



EPA's Computational Toxicology Community of Practice

October 22, 2020

11:00 am – noon EST

Celebrating Children's Health Month

Researching Developmental Toxicity Models

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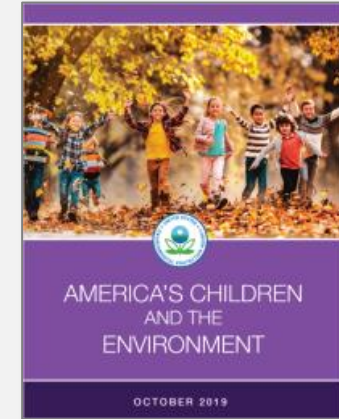
ORCID 0000-0002-5036-596x

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

October is Children's Health Month:

Raising Children's Environmental Health Awareness

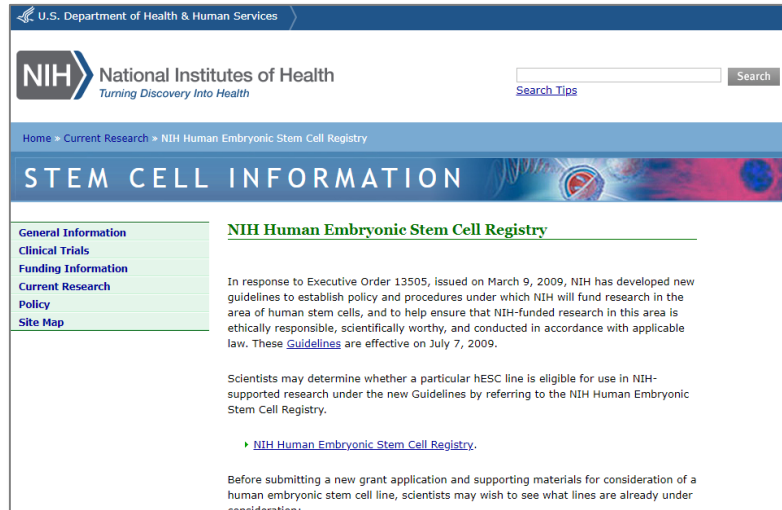
- Children's health outcomes result from multiple causes:
 - genetics, nutrition, socioeconomic factors
 - prenatal and childhood exposure to environmental factors.
- Adverse birth outcomes:
 - preterm birth, low birth weight, birth defects, infant mortality
 - USA occurrences: ~3% birth defects and 11% low birth weight.
- Assessing developmental toxicity during pregnancy:
 - critical role in setting health and environmental policy
 - teratogenesis is a major concern in animal models of human relevance (e.g., pregnant rats, rabbits).
- New Approach Methodologies (NAMs):
 - shifting developmental hazard detection to *in vitro* data, *in silico* models, and biological pathways
 - embryonic stem cell (ESC) lines are among the most promising alternatives to pregnant animal testing.



<https://www.epa.gov/children/>

- Respiratory diseases
- Childhood cancer
- Neurodevelopmental disorders
- Obesity
- Adverse birth outcomes

Disclosure: *research on pluripotent stem cell lines*



<https://stemcells.nih.gov/research/registry.htm>

Compliance: All work involving human embryonic stem (hES) cell lines is compliant with Executive Order 13505 (issued 2009) to ensure that is ethically responsible, scientifically worthy, and conducted in accordance with applicable law.

Funding: This research was performed under EPA's *Chemical Safety for Sustainability Research Program, Research Area 5 'Virtual Tissue Models' (VTMs)*.

Our hESC cell lines are registered in the NIH Human Embryonic Stem Cell Registry:

WA09 (H9): NIH Approval Number: NIHhESC-10-0062

RUES2: NIH Approval Number: NIHhESC-09-0013



Stemina Biomarker Discovery
EPA contract EP-D-13-055

Other pluripotent stem cell lines:

Episomal hiPSC Line (Gibco-A18945)

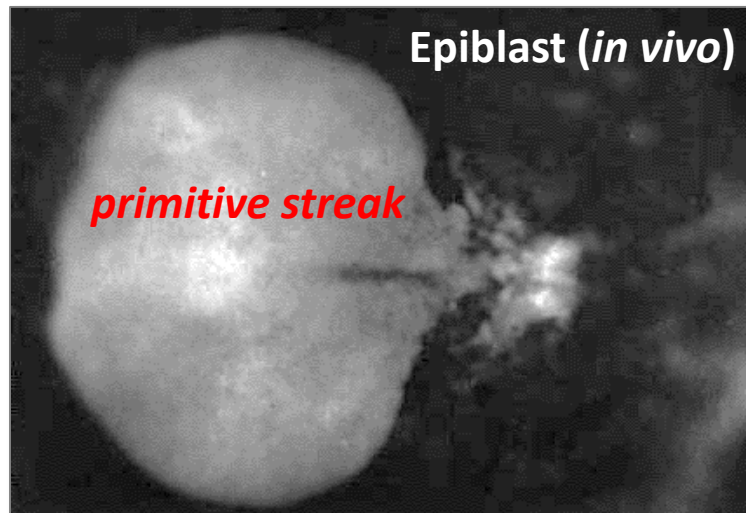
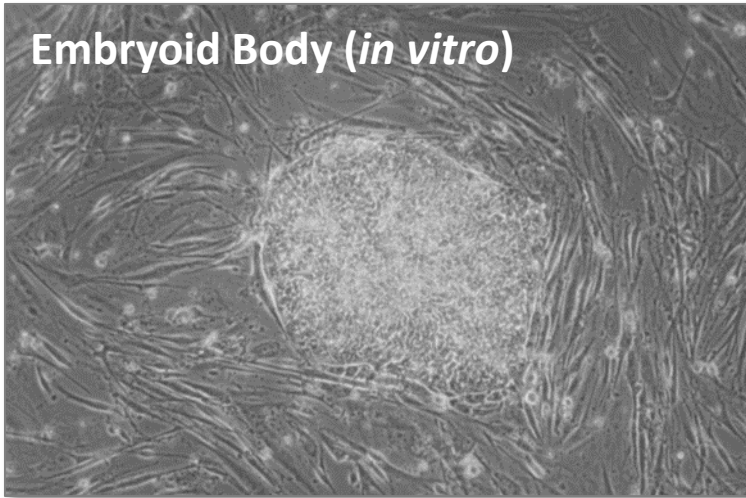
Endodermal hiPSC line (Allele Biotech #ABPSC-HDFAIPS)

J1 mouse ESC line (ATCC® SCRC-1010™)



Vala Sciences, Inc.
EPA contract EP-D-13-054

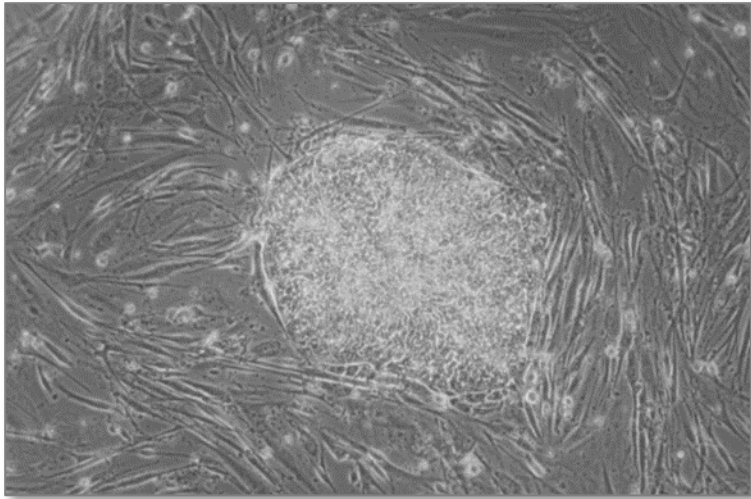
Novel features of pluripotent hESC lines:



- **Self-renewal:** cells proliferate and replicate themselves indefinitely when cultured under appropriate conditions.
- **Differentiation:** maintain the potential to:
 - i) form most cells of the embryo/fetus (pluripotency)
 - ii) self-organize into rudimentary structures (autopoiesis).
- **Embryology:** hESC monolayers recapitulate the 'epiblast' of an early embryo (GD 5-7 mouse, week 2-3 human).

Pluripotent ESC lines capture many key features of embryogenesis that are uniquely covered in guideline prenatal developmental toxicity studies.
(e.g., OECD TG 414, organogenesis in pregnant rat/rabbit)

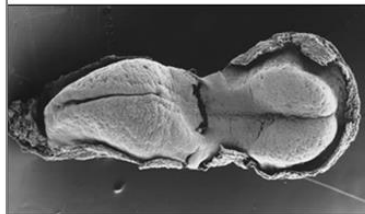
Will a primitive hESC type live up to the challenge of NAMs?



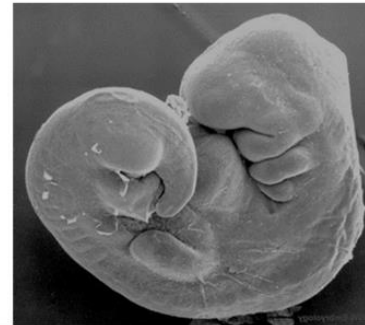
?

TIMELINE OF THE HUMAN EMBRYONIC PERIOD

Week 3
(Carnegie Stage 8)



Week 4
(Carnegie Stage 13)



Week 8
(Carnegie Stage 20)



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

- does not encompass the full complexity of anatomical development;
- blind to the precise spatial-temporal control of cell-cell interactions *in vivo* ;
- misses developmental effects secondary to maternal or placental toxicity;
- uncertainty of post-organogenesis vulnerability and post-natal manifestations;
- cross-species extrapolation (mESC to human, hESC to animals);
- limited xenobiotic metabolism and other ADME considerations (toxicokinetics);
- uncertainties in translatability to the intact embryo (toxicodynamics).

A lot of work has been done using mESC and hESC lines *in vitro*

Detailed literature review of conceptual and practical considerations that must be given for readiness of mESC and hESC data in regulatory toxicology (e.g, NAMs).

- chemical domain
- readout endpoints
- standardized protocols
- reproducibility
- biological domain
- predictive power

PLURIPOTENT STEM CELL ASSAYS: MODALITIES AND APPLICATIONS FOR PREDICTIVE DEVELOPMENTAL TOXICITY

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

SUMMARY

I. GENERAL INTRODUCTION

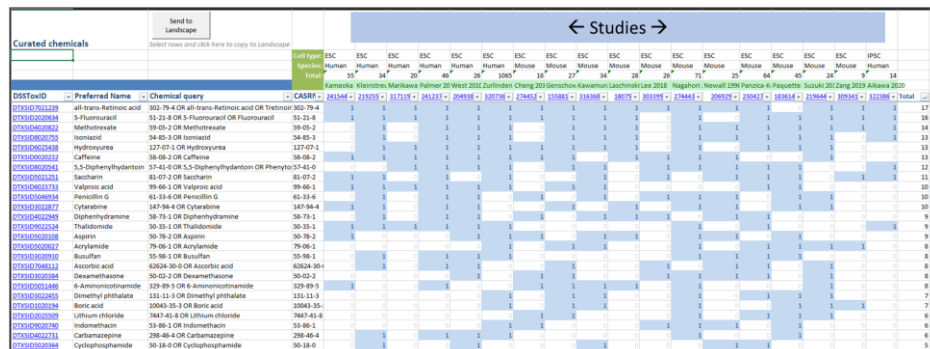
- Regulatory test guidelines for developmental toxicity
- The embryonic stem cell test as an alternative test in developmental toxicology
- A systematic scoping review of embryonic stem cell tests
- The application of EST within the regulatory perspective

II. THE PRINCIPLES OF DEVELOPMENTAL TOXICOLOGY

- Apical endpoints

ESC testing data available in the open literature

1,533 PubMed → 333 (AI relevance) → curation



The screenshot shows a dashboard with a table of chemicals and their testing results. The table has columns for Chemical Name, CAS#, and various study identifiers (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18). The chemicals listed include all-trans-retinoic acid, 5-fluorouracil, Methotrexate, Isotretinoin, Hydroxyurea, Caffeine, 5,3-Dihydroxyflavone, Saccharin, Valproic acid, Penicillin G, Cytarabine, Diphenylhydantoin, Thalidomide, Aspirin, Acetaminophen, Butoxifen, Acetic acid, Dexamethasone, 6-Aminocaproic acid, Dimethyl phthalate, Boric acid, Lithium chloride, Indomethacin, Carbamazepine, and Cyclophosphamide.

- **1,250 annotated chemicals:**
18 publications tested ≥ 10 compounds (primary)
174 publications tested 1-9 (evidentiary support)
- **Most overlapping compounds:**
all-trans retinoic acid (17), 5-fluorouracil (16),
and methotrexate (14) - all strong teratogens.
- **Most common use categories:**
pharma: anti-infective agents, enzyme inhibitors
chemical: pesticides.


N Baker, K Tsaion: Abstract Sifter, SWIFT,
MeSH terms, Chemicals Dashboard, ...
working in unison

Now includes ToxCast_STM dataset

devTOX^{qP} assay: *Stemina Biomarker Discovery, EPA contract EP-D-13-055*


Birth Defects Research Part B

Developmental and Reproductive Toxicology


Explore this journal >

Original Article


Establishment and Assessment of a New Human Embryonic Stem Cell-Based Biomarker Assay for Developmental Toxicity Screening

Jessica A. Palmer , Alan M. Smith, Laura A. Egnash, Kevin R. Conard, Paul R. West, Robert E. Burrier, Elizabeth L.R. Donley, Fred R. Kirchner

First published: August 2013 [Full publication history](#)


DOI: 10.1002/bdrb.21078 [View/save citation](#)

Cited by (CrossRef): 18 articles [Check for updates](#) [Citation tools](#)



View issue TOC
Volume 98, Issue 4
August 2013
Pages 343–363

Pluripotent H9 human embryonic stem cell metabolomics assay that “... identified the potential developmental toxicants in the test set with 77% accuracy (57% sensitivity, 100% specificity).”

Palmer et al. (2013) Birth Defects Res

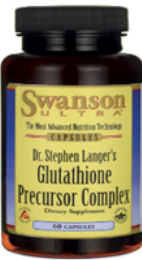


Ornithine release
urea cycle, polyamine &
pyrimidine synthesis.



TI = ORN/CYSS

Cystine utilization
glutathione synthesis,
redox cycling.



Using the H9 devTOX^{qP} platform to profile the ToxCast library

OXFORD SOT Society of Toxicology
academic.oup.com/toxsci

TOXICOLOGICAL SCIENCES, 174(2), 2020, 189-209
doi: 10.1093/toxsci/kfz094
Advance Access Publication Date: February 19, 2020
Research Article

Profiling the ToxCast Library With a Pluripotent Human (H9) Stem Cell Line-Based Biomarker Assay for Developmental Toxicity

Todd J. Zurlinden¹, Katherine S. Saili¹, Nathaniel Rush¹, Parth Kothiyi¹, Richard S. Judson¹, Keith A. Houck¹, E. Sidney Hunter¹, Nancy C. Baker¹, Jessica A. Palmer¹, Russell S. Thomas¹, and Thomas B. Knudsen¹

¹National Center for Computational Toxicology (NCCT) and ²National Health and Environmental Effects Research Laboratory (NHEERL), Office of Research and Development (ORD), U.S. Environmental Protection Agency (USEPA), Research Triangle Park, North Carolina 27711; ³Leidos, Research Triangle Park, North Carolina 27711; and ⁴Stemina Biomarker Discovery, Inc., Madison, Wisconsin 53719

*To whom correspondence should be addressed at National Center for Computational Toxicology (2025-01), U.S. Environmental Protection Agency, Research Triangle Park, NC 27711. Fax: 919-541-1194. E-mail: knudsen.thomas@epa.gov.

Disclaimers: The views expressed in this article are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

ABSTRACT

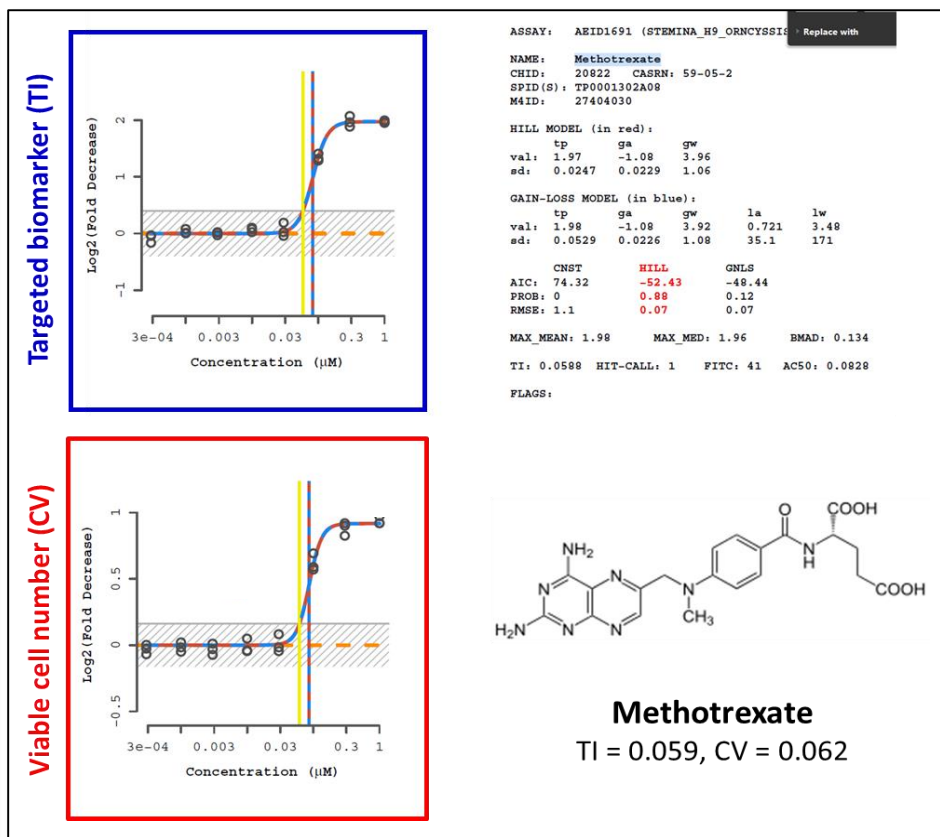
The Stemina devTOXquickPredict platform is a human pluripotent stem cell-based assay that predicts the developmental toxicity potential based on changes in cellular metabolism following chemical exposure [Palmer, J. A., Smith, A. M., Egnash, L. A., Conard, K. R., West, P. R., Burrier, R. E., Donley, E. L. R., and Kirchner, F. R. (2013). Establishment and assessment of a new human embryonic stem cell-based biomarker assay for developmental toxicity screening. *Birth Defects Res. B Dev. Reprod. Toxicol.* 98, 343-363]. Using this assay, we screened 1065 ToxCast phase I and II chemicals in single-concentration or concentration-response for the targeted biomarker (ratio of ornithine to cystine secreted or consumed from the media). The dataset from the Stemina (STM) assay is annotated in the ToxCast portfolio as STM. Major findings from the analysis of ToxCast_STM dataset include (1) 19% of 1065 chemicals yielded a prediction of developmental toxicity, (2) assay performance reached 79%-82% accuracy with high specificity (> 84%) but modest sensitivity (< 67%) when compared with *in vivo* animal models of human prenatal developmental toxicity, (3) sensitivity improved as more stringent weights of evidence requirements were applied to the animal studies, and (4) statistical analysis of the most potent chemical hits on specific biochemical targets in ToxCast revealed positive and negative associations with the STM response, providing insights into the mechanistic underpinnings of the targeted endpoint and its biological domain. The results of this study will be useful to improving our ability to predict *in vivo* developmental toxicants based on *in vitro* data and *in silico* models.

Keywords: predictive toxicology; developmental toxicity; embryonic stem cells.

In 2007, the National Research Council published *Toxicity Testing in the 21st Century: A Vision and a Strategy* (National Research Council, 2007). This report addressed the potential for automated high-throughput screening (HTS) and high-content screening (HCS) assays and technologies to identify chemically induced biological activity in human cells and to develop predictive models of *in vivo* biological response that would ignite a shift from traditional animal endpoint-based testing to human pathway-based risk assessment (Collins et al., 2008). Concurrent with the NRC 2007 report, the U.S. Environmental Protection

Published by Oxford University Press on behalf of the Society of Toxicology 2020. This work is written by US Government employees and is in the public domain in the US.

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- 1065 ToxCast Ph I/II chemicals at single-conc. or multi-conc.;
- data pipelined to *in vitro-db_v3* database (>1125 features);
- available in EPA's CompTox Chemicals Dashboard;
- **ToxCast_STM** dataset includes controls for data quality.

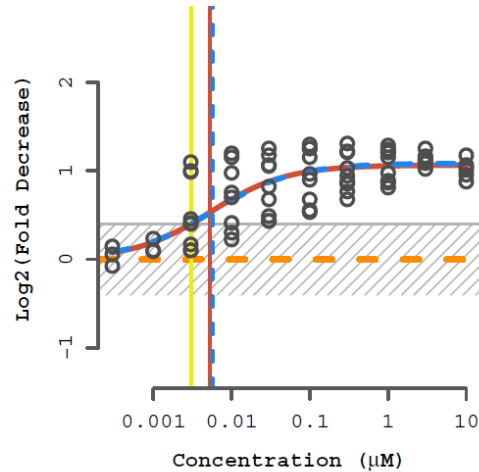
<https://comptox.epa.gov/dashboard>

19% positivity rate, indicative of teratogenic potential as a potential developmental hazard prediction.

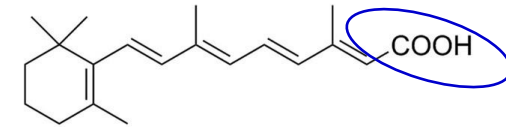
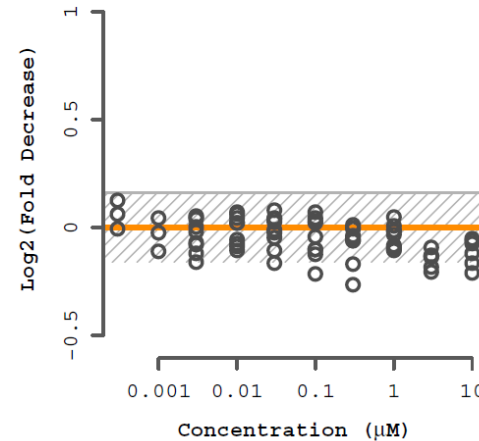
Zurlinden et al. (2020) *Toxicol Sci*

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

Targeted biomarker (TI)



Viable cell number (CV)

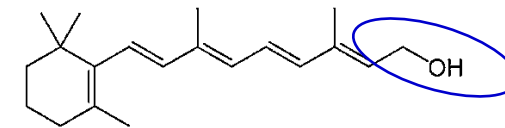
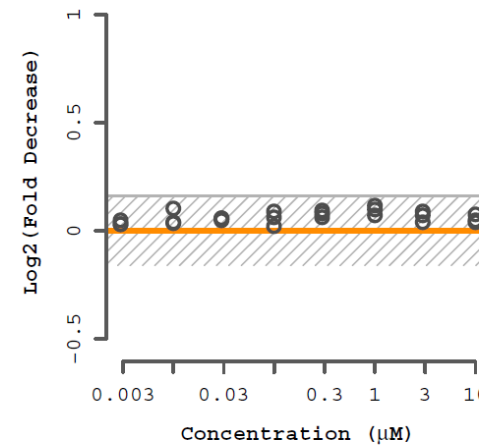
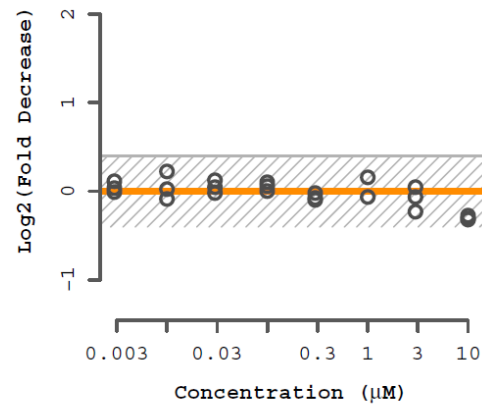


all trans Retinoic acid

TI = 0.003 μM , CV = NA

dLEL rat = 2.5 mg/kg/day (> mLEL)

dLEL rabbit = 0.5 mg/kg/day (= mLEL)



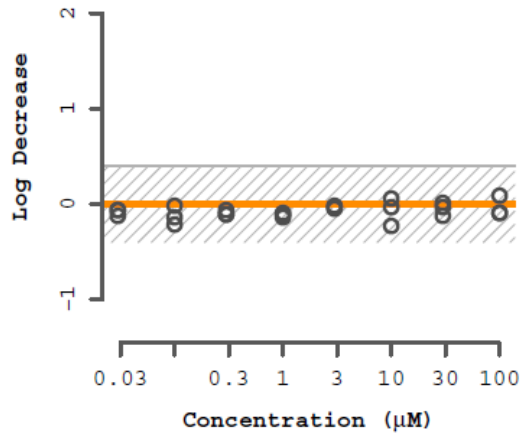
Retinol (vitamin-A)

TI = NA, CV = NA

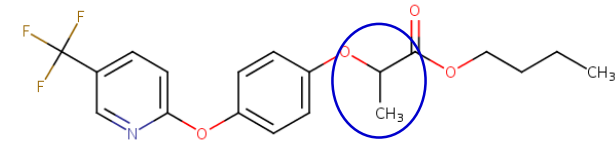
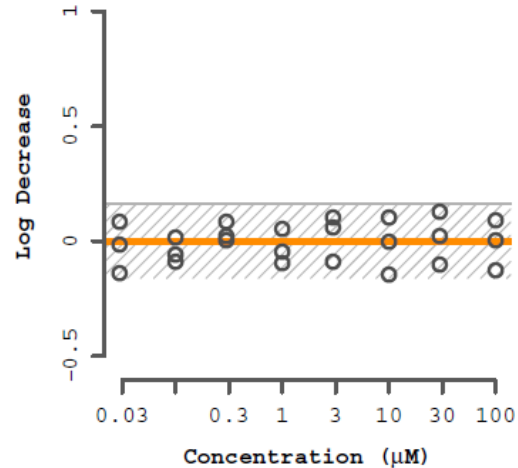
(True Negative)

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

Targeted biomarker (TI)

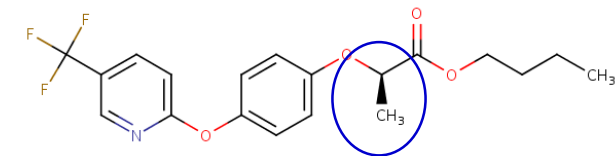
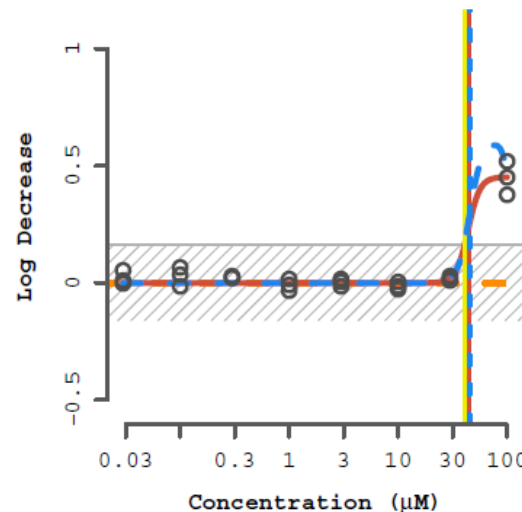
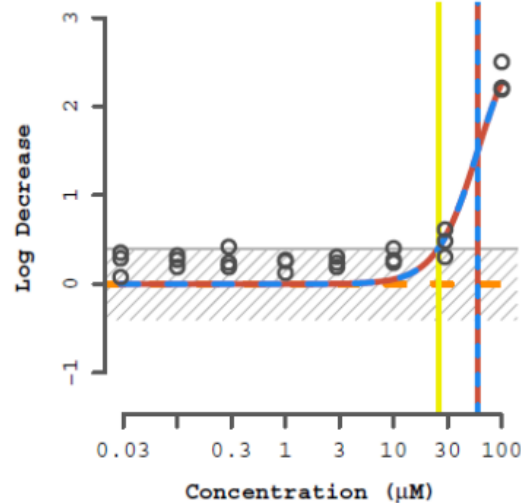


Viable cell number (CV)



Fluazifop butyl

TI = not active, CV = no effect
dLEL rat = 10 mg/kg/day (< mLEL)
dLEL rabbit = 90 mg/kg/day (mLEL)

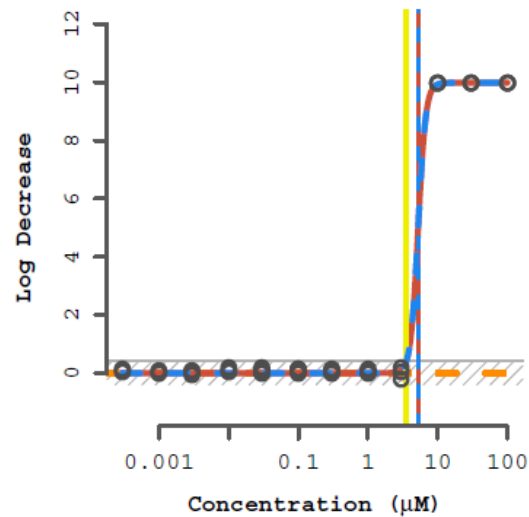


Fluazifop-*P*-butyl

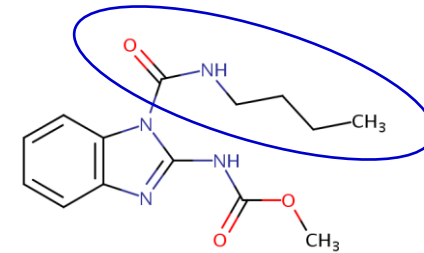
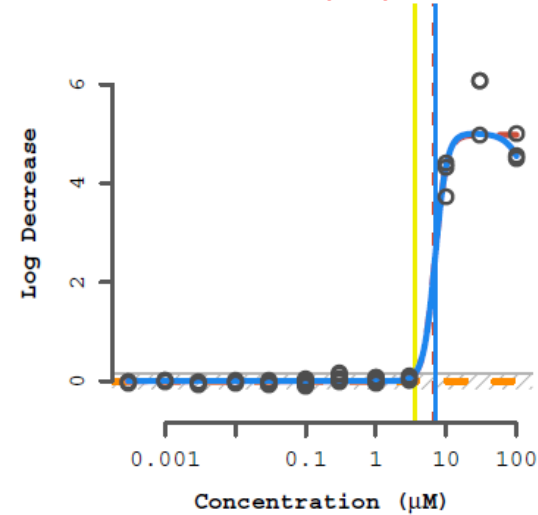
TI = 26 μM, CV = 40.8 μM
dLEL rat = 5 mg/kg/day (< mLEL)
dLEL rabbit = 50 mg/kg/day (mLEL)

Example: Benomyl and its conversion product (Carbendazim)

Targeted biomarker (TI)

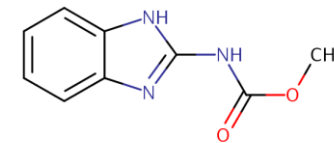
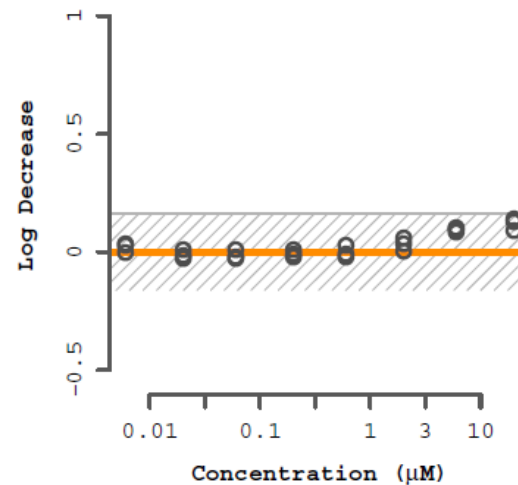
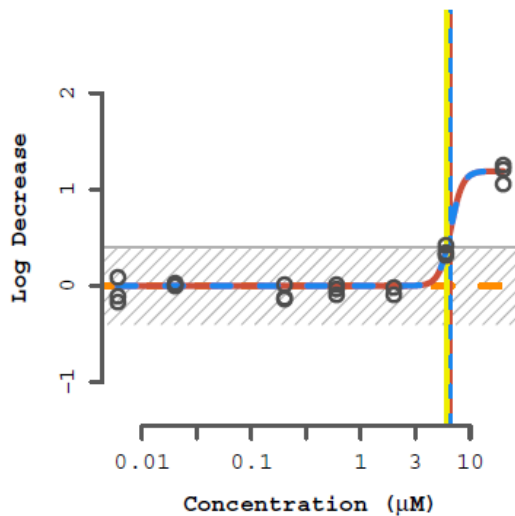


Viable cell number (CV)



Benomyl

TI = 3.53 μM , CV = 3.63 μM
dLEL rat = 62.5 mg/kg/day (< mLEL)
dLEL rabbit = 180 mg/kg/day (mLEL)

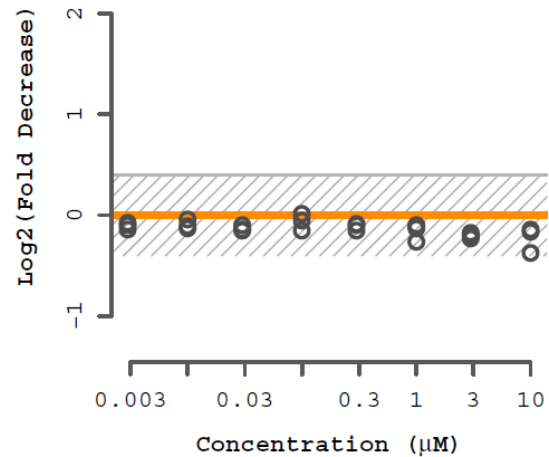


Carbendazim

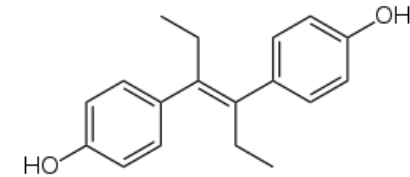
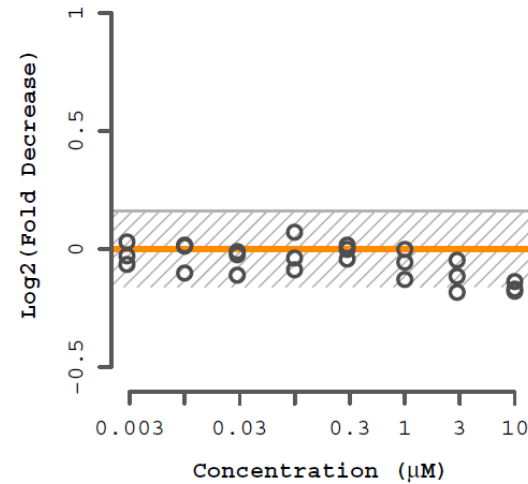
TI = 6.12 μM , CV = no effect
dLEL rat = 20 mg/kg/day (< mLEL)
dLEL rabbit (no ToxRefDB entry)

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

Targeted biomarker (TI)

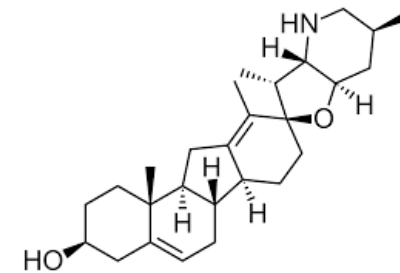
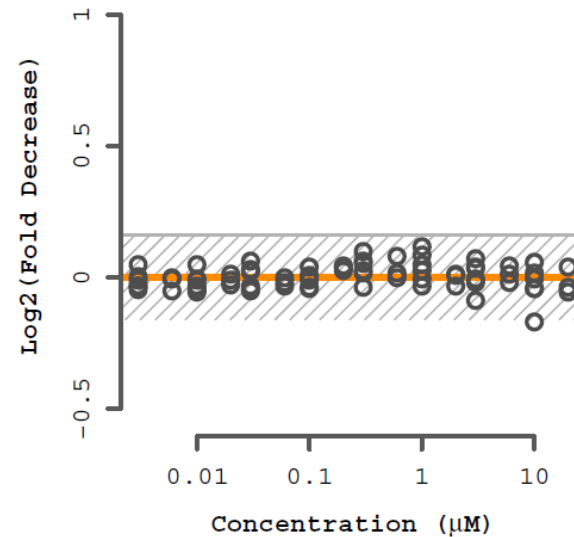
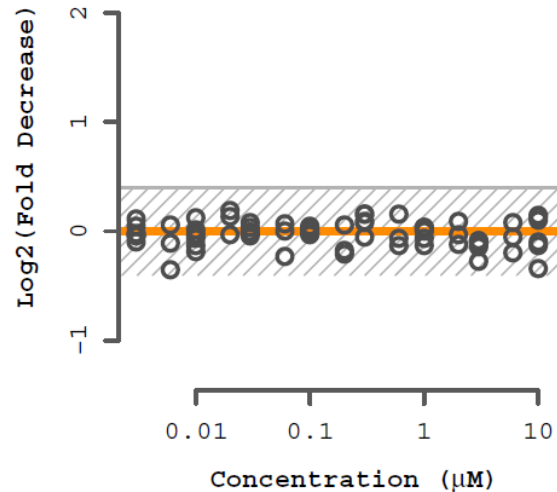


Viable cell number (CV)



Diethylstilbestrol (DES)

TI = NA, CV = NA
dLEL rat = 0.03 mg/kg/day (= mLEL)
(no rabbit data in ToxRefDB)

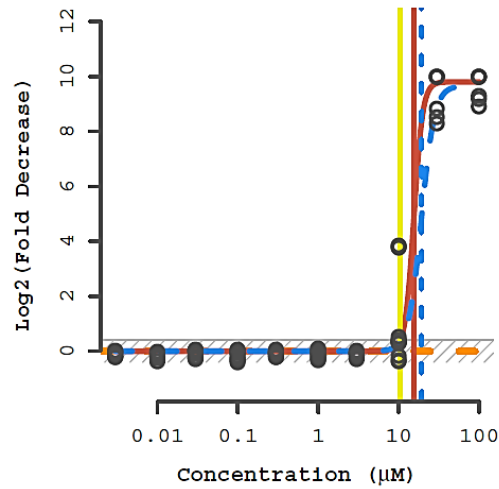


Cyclopamine

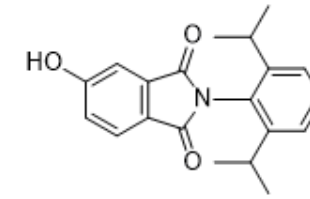
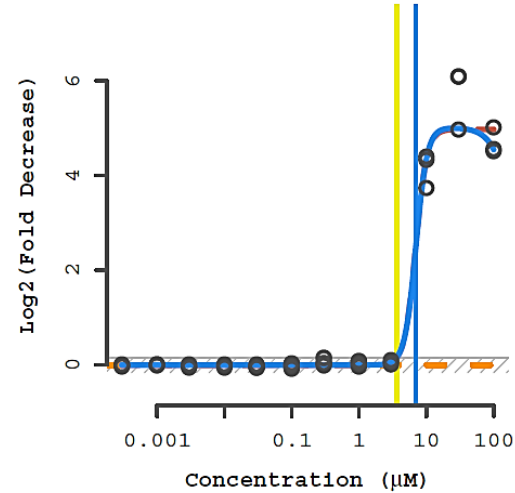
TI = NA, CV = NA

Example: *pharmacological angiogenesis inhibitors*

Targeted biomarker (TI)



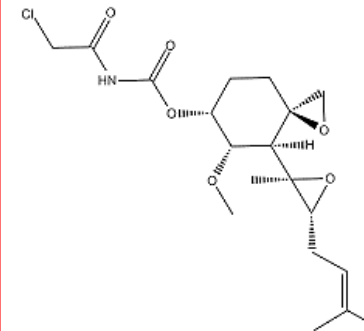
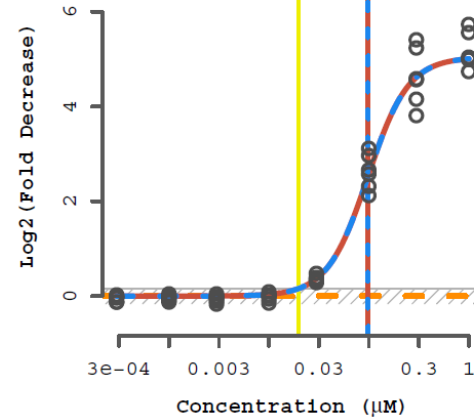
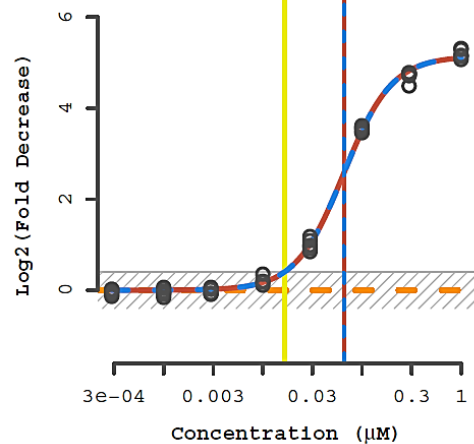
Viable cell number (CV)



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)

Does the STM biomarker really predict a teratogenic threshold?

Colleagues at Dow Chemical's Predictive Toxicology group, led by Ed Carney[†], tested T.I. predictions for the two structurally diverse angiogenic inhibitors using a 48h rat WEC platform.

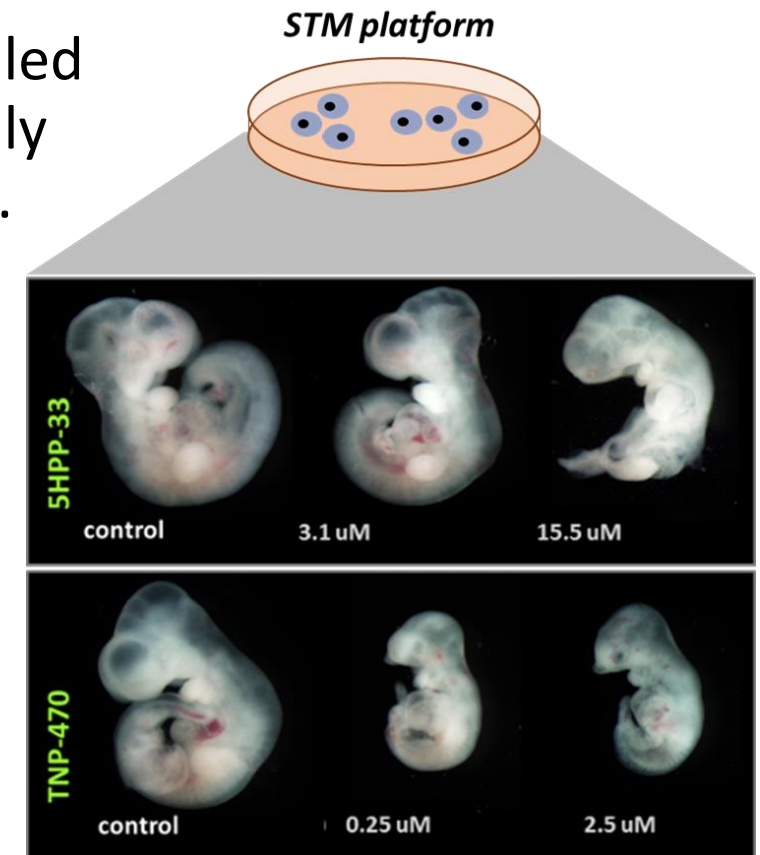
5HPP-33:

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

TNP-470:

- T.I. predicted by hESC was 0.02 μM
- AC50 observed at 0.04 μM (dysmorphogenesis)

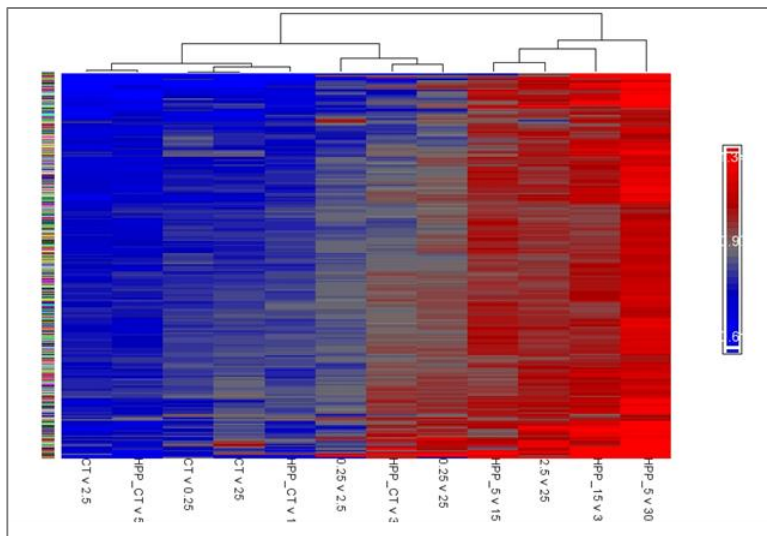
Phenotypic consequences differed but the threshold T.I. predicted by the ToxCast_STM data was just under the AC50 observed.



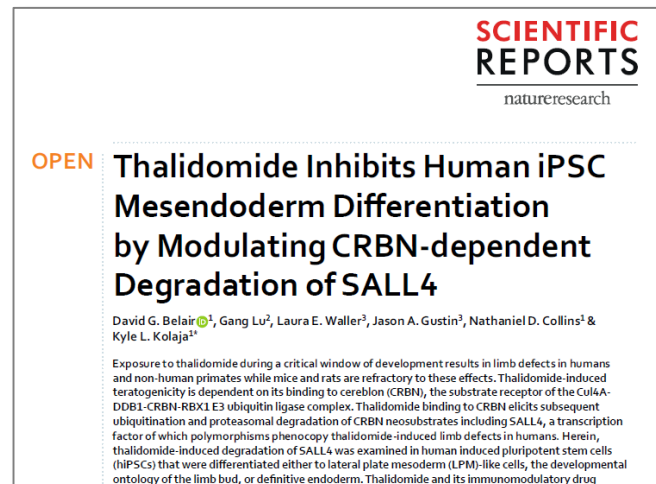
Ellis-Hutchings et al. (2017) Reprod Toxicol

Does the STM biomarker predict something new? *sort of...*

- We used RNA-seq profiling to examine how the exposed embryos reacted to threshold concentrations of 5HPP-33 and TNP-470 after 4 hr exposure in rat WEC.
- Several pathways uniquely affected by each compound, but some in common:
 - *spliceosome-RNA metabolism*
 - *proteasome-ubiquitination*



Saili, Franzosa et al. (2019) *Curr Opin Tox*



Belair et al. (2020) *Scientific Reports*

Thalidomide's teratogenicity triggered by cereblon (CRBN) → proteasomal degradation of SALL4 in human iPSC progenitors of limb-bud mesoderm.

Performance check

- Qualification on 42 well-curated reference compounds often used to validate alternative DevTox platforms¹.
- Balanced Accuracy (BAC) = 82% (0.65 sensitivity, 1.00 specificity).
- These performance measures are consistent with the original pharma-trained model [Palmer et al. 2013]

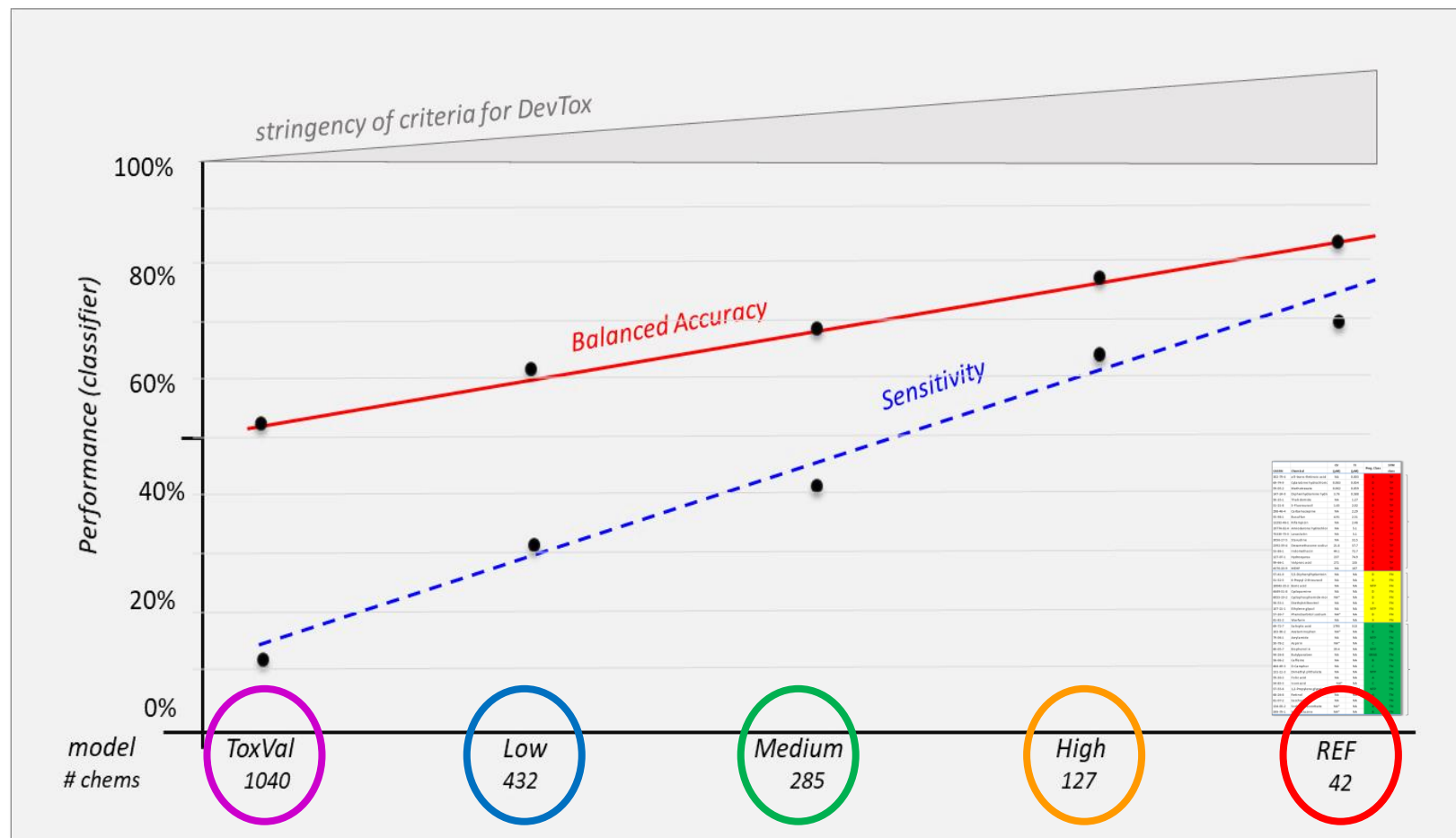
Many assays that have undergone a validation exercise were tested with a limited set of data-rich chemicals, often inflating predictive capacity of 80% or higher. *This situation has hampered regulatory acceptance of individual alternative assays.*

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

CASRN	Chemical	CV (μ M)	TI (μ M)	Preg. Class	STM class	
302-79-4	all-trans-Retinoic acid	NA	0.003	X	TP	True Positive
69-74-9	Cytarabine hydrochloric	0.083	0.054	D	TP	
59-05-2	Methotrexate	0.062	0.059	X	TP	
147-24-0	Diphenhydramine hydro	3.76	0.588	B	TP	
50-35-1	Thalidomide	NA	1.27	X	TP	
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 sodium	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	False Negative
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	Phenobarbital sodium	NA*	NA	D	FN	True Negative
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	

Zurlinden et al. (2020)

Performance evaluation against ToxRefDB chemicals



Scaling Criteria (ToxRefDB)

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

Predictivity of the hESC biomarker declines as fetal outcomes gain less concordance between rat-rabbit studies, and concurrent maternal toxicity.

Implications of weak sensitivity?

- Predictive power of an assay depends on its biological relevance as well as the standard against which the prediction is validated:
 - ORN/CYSS biomarker may be missing important pathways;
 - pluripotent H9 cells may be missing relevant developmental processes.



With ~1K *in vitro* assays in ToxCast/Tox21, can we use machine learning and mechanistic models to better characterize the biological domain of applicability?

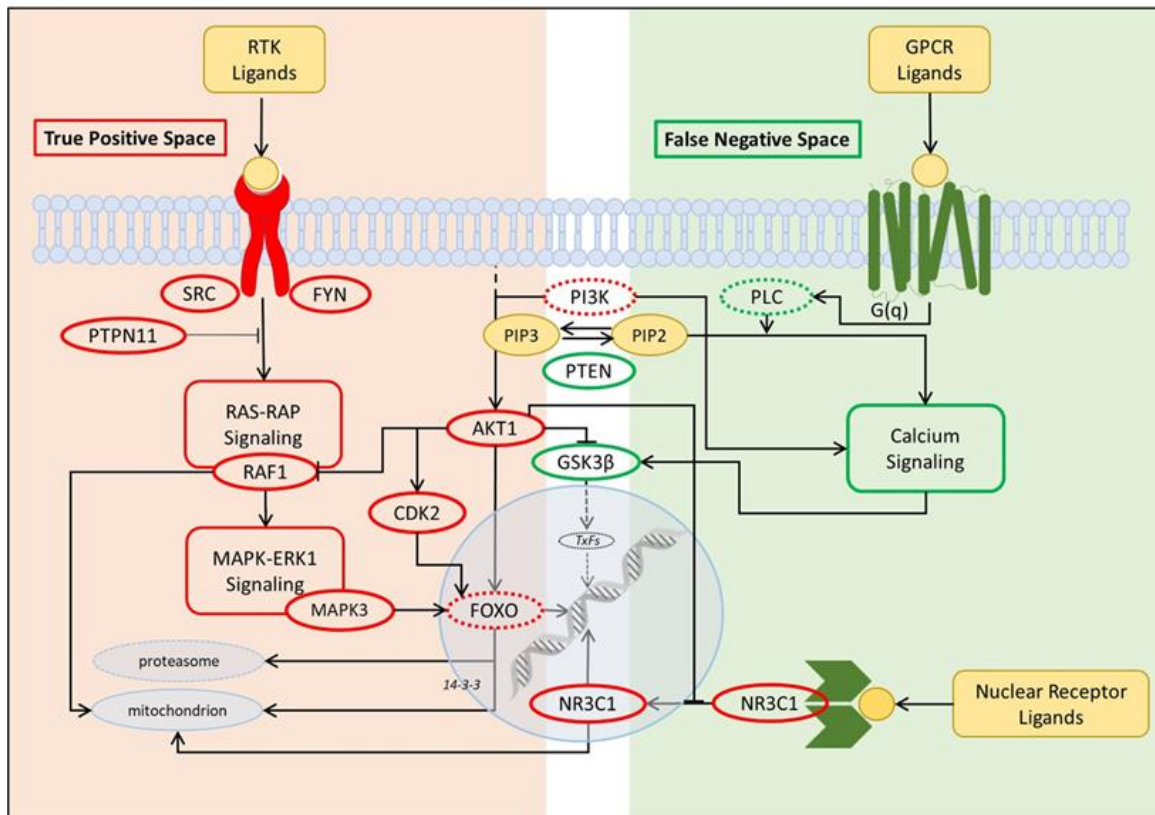


What human relevant pathways are detected or not?

Sensitive Domain

Insensitive Domain

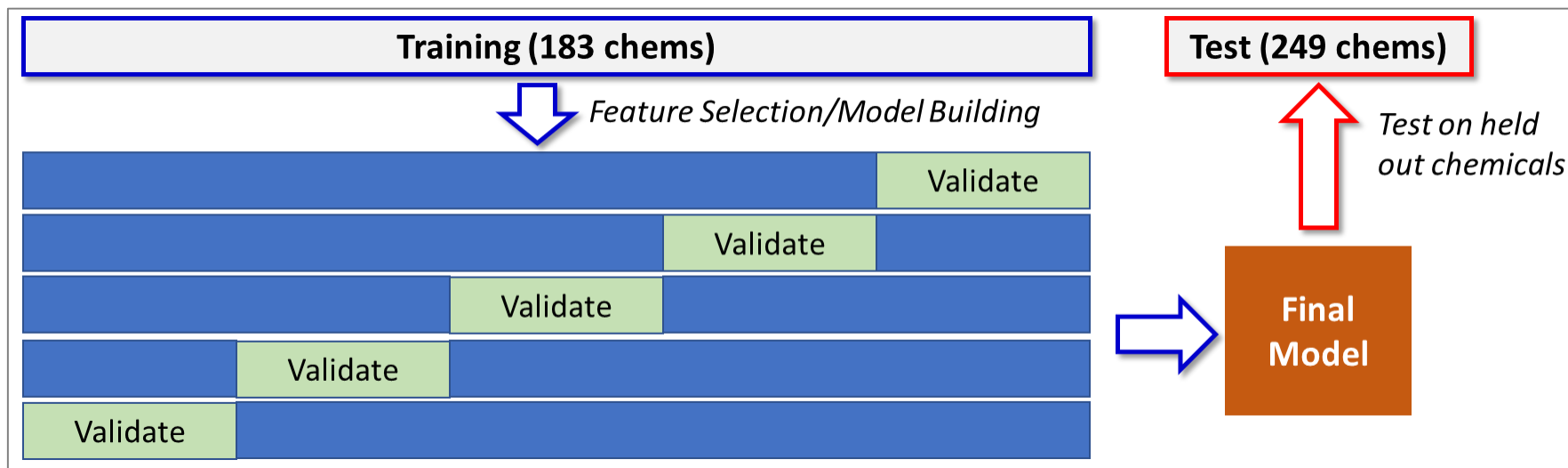
Annotation System	Keystone Pathway / Process	# MIEs	Class
GOTERM_BP_DIRECT	GO:0014066~regulation of phosphatidylinositol 3-kinase signaling	6	TP
KEGG_PATHWAY	hsa04068:FoxO signaling pathway	8	TP
KEGG_PATHWAY	hsa04510:Focal adhesion	13	TP
GOTERM_BP_DIRECT	GO:0007200~phospholipase C-activating G-protein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



- Mapped ToxCast_STM correlations for well-curated chemicals onto 337 ToxCast_NVS targets;
- flow of regulatory information to AKT/FOXO signaling in the sensitive domain (**true positives**);
- G(q) signaling and steroid hormone signaling in the insensitive domain (**false negatives**).

Positive predictivity in the hESC model is driven to FOXO, a key regulator of proteasome-ubiquitination.

Let's try again ... but with all additional ToxCast assays



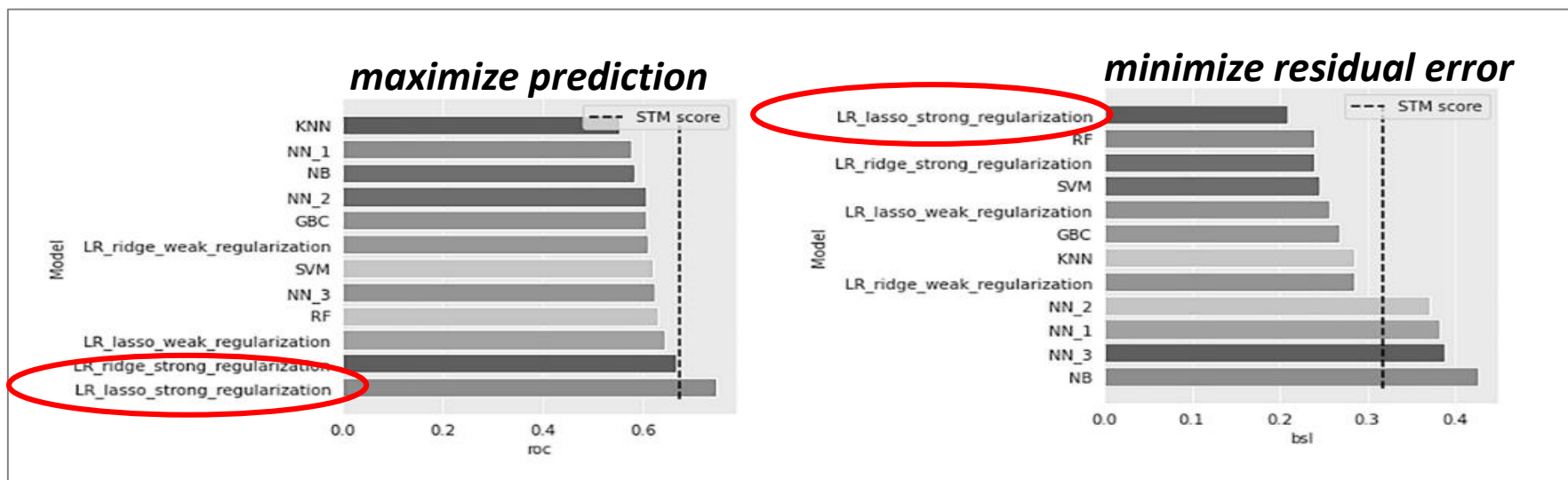
Unsupervised feature reduction (107)

Algorithms

KNN	K Nearest Neighbors
NB	Naive Bayes
SVM	Support Vector Machine
NN	Neural Network (n hidden layers)
RF	Random Forest
LR	Logistic Regression
GBC	Gradient Boosting Classification

Bayesian regularization - used to shrink assay selection based on residual error.

(----) ROC AUC for DevTox prediction using STM hit call alone.



Training model (STM + 7 model)

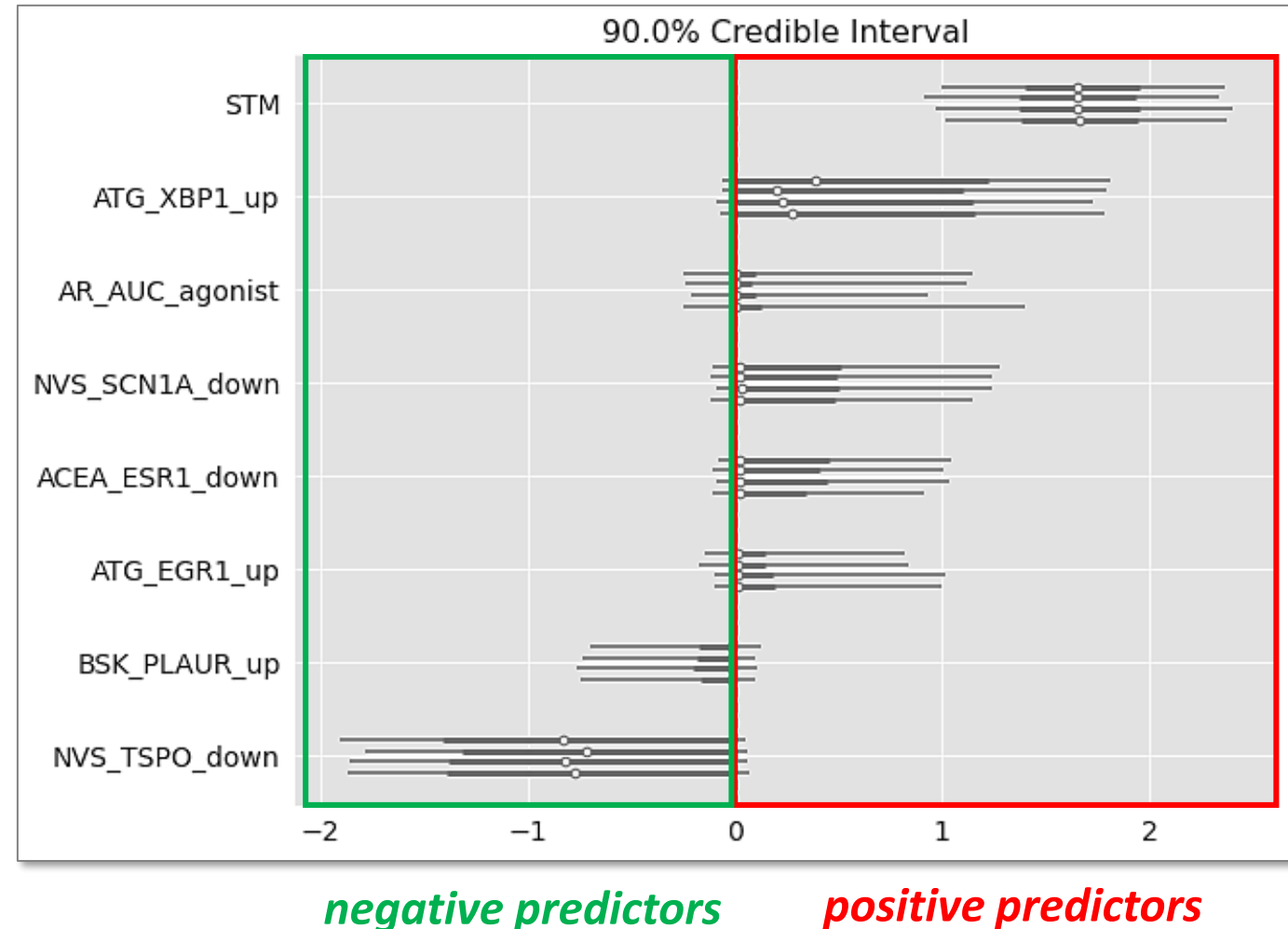
ToxCast_STM is strongest predictor of prenatal developmental toxicity in ToxCast/Tox21;

XBP1 induces UPR and independently targets FOXO1 for proteasomal degradation;

SCN1A picked up some false negatives (e.g., cyclopamine);

Estrogenic and androgenic pathways are outside the STM biological domain;

TSPO mitochondrial 18K translocator that pharmacologically represses XBP1 activation.



Does the integrated statistical model perform better than ESCs?

Model	Curation	n	sens	PPV	BAC
STM	none	432	0.325	0.803	37.3
	low	432	0.364	0.567	60.4
	medium	285	0.512	0.537	66.5
	high	127	0.667	0.556	72.3
	very high	42	0.654	1.000	82.0
STM+7	train	183	0.593	0.836	77.5
	test	249	0.300	0.469	54.5
Rat subset	90%CI > 0.5	135	0.480	0.783	55.2
	90%CI > 0.7	68	0.736	0.848	60.1
	90%CI > 0.8	23	0.813	0.867	74.6
Rabbit subset	90%CI > 0.5	110	0.429	0.574	50.2
	90%CI > 0.7	58	0.710	0.595	58.3
	90%CI > 0.8	31	0.722	0.650	59.8

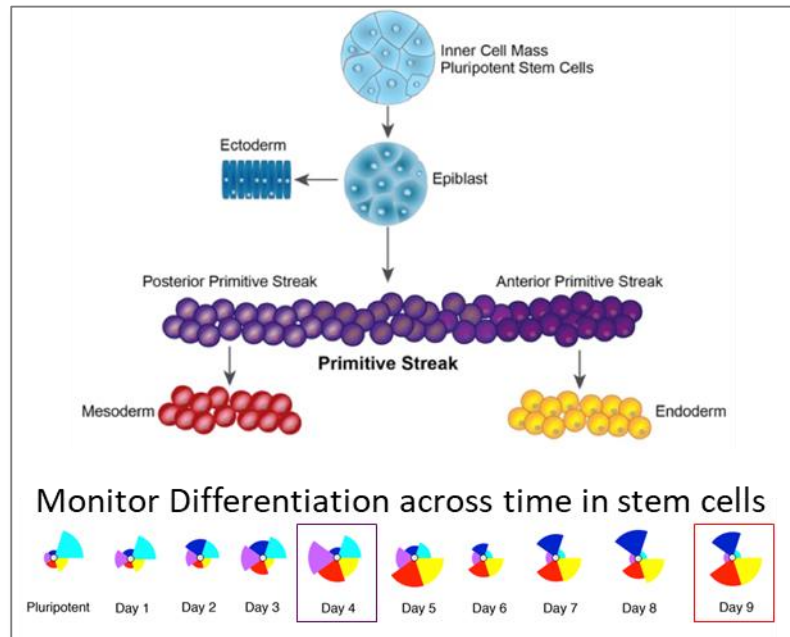
Scaling Criteria (ToxRefDB)

- dLEL < 1000 mg/kg/day
- dLEL \leq 200 mg/kg/day
- dLEL < mLEL, rat OR rabbit
- concordant response, rat AND rabbit
- BM-42 reference
- Train with most confident compound set
- Test on the remainder

A subset of test chemicals with higher confidence in the model can achieve training-level performance.

Augmentation correlated with the mouse J1 cell (mESC) assay

Goosecoid (GSC) gastrulation marker (day4) and Cardiomyocyte differentiation (day 9)



Correlation of ToxCast Gene Targets and effects on mESCs

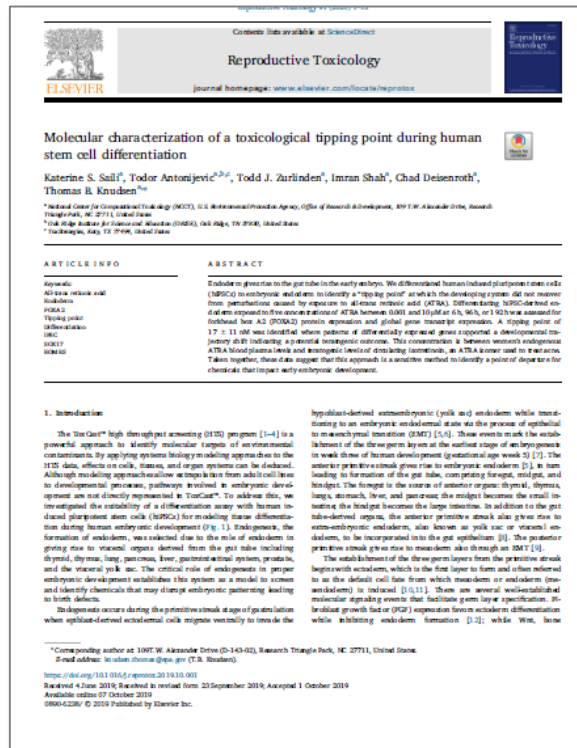
ADRA2C		D4_Cell-*	NFE2L2	GSC_Differentiation-*	D4_Cell-*
AHR	GSC_Differentiation-*	D4_Cell-*	NOS1	GSC_Differentiation-*	
AR	GSC_Differentiation-*	D4_Cell-*	NR1I2	GSC_Differentiation-*	D4_Cell-*
CEBPB		D4_Cell-*	NR4A2		D4_Cell-*
CREB3	GSC_Differentiation-*	D4_Cell-*	OXTR		D4_Cell-*
CYP1A1	GSC_Differentiation-*		POU2F1	GSC_Differentiation-*	D4_Cell-*
CYP1A2	GSC_Differentiation-*		PPARA		D4_Cell-*
CYP2C19	GSC_Differentiation-*	D4_Cell-*	RARA	GSC_Differentiation-*	D4_Cell-*
CYP2C9	GSC_Differentiation-*		RORA		D4_Cell-*
CYP2D6	GSC_Differentiation-*		RXR	GSC_Differentiation-*	D4_Cell-*
DT40	GSC_Differentiation-*	D4_Cell-*	SCN1A	GSC_Differentiation-*	D4_Cell-*
EGR1		D4_Cell-*	SMAD1	GSC_Differentiation-*	D4_Cell-*
ELANE	GSC_Differentiation-*	D4_Cell-*	SOX1		D4_Cell-*
ESR1	GSC_Differentiation-*	D4_Cell-*	SP1	GSC_Differentiation-*	
FOS	GSC_Differentiation-*	D4_Cell-*	SREBF1	GSC_Differentiation-*	D4_Cell-*
JUN	GSC_Differentiation-*	D4_Cell-*	TACR1		D4_Cell-*
MAOA		D4_Cell-*	THR	GSC_Differentiation-*	
MMP3		D4_Cell-*	TSPO	GSC_Differentiation-*	
MTF1		D4_Cell-*	XBP1	GSC_Differentiation-*	D4_Cell-*

Potency also correlated with effects on mESC growth and differentiation.

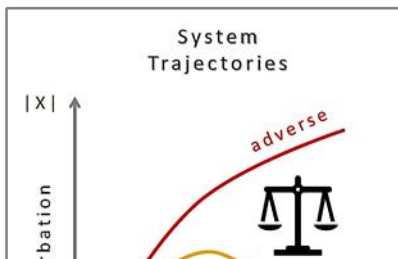
Molecular characterization of a Toxicological Tipping Point



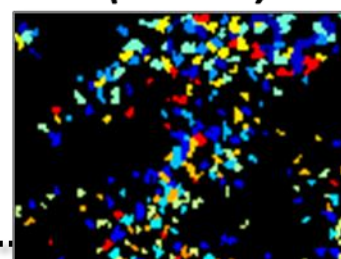
- hESCs tracked in mesendodermal lineage classified teratogenicity based on SOX17 (87-94% accuracy) [Kameoka et al. 2014];
- all-trans* retinoic acid (ATRA) is an endogenous signal in mesendodermal patterning (T.I. = 19 nM in hiPSCs [Palmer et al. 2017]);
- what would a 'toxicological tipping point' on hiPSC differentiation look like with ATRA at the molecular level?



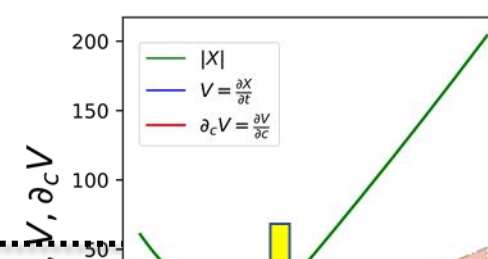
ATRA conc x time



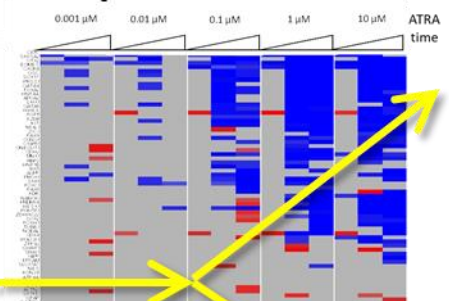
HCI (FOXA2)



TP = 17 nM x 96 hr




↓ endoderm
↑ mesoderm



The tipping point reflects an imbalance on genes regulated by *Eomesodermin (EOMES)*, a T-box family member that drives endodermal specification and mesodermal delamination during primitive streak formation (gastrulation).

EOMES is regulated by FOXO: *at least in memory T-cells!*

 NIH Public Access
Author Manuscript
Immunity. Author manuscript; available in PMC 2013 March 23.
Published in final edited form as:
Immunity. 2012 March 23; 36(3): 374–387. doi:10.1016/j.immuni.2012.01.015.

Transcription factor Foxo1 represses T-bet mediated effector functions and promotes memory CD8⁺ T cell differentiation

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SUMMARY

The evolutionary conserved Foxo transcription factors are important regulators of quiescence and longevity. Although, Foxo1 is known to be important in regulating CD8⁺ T cell trafficking and homeostasis, its role in functional differentiation of antigen stimulated CD8⁺ T cells is unclear. Herein, we demonstrate that inactivation of Foxo1 was essential for instructing T-bet transcription factor-mediated effector differentiation of CD8⁺ T cells. The Foxo1 inactivation was dependent on mTORC1 kinase, as blockade of mTORC1 abrogated mTORC2 mediated Akt (Ser473) kinase phosphorylation, resulting in Foxo1-dependent switch from T-bet to Eomesodermin transcription factor activation and increase in memory precursors. Silencing Foxo1 ablated interleukin-12 and rapamycin enhanced CD8⁺ T cell memory responses, and restored T-bet mediated effector functions. These results demonstrate an essential role of Foxo1 in actively repressing effector or terminal differentiation processes to promote memory CD8⁺ T cell development, and identify the functionally diverse mechanisms utilized by Foxo1 to promote quiescence and longevity.



‘... Foxo1-dependent switch from T-bet to Eomesodermin transcription factor activation...

Cancer Immunology, Immunotherapy (2018) 67:691–702
<https://doi.org/10.1007/s00262-018-2120-5>

ORIGINAL ARTICLE

 CrossMark

N-acetyl cysteine protects anti-melanoma cytotoxic T cells from exhaustion induced by rapid expansion via the downmodulation of Foxo1 in an Akt-dependent manner

Matthew J. Scheffel¹ · Gina Scurti² · Megan M. Wyatt¹  · Elizabeth Garrett-Mayer³ · Chrystal M. Paulos¹ · Michael I. Nishimura² · Christina Voelkel-Johnson¹ 

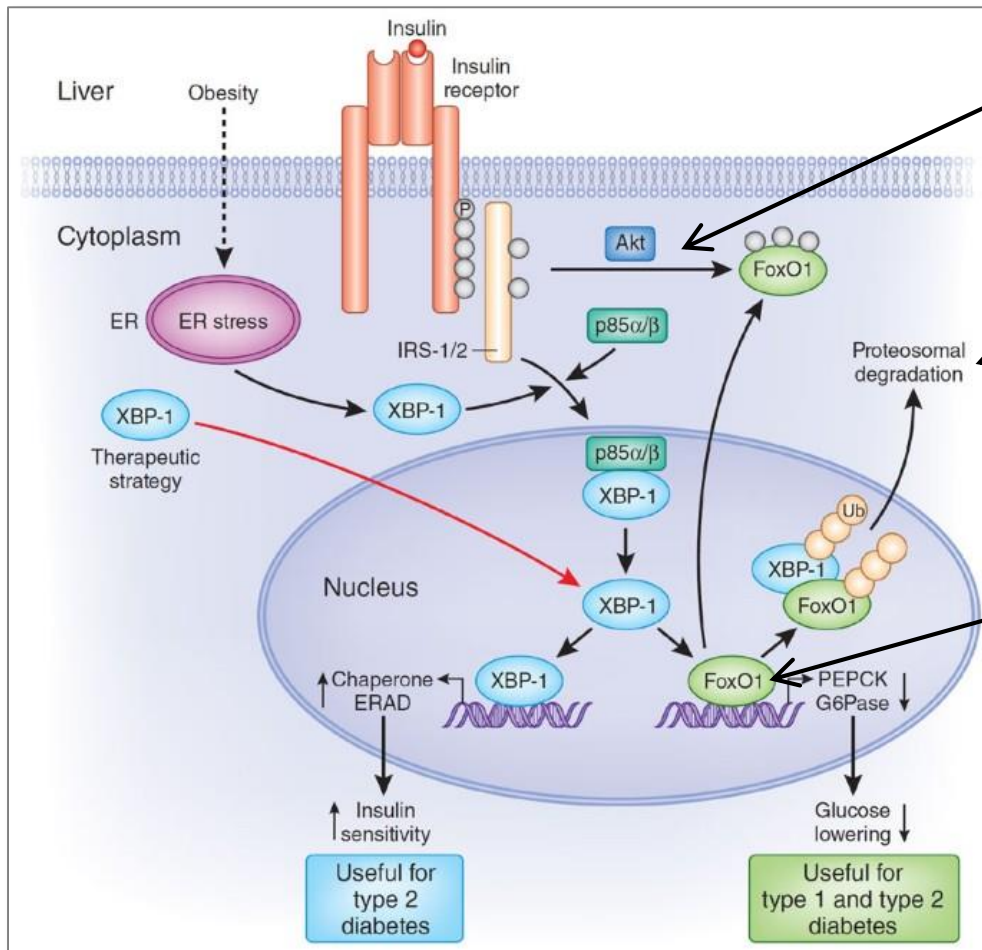
Received: 30 August 2017 / Accepted: 22 January 2018 / Published online: 2 February 2018
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Abstract

Therapeutic outcomes for adoptive cell transfer (ACT) therapy are constrained by the quality of the infused T cells. The rapid expansion necessary to obtain large numbers of cells results in a more terminally differentiated phenotype with decreased durability and functionality. N-acetyl cysteine (NAC) protects against activation-induced cell death (AICD) and improves anti-tumor efficacy of Pmel-1 T cells in vivo. Here, we show that these benefits of NAC can be extended to engineered T cells and significantly increases T-cell survival within the tumor microenvironment. The addition of NAC to the expansion protocol of human TIL138381 TCR-transduced T cells that are under evaluation in a Phase I clinical trial, demonstrated that findings in murine cells extend to human cells. Expansion of TIL138381 TCR-transduced T cells in NAC also increased their ability to kill target cells in vitro. Interestingly, NAC did not affect memory subsets, but diminished up-regulation of senescence (CD57) and exhaustion (PD-1) markers and significantly decreased expression of the transcription factors EOMES and Foxo1. Pharmacological inhibition of the PI3K/Akt pathway ablates the decrease in Foxo1 induced by NAC treatment of activated T cells. This suggests a model in which NAC through PI3K/Akt activation suppresses Foxo1 expression, thereby impacting its transcriptional targets EOMES, PD-1, and granzyme B. Taken together, our results indicate that NAC exerts pleiotropic effects that impact the quality of TCR-transduced T cells and suggest that the addition of NAC to current clinical protocols should be considered.

‘... PI3K/Akt activation suppresses Foxo1 expression, thereby impacting its transcriptional targets EOMES ...

Does regulation of proteasomal function explain hESC positivity?



AKT/IP3K signaling to FOXO1 was the winning pathway in correlating NVS biochemical targets to STM positivity

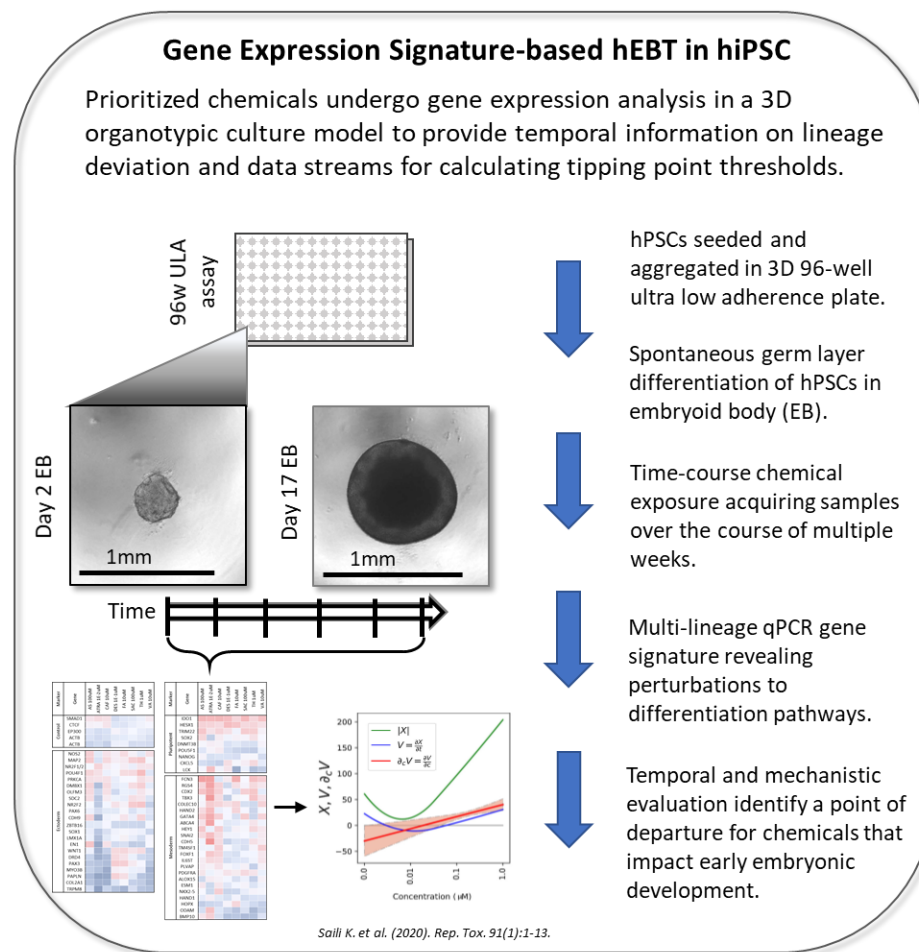
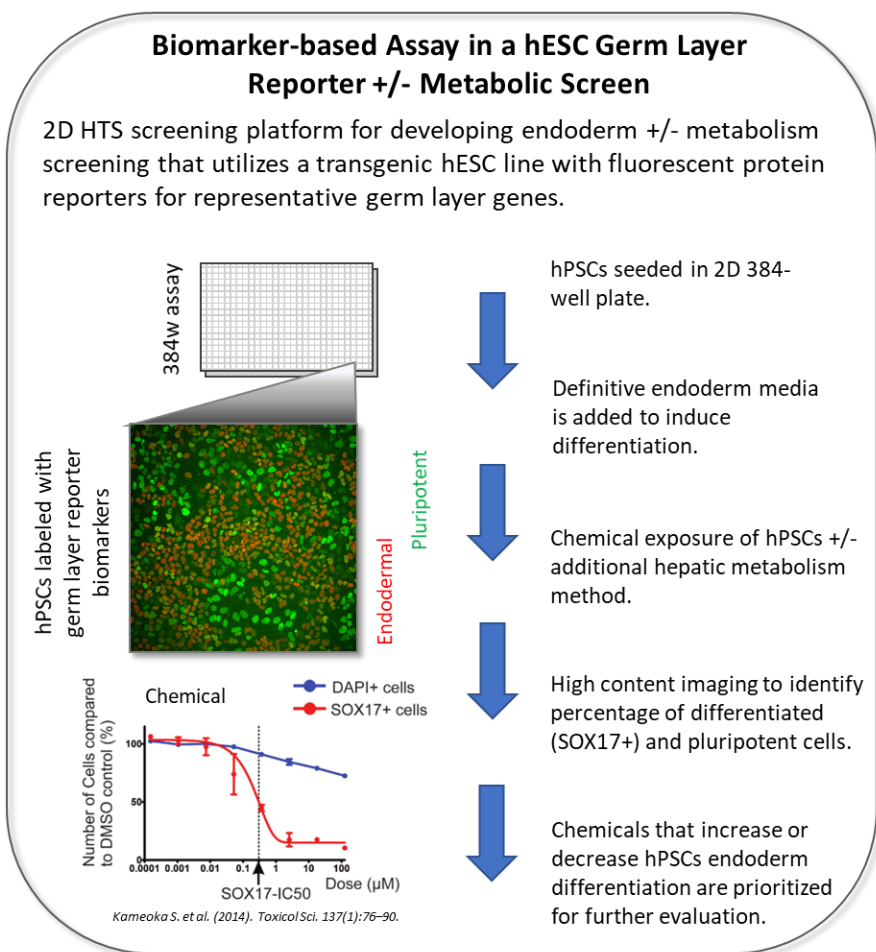
RNAseq rat WEC found the proteasome-ubiquitination as an early feature for 2 chemicals predicted positive

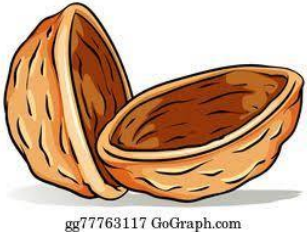
Activation of XBP1 was second only to STM as the winning feature across the broader ToxCast portfolio

HYPOTHESIS: hESC-positivity linked to altered homeostatic control of the FoxO1 axis as a negative regulator of hESC differentiation.

Ueki and Kadowaki (2011) Nature

Expanding the molecular landscape of tipping points





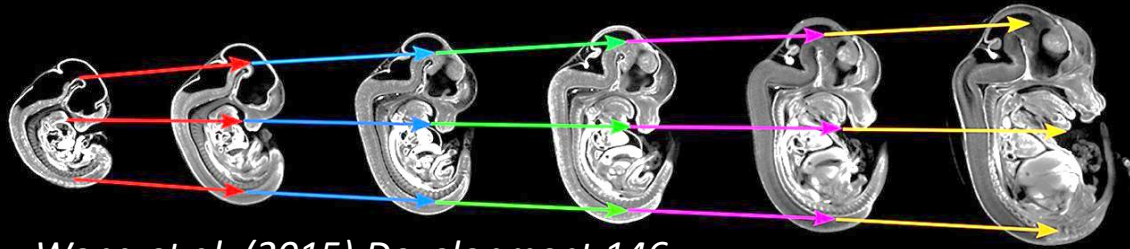
Computational models for NAMs:

predicting developmental toxicity and translation to human pregnancy

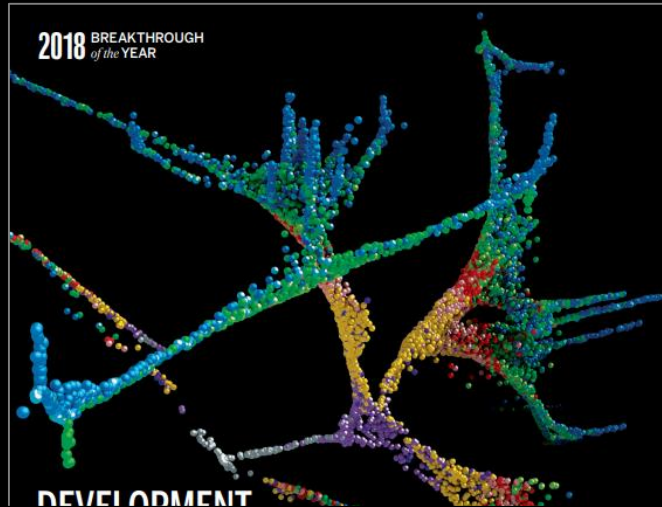
- HTS data-driven models can confidently predict developmental toxicity potential in pregnant rat/rabbit study designs; but ...
- ... there are limitations of mESC and hESC platforms for classifying teratogenic across a mechanistically diverse landscape of chemicals (pathways, metabolism, complexity, ...);
- bringing embryology into the fold will further improve mechanistic understanding in translating NAM data to probabilistic effects and early lifestage phenotypes.

I'll end with two new ways to evaluate the embryo suggesting why sophisticated computer models are needed in the immediate future.

Cell State Manifold: *single-cell transcriptome profiling*



Wong et al. (2015) *Development* 146



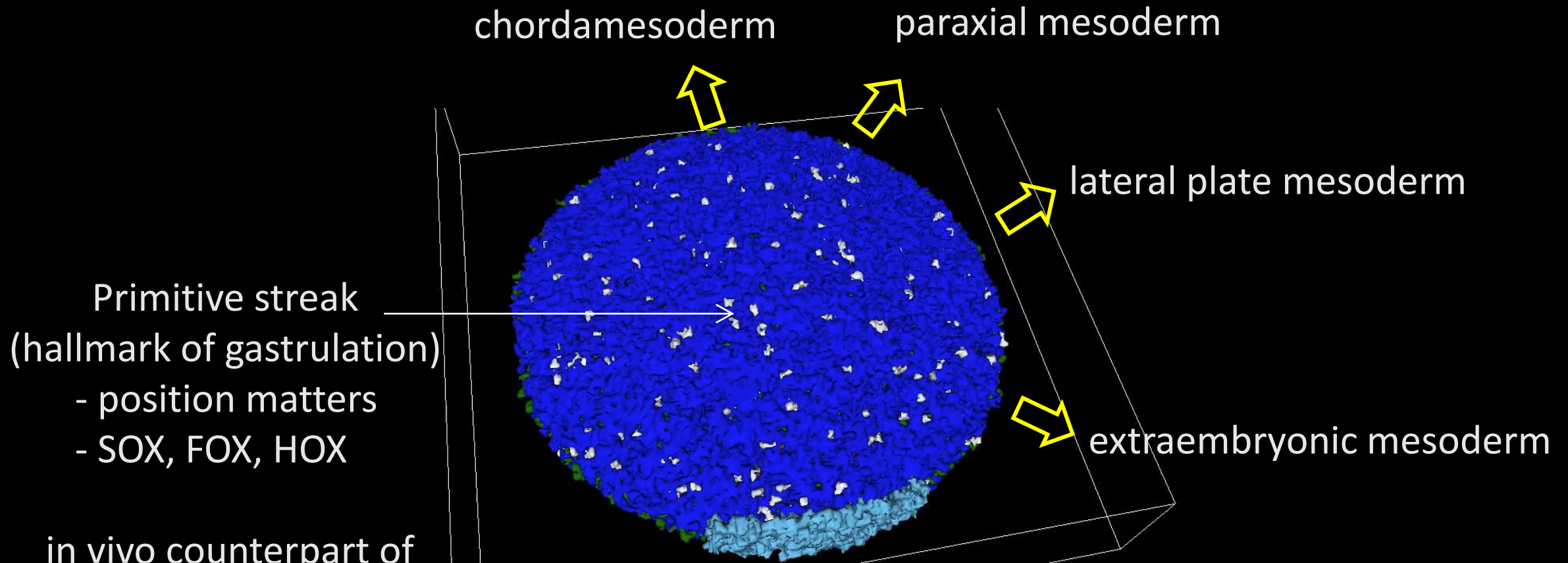
Cao et al. (2019) *Nature* 566



Video from Malte Spielmann, with thanks

A key challenge for science and technology is to critically define sentinel cells in a perturbed system that propagate chemical injury to toxicological tipping point, ultimately manifesting as a structural defect or altered physiological function.

Epiblast: *spatial dynamics of mesodermal birth*



"It is not birth, marriage, or death, but **gastrulation** which is truly the most important time in your life." - *Lewis Wolpert*

Acknowledgements



<http://www2.epa.gov/sites/production/files/2015->

Virtual Tissues Team (CCTE/BCTD)

Todor Antonijevic
Nancy Baker – Leidos/SCDCD
John Cowden (MI)
Chad Deisenroth
John Gamble (ORISE)
Jill Franzosa
Sid Hunter
Richard Judson
Nicole Kleinstreuer (now NTP/NICEATM)
Kate Saili (now OAQPS)
Imran Shah
Richard Spencer – General Dynamics
Rusty Thomas
Doug Young (MI)
Todd Zurlinden (now CPHEA)

Stemina Biomarker Discovery, Madison WI

Michael Colwell
Jessica Palmer

Dow Chemical Company, Midland MI

Ed Carney
Rob Ellis-Hutchings
Raja Settivardi

DRP team for EST

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