

# Evaluation for developmental toxicity using human iPS cells

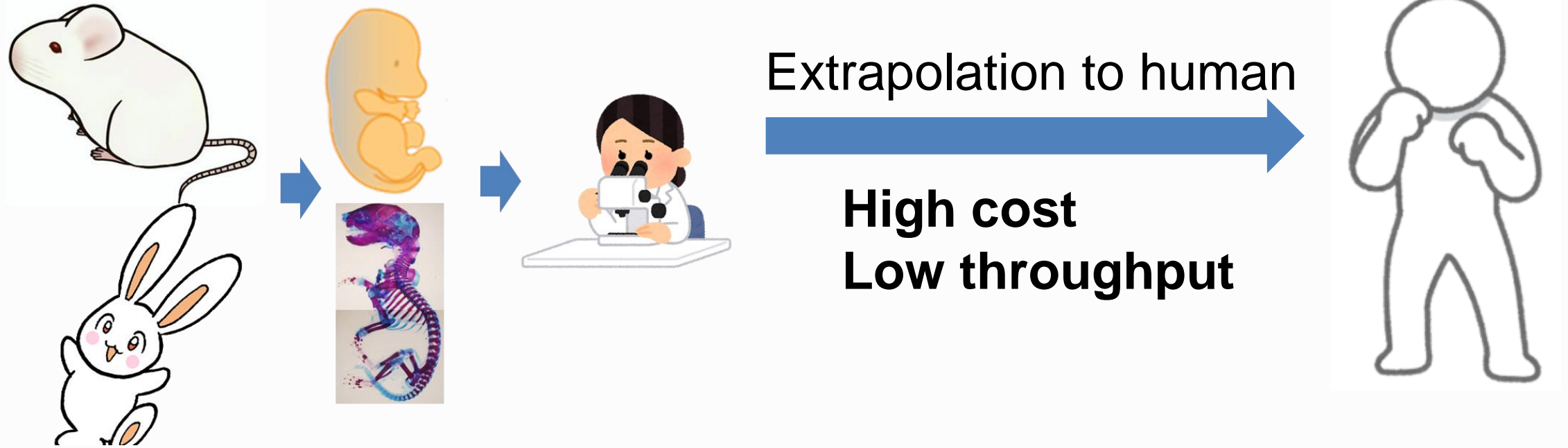
**Yusuke OKUBO**

National Institute of Health Sciences (JAPAN)



# Fundamental challenge in developmental toxicity testing

**Low sensitivity stemming from species differences.**



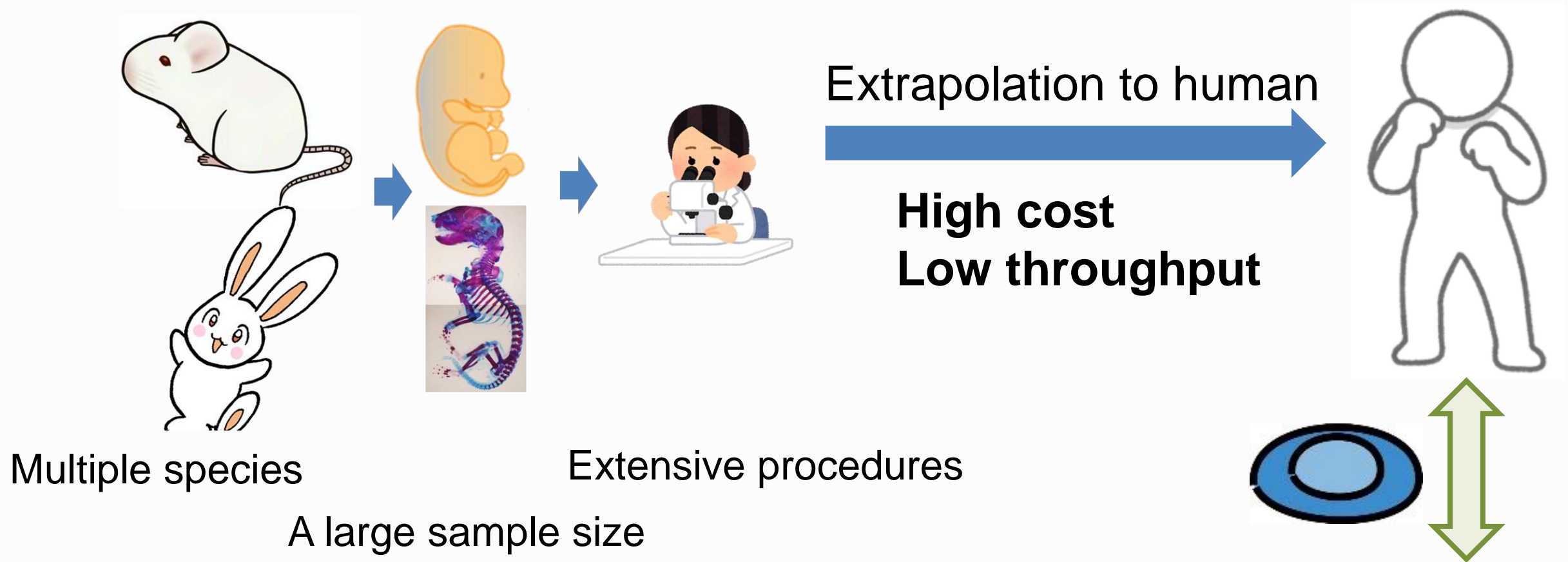
Multiple species

Extensive procedures

A large sample size

# Fundamental challenge in developmental toxicity testing

**Low sensitivity stemming from species differences.**



Utilizing human cells, especially ES/iPS cells

# Lengthy and complexity of developmental processes

## Human development



## Developmental processes

- Temporal dependency
- Proliferation
- Differentiation
- Migration
- Cell-cell interaction
- Tissue formation
- Organogenesis



# Lengthy and complexity of developmental processes

## Human development



## Developmental processes

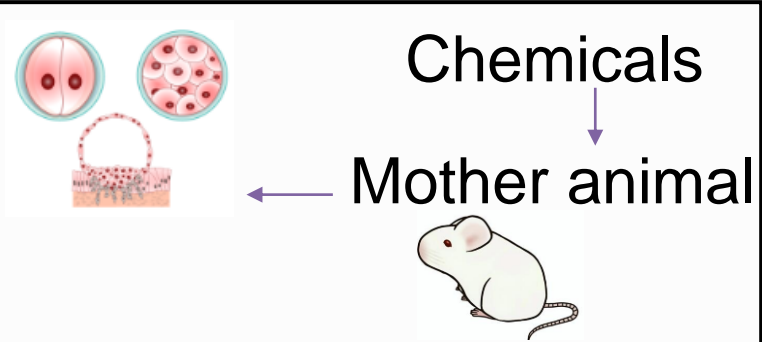
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Evaluation for all  
developmental processes

# Current developmental toxicity testing

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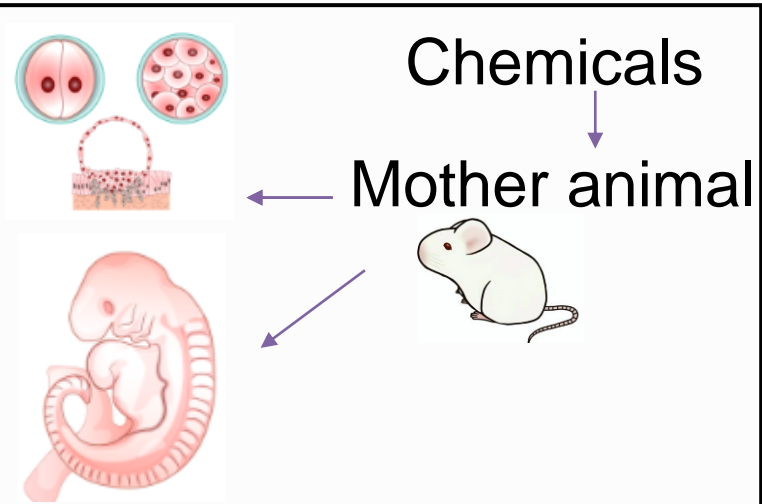
## Animal testing



# Current developmental toxicity testing

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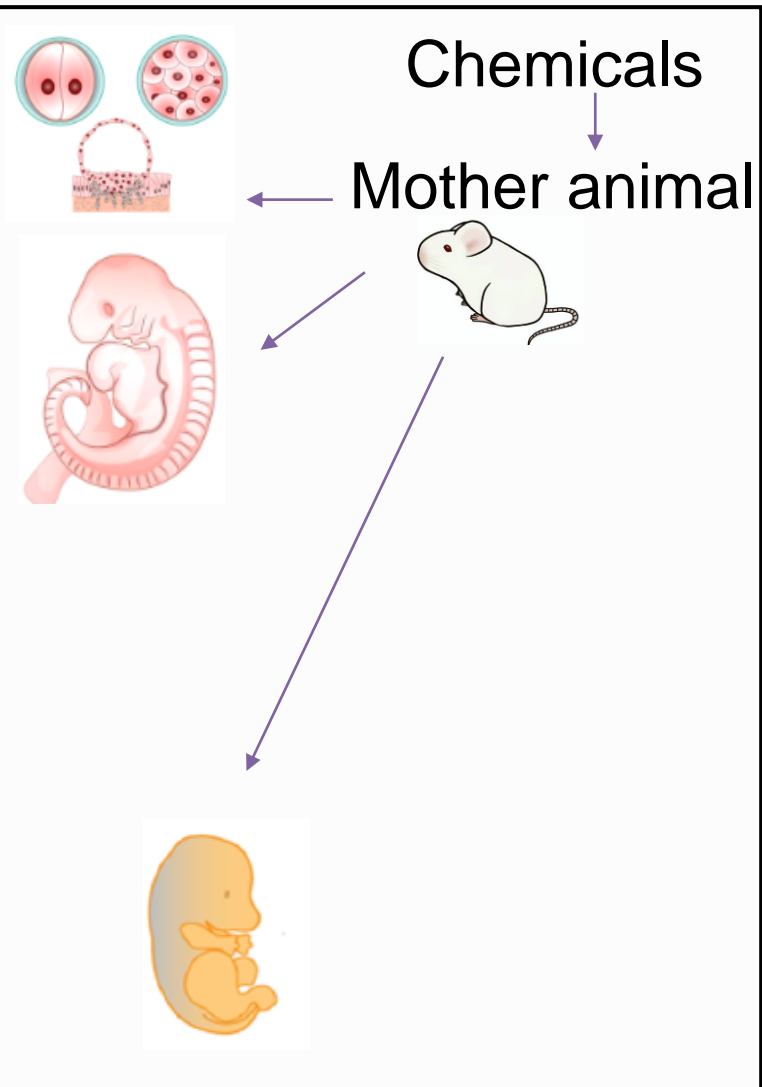
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# Current developmental toxicity testing

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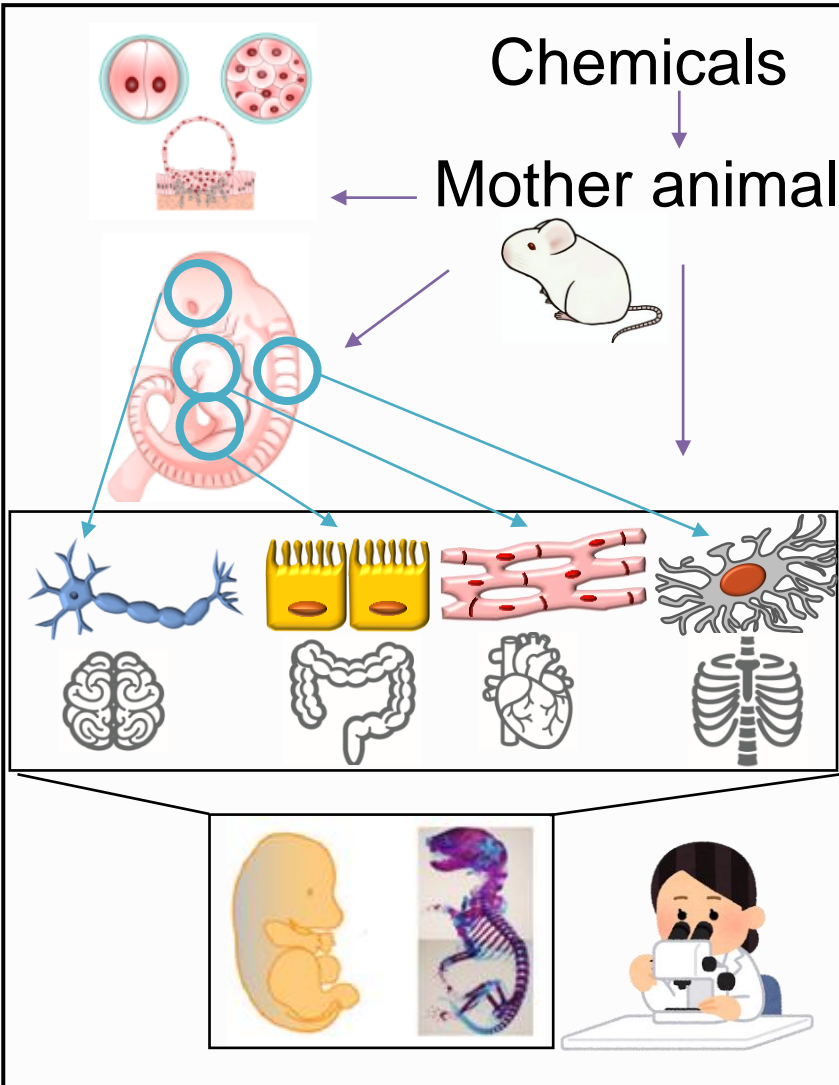
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# Current developmental toxicity testing

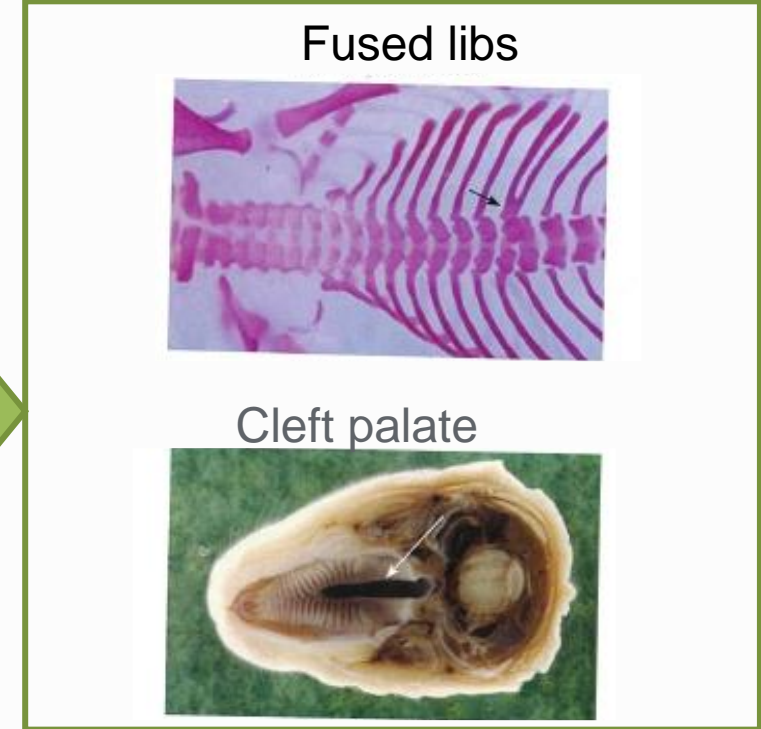
## Animal testing



## Developmental processes

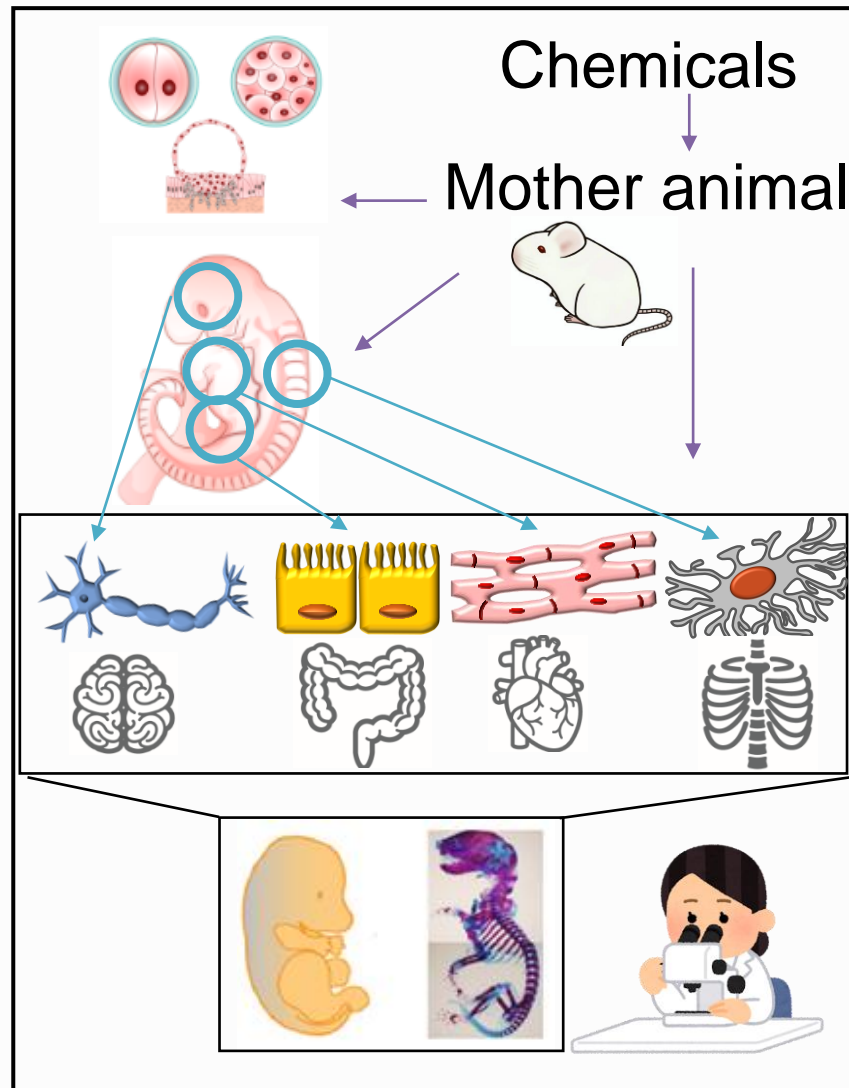
- Temporal dependency
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- 
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## Examples of malformation



# Current developmental toxicity testing

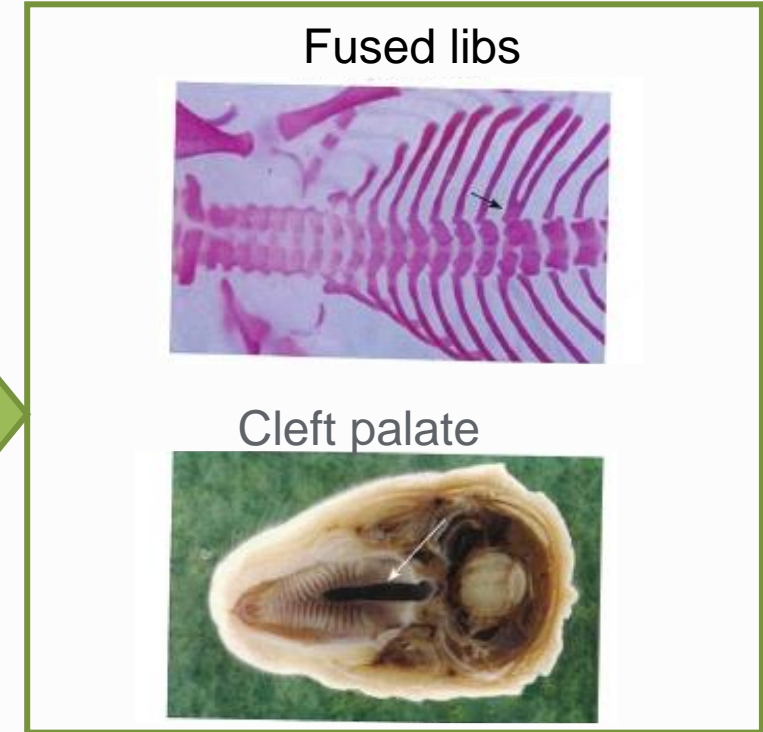
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## Developmental processes

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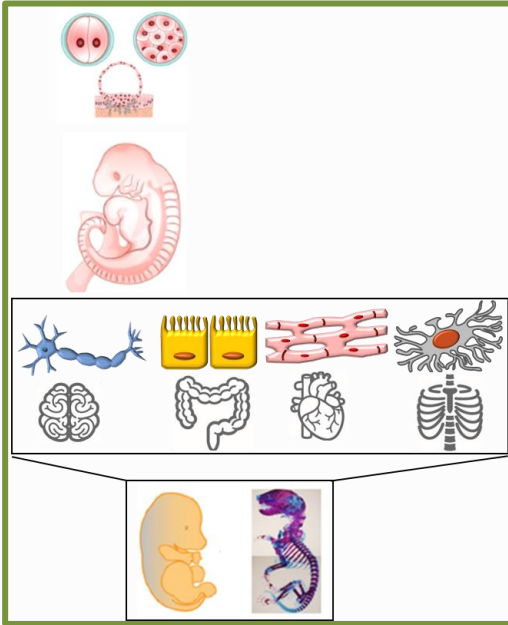
## Examples of malformation



Evaluating developmental toxicity based on the outcome of chemical induced effects.

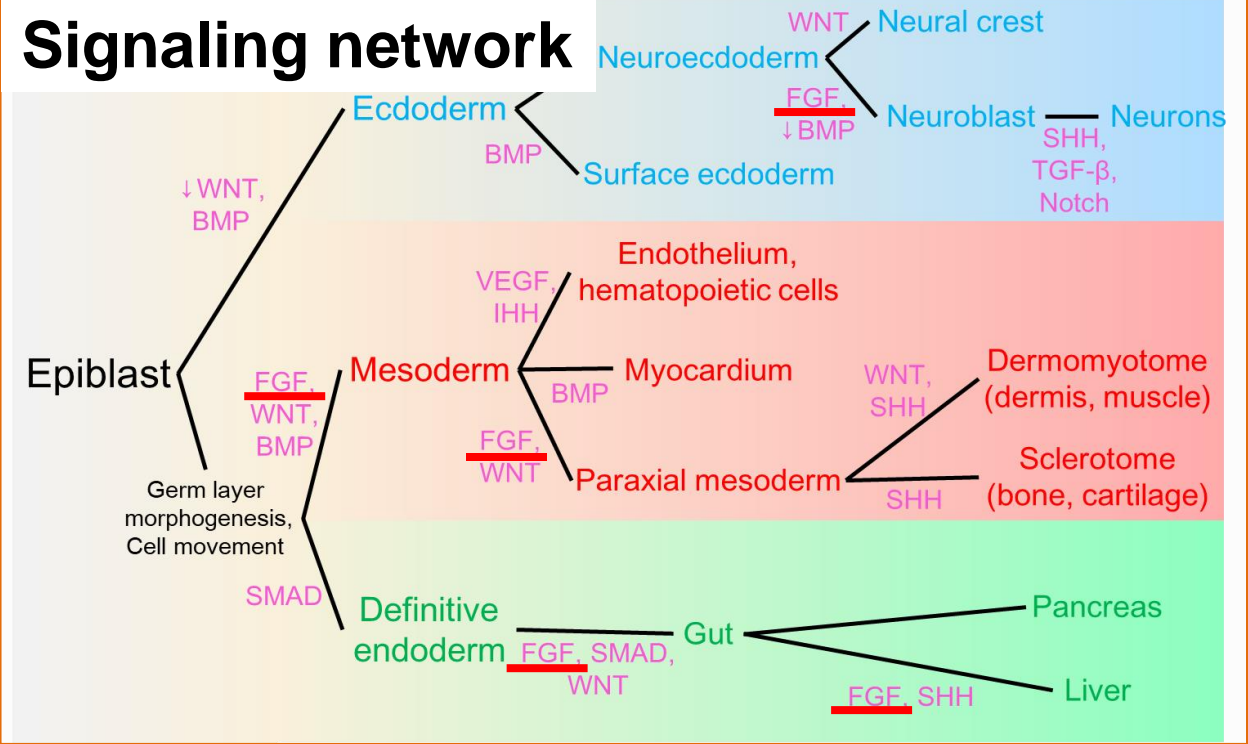
# Developmental processes are regulated by signal transduction

## Developmental processes



- Temporal dependency
- Proliferation
- Differentiation
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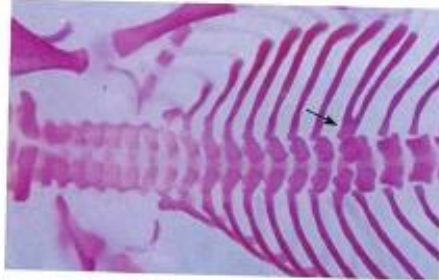
## Signaling network



# Developmental processes are regulated by signal transduction

## Examples of malformation

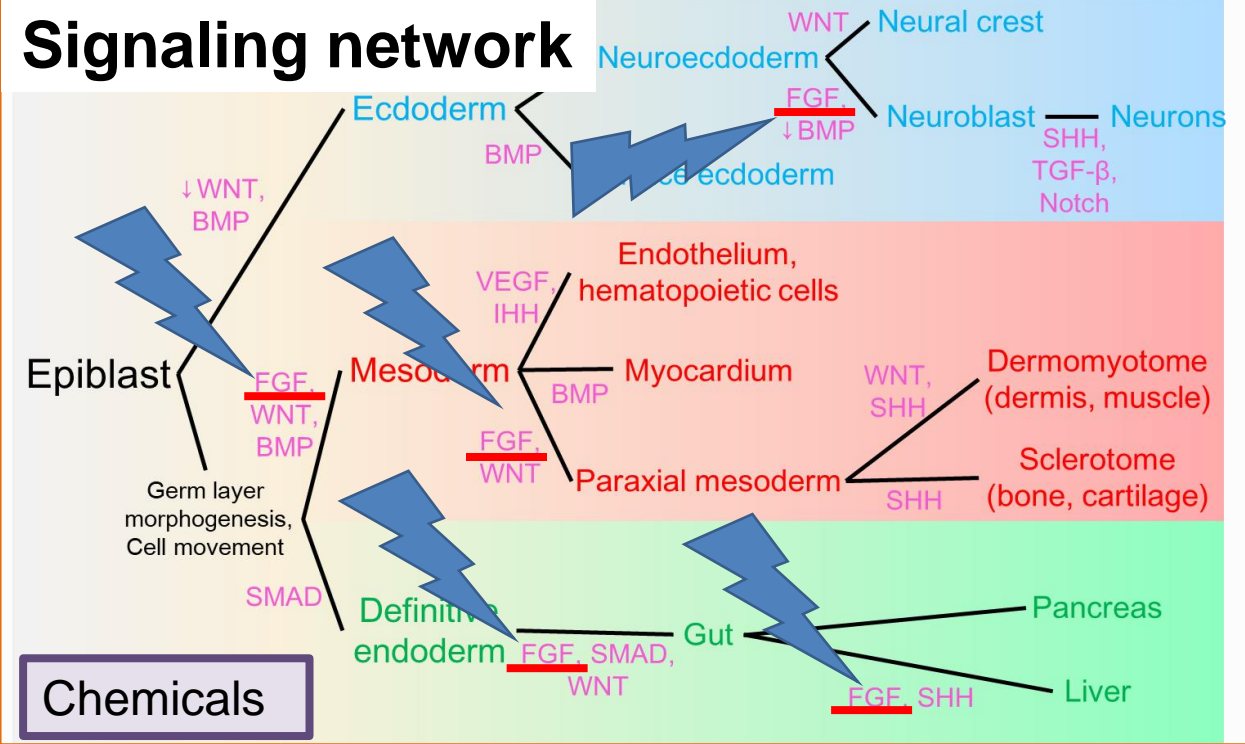
Fused ribs



Cleft palate



## Signaling network



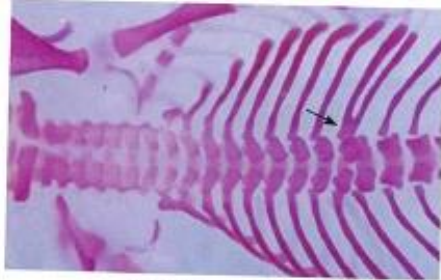
Hypothesis: Malformation ← Signal disruption (directly )



# Developmental processes are regulated by signal transduction

## Examples of malformation

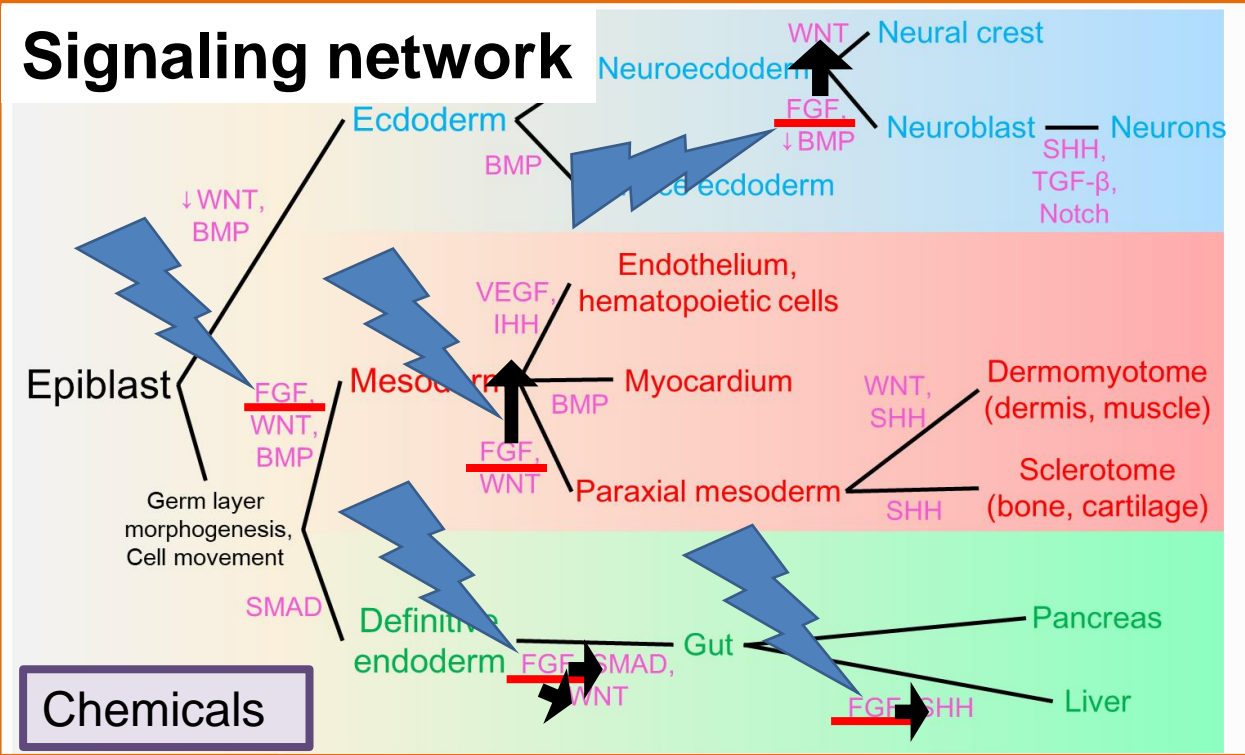
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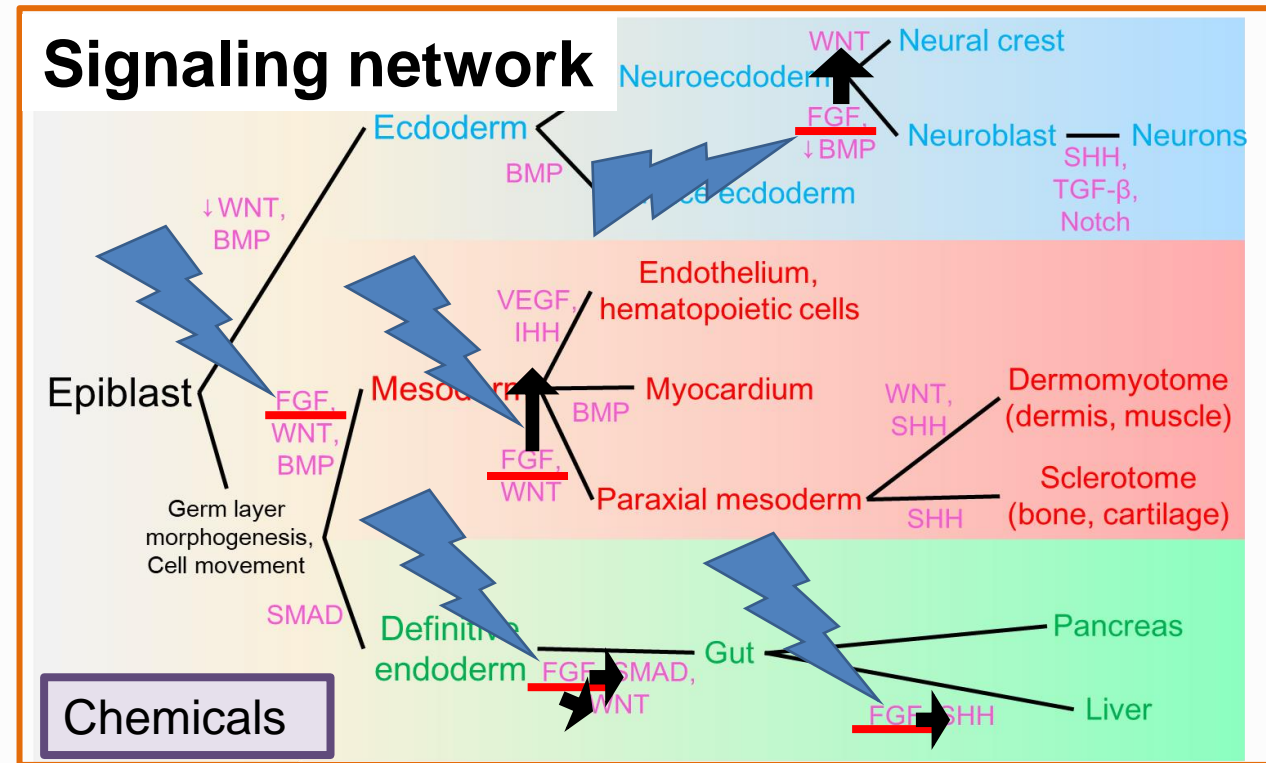
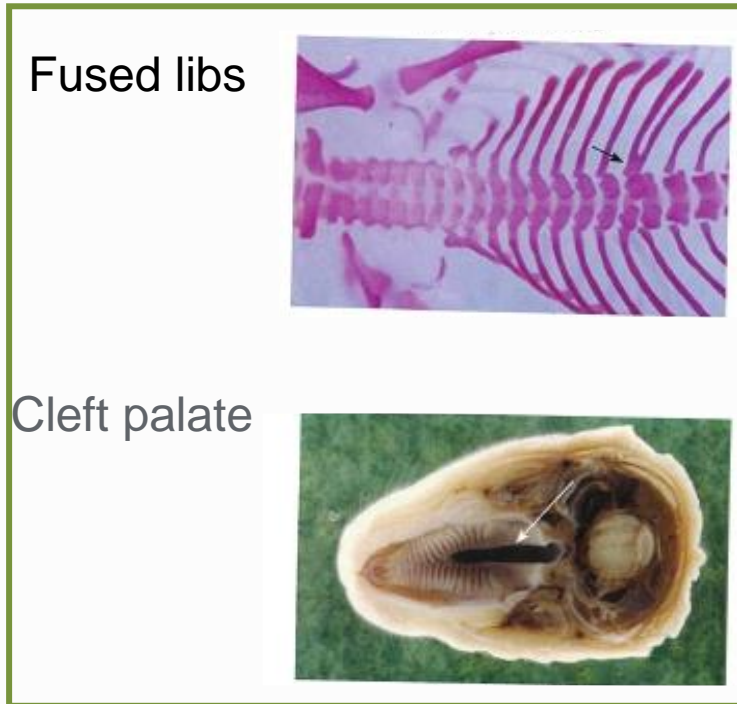
## Signaling network



Hypothesis: Malformation  Signal disruption (directly or indirectly)

# Developmental processes are regulated by signal transduction

## Examples of malformation

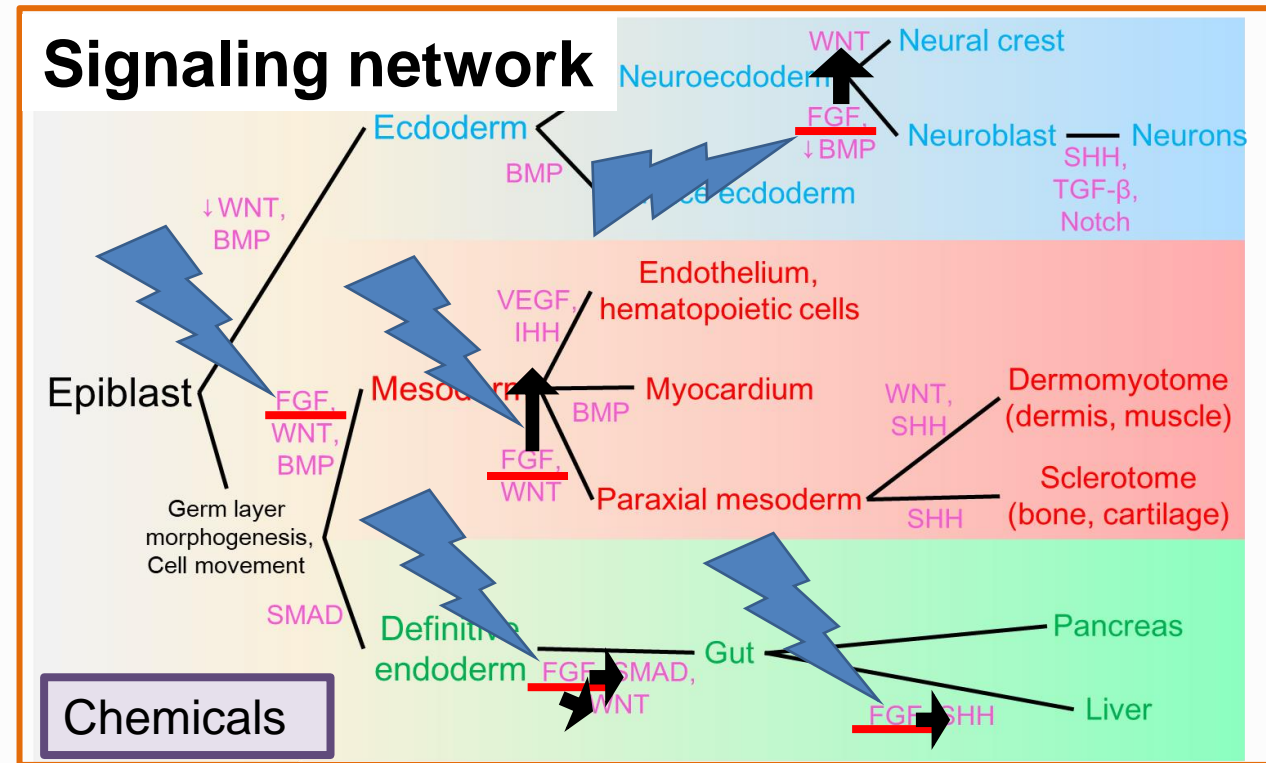
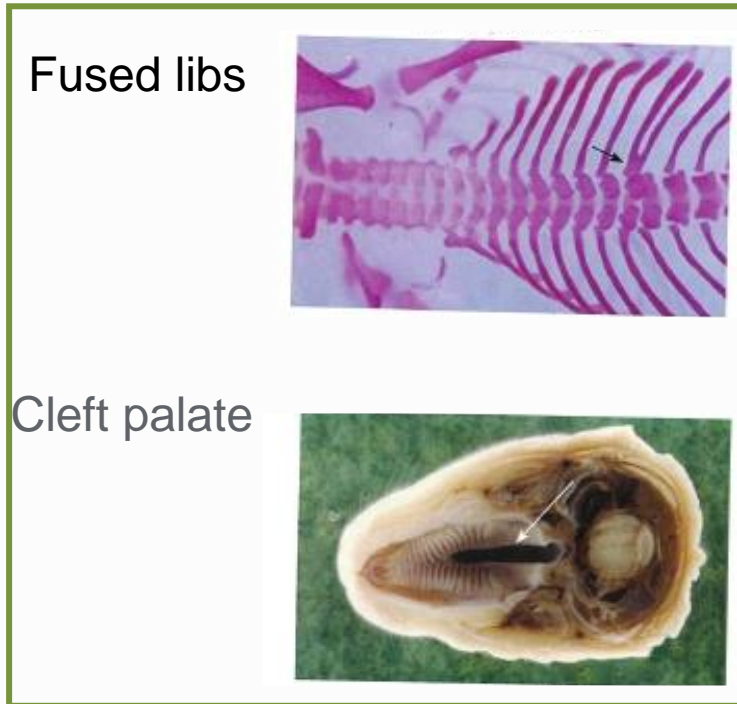


Hypothesis: Malformation  Signal disruption (directly or indirectly)  
Can we evaluate developmental toxicity based on signal disruption?



# Developmental processes are regulated by signal transduction

## Examples of malformation

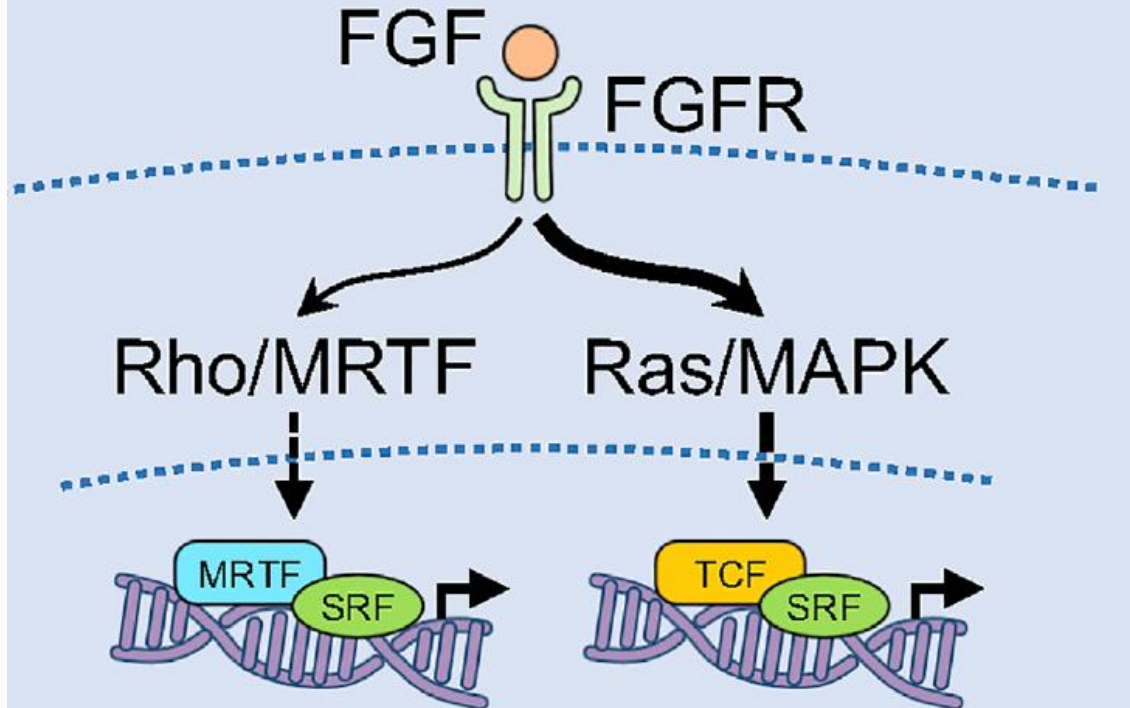


Hypothesis: Malformation  Signal disruption (directly or indirectly)  
Can we evaluate developmental toxicity based on signal disruption?

 Trying to detect the dynamics of signal disruption by chemicals.

# Method : Establishment of FGF-SRF signal reporter cell line

## FGF-SRF signaling pathway

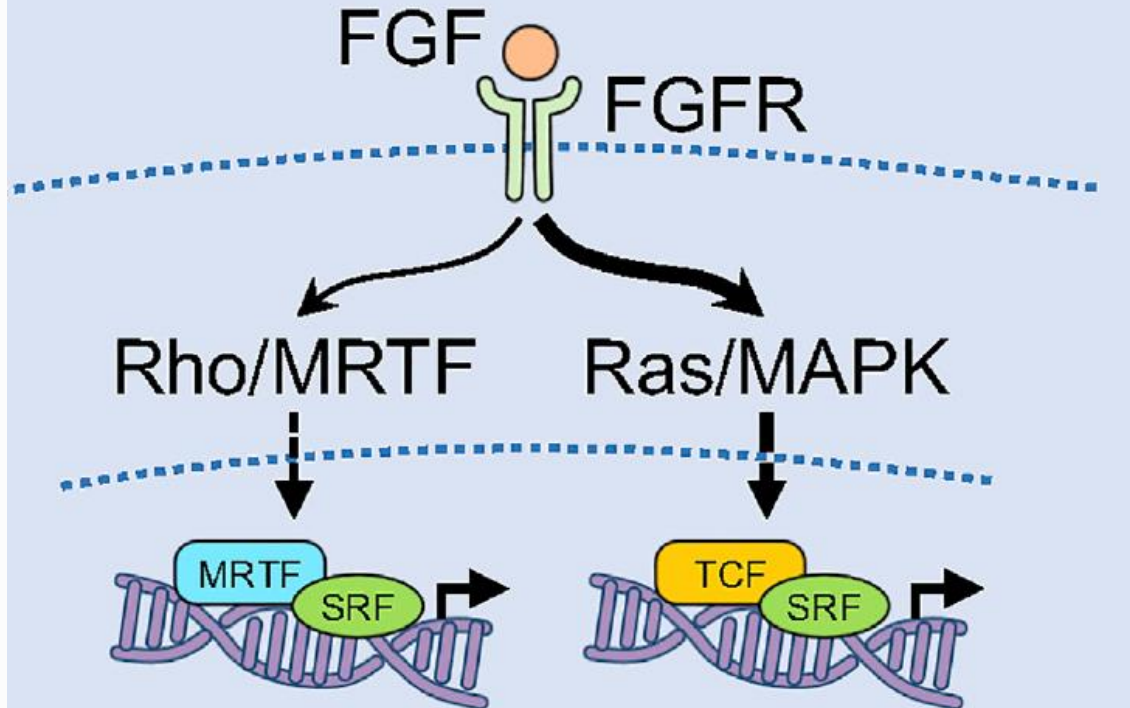


(Kanno *et al.*, *iScience*)

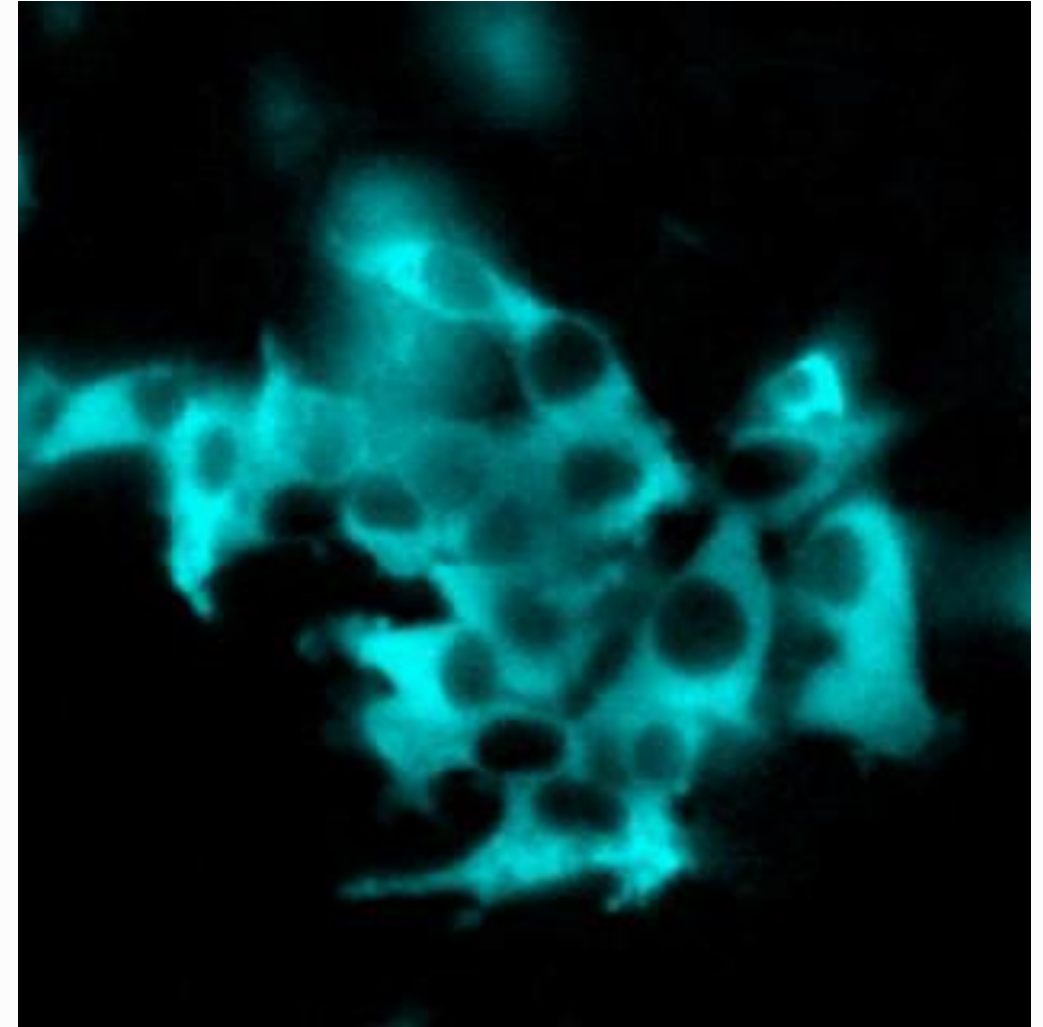
# Method : Establishment of FGF-SRF signal reporter cell line

Live-cell luciferase assay using NanoLuc

## FGF-SRF signaling pathway



(Kanno *et al.*, *iScience*)

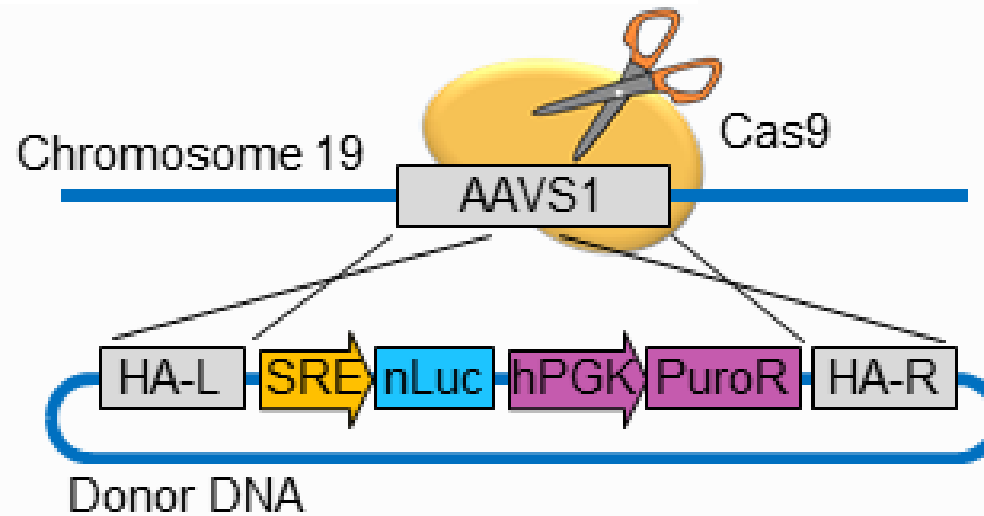


(Promega)

# Method : Establishment of FGF-SRF signal reporter cell line

Knock-in the construct into AAVS1 locus

Human iPSCs 201B7 line



AAVS1 locus:

A region in the human genome where inserted gene is stably expressed and do not interfere with the expression of other genes

Live-cell luciferase assay system

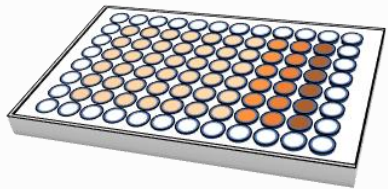
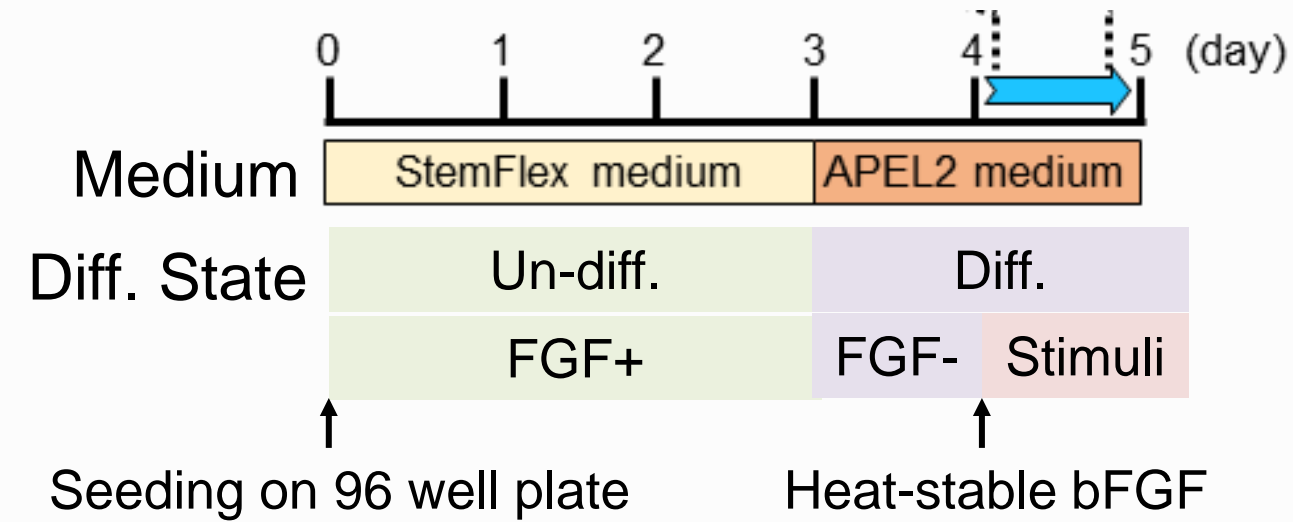
## Method : Evaluated chemicals (ECVAM validation study on in vitro embryotoxicity tests)

**The maximum concentration used was below the IC50 value; otherwise it was determined by maximum dissolved volume.**

Developmental toxicity	Test chemicals	Abbreviation	CAS No.	Vehicle	Max Conc. (µg/mL)
Positive	all-trans-Retinoic acid	ATRA	302-79-4	DMSO	0.010
	Hydroxyurea	HU	127-07-1	PBS	<u>149</u>
	Methoxyacetic acid	MAA	625-45-6	PBS	<u>683</u>
	Methylmercury chloride	MeHg	115-09-3	DMSO	<u>1.20</u>
	Methotrexate hydrate	MTX	133073-73-1	DMSO	5.00
	Sodium salicylate	SA	54-21-7	PBS	<u>666</u>
	Valproic acid	VPA	99-66-1	DMSO	100
	6-Aminonicotinamide	6-AN	329-89-5	DMSO	1.00
	Boric acid	BA	10043-35-3	PBS	250
	5-Bromo-2'-deoxyuridine	BrdU	59-14-3	DMSO	50.0
	5,5-Dimethyl-2,4-oxazolidinedione	DMO	695-53-4	PBS	840
	Lithium chloride	LiCl	7447-41-8	PBS	250
Negative	Acrylamide	AcA	79-06-1	PBS	<u>454</u>
	D-Camphor	CAM	464-49-3	DMSO	50.0
	Diphenhydramine hydrochloride	DHM	147-24-0	PBS	<u>262</u>
	Dimethyl phthalate	DMP	131-11-3	DMSO	100
	Penicillin G sodium salt	PenG	69-57-8	PBS	1,000
	Sodium saccharin	SAC	82385-42-0	PBS	1,000

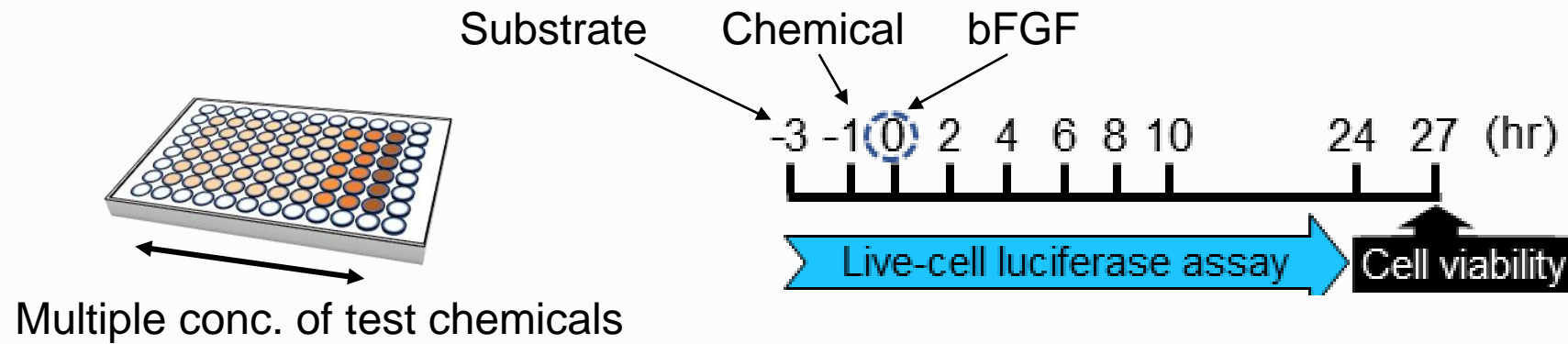
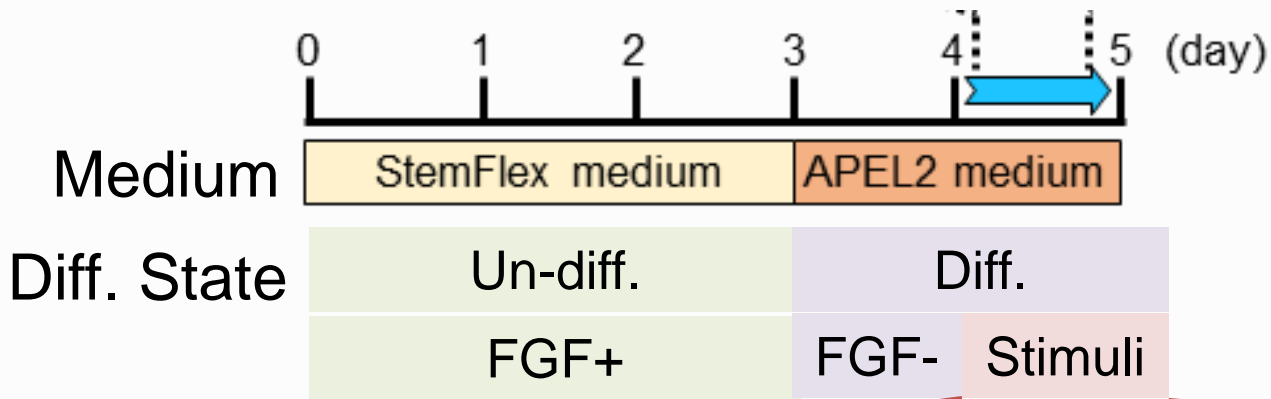
(Kanno *et al.*, *iScience*)

# Experimental procedure to detect the dynamics of signal disruption

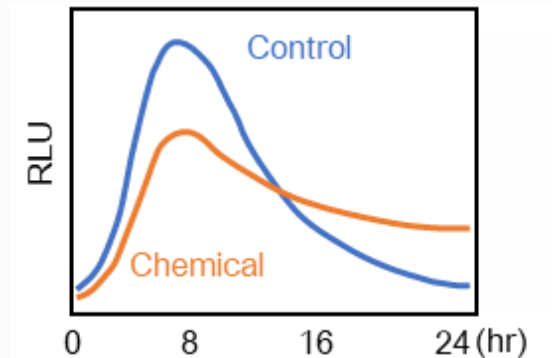




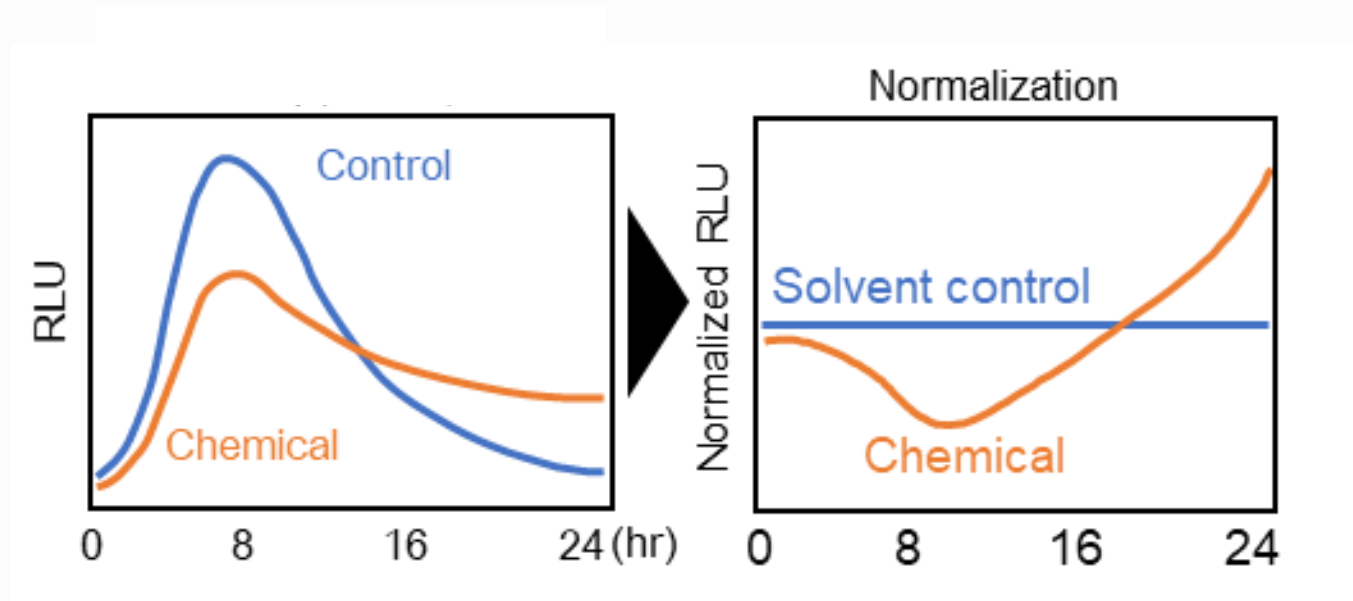
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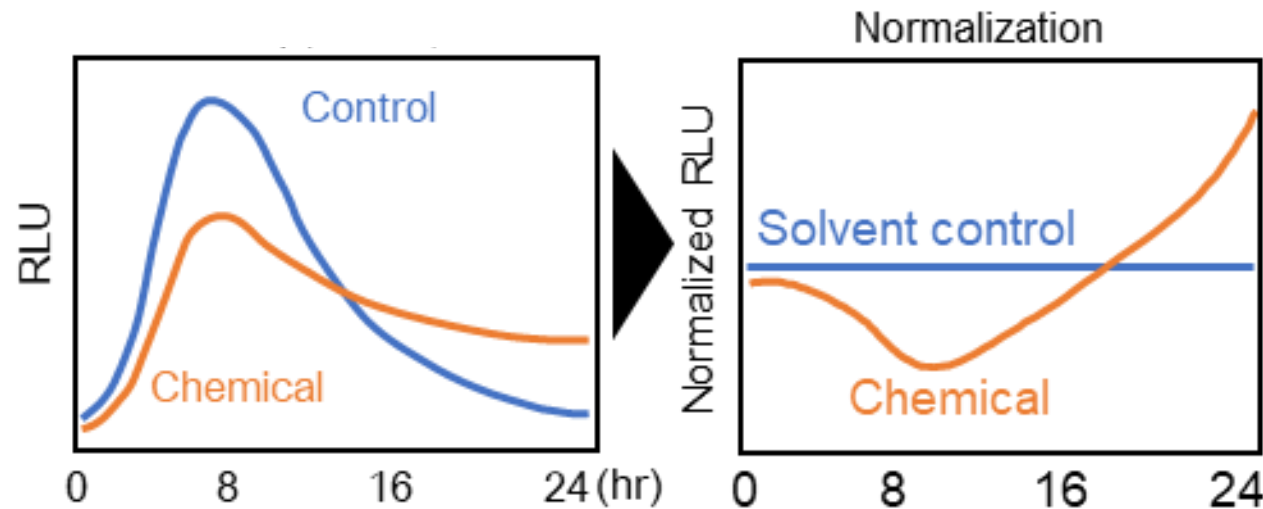
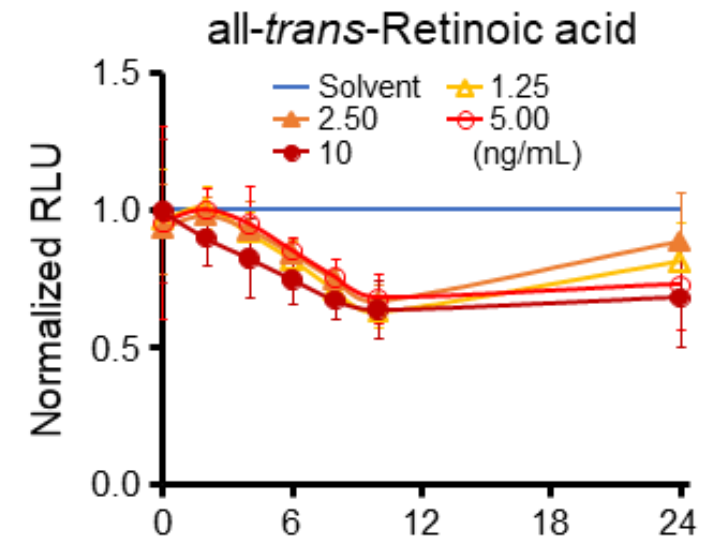
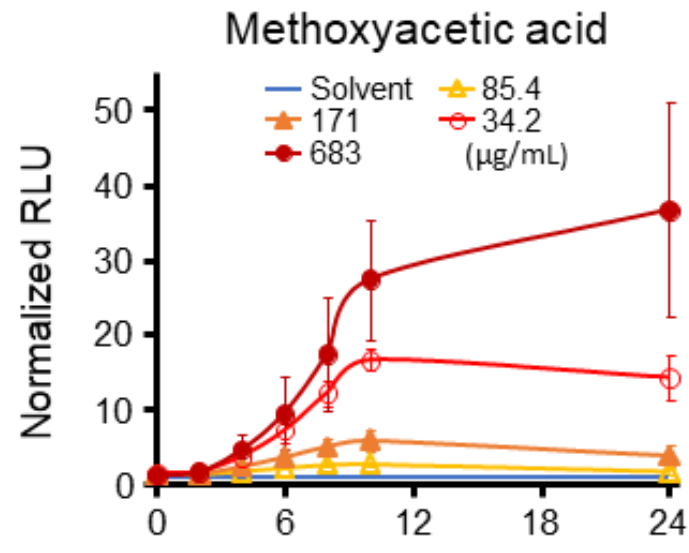
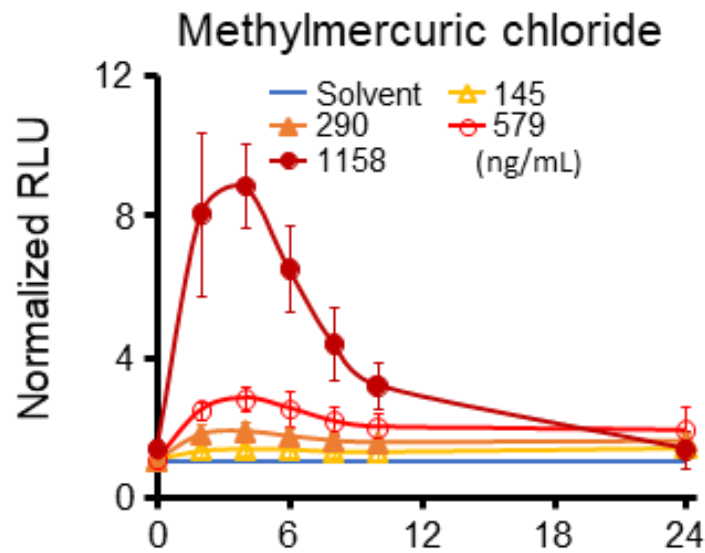
## Signal dynamics



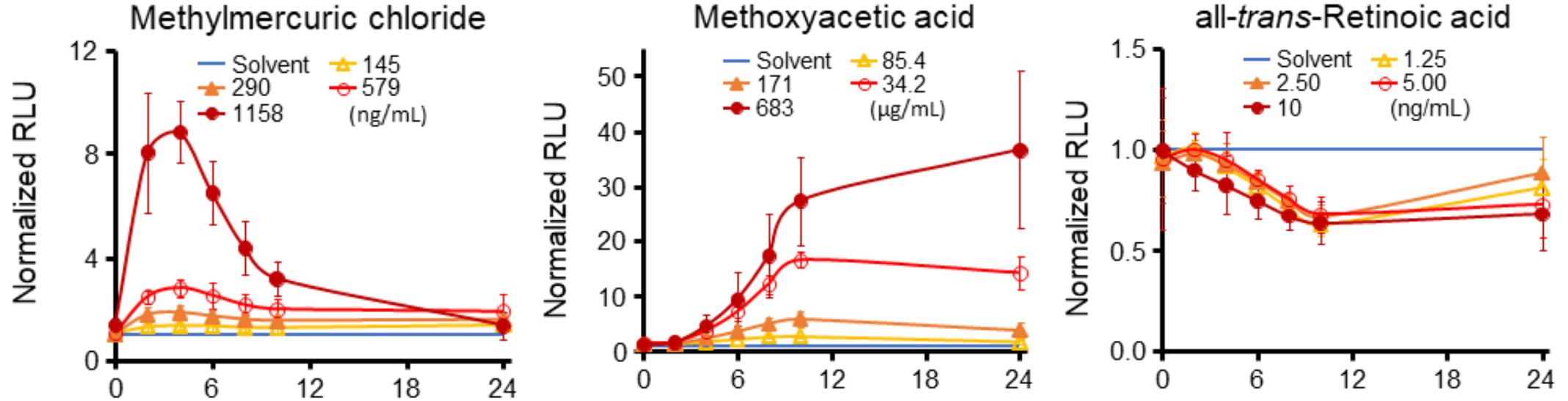
FGF-SRF signal was disrupted at different timing for each chemical.



# FGF-SRF signal was disrupted at different timing for each chemical.

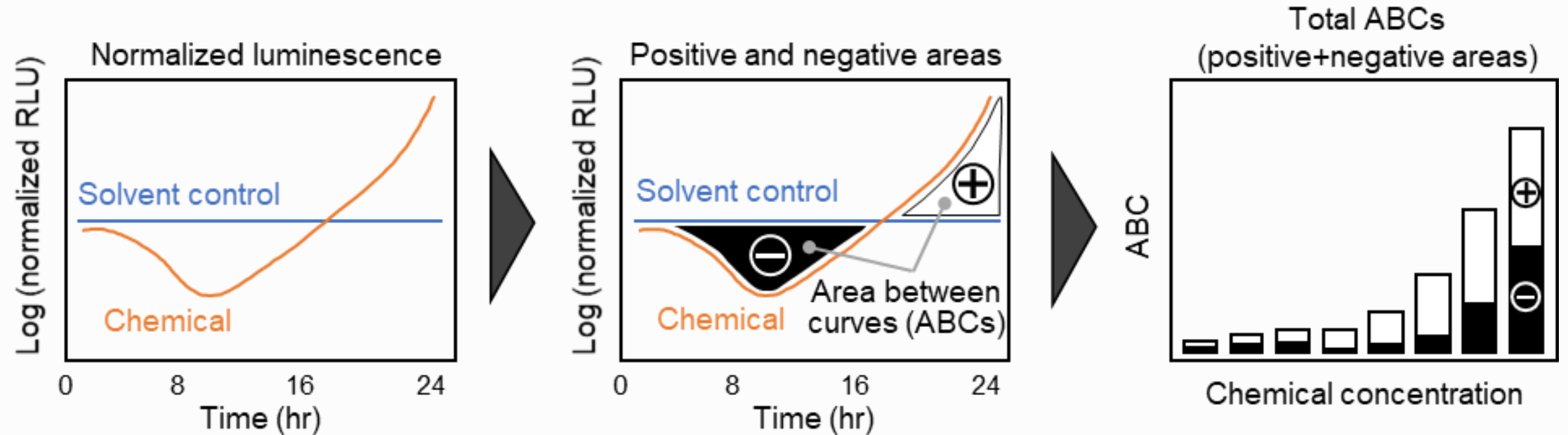


## FGF-SRF signal was disrupted at different timing for each chemical.

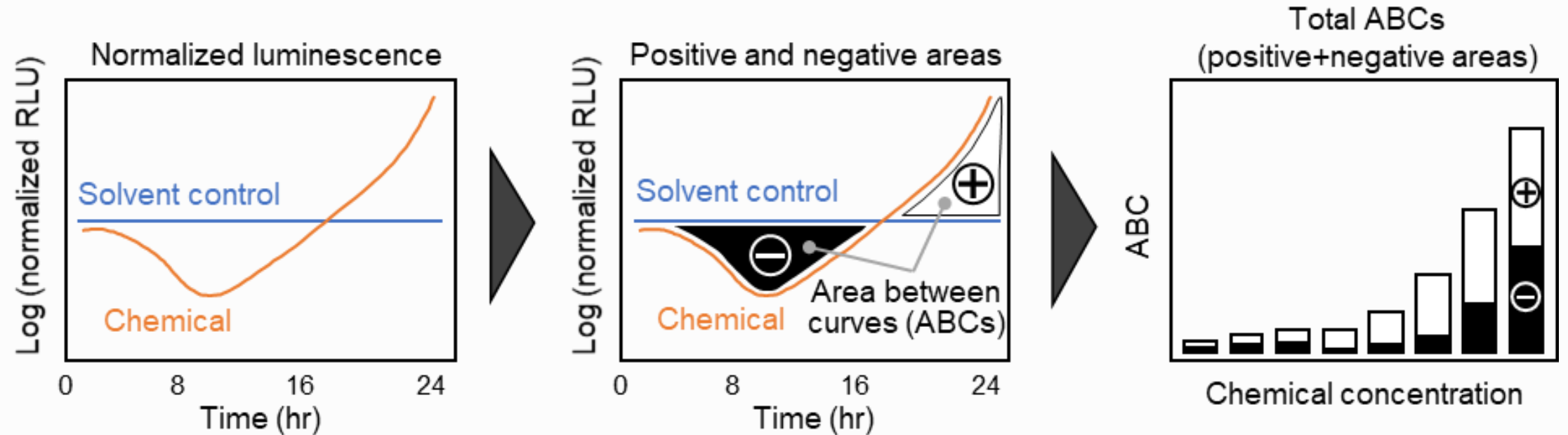


The impact of signal disruption need to capture the dynamics.

# Results : Area between the curve (ABC)

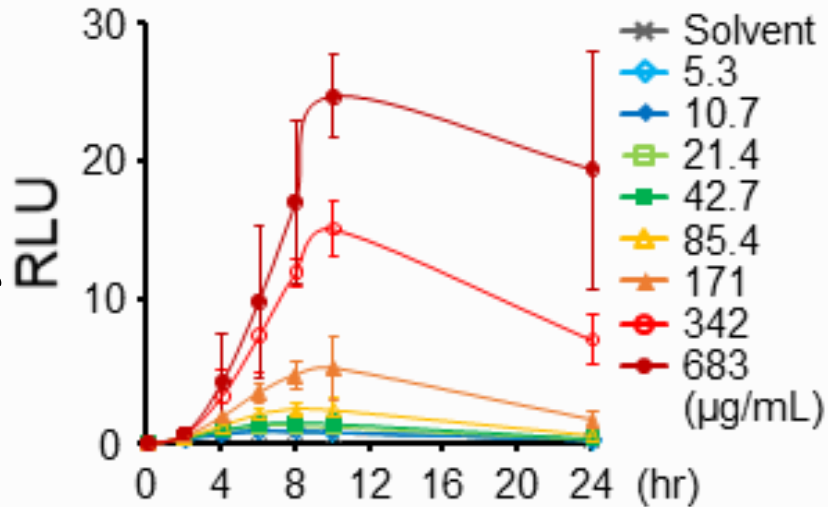


# Results : Area between the curve (ABC)

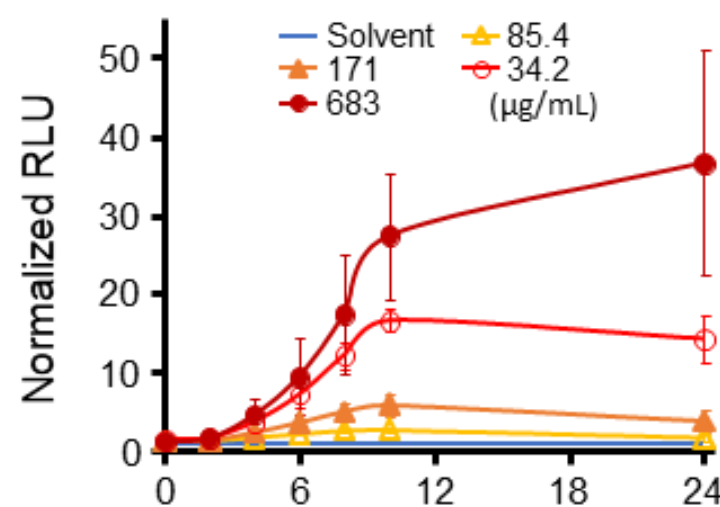


Methoxyacetic acid

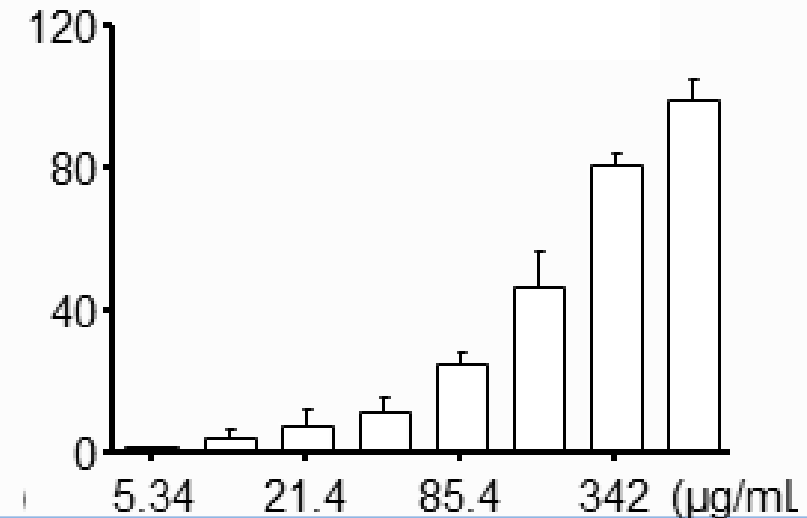
## Raw data



## Normalized data

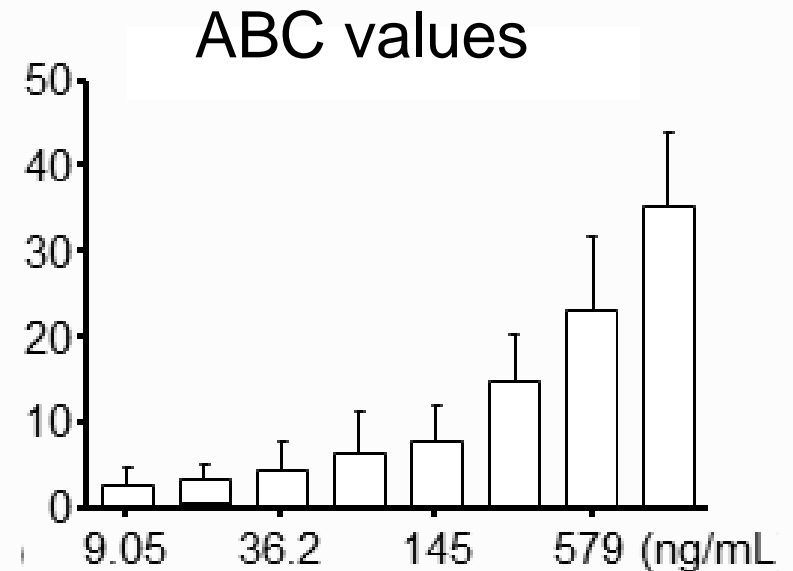
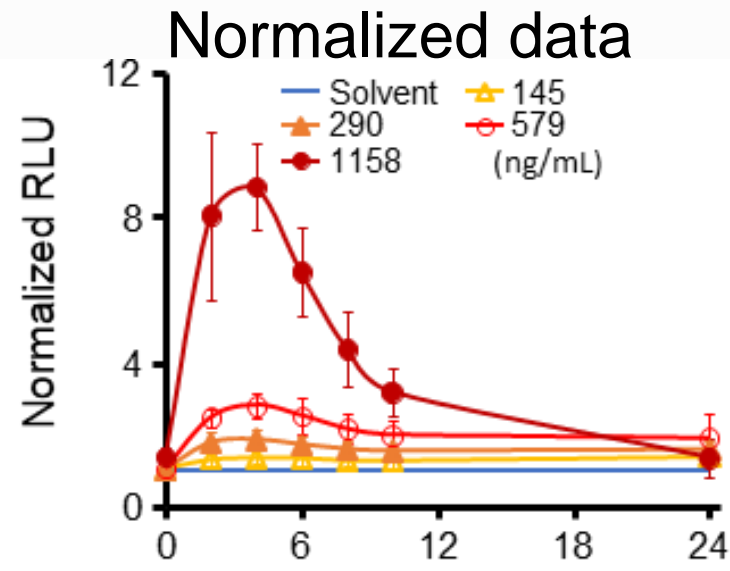
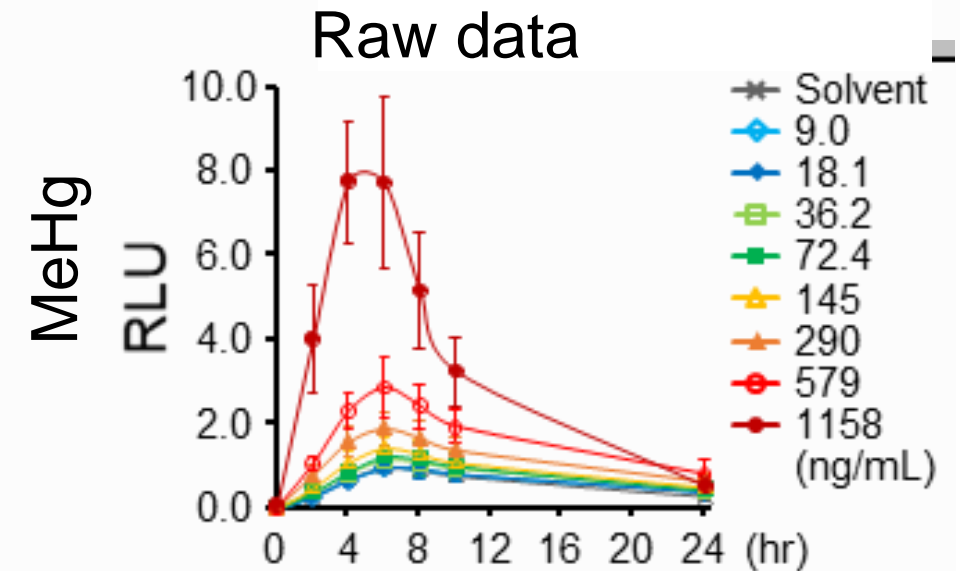
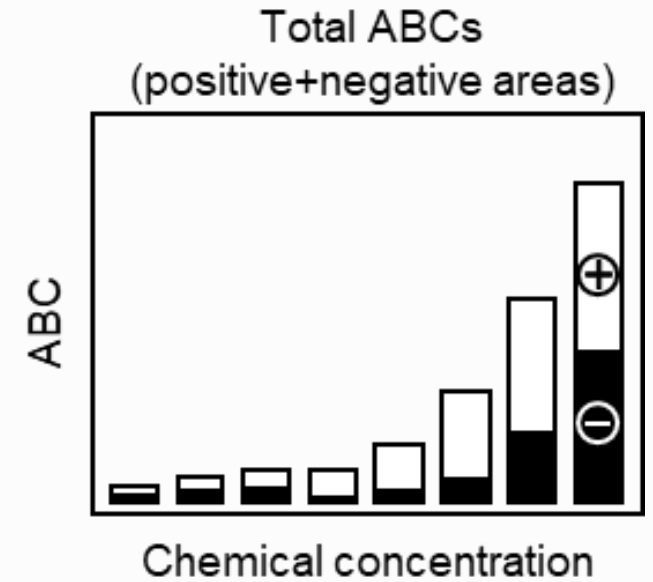
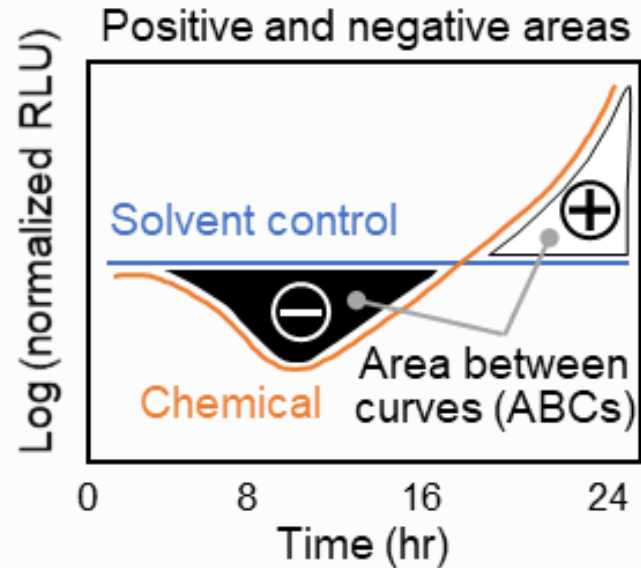
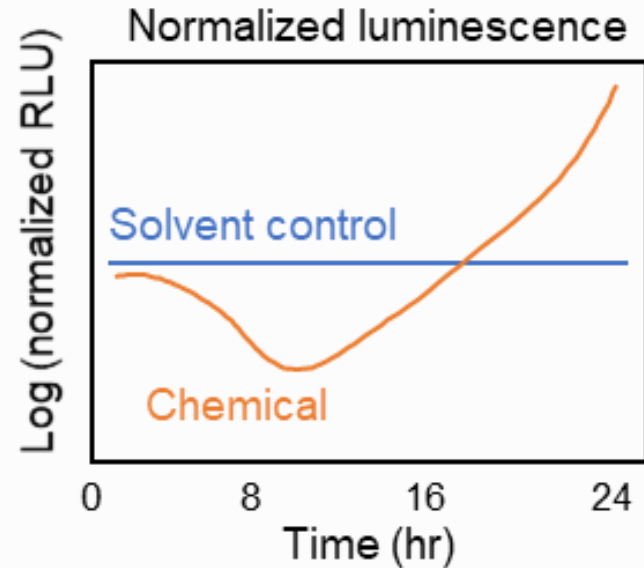


## ABC values

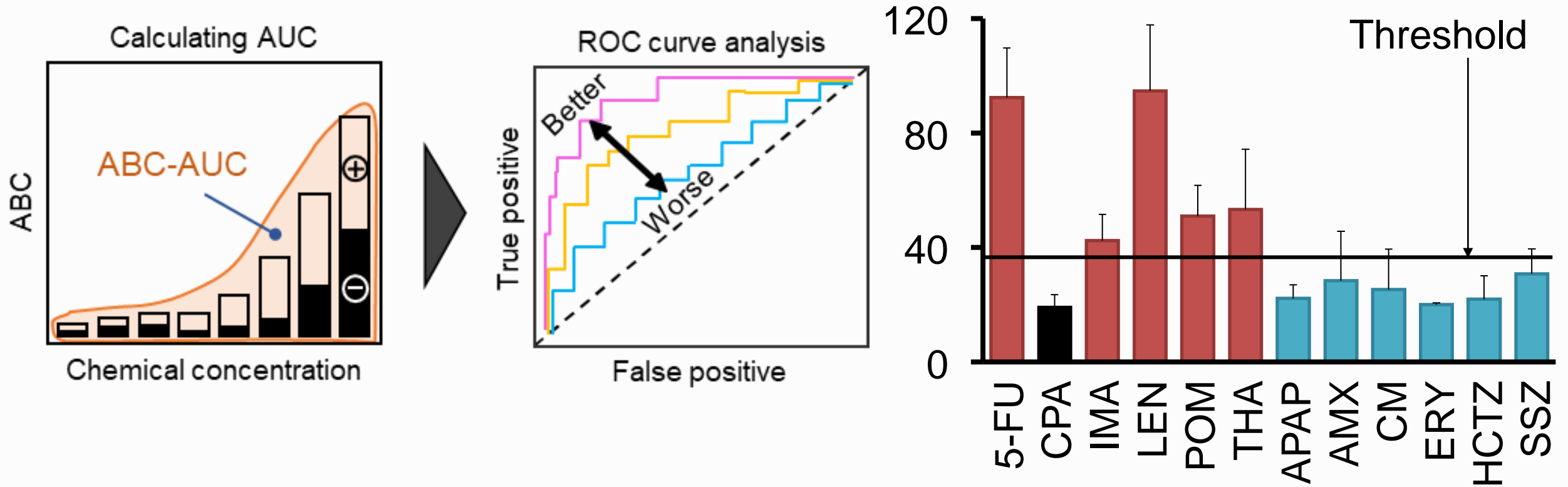




# Results : Area between the curve (ABC)



# Results : ROC curve analysis



# Summary of evaluated results: 21 toxicants and 14 non-toxicants

	Developmental toxicity +	MAX conc.	Developmental toxicity -	MAX conc.
1	5,5-Dimethyl-2,4-oxazolidinedione	840 ug/mL	Acetaminophen	20 ug/mL
2	5-Bromo-2'-deoxyuridine	50 ug/mL	Acrylamide	454 ug/mL
3	5-Fluorouracil*	14.1 ug/mL	Amoxicillin Trihydrate	25 ug/mL
4	6-Aminonicotinamide	1 ug/mL	Cefotaxime	10 ug/mL
5	all- <i>trans</i> -Retinoic acid*	0.01 ug/mL	Cimetidine	30 ug/mL
6	Boric acid	250 ug/mL	D-Camphor	50 ug/mL
7	Cyclophosphamide Monohydrate*	400 ug/mL	Dimethyl phthalate	100 ug/mL
8	Hydroxyurea*	149 ug/mL	Erythromycin	15 ug/mL
9	Imatinib (mesylate)*	12.5 ug/mL	Hydrochlorothiazide	20 ug/mL
10	Lenalidomide	35 ug/mL	L-Ascorbic Acid	1000 ug/mL
11	Lithium chloride	250 ug/mL	Penicillin G sodium salt	1000 ug/mL
12	Methotrexate hydrate*	5 ug/mL	Progesterone	10 ug/mL
13	Methoxyacetic acid	683 ug/mL	Sodium saccharin	1000 ug/mL
14	Methylmercury chloride	1.2 ug/mL	Sulfasalazine	100 ug/mL
15	Misoprostol	50 ug/mL		
16	Phenytoin*	5 ug/mL		
17	Pomalidomide *	2.5 ug/mL		
18	Sodium salicylate	666 ug/mL		
19	(+/-)-Thalidomide*	12 ug/mL		
20	5-OH Thalidomide	12.5 ug/mL		
21	Valproic acid *	100 ug/mL		

\* ICH S5 (R3) Reference Compound Positive Control Examples for Qualifying Alternative Assays

**Accuracy: 89%**

(Specificity: 100%, Sensitivity: 81%)

Thalidomide, as well as its metabolite and derivatives, showed positive results.

Table 1. Alternative Models in Developmental Toxicity Testing

Model	Accuracy	References
Mouse embryonic stem cell test	78%	Genschow et al. (2002)
Rat MM test	70%	Genschow et al. (2002)
Rat WEC assay	80%	Genschow et al. (2002)
Zebrafish embryotoxicity test	72%	Chapin et al. (2008)
Frog embryo teratogenesis assay	NA	Bantle et al. (1989)

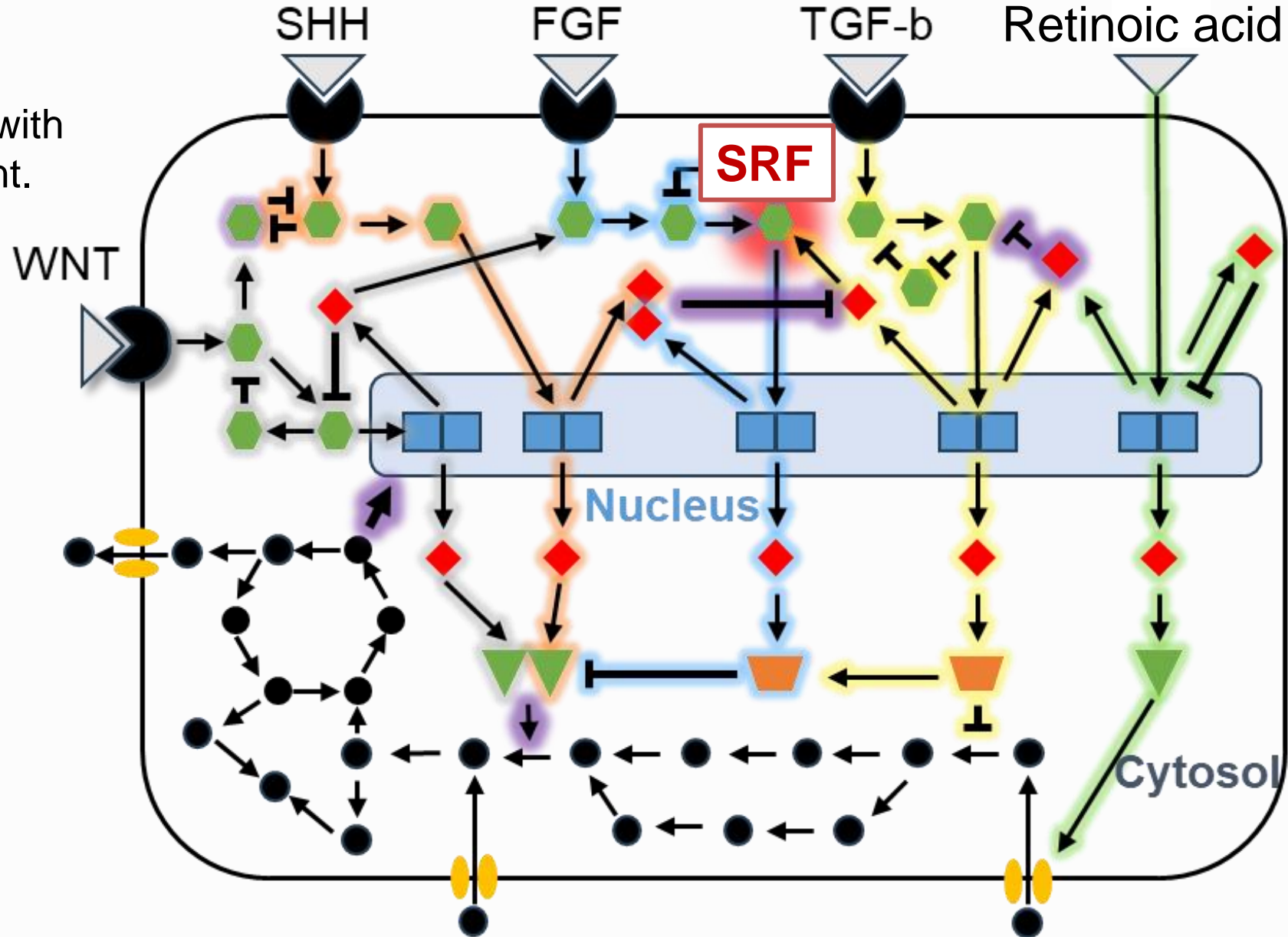
(TOXICOLOGICAL SCIENCES, 165(1), 2018, 31-39)

*iScience*, 2022. *JBB*, 2022. *STAR Protocols*, 2022.

# Hypothesis of the high accuracy results

## Conceptual diagram

Major signaling pathways interact with each other throughout development.

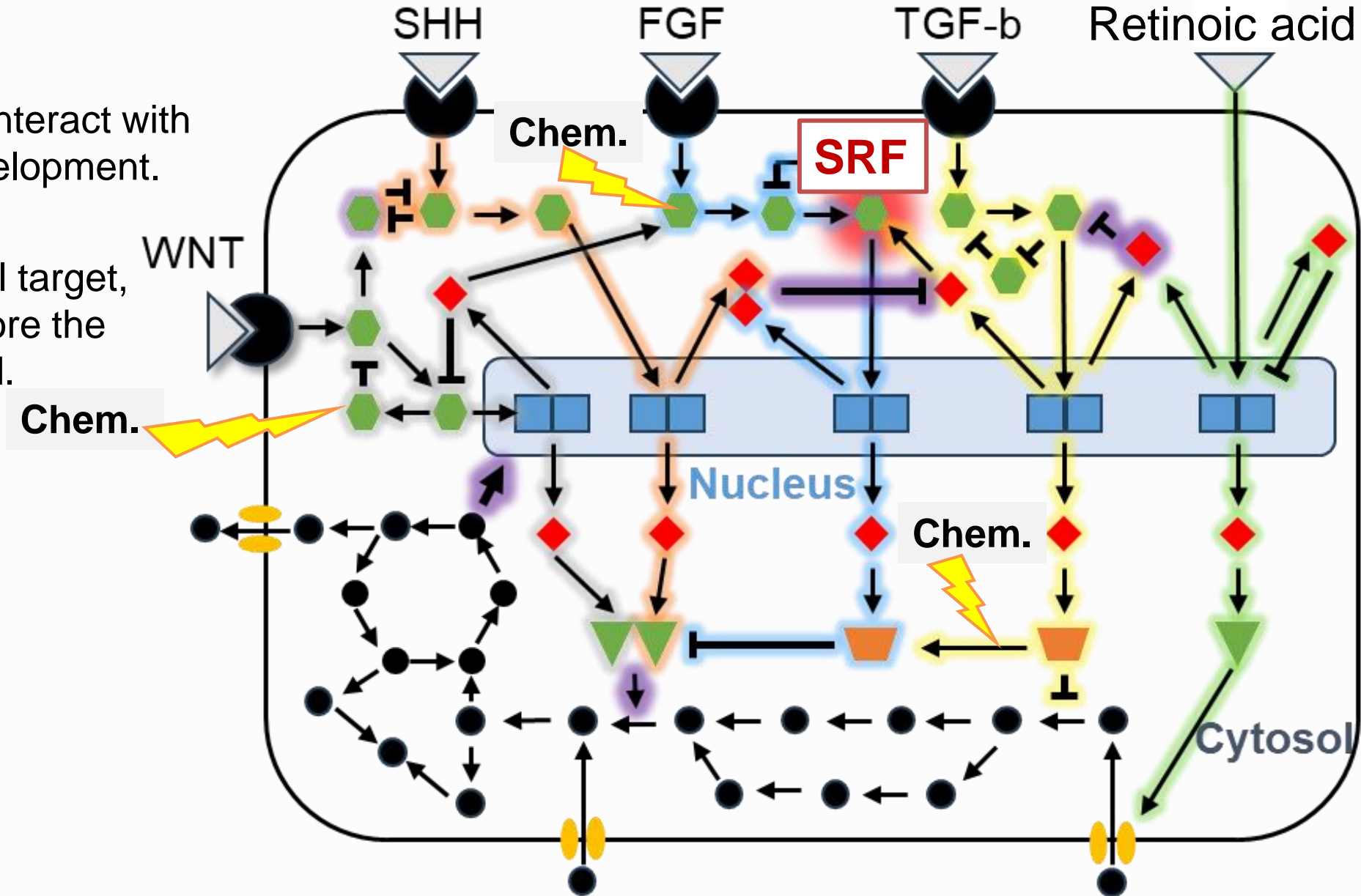


# Hypothesis of the high accuracy results

## Conceptual diagram

Major signaling pathways interact with each other throughout development.

Depending on the chemical target, there is time variability before the FGF-SRF signal is affected.





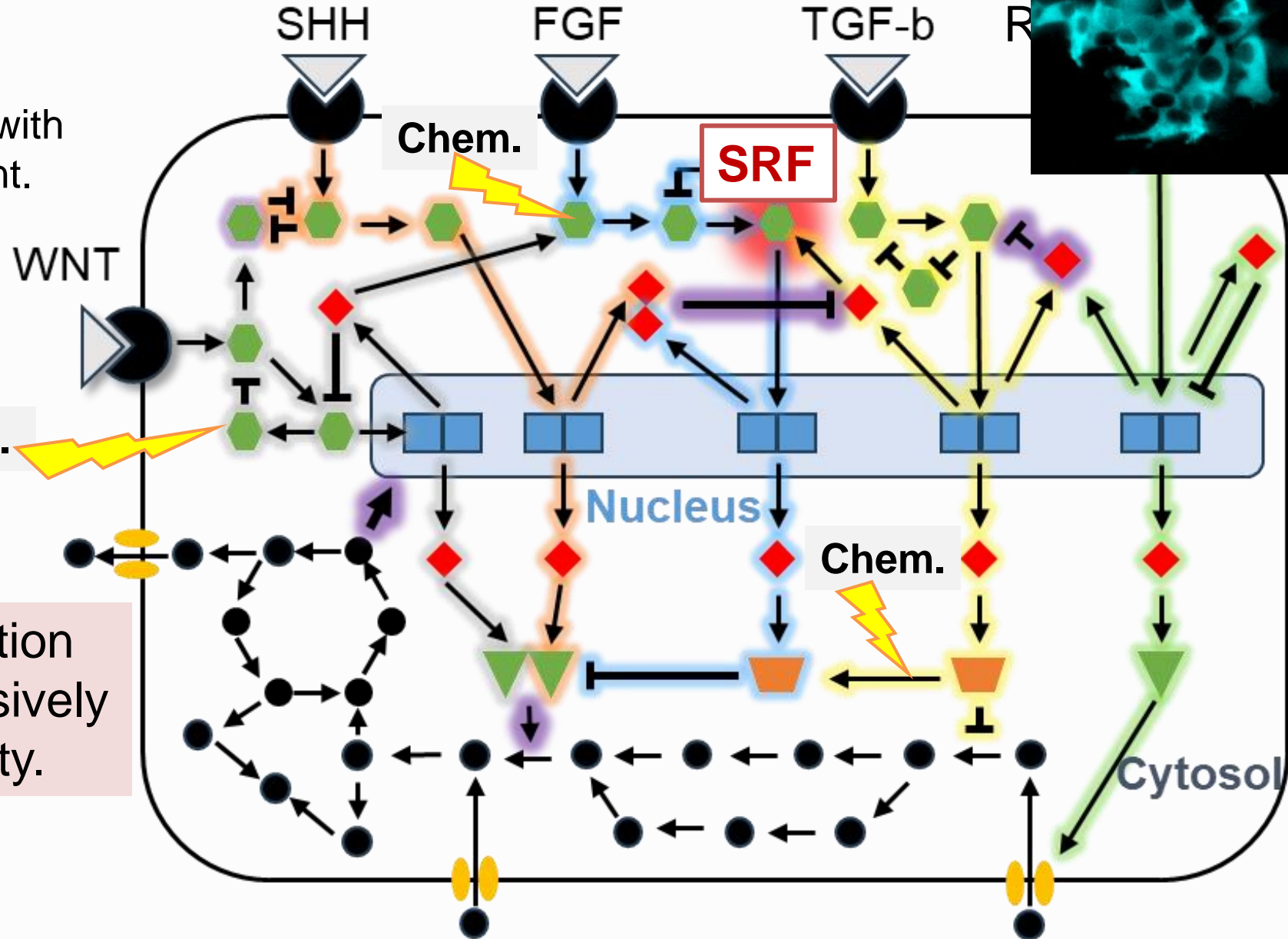
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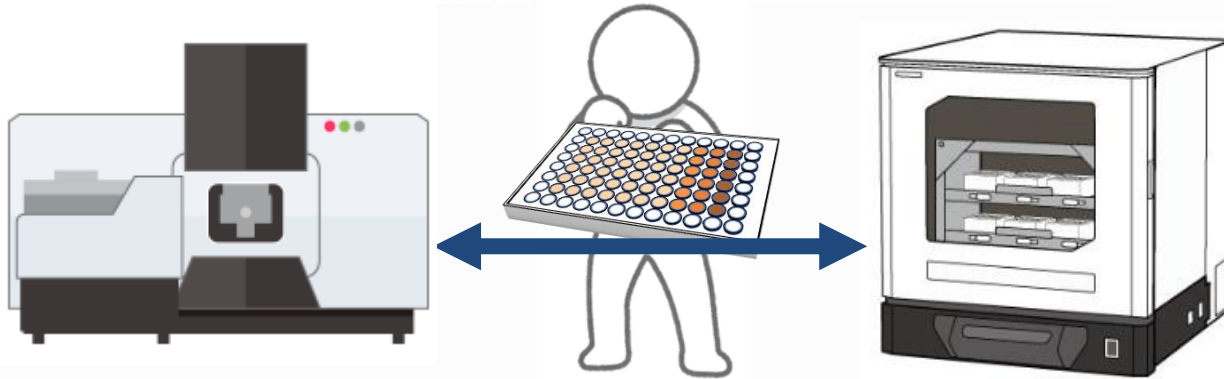
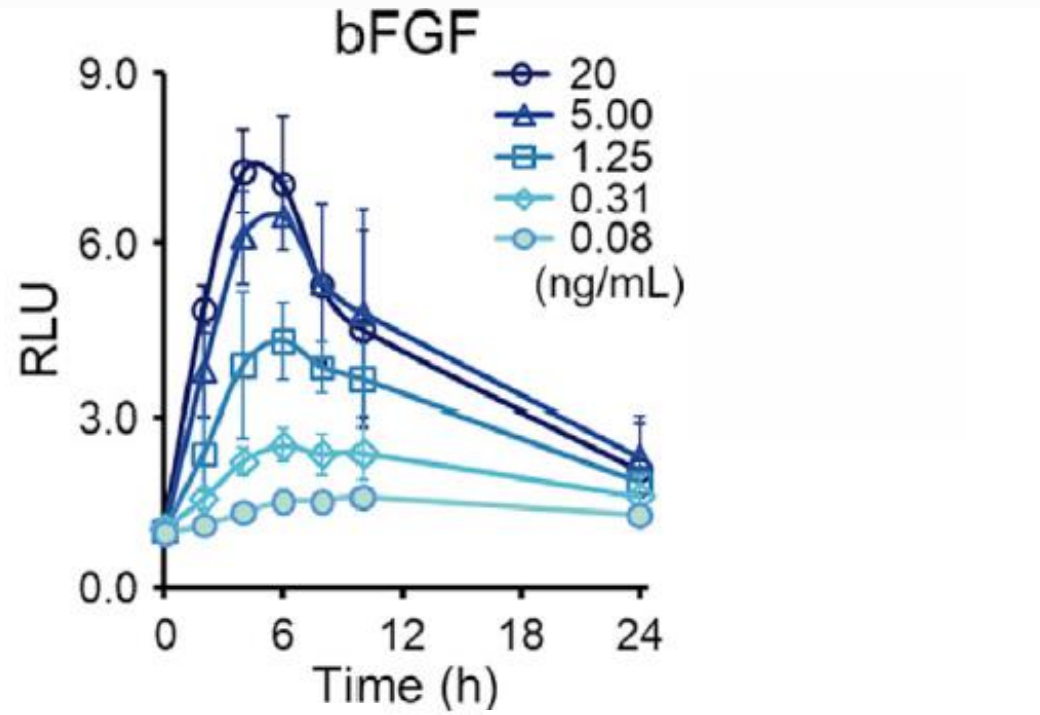
Understanding signal disruption dynamics might comprehensively assess developmental toxicity.





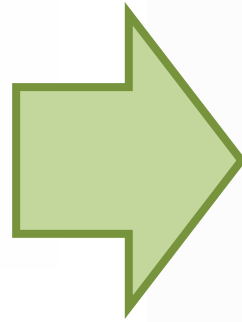
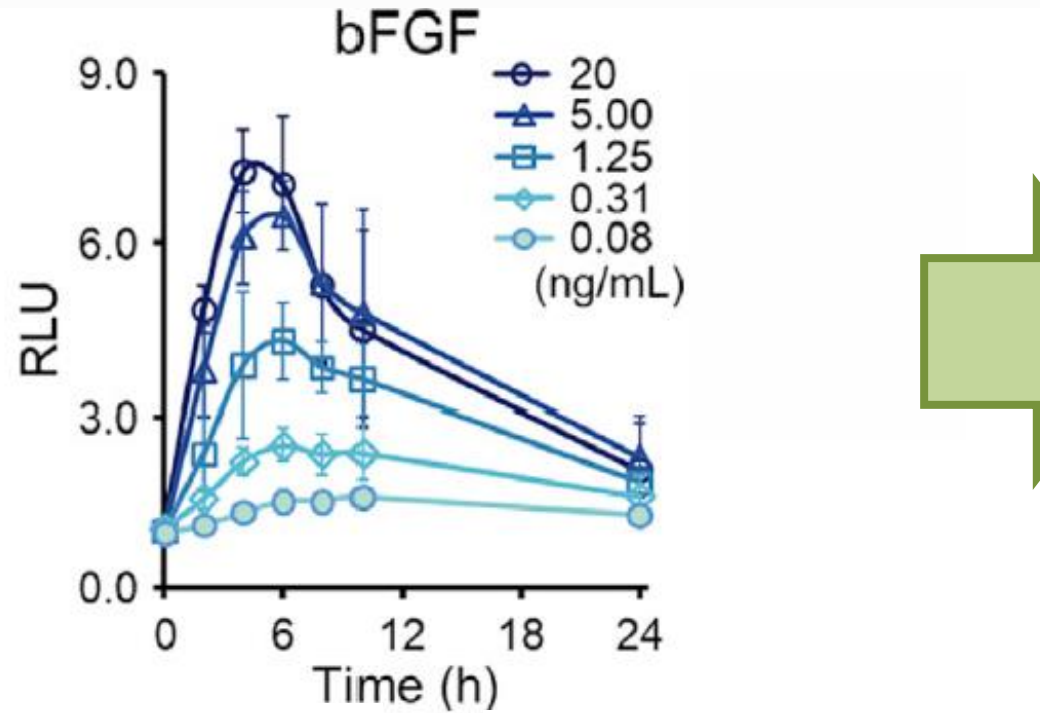
# Generalizing our experimental system and improving its throughput

## Manual measurement

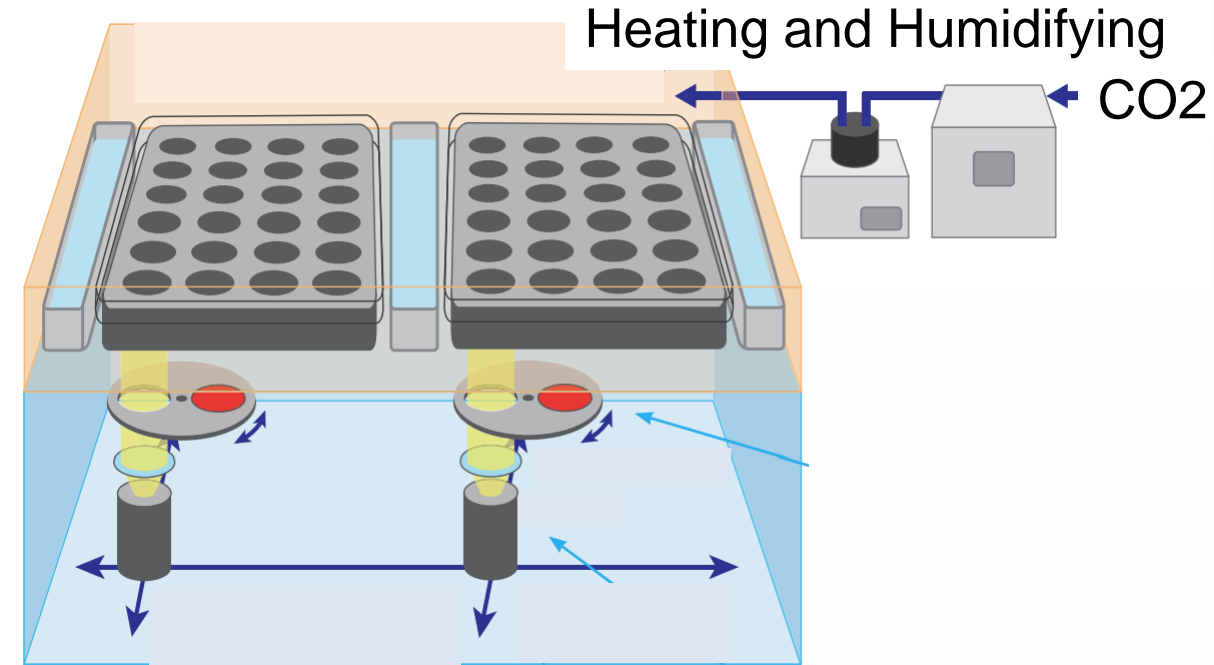


# Generalizing our experimental system and improving its throughput

## Manual measurement



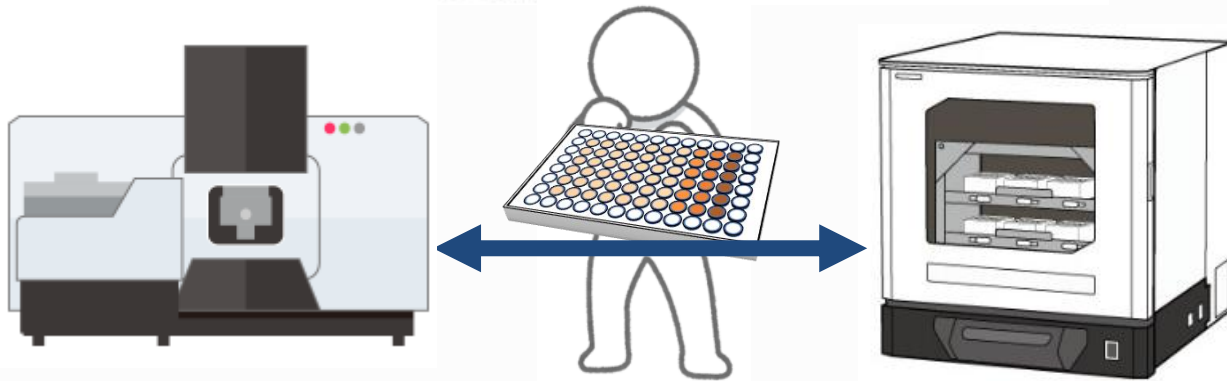
## Automatic measurement



Kronos HT

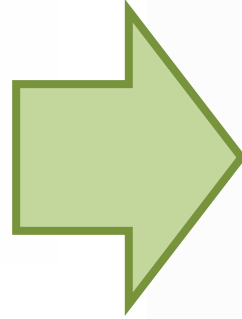
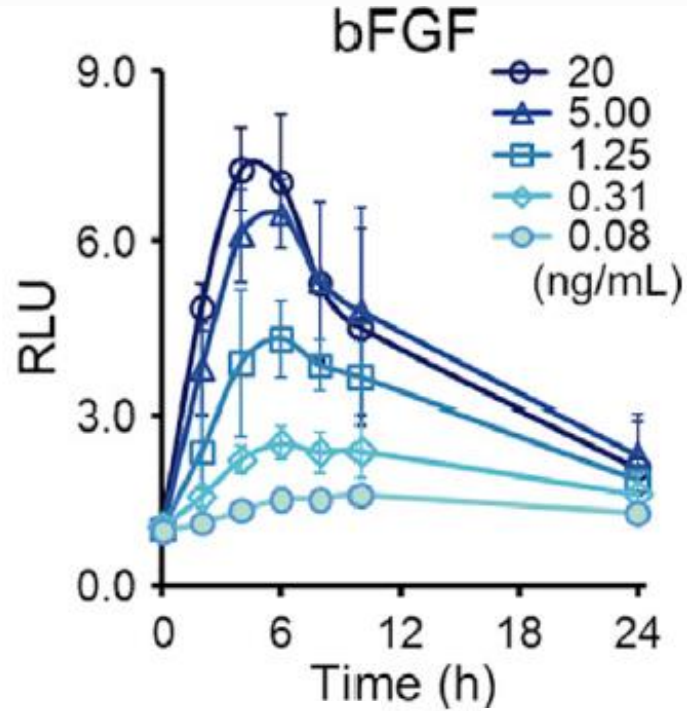


Dr. Nakajima

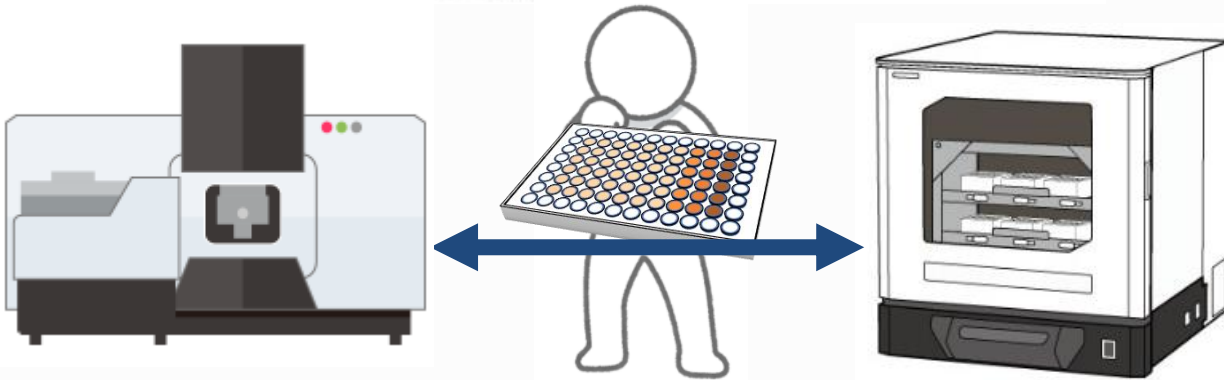
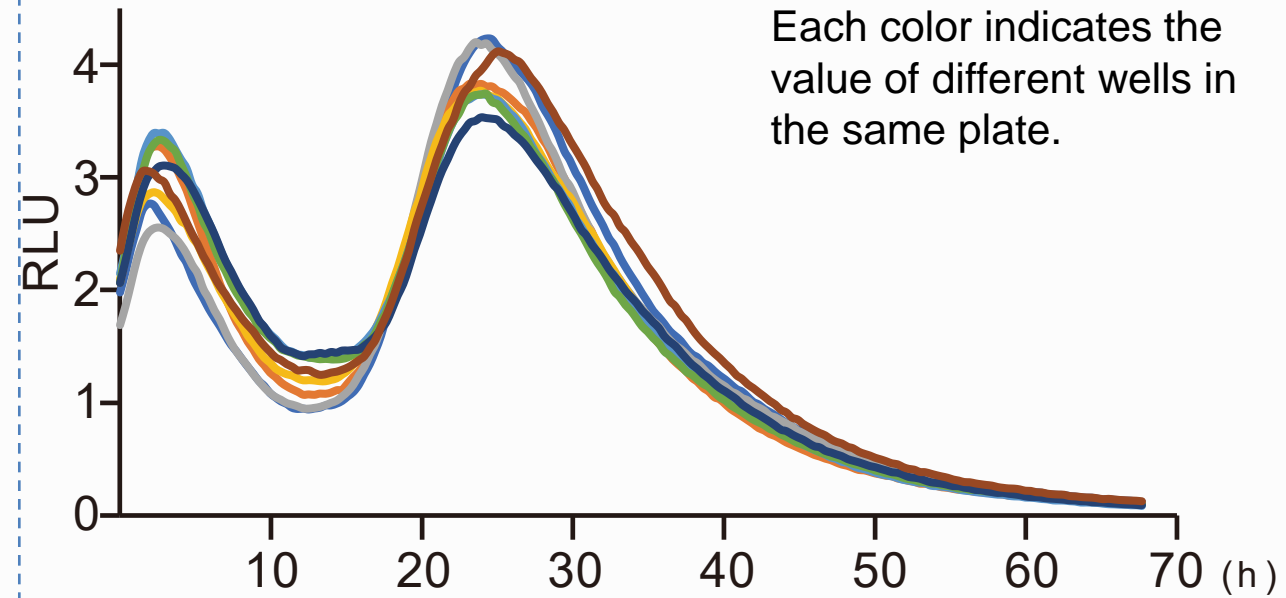


# Generalizing our experimental system and improving its throughput

## Manual measurement



## Automatic measurement



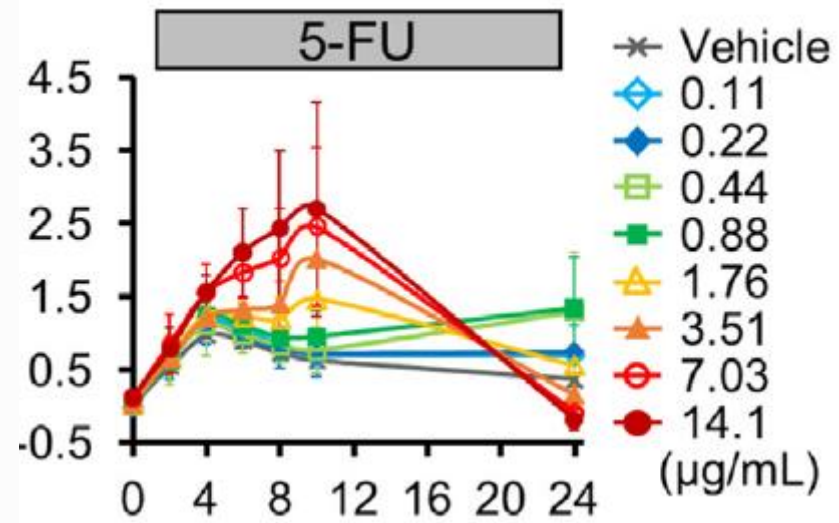
Kronos HT



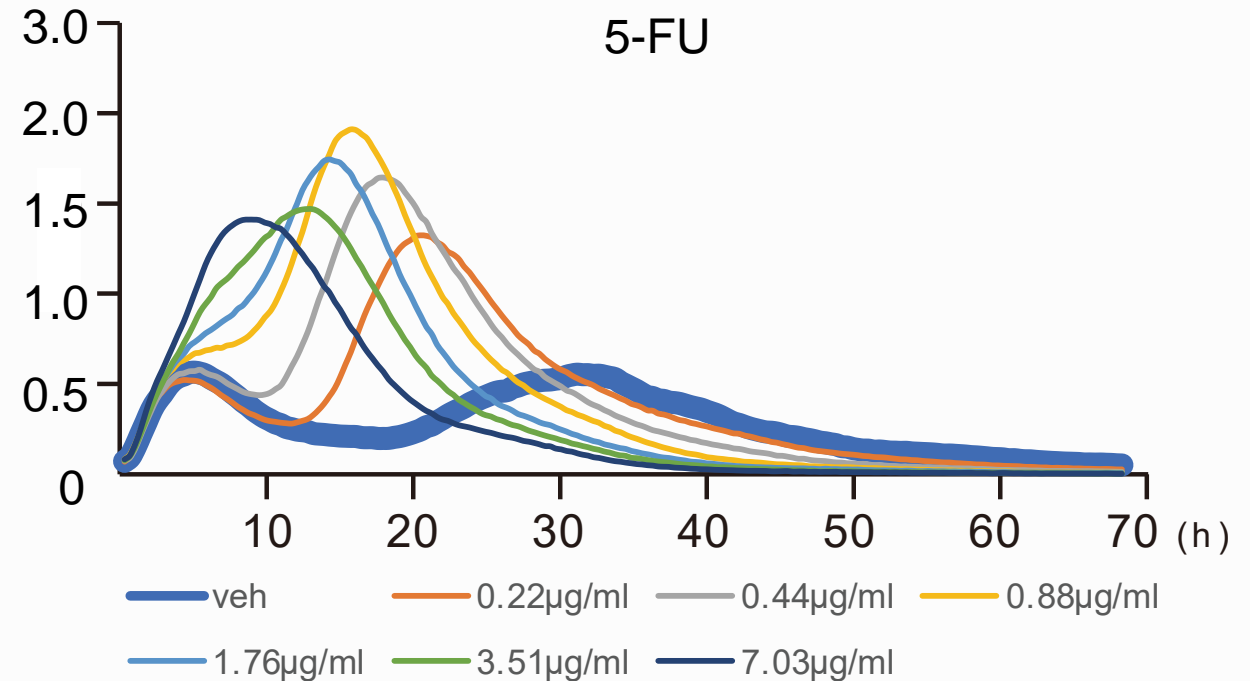
Dr. Nakajima

# Changes in concentration-dependent signal disruption dynamics

## Manual measurement

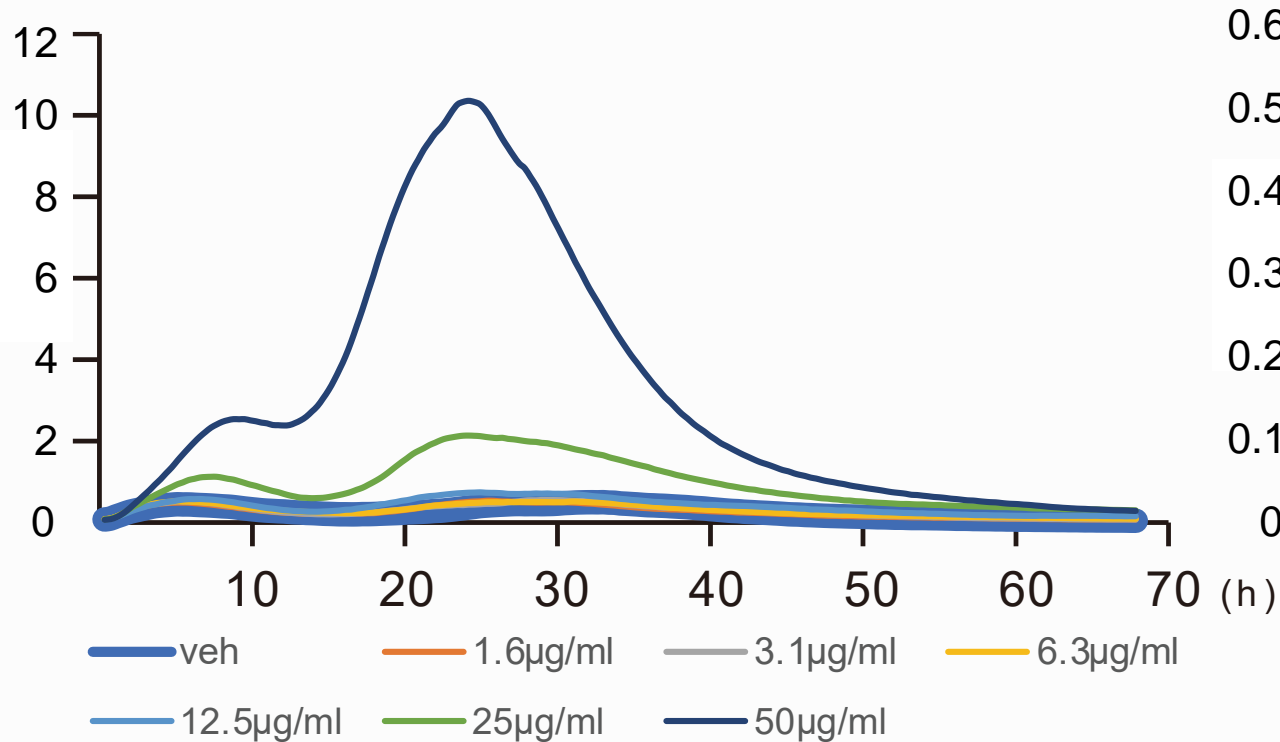


## Real-time measurement

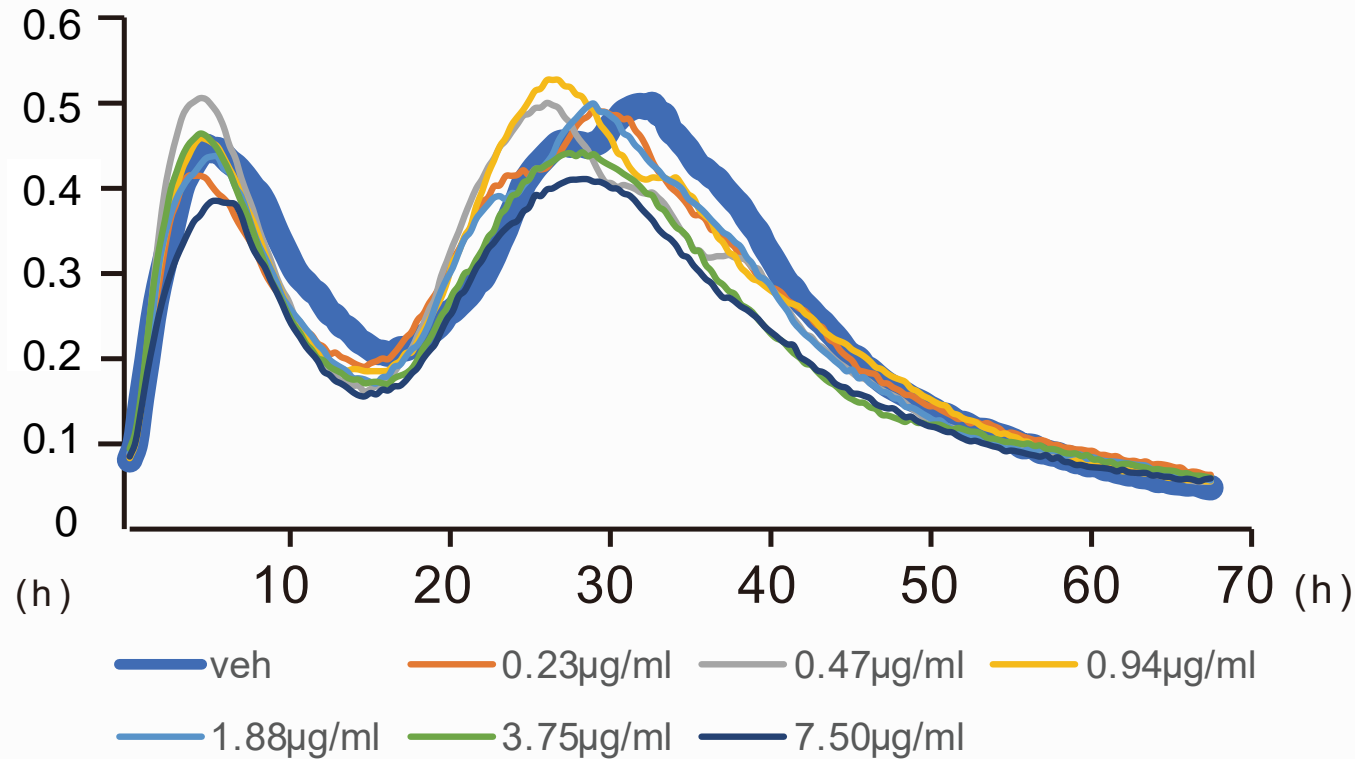


# Generalizing our experimental system and improving its throughput

Developmental toxicant:  
Valproic acid

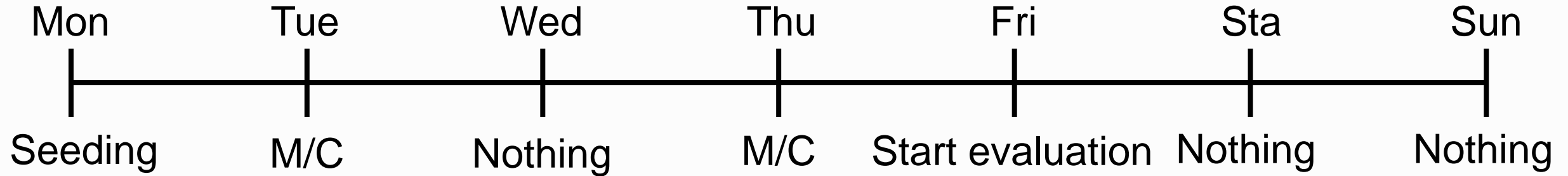


Non-Developmental toxicant:  
Cimetidine



**Real-time measurements enable detailed analysis of signal disruption dynamics.**

## Real-time measurement procedure



Next week,  
Stop Kronos

This method offers a high-throughput, cost-effective screening for accurate developmental toxicity testing that can be completed within one week.

# Conclusion

We've developed an in vitro developmental toxicity test with high accuracy and throughput, utilizing the dynamics of FGF-SRF signaling disruption caused by chemicals.

## Future directions

1. Increase the number of test substances.
2. Clarify the molecular mechanisms behind the detection of developmental toxicity in our assay.
3. Investigate downstream pathways other than SRF in FGF signaling and disruptions in other signals."



# Acknowledgement



Yokohama National University :  
Junji Fukuda,

Kashu Mizota, Rintaro Ohara

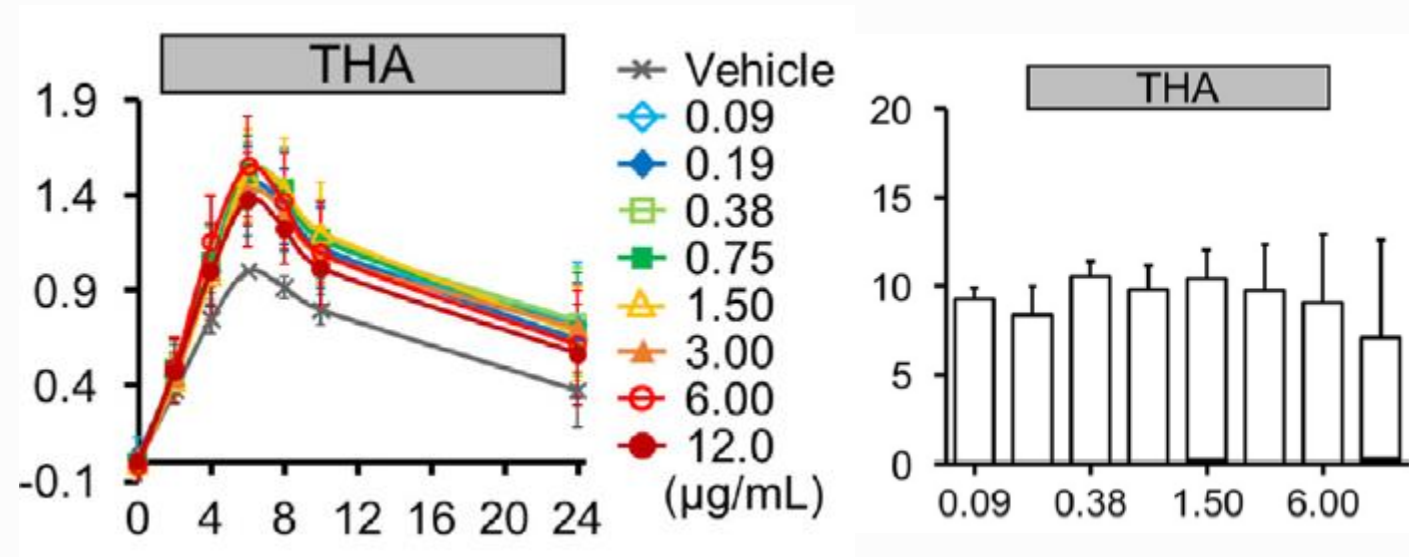
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Yoko Hirabayashi, Hajime Kojima, Takao Ashikaga,  
Mitsuaki Shibata, Satoshi Kitajima



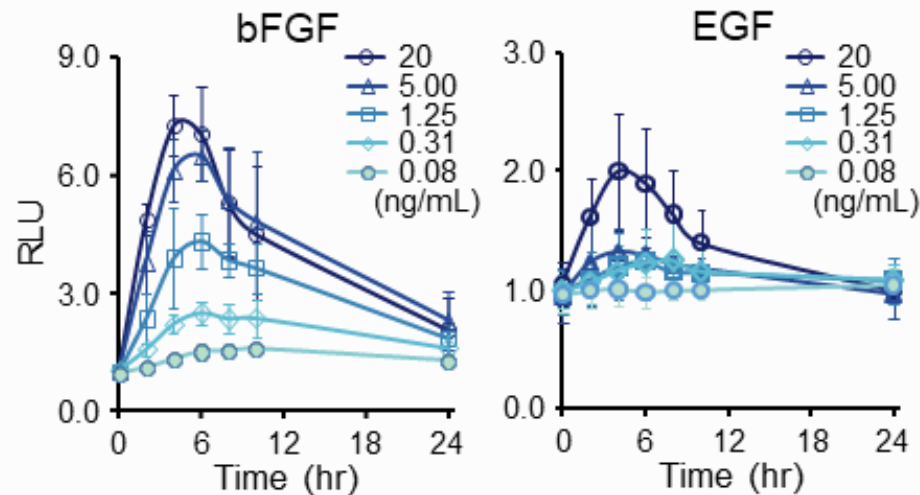
# Clinical data

Human :  $C_{MAX}$  0.62  $\mu\text{g/mL}$

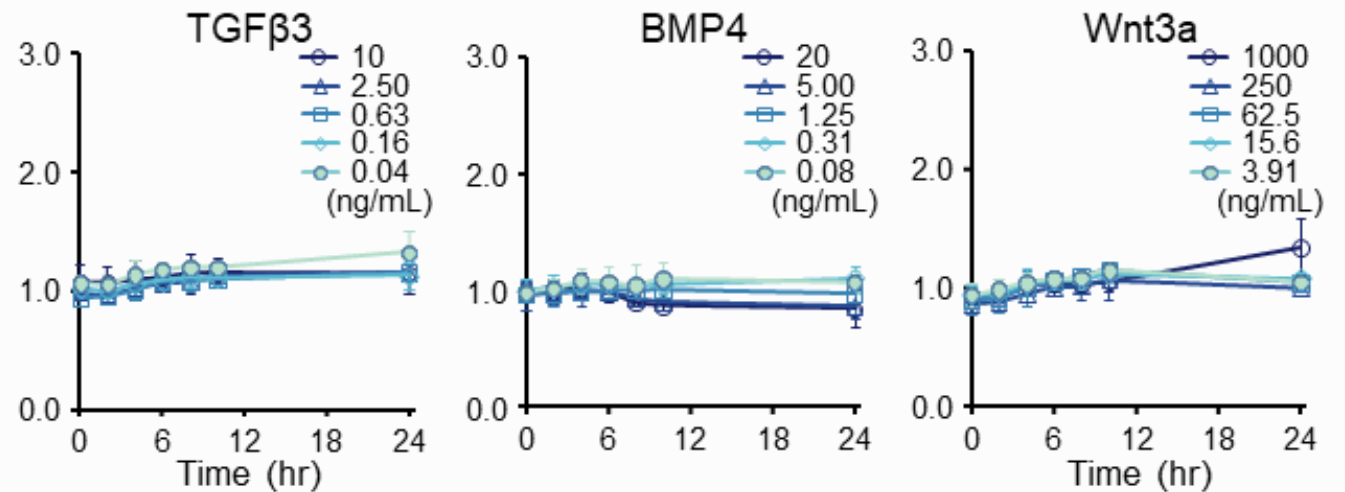


# Validating RTK-SRF signal reporter system in live cells

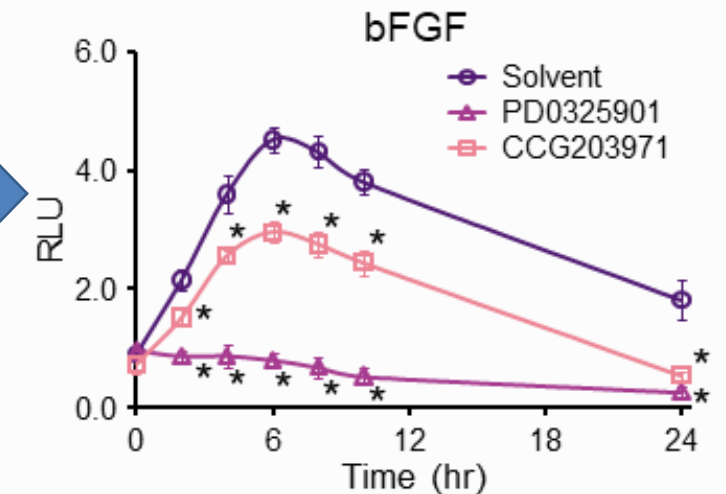
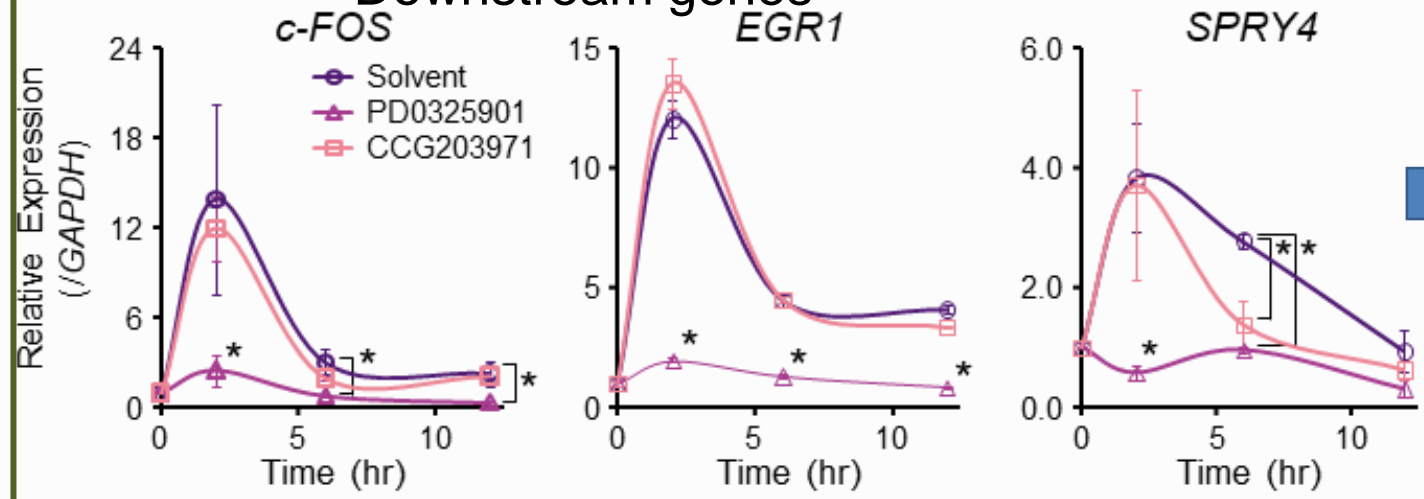
## Ligands



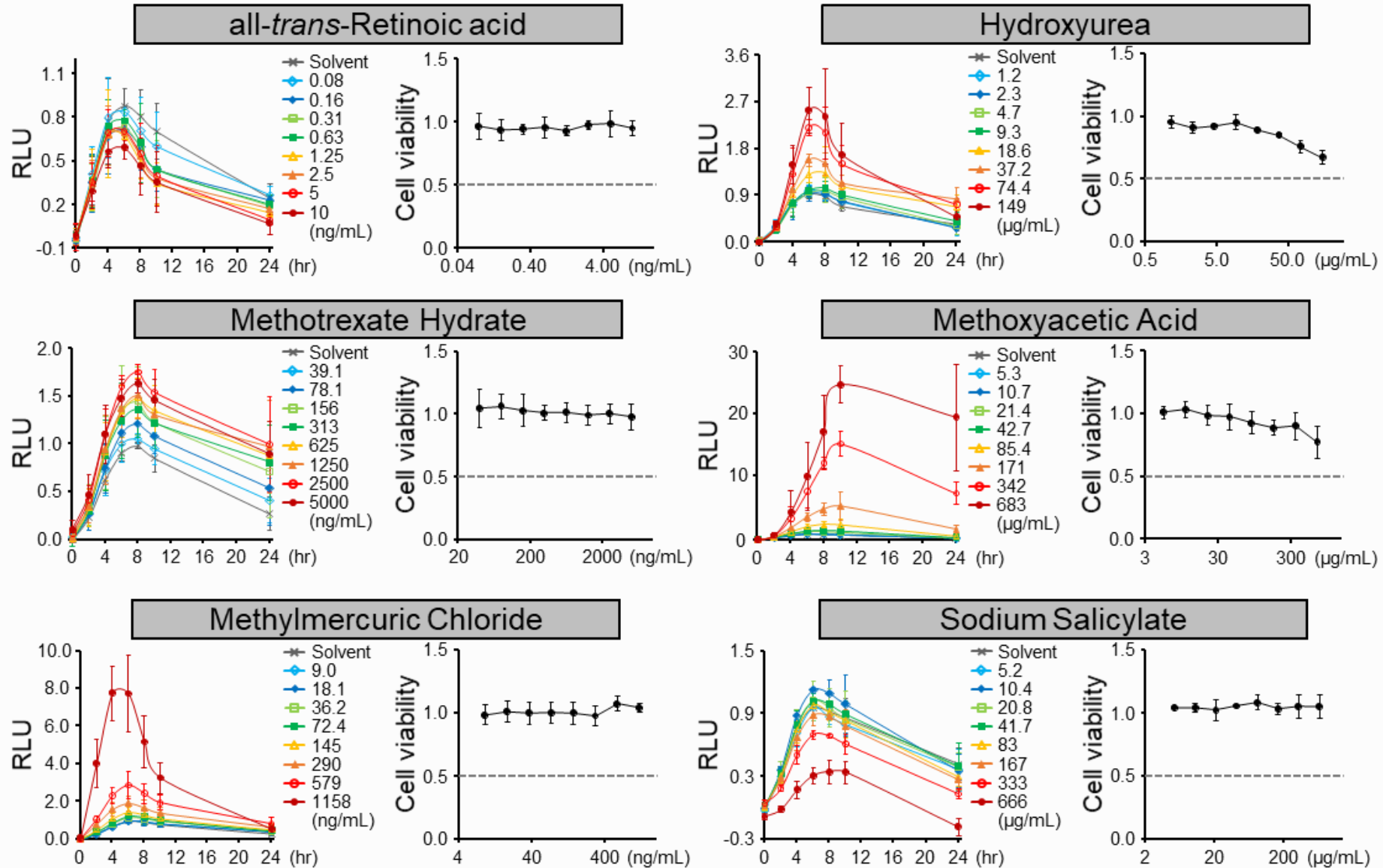
## Not ligands



## Downstream genes

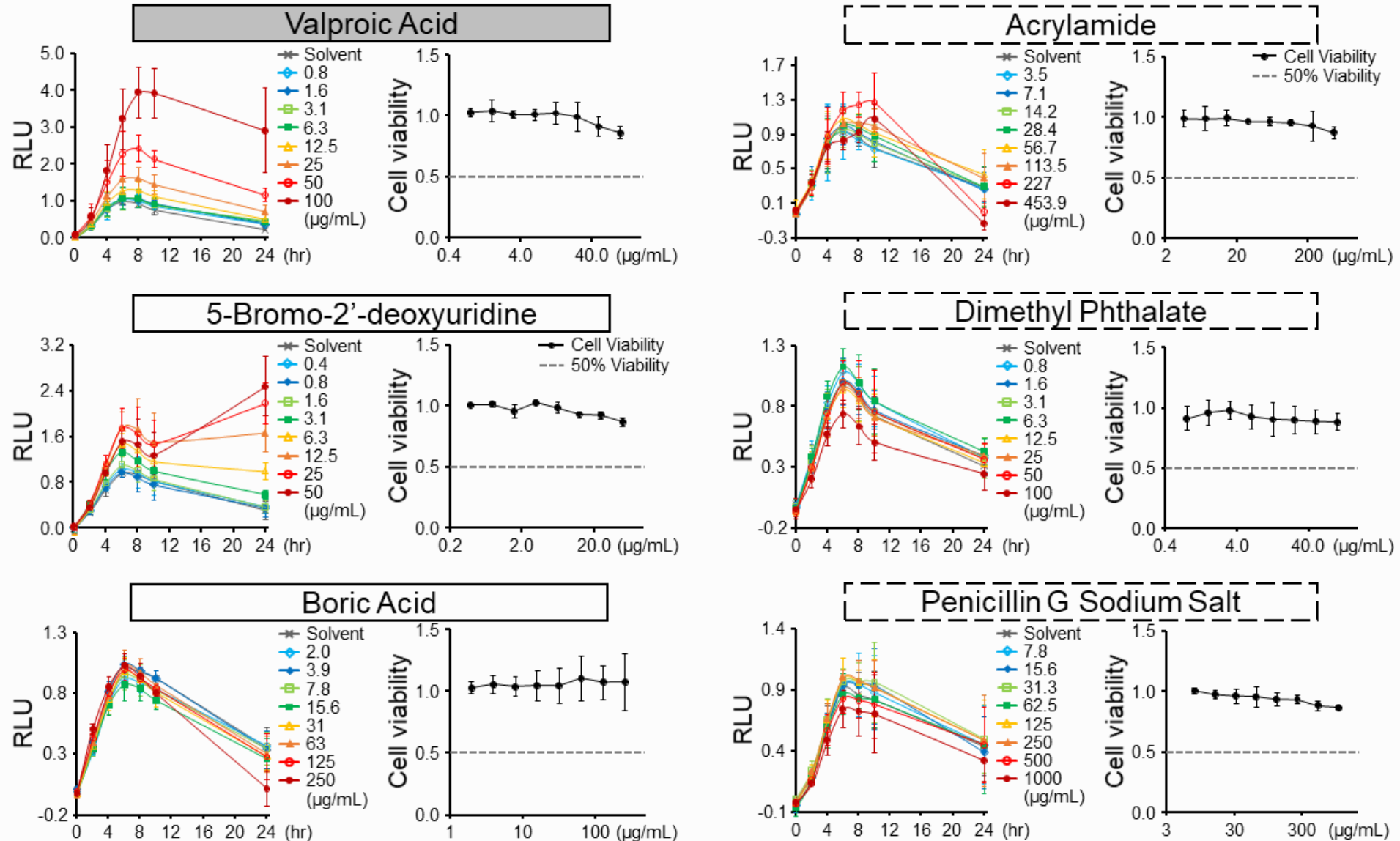


# Results : FGF-SRF signal reporter assay



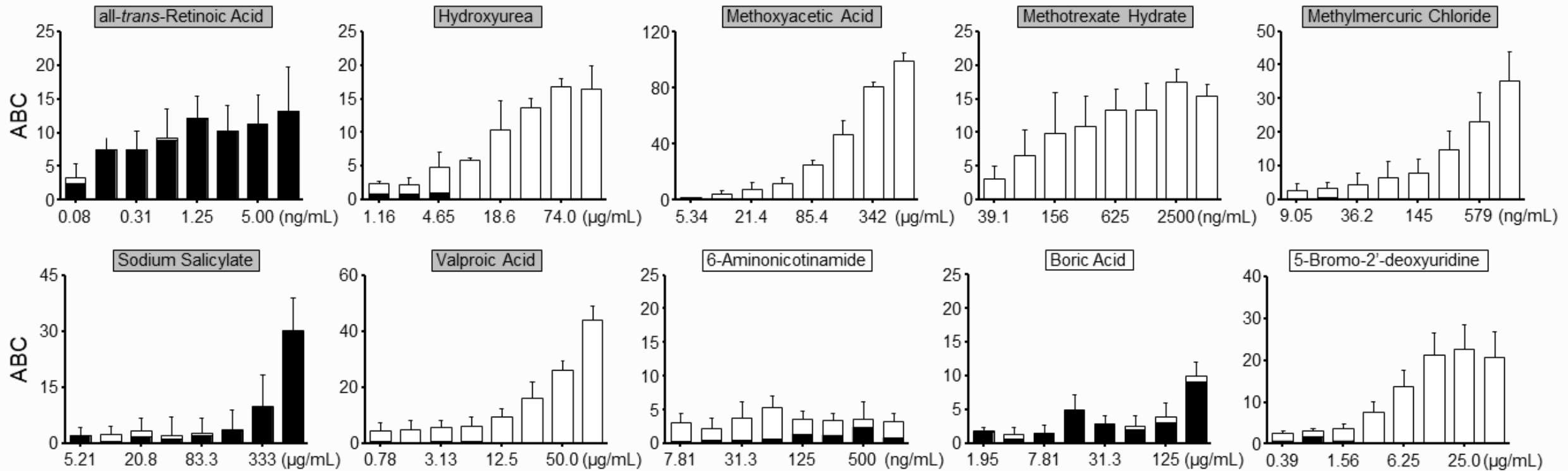
Solid gray line: developmental toxicants causing limb deformities, solid white line: other developmental toxicants, dotted white line: non-developmental toxicants

# Results : FGF-SRF signal reporter assay



Solid gray line: developmental toxicants causing limb deformities, solid white line: other developmental toxicants, dotted white line: non-developmental toxicants

# Results : Area between the curve (ABC)





# Results : Area between the curve (ABC)

