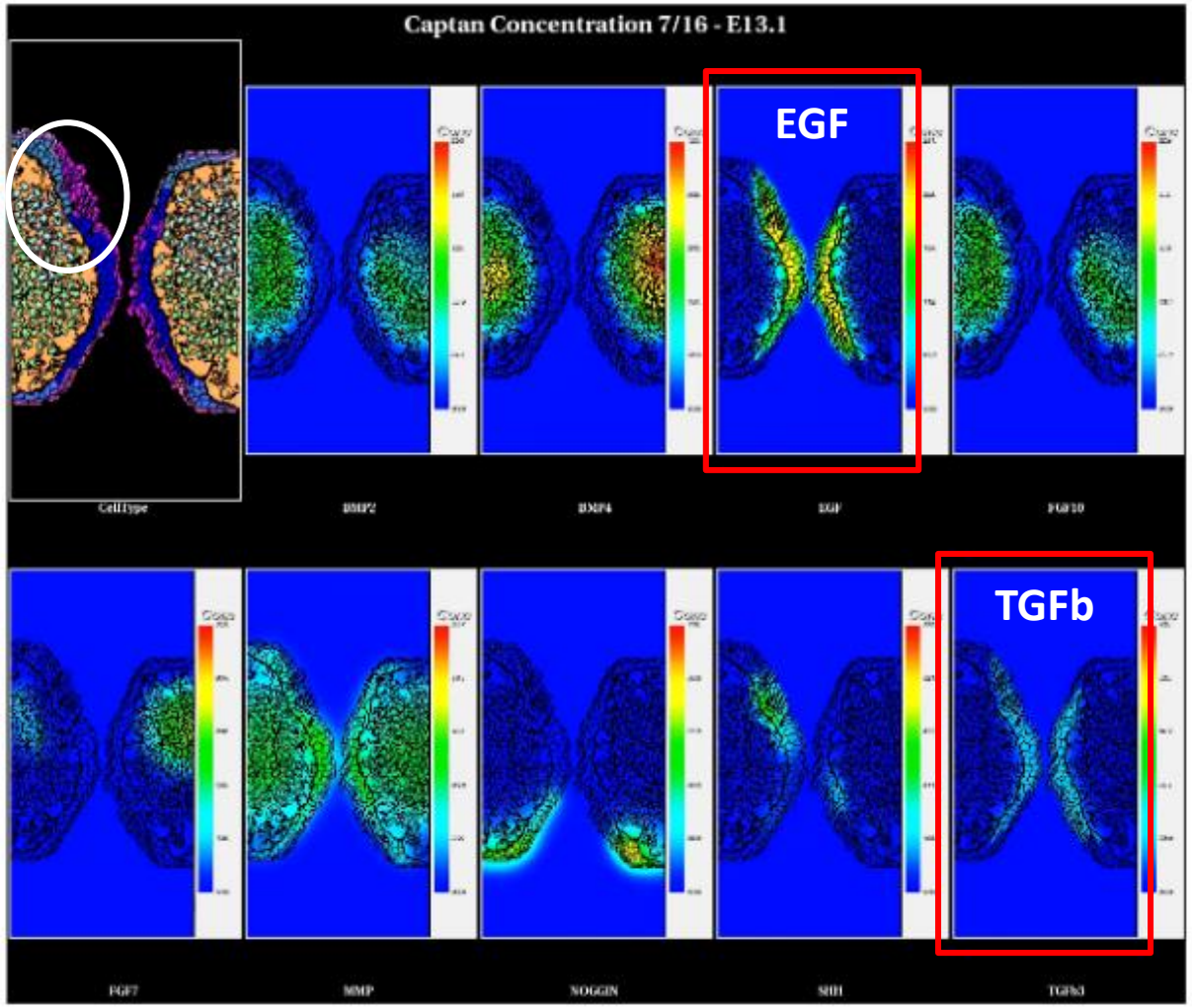
CompTox exposure prediction
0.88×10^{-7} mg/kg/day

Pathogenesis: simulating the prefusion alterations

pre-critical dose



post-critical dose



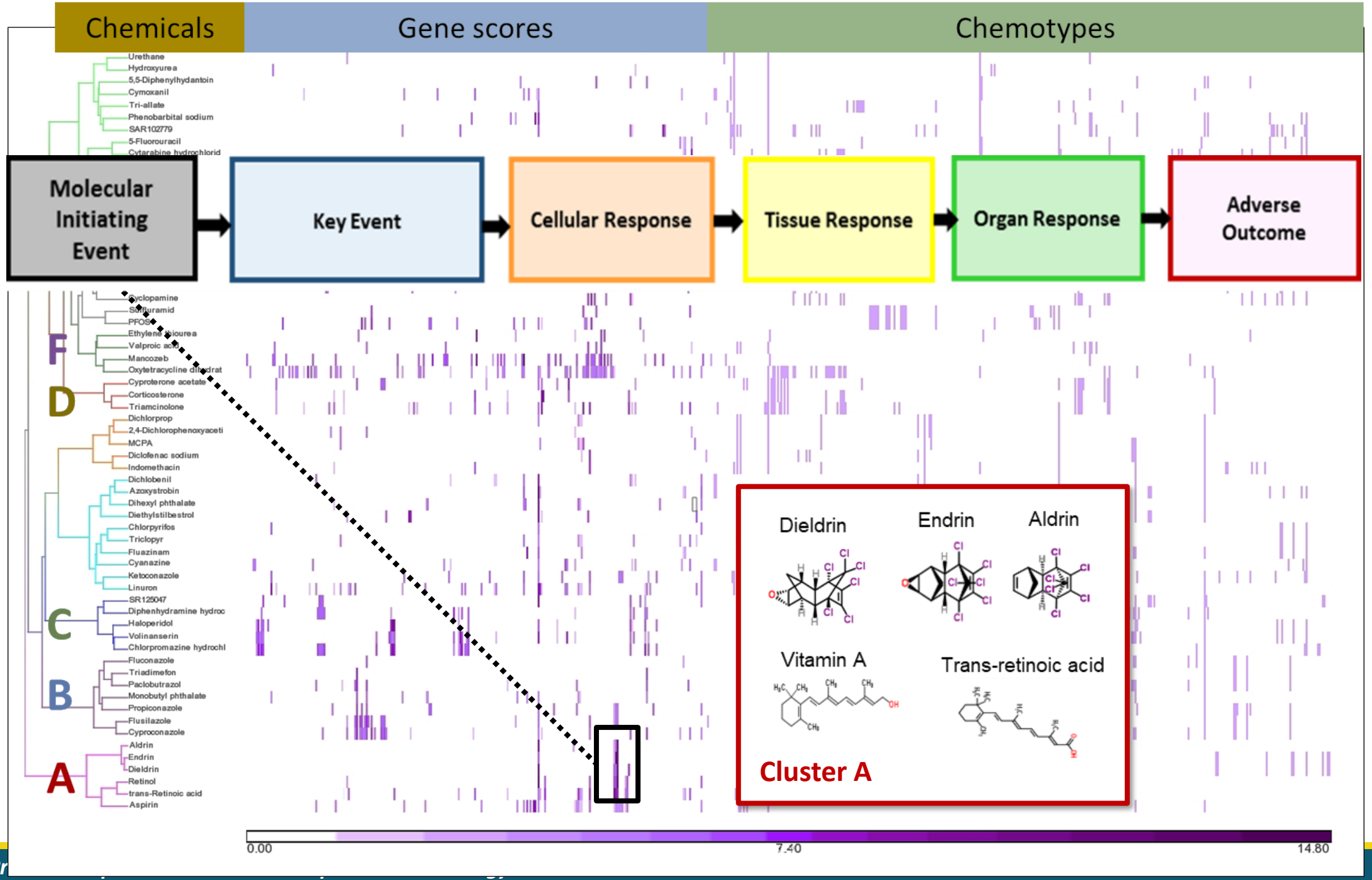
Cleft palate: multiple mechanisms inferred from ToxCast

ToxCast Chemicals summarized by ToxCast gene score and chemotype for machine-learning

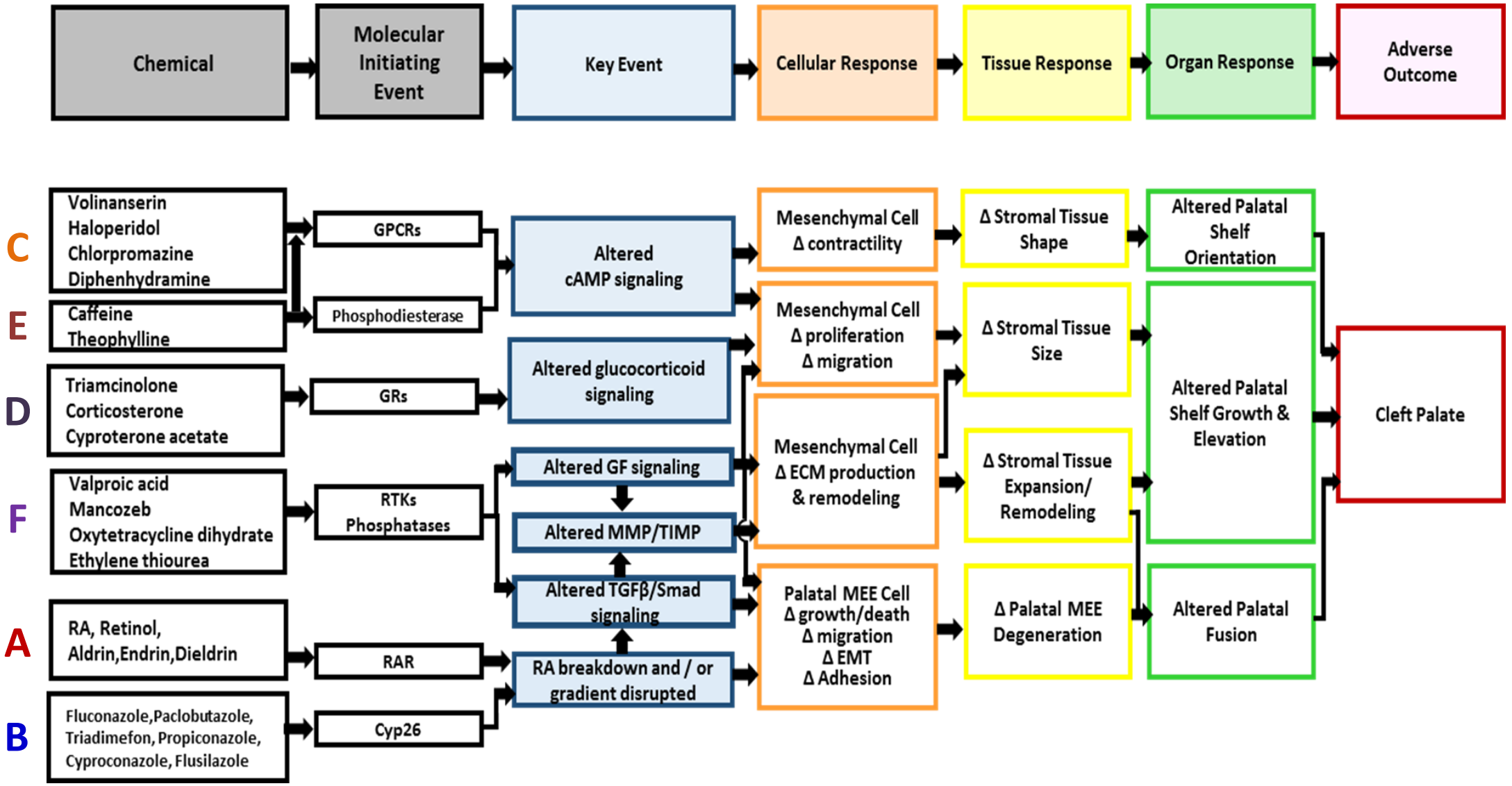
Animal studies 63 of 500 chemicals associated with cleft palate in ToxRefDB or biomedical literature

AOP clusters 6 mechanistic pathways inferred from integration of HTS data with chemical structure.

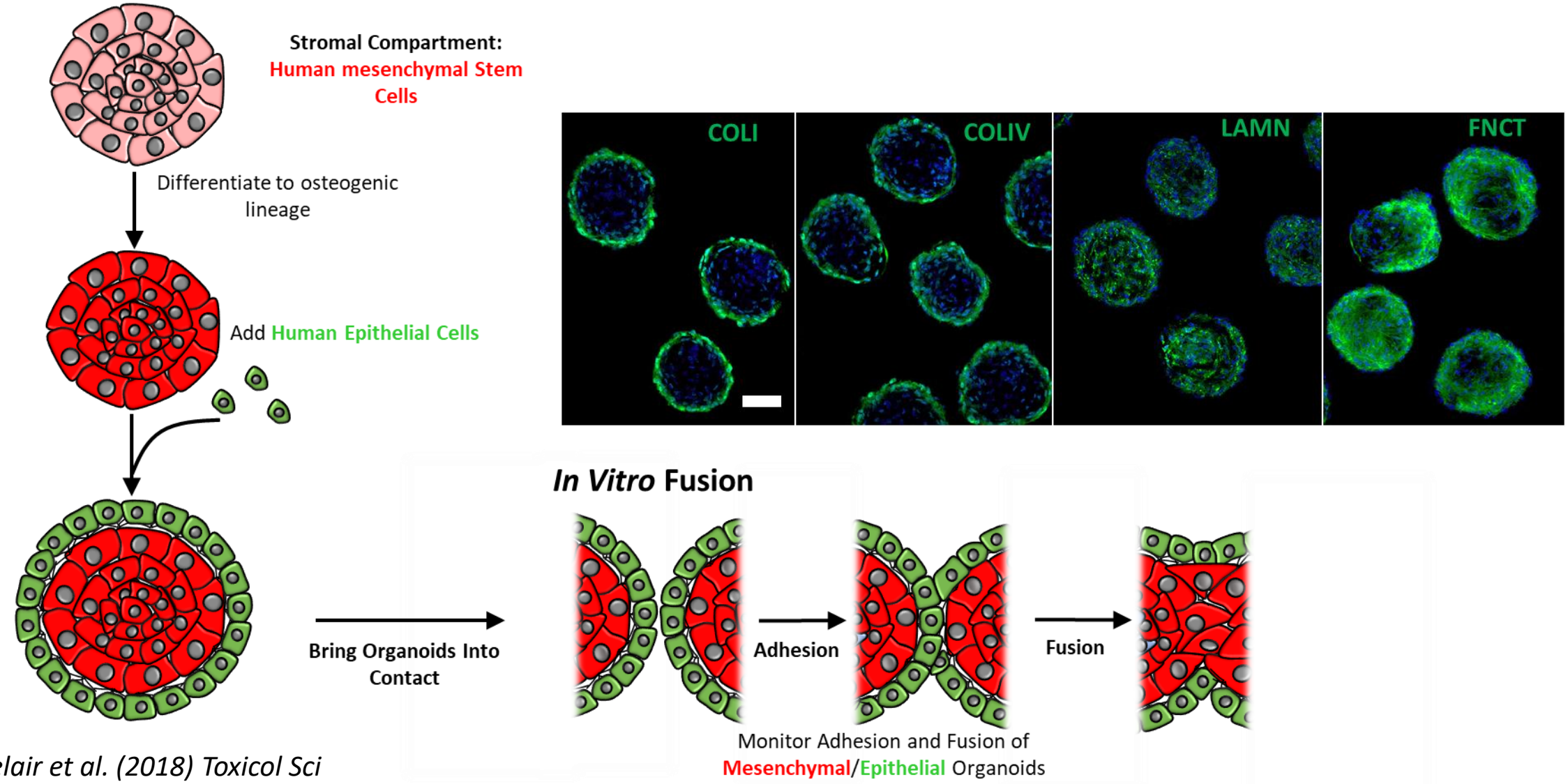
SOURCE: Baker et al. (manuscript)


Chemicals
Gene scores
Chemotypes
Molecular Initiating Event
Key Event
Cellular Response
Tissue Response
Organ Response
Adverse Outcome
Urethane
Hydroxyurea
5,5-Diphenylhydantoin
Cymoxanil
Tri-allate
Phenobarbital sodium
SAR102779
5-Fluorouracil
Cytarabine hydrochlorid
Cyclopamine
Sulfuramid
PFOS
Ethylenethiourea
Valproic acid
Mancozeb
Oxytetracycline dihydrat
Cyproterone acetate
Corticosterone
Triamcinolone
Dichlorprop
2,4-Dichlorophenoxyaceti
MCPA
Diclofenac sodium
Indomethacin
Dichlobenil
Azoxystrobin
Dihexyl phthalate
Diethylstilbestrol
Chlorpyrifos
Triclopyr
Fluazinam
Cyanazine
Ketoconazole
Linuron
SR125047
Diphenhydramine hydroc
Haloperidol
Volinanserin
Chlorpromazine hydrochl
Fluconazole
Triadimefon
Paclobutrazol
Monobutyl phthalate
Propiconazole
Flusilazole
Cyproconazole
Aldrin
Endrin
Dieldrin
Retinol
trans-Retinoic acid
Aspirin
F
D
C
B
A
Dieldrin
Endrin
Aldrin
Vitamin A
Trans-retinoic acid
Cluster A
0.00
7.40
14.80

AOP clusters: inferred from chemical structure-bioactivity profiles

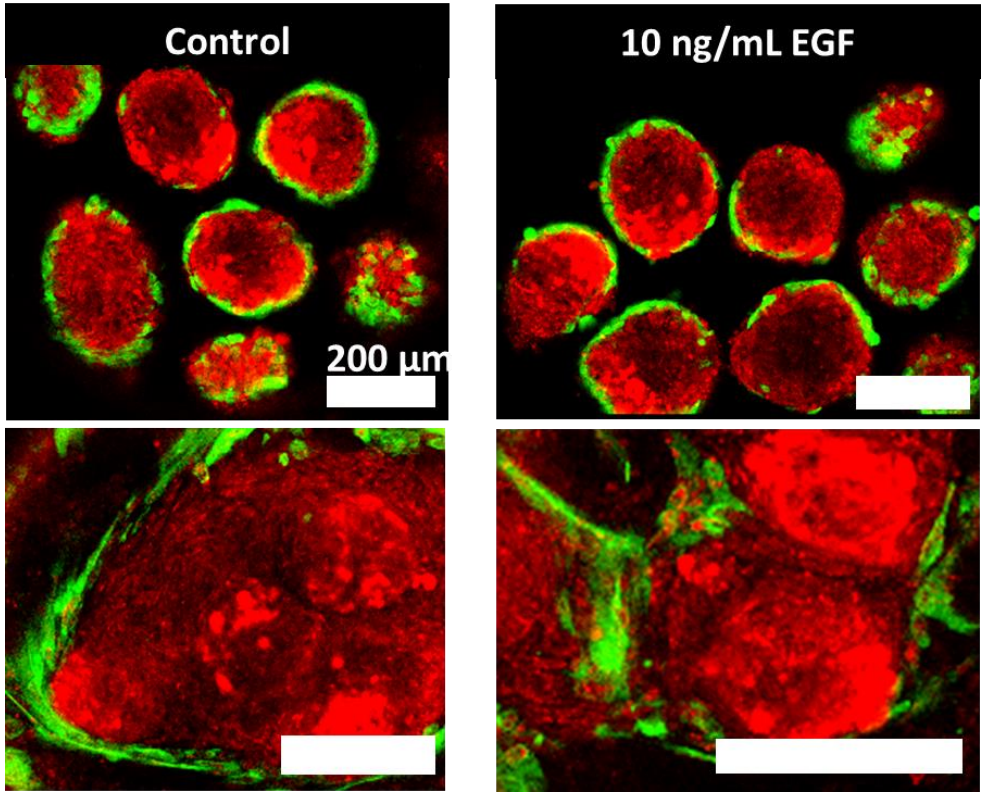


Fusion-competent organoids



SOURCE: Belair et al. (2018) Toxicol Sci

Fusion is delayed by excessive EGF Signaling

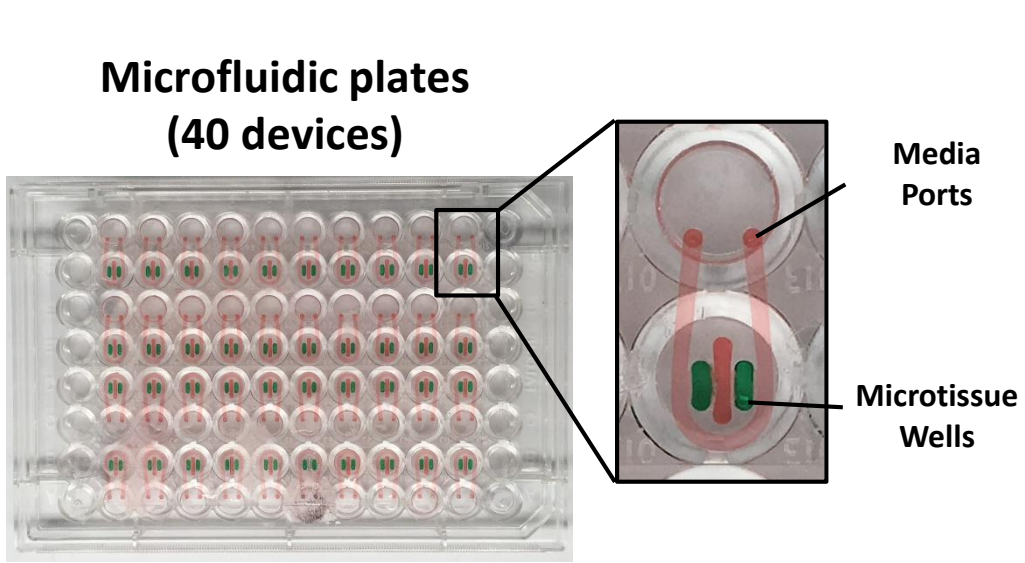


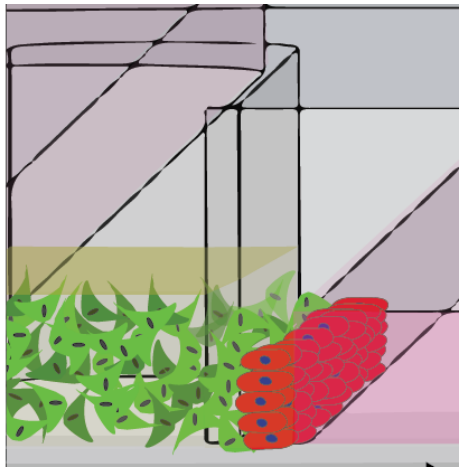
Chemical	Perturbation Days 0-2	Perturbation Days 0-4	Cytotoxicity
TCDD			
All-Trans Retinoic Acid (ATRA)		✓	✓
Dexamethasone			
Corticosterone			
Triamcinolone		✓	
Valproic Acid	✓		
Fluconazole			
Caffeine			
Nicotine			
Tributyltin	✓	✓	✓
Triadimefon			
Theophylline	✓		

- 2,400 spheroids per batch (120 wells per week) to assess tissue fusion in a human cell-based system.
- Process is sensitive to pharma compounds acting on various pathways (EGF, IGF, FGF, HGF, BMP);
- Sensitive to chemicals (ATRA, TBT, VPA, Theophylline, Triamcinolone) via viability or epithelial migration.

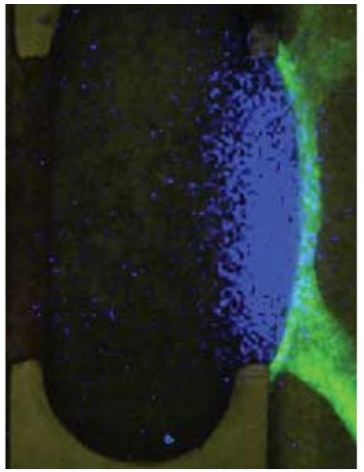
SOURCE: Belair et al. (2018) Toxicol Sci

Microphysiological system: reverse-engineering E/M interactions during outgrowth

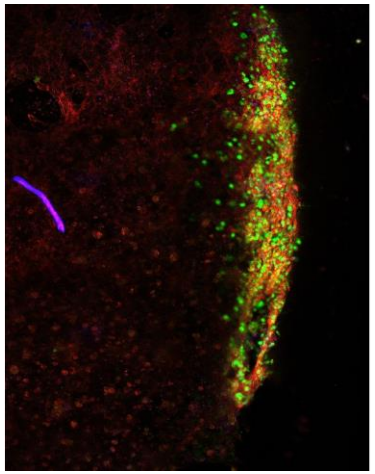




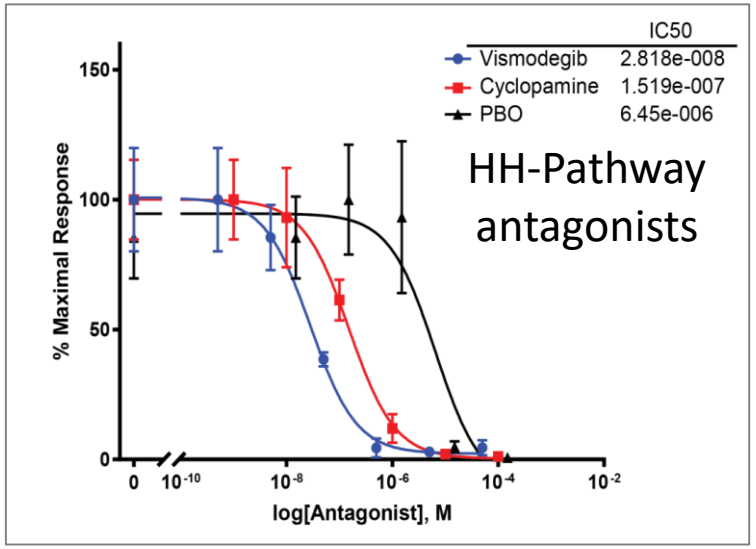
Gli-luciferase



Ki67 proliferation

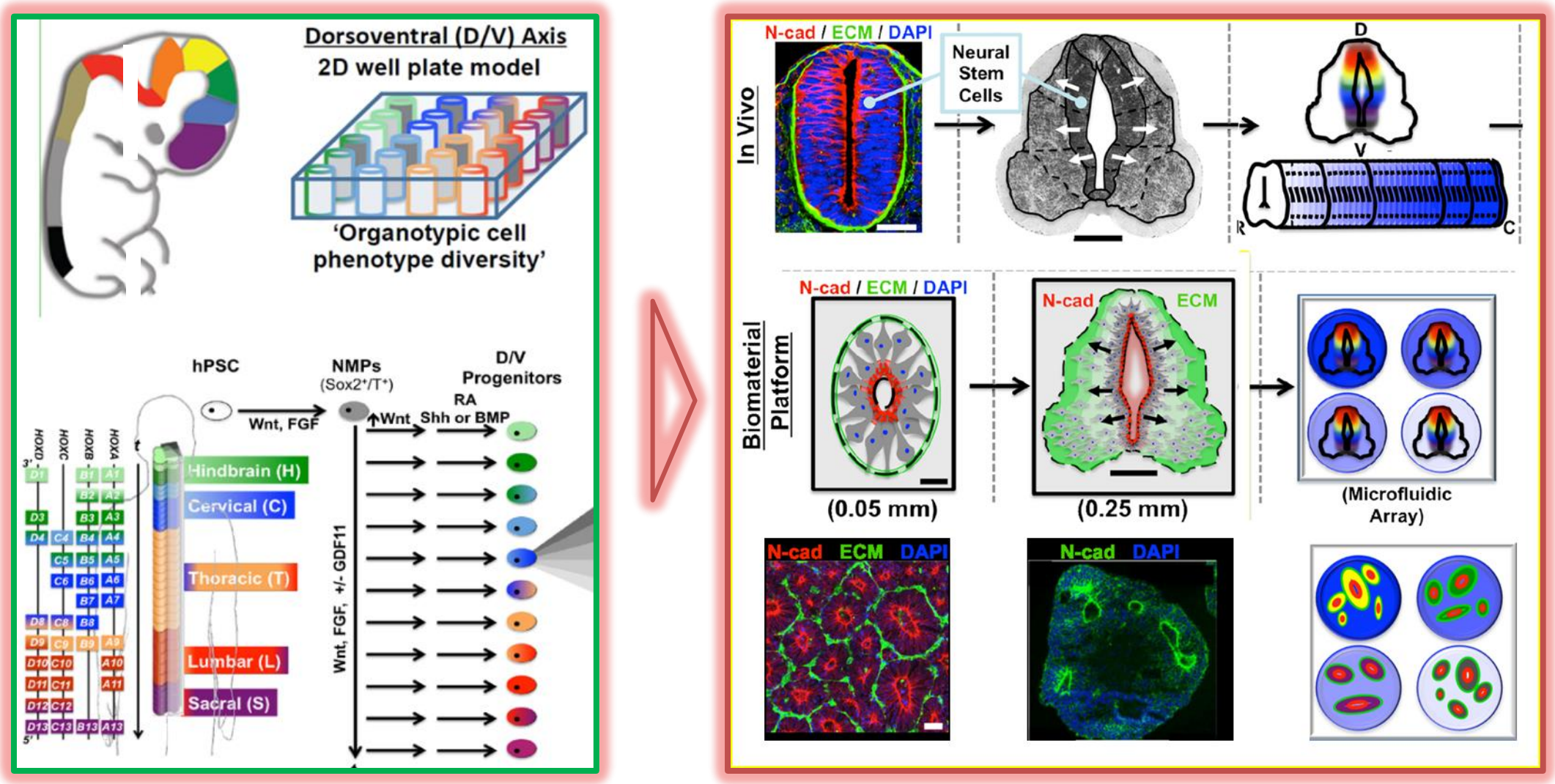


- 3D epithelial / mesenchymal organization
- SHH gradient directs Gli1-outgrowth
- HTS and HCI amenable
- fluorescent and luminescent readouts



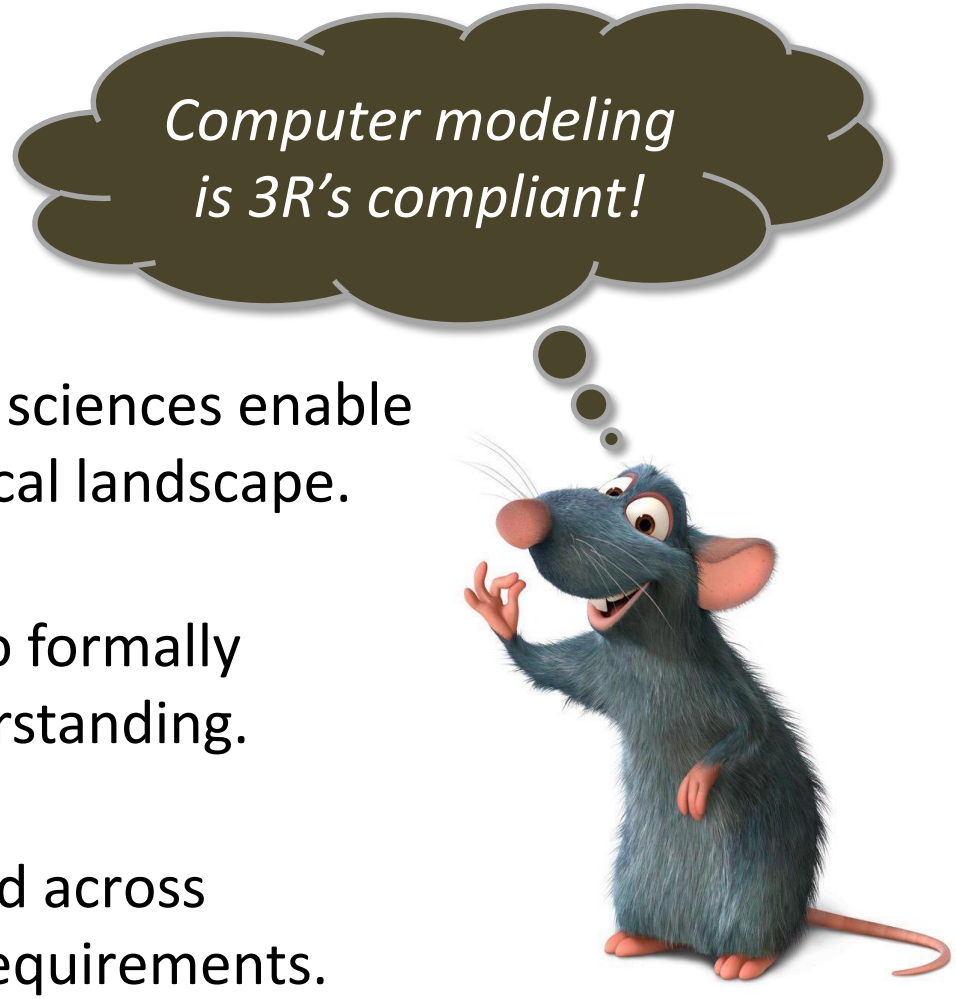
SOURCE: Brian Johnson, U Wisconsin (HMAPS)

Micropatterning: regionally-diverse stem cell arrays for the human neural tube



SOURCE: Randolph Ashton, U Wisconsin H-MAPS Center [Lippman et al. 2015; Knight and Ashton 2015]

In a nutshell …



https://www.pinterest.com/courtney1882/disney-ratatouille/

- Advances in biomedical, engineering, and computational sciences enable high-throughput screening (HTS) to profile the toxicological landscape.
- Surfeit of HTS data now in hand, a practical need arises to formally translate this information into actionable biological understanding.
- Information must be collected, organized, and assimilated across multiple levels of biological organization to meet these requirements.
- Computational systems and human organoids models are uniquely positioned to help shift decision-making to mechanistic prediction.

Pondering the way forward …

Translational: what do synthetic models of human development - both computational and organoids - bring to future of DART testing?

Investigational: how smart must these models be (A.I.) to support decision-making in the animal-free (3Rs) zone?

Operational: what best practices are needed to implement synthetic models into an integrative decision framework (eg, AOP-based IATAs)?

Communication: what are the practical considerations for science, engineering, and stakeholder engagement (academics, government, industry, NGOs, policy, …)?

National Center for Computational Toxicology

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- James Glazier – Indiana U
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- David Belair – NHEERL/TAD
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- Nicole Kleinstreuer (now NTP/NICEATM)
- Shane Hutson – Vanderbilt U
- Bill Murphy – U Wisconsin
- Brian Johnson – U Wisconsin

EPA STAR OCM-PT Centers

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- Bill Murphy – U Wisconsin (H-MAPS)
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- Ivan Rusyn – Texas A&M U (CT-AOP)