Adaptation in the TGFβ pathway and embryonic patterning.

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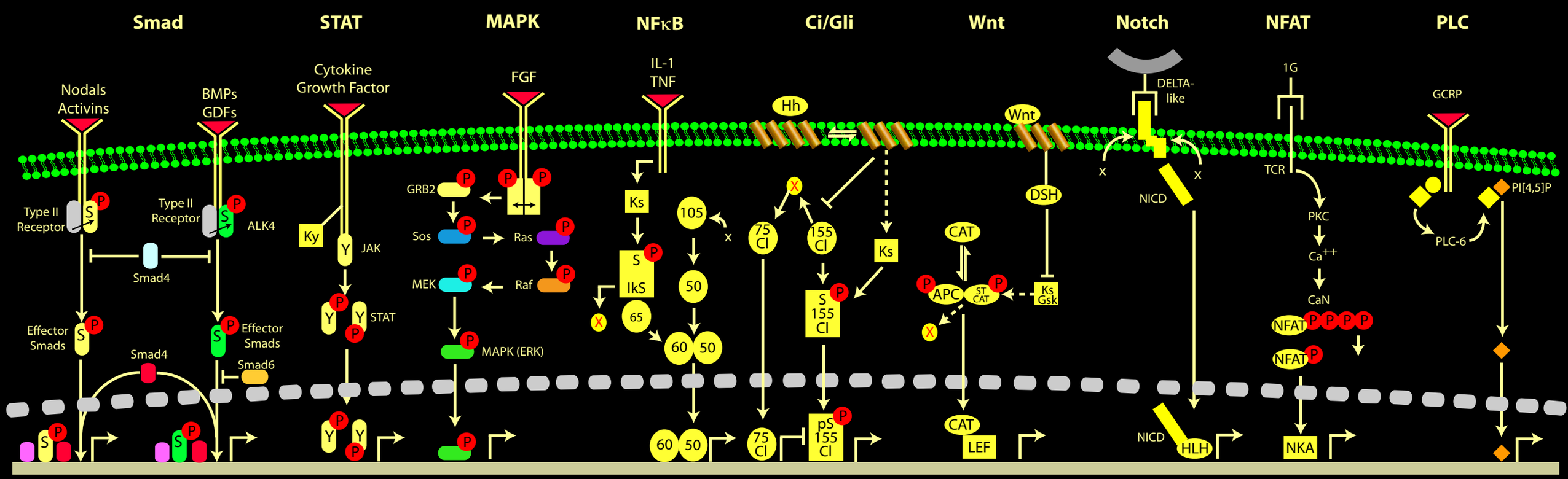
Qixiang Zhang and Alin Vonica

PNAS July 2012 & unpub.

What’s the problem?

Signaling pathways involved in cell fate determination

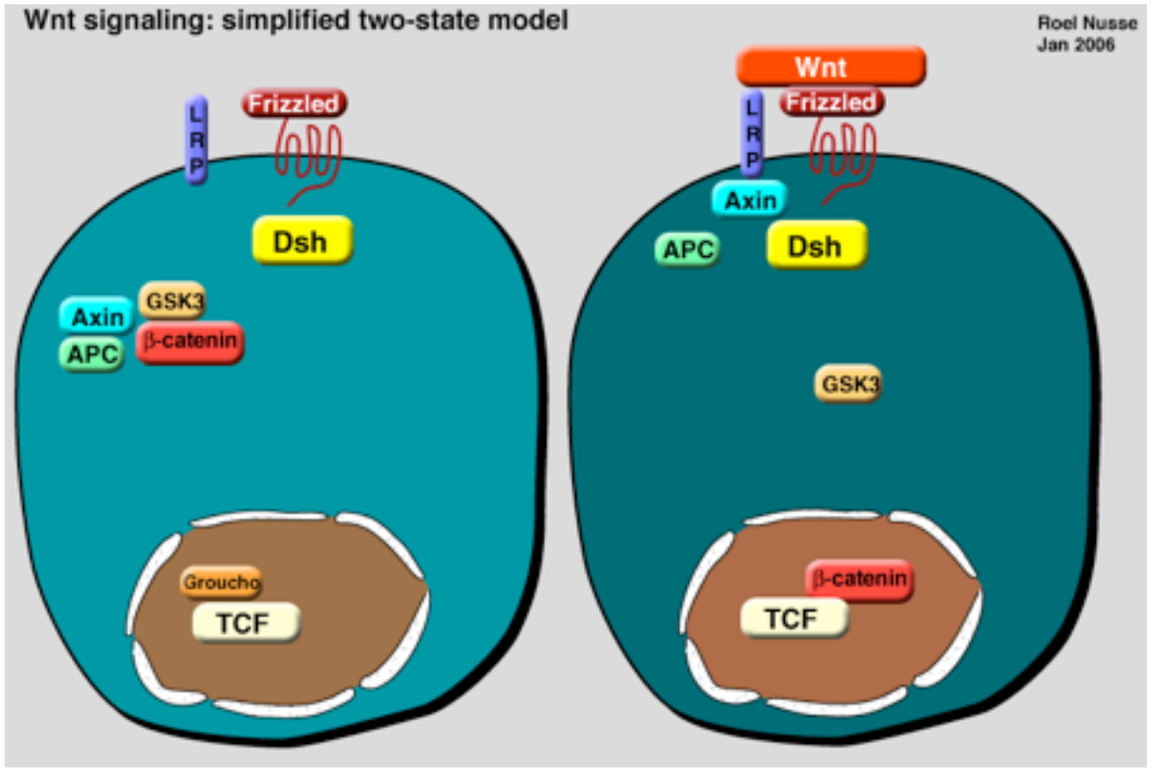


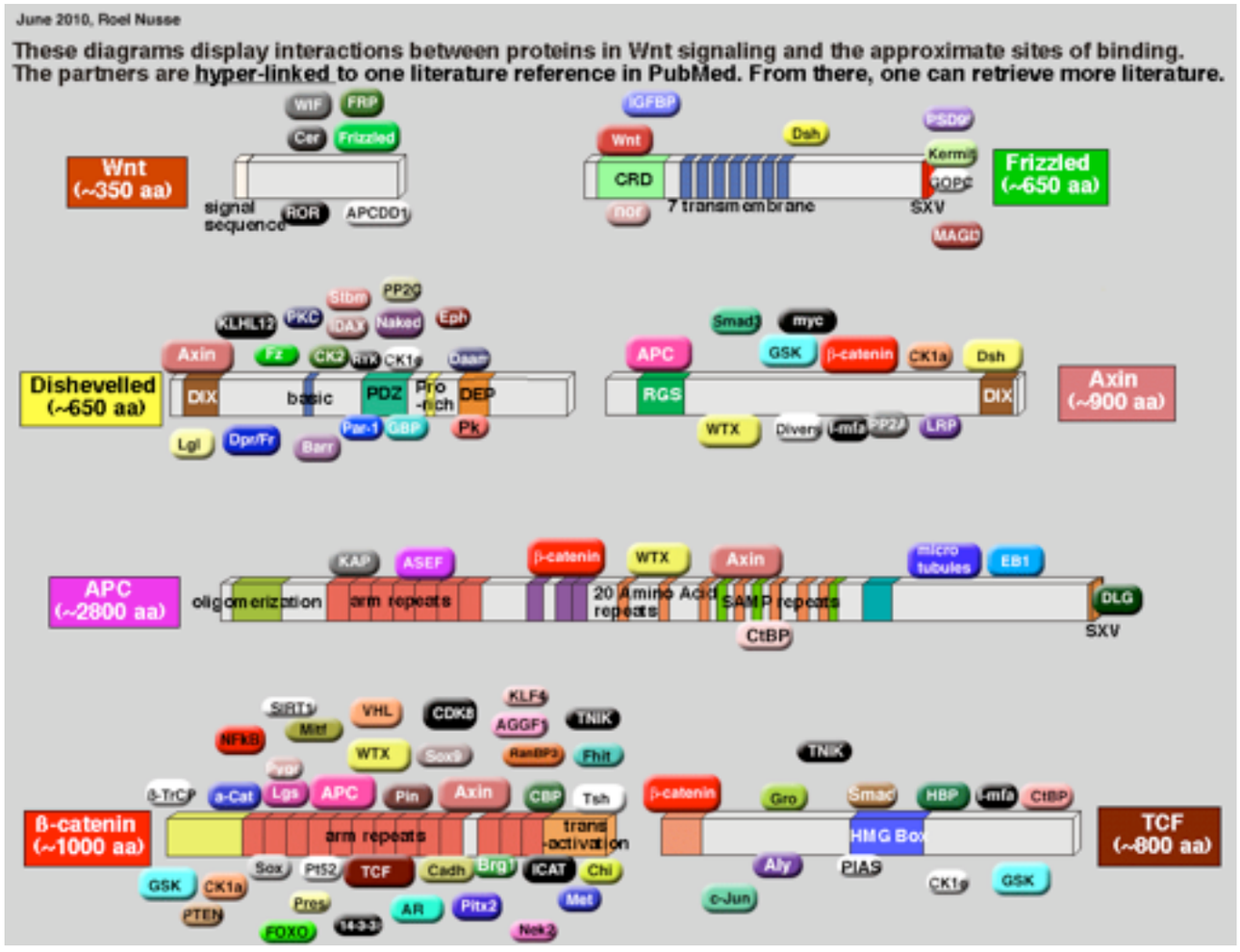
Transcription factors involved in cell fate determination operate under the controls of signaling pathways

(Brivanlou and Darnell 2002, Science)

Signaling Pathways are Complex

Wnt's for dummies





What's the problem?

Pathways reused

TGFβ operates blastula....immune.....cancer

NFκB (inflammation) many inputs to many outputs

Are they simple ON/OFF switches, or rheostats from ligand to TF?

Periodic table for <u>phenotype</u>?

What pathways operate in what contexts?

What pathways work together?

Evolution artifact or signal processing?

(Gene centric models at best metaphors:)

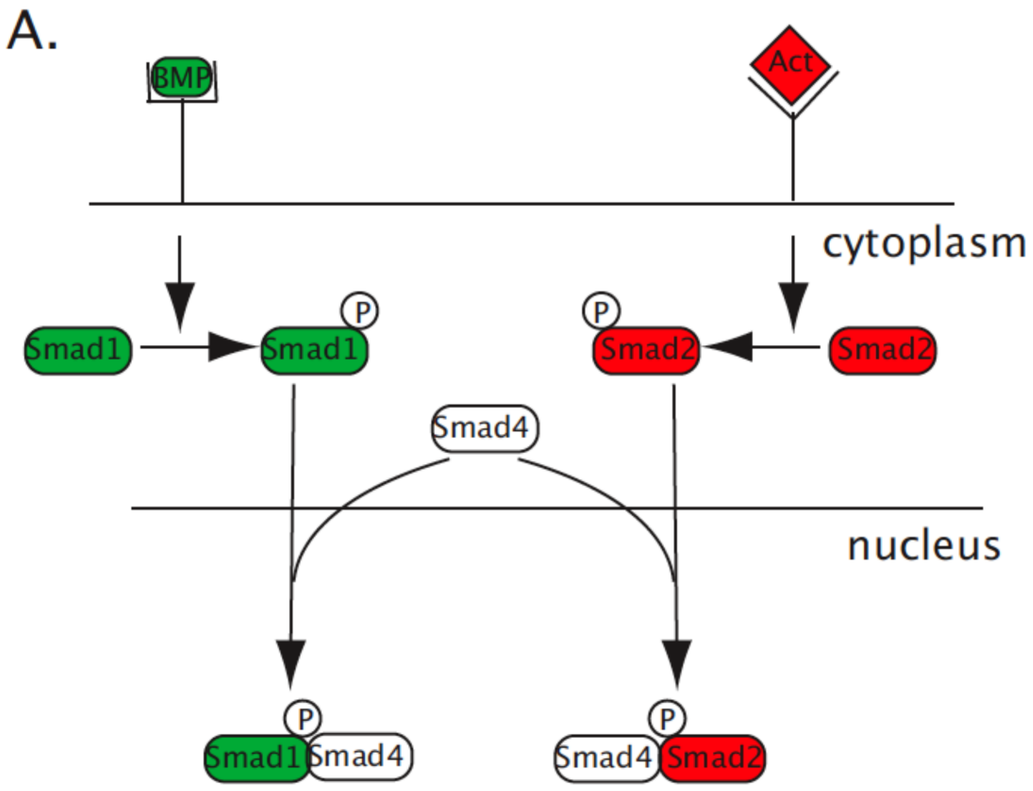
Ignores cell biology, assumes cell well mixed!

Impossible to get all parameters (can not mix sources)

Single cell ≠ population (e.g., graded response?)

(Are linear pathways an artifact of genetics + biochem?)

TGFβ pathway (simplified)



Receptor Smads (e.g. 1,2) are activated by specific receptors + Smad4 -> nucleus.

Genetic and Biochemical Studies:

1. R-Smad phosphorylation and nuclear accumulation stably reflect ligand levels

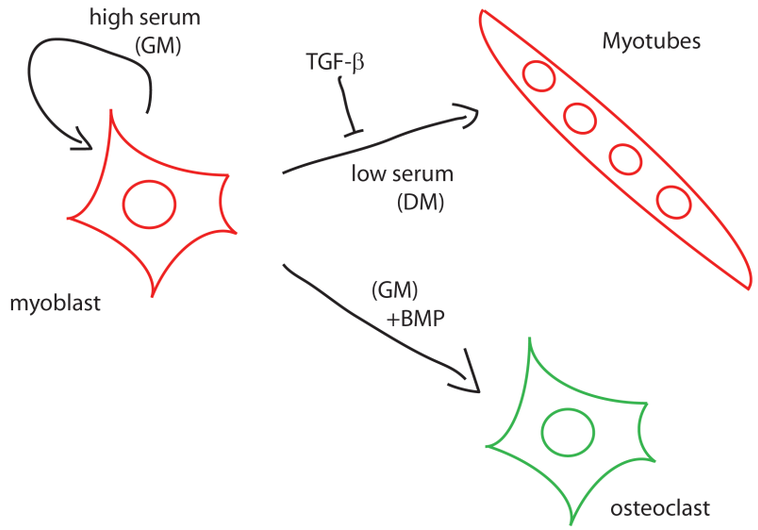
2. R-Smads nuclear synonymous with transcription

3. Termination of signaling caused by degradation or dephosphorylation of R-Smads

Test with dynamic studies

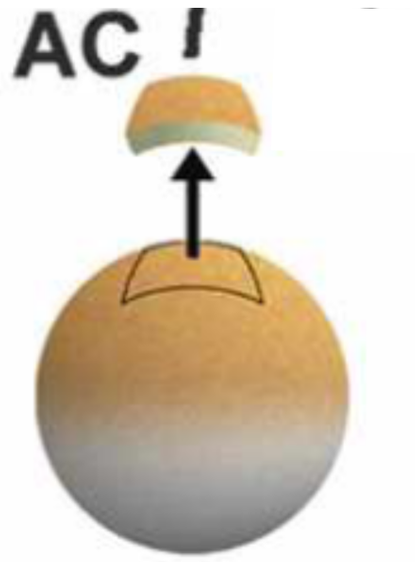
Systems for TGFβ

C2C12 cell line (myoblast)



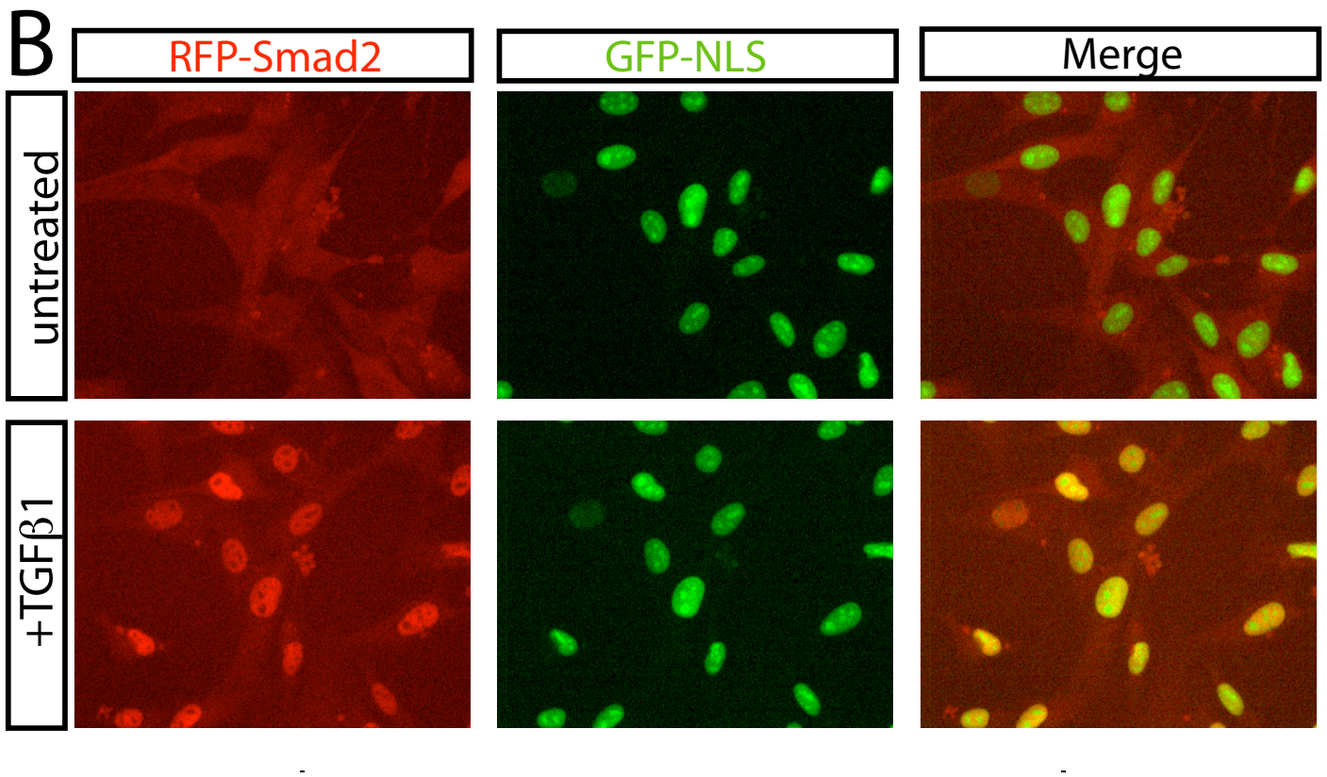
HaCaT (keratinocyte)

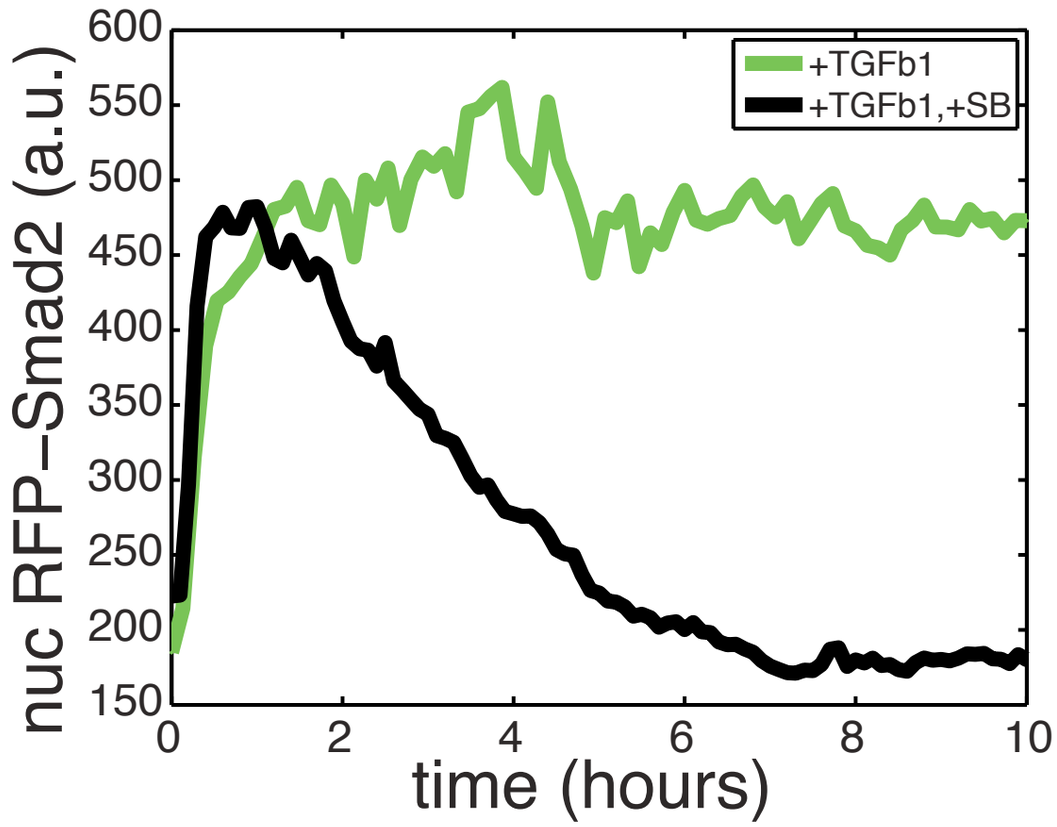
Xenopus animal caps
(fated to epidermis,
+- morphogens -->
neural, mesoderm)



(hESC)

Smad2 -> nuclear with stimulation





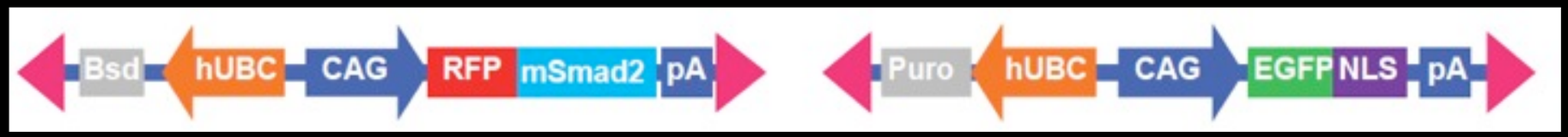
Stably integrate RFP-Smad2, GFP-NLS
Controls: level, response vs WT via IF

T=0 add ligand to dish
Nuc-Smad2 follows ligand

SB inhibits receptor kinase
kills response

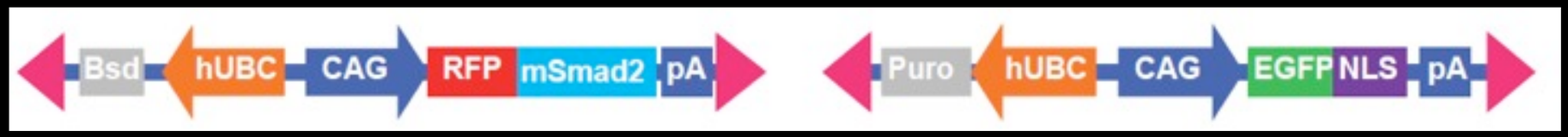
Control: Same response via antibodies to pSmad2 for endogenous cells

R-Smad activation is stable and graded

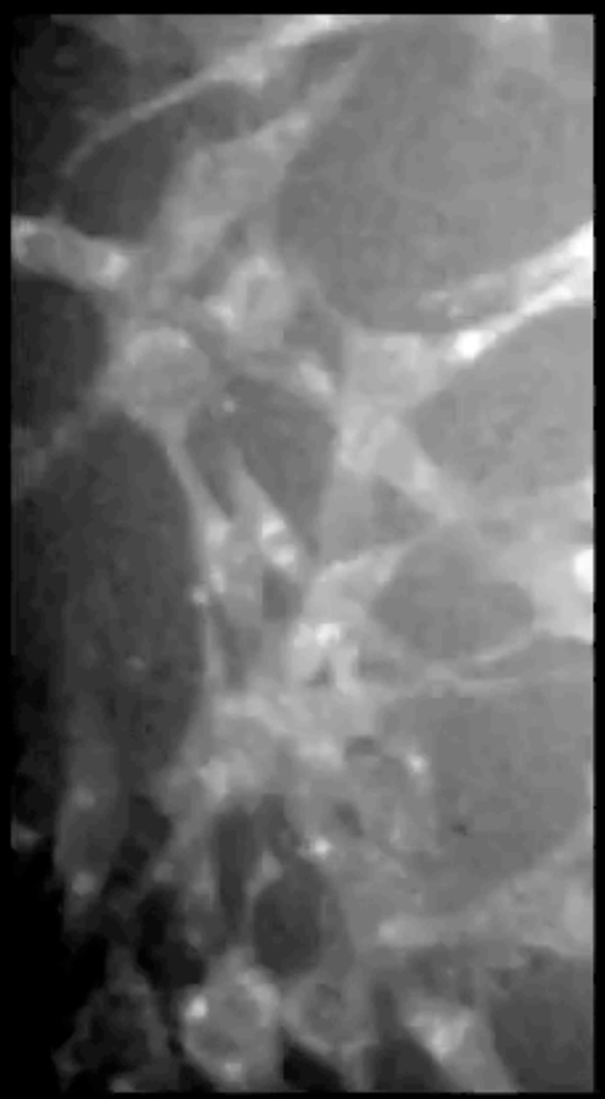


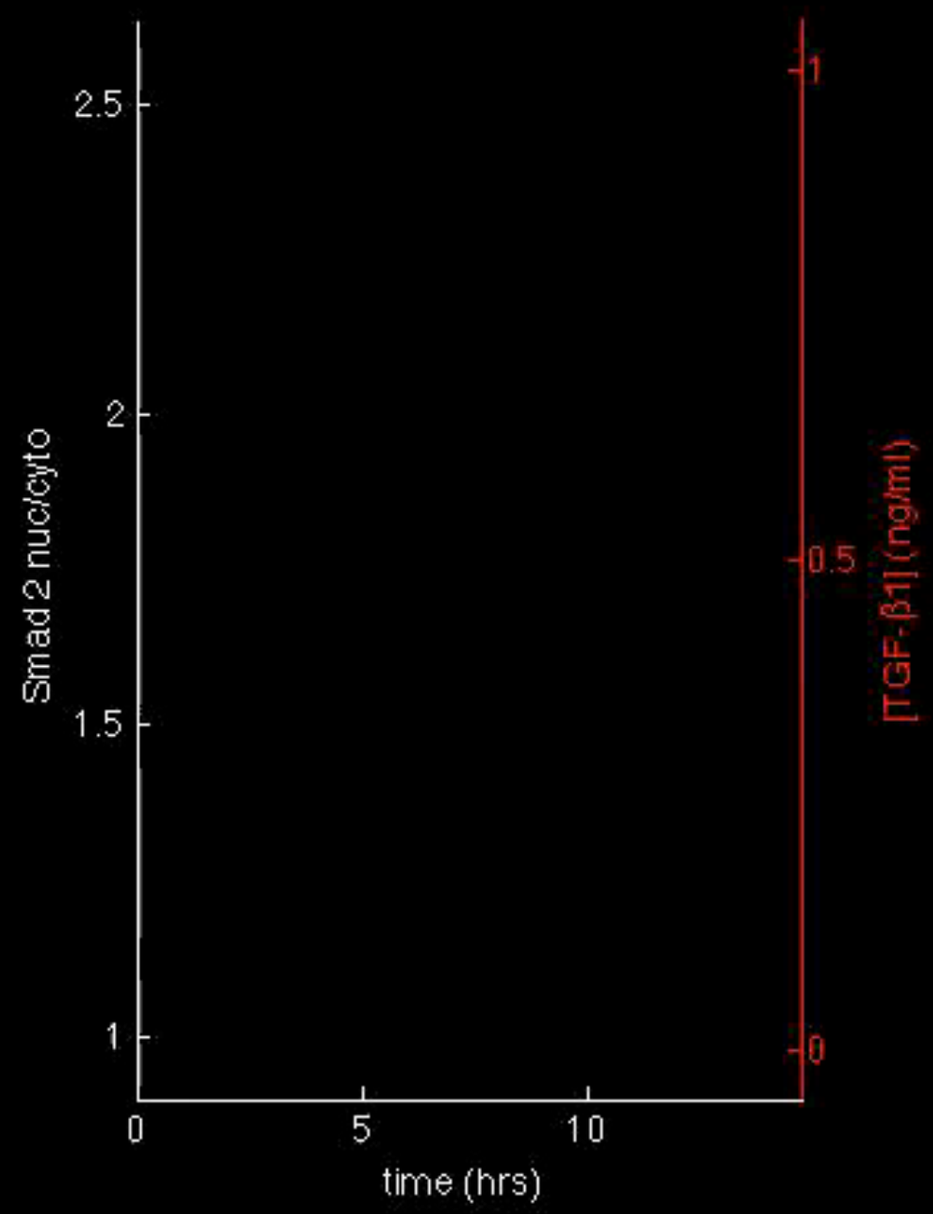
RFP-Smad2

R-Smad activation is stable and graded

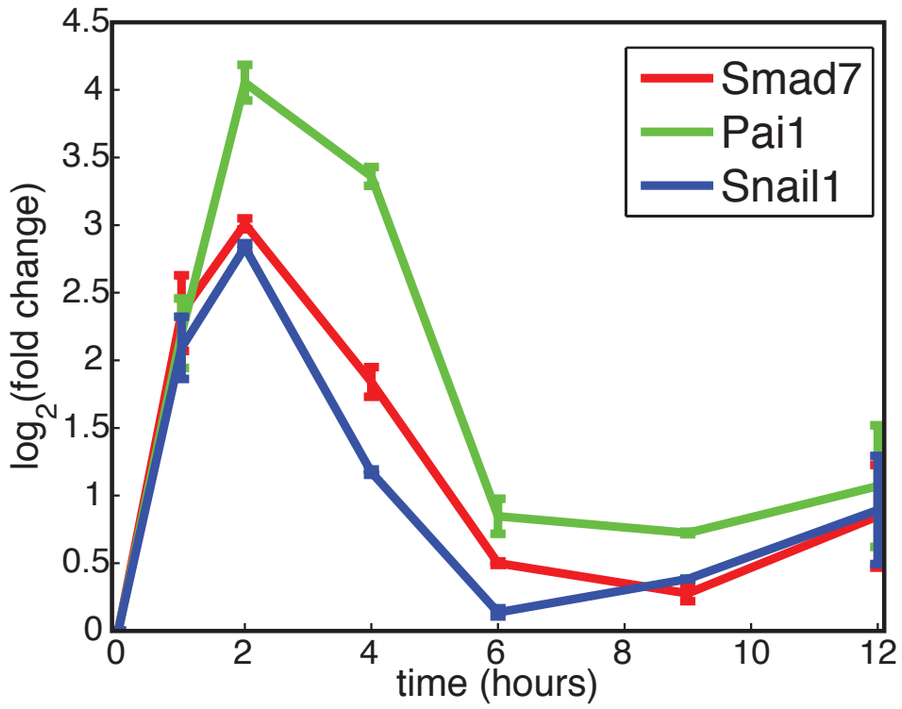


RFP-Smad2

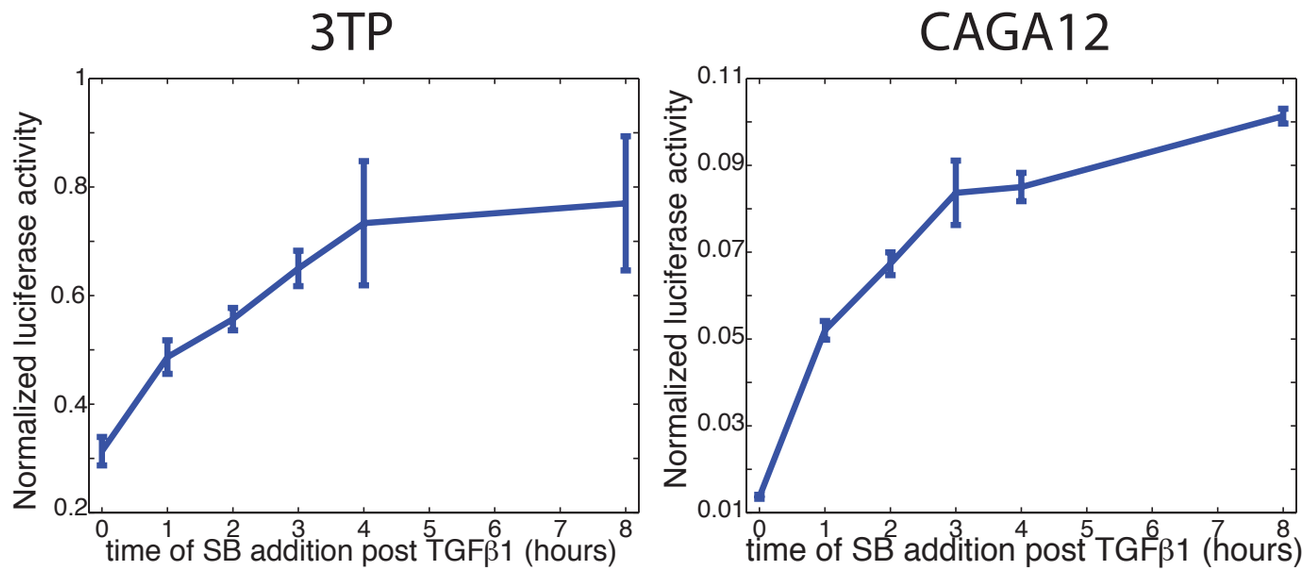




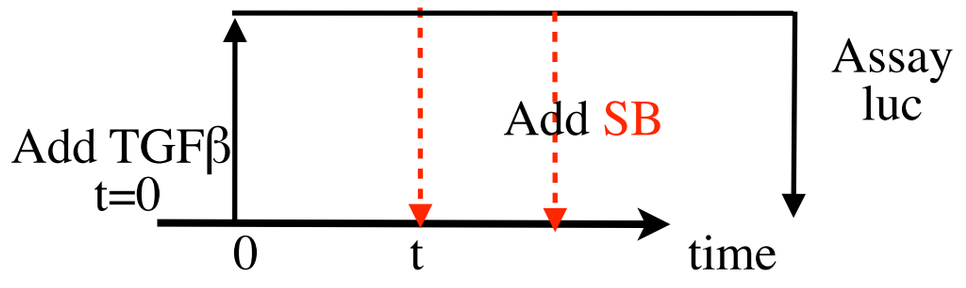
Transcription terminates during continuous stimulation



qRT-PCR on standard TGFβ targets (nb log scale)

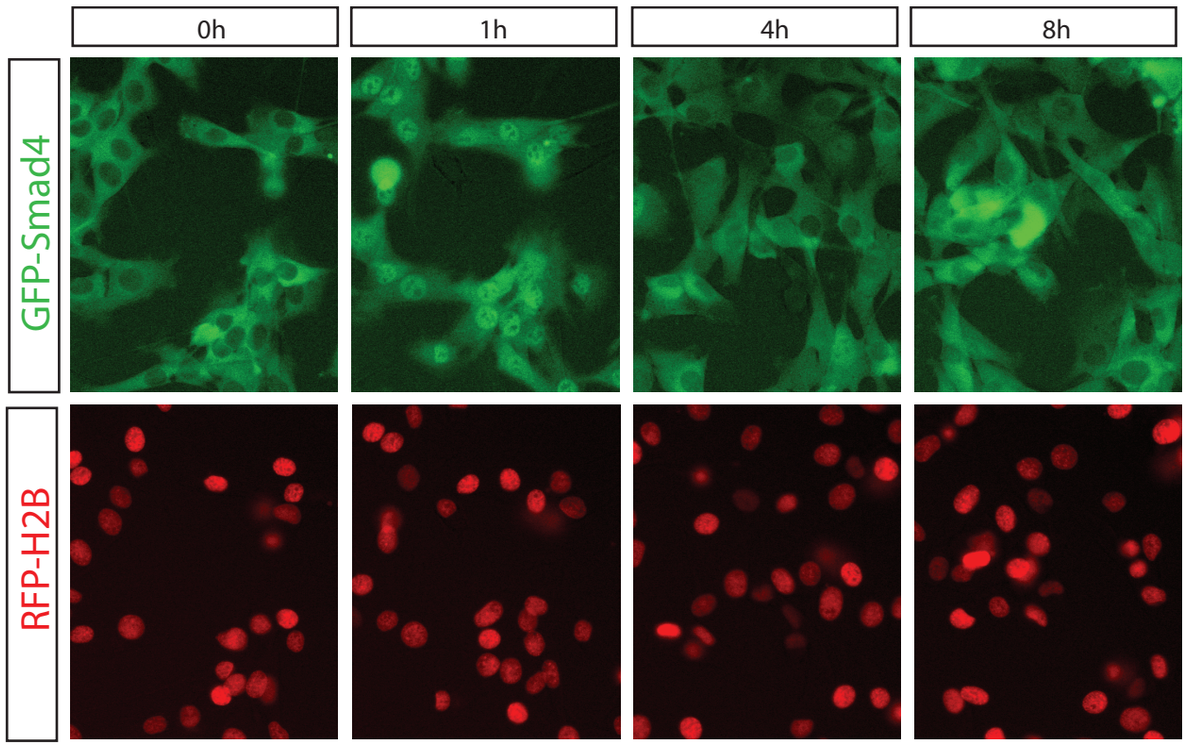


luciferase assay from synthetic promoters. output does not increase after ~4 hrs

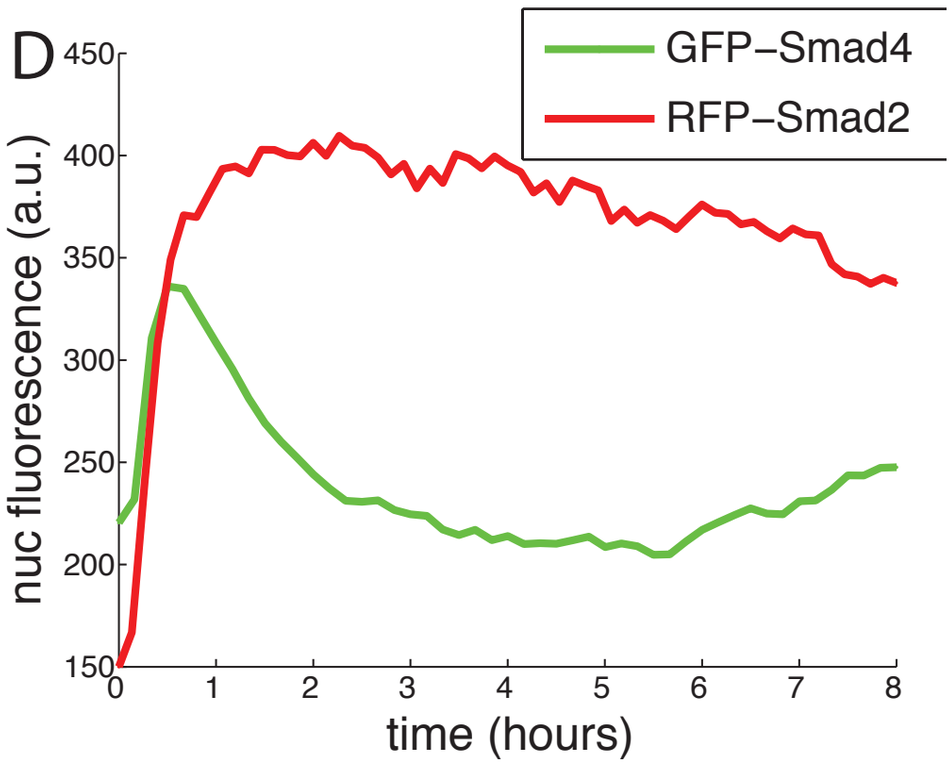


TGFβ pathway adapts!

Nuclear Smad4 response tracks transcription



Integrate GFP-Smad4, RFP-H2B, Smad2



nuc Smad4 vs time for popul
single cells: homogen, graded

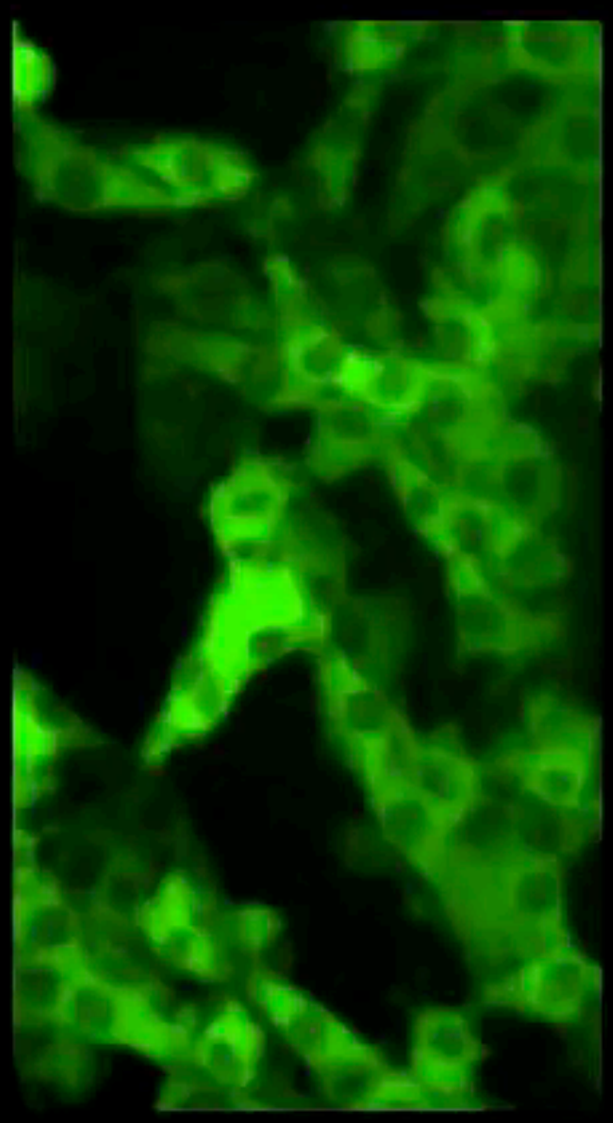
Controls: dynamics pSmad2 IF ~ WT, qRT-PCR ~ WT
HaCaT cells dense cultures endog Smad4 IF

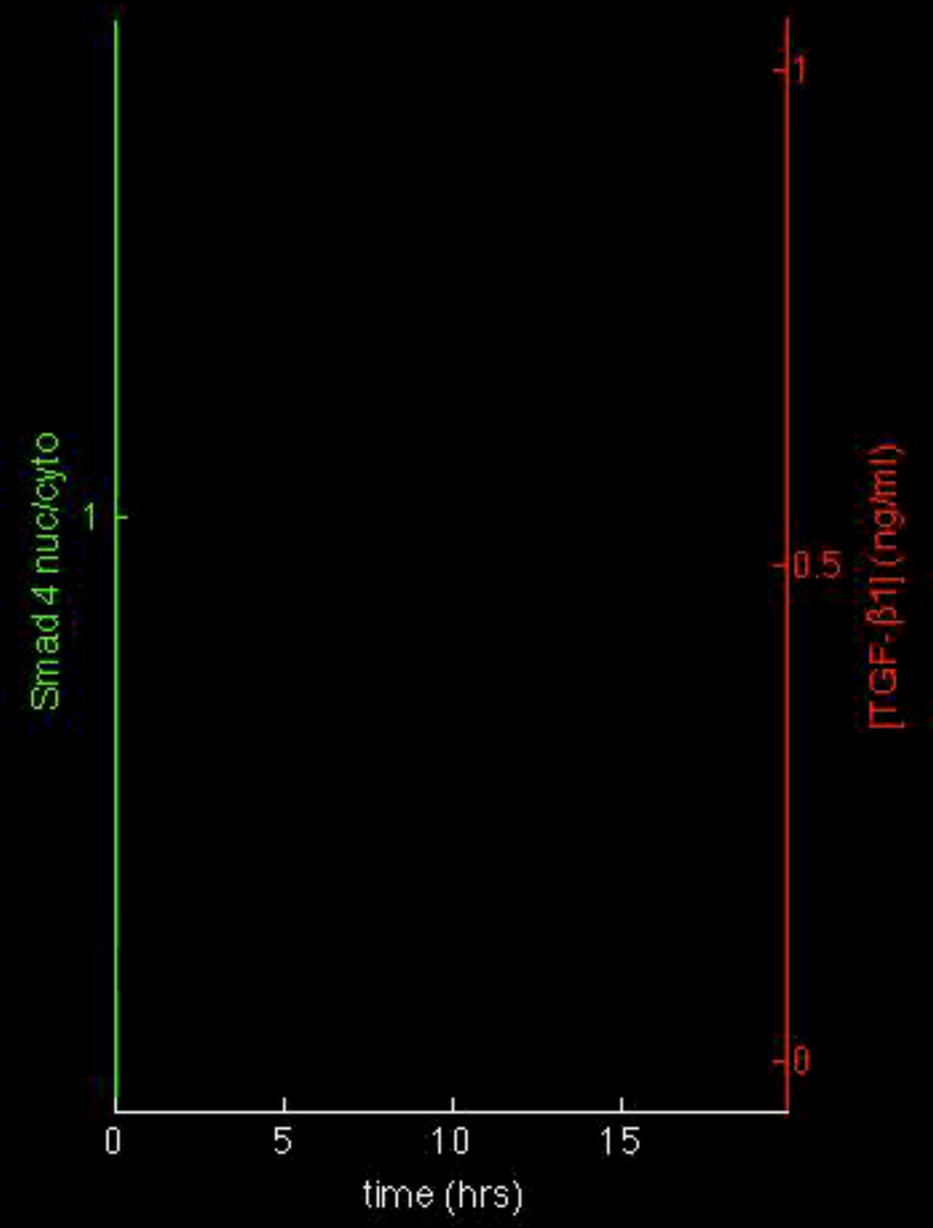
Smad4 is adaptive in response to a step increase in ligand concentration

GFP-Smad4

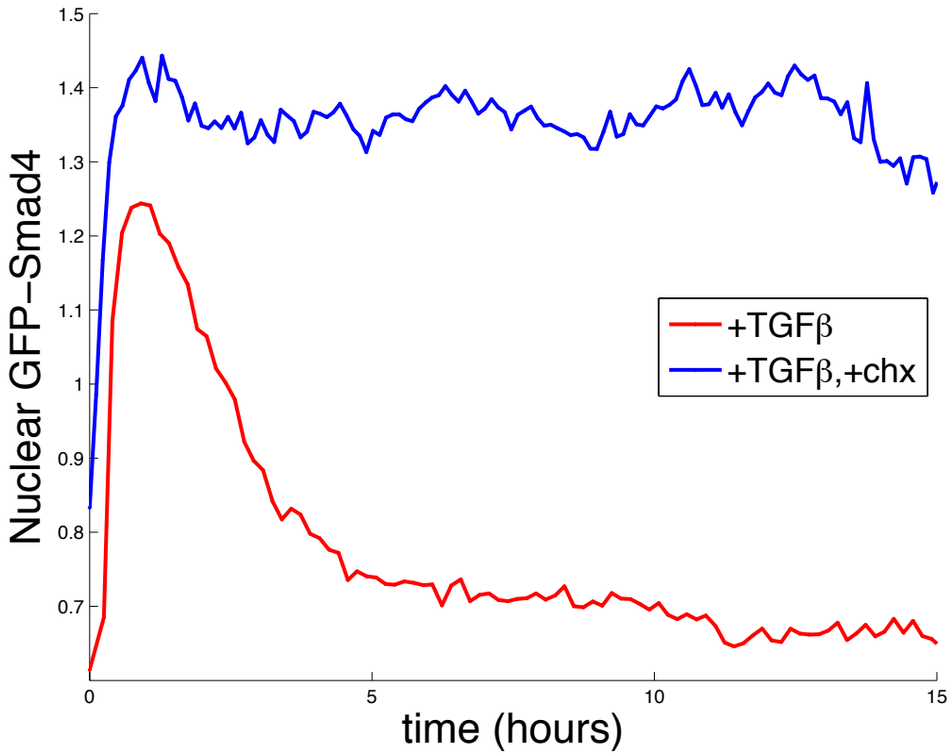
Smad4 is adaptive in response to a step increase in ligand concentration

GFP-Smad4

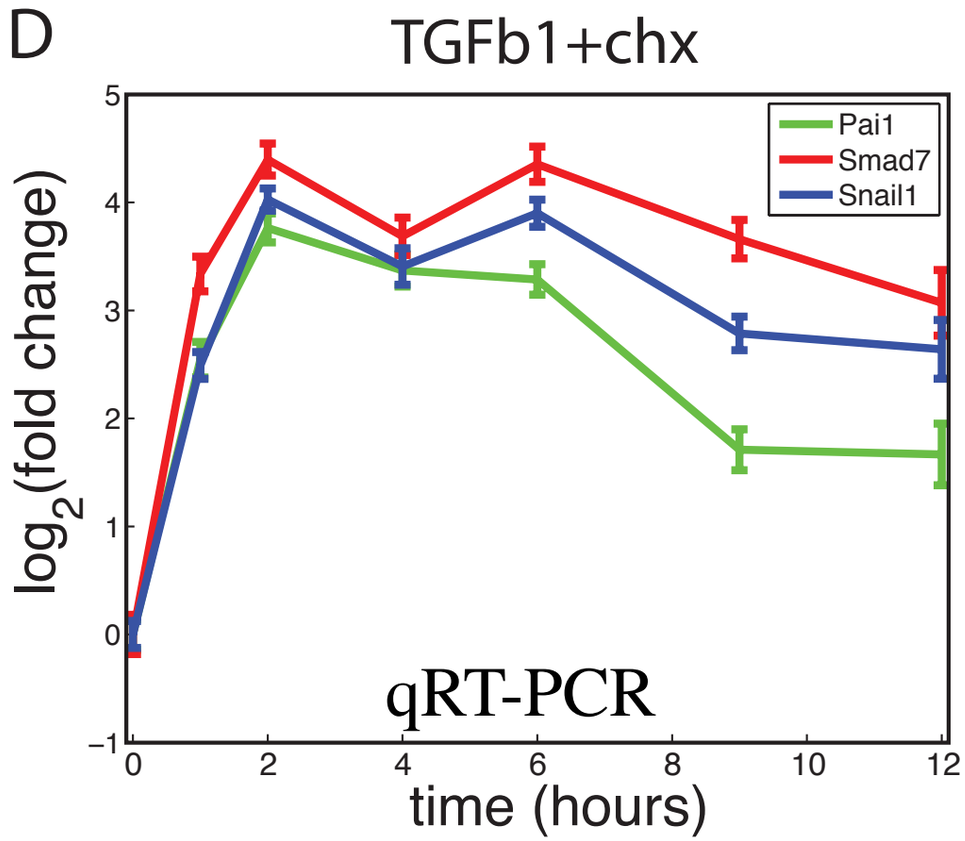




Adaptation requires transcription



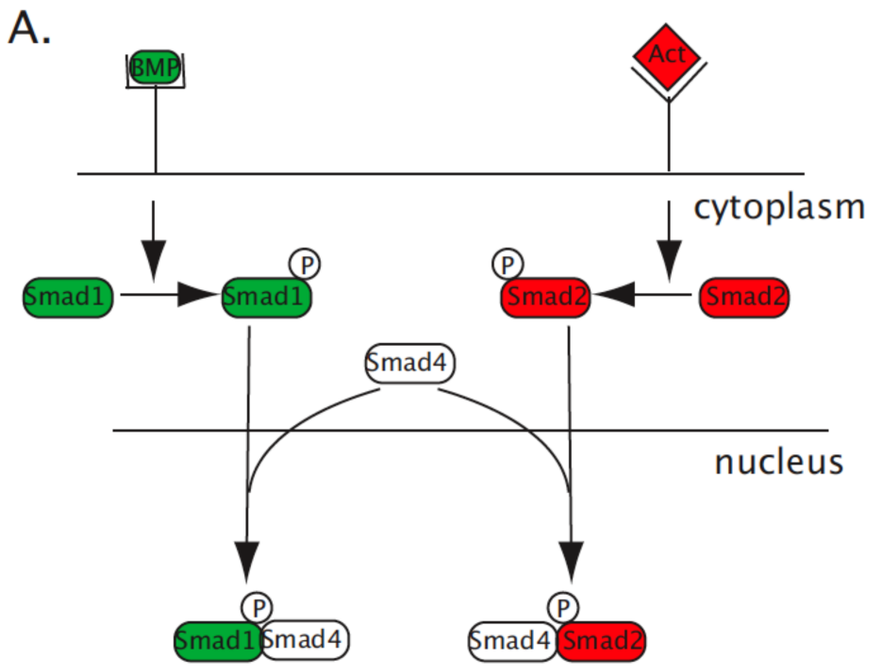
with CycloHex Smad4
remains nuclear

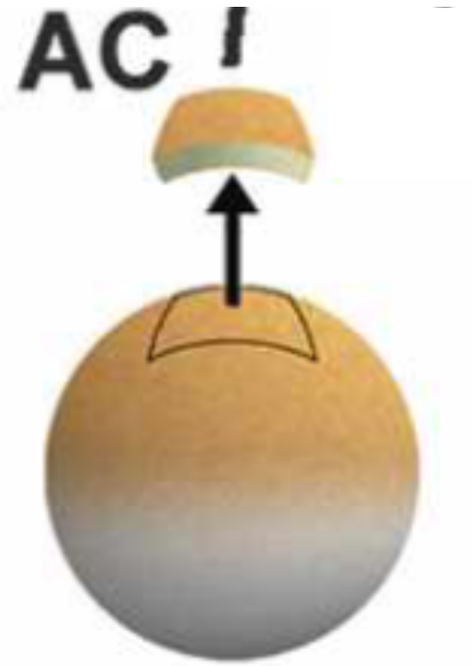


Transcription lasts
longer, follows Smad4

--> Adaption not due to inhibitory ligands (eg *lefty*)
or inhibitory Smads, since would affect pSmad2

Imaging Smad proteins in *Xenopus* Animal Caps





Response to endogenous BMP

Block BMP receptor add activin same behavior

rSmad nuclear, but Smad4 bursts ~20min ~cellcycle time

Animal caps multi-potent tissue

Inject mRNA constructs to 2 cell stage, cut at ~4000 cell stage, image

Behavior of Smads in *Xenopus* animal caps

GFP-Smad1

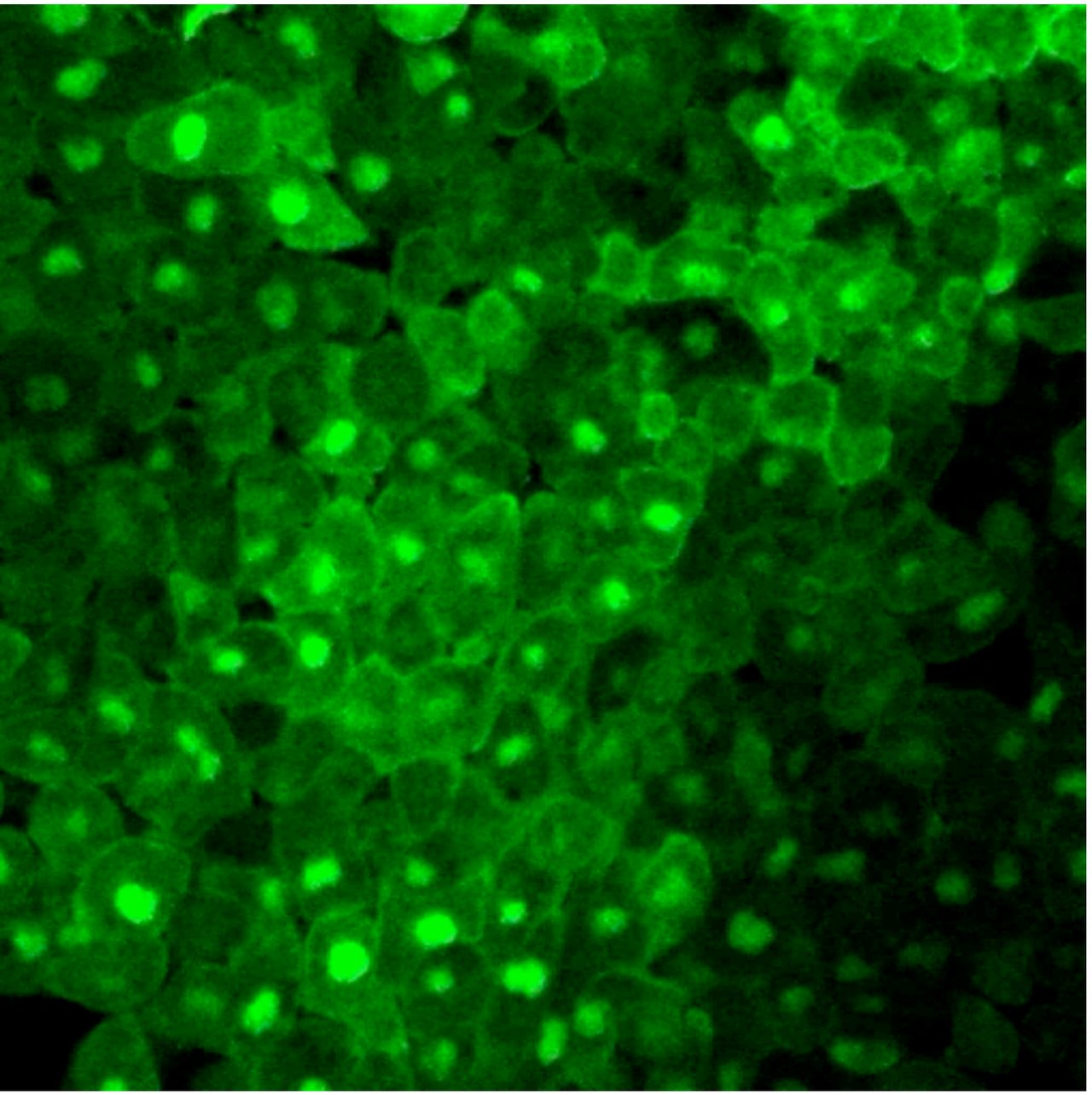
Venus-Smad4

GFP-Smad1 is stably localized in the nucleus of cells....

... butVenus-Smad4 pulses in and out of the nucleus

Behavior of Smads in *Xenopus* animal caps

GFP-Smad1



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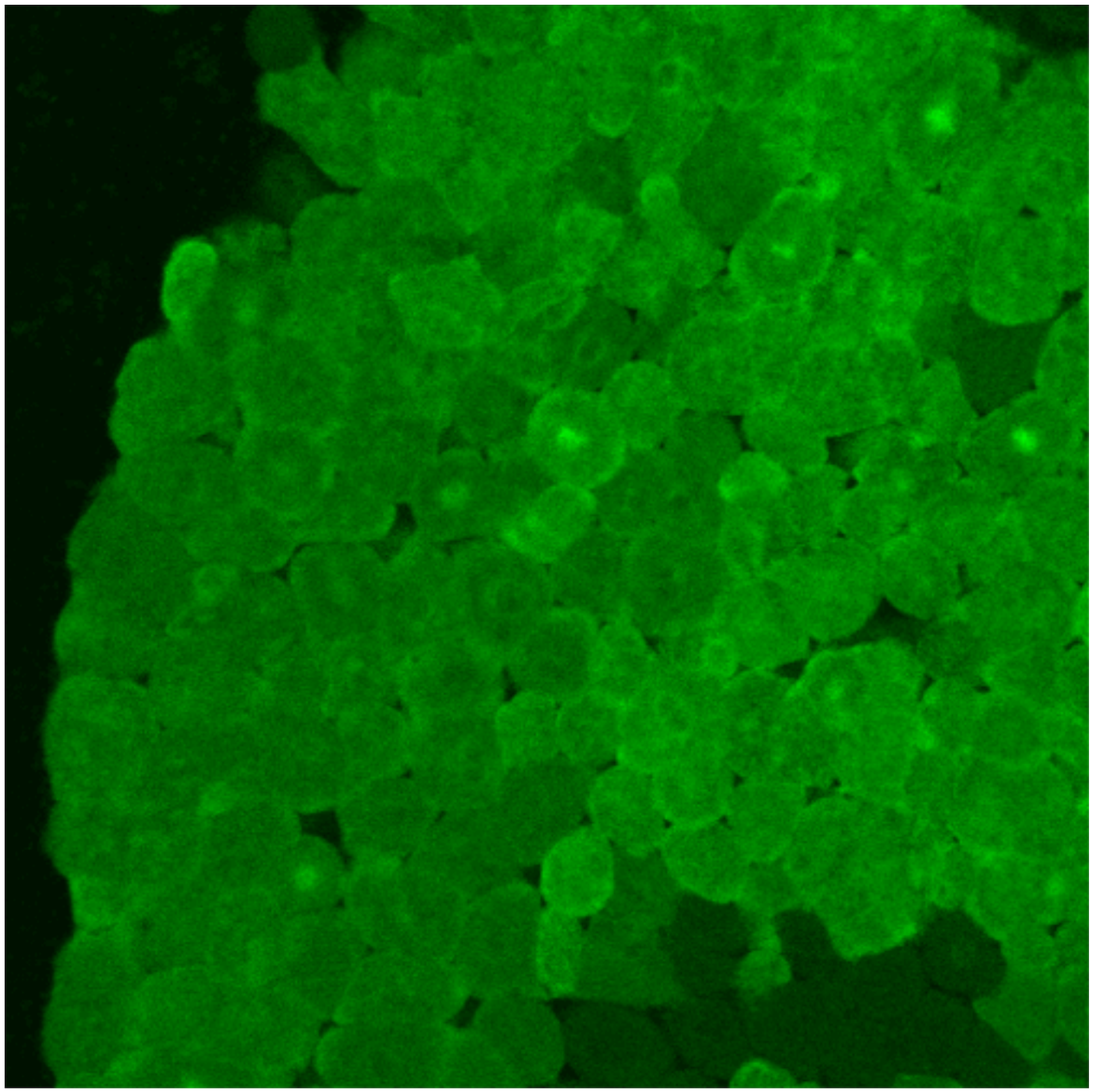

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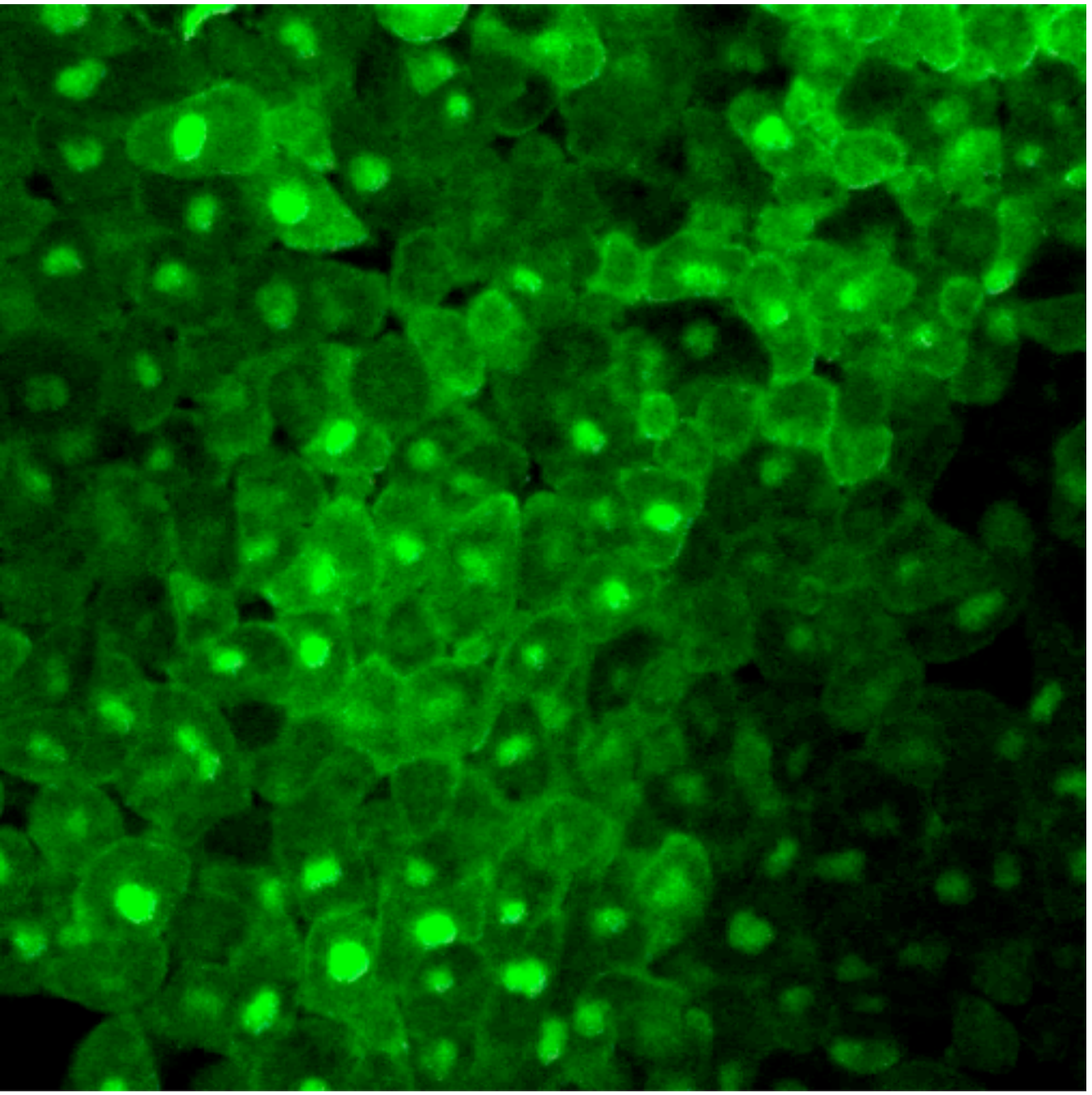
Behavior of Smads in *Xenopus* animal caps

GFP-Smad1



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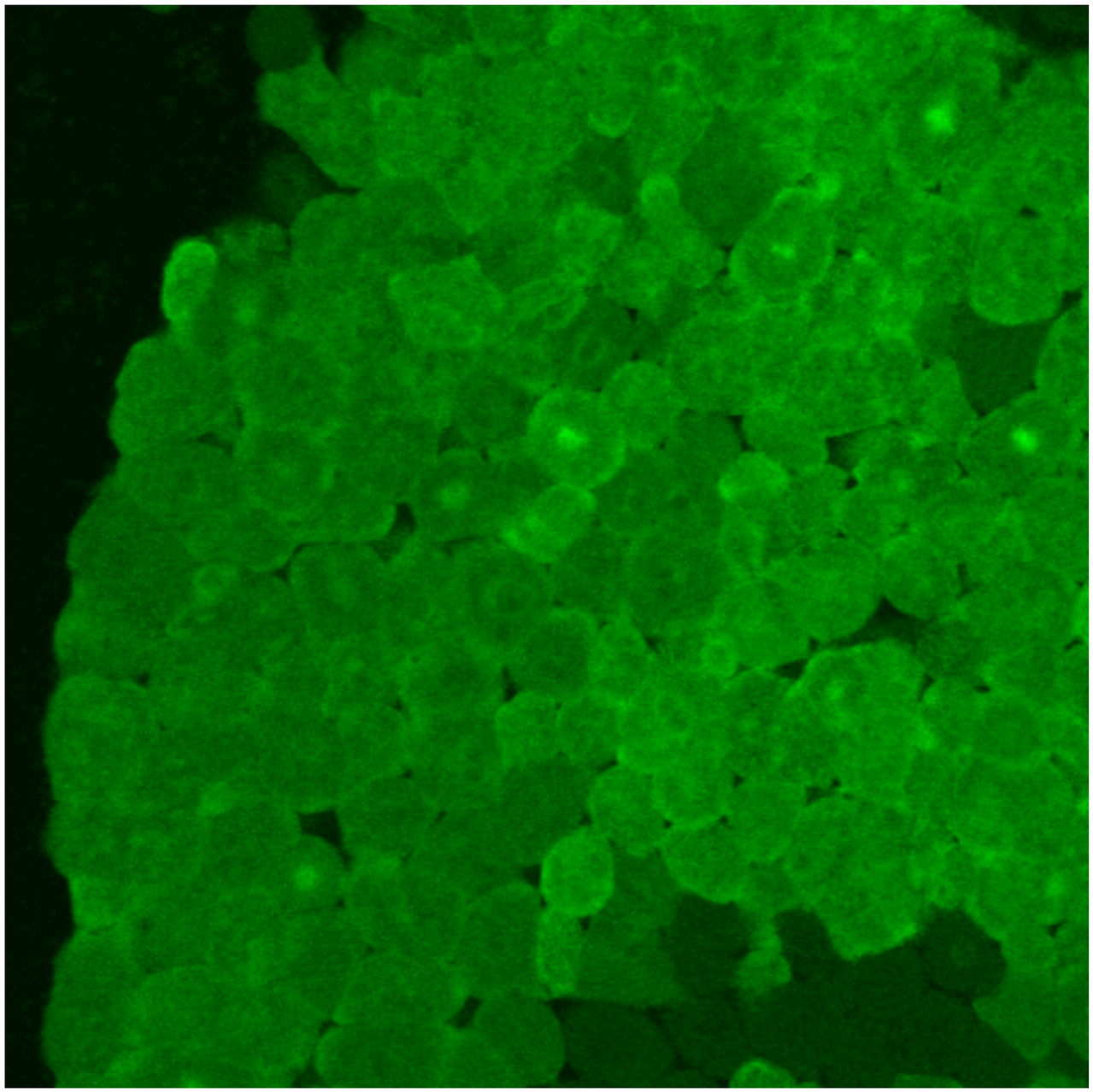
Venus-Smad4



... butVenus-Smad4 pulses in and out of the nucleus

What's new?

TGFβ activity == Smad4; pR-Smads necessary not suffic.
Functional Complex= [adaptation module] * [graded receptor]

~ 4*10^4 papers on TGFβ was adaptation seen?? NO
* end point assays
* population measurements for incoherent cells
* cell density can affect response in HaCaT
* used CHX to get direct effects
* cell type?

What's causing nuclear Smad4 bursts in the embryo??
ligand/inhibitors move between cells?
autocrine?
tied to transcription
not via TGFβ inhib ligands or inhibitory Smads

Implications of adaptive pathway for embryonic patterning

Patterning by static morphogen (signal) the exception

Fly:

Wing disk: dpp and size control (d ln(dpp)/dt)

Eye: morphogenic furrow moves as wave

Vertebrates:

Dorsal-ventral by TGFβ activin/nodal (Schohl ... Whitman..

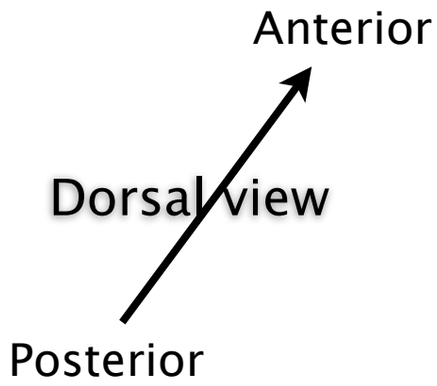
A-P Hox genes gastrulation time->space (Wacker)

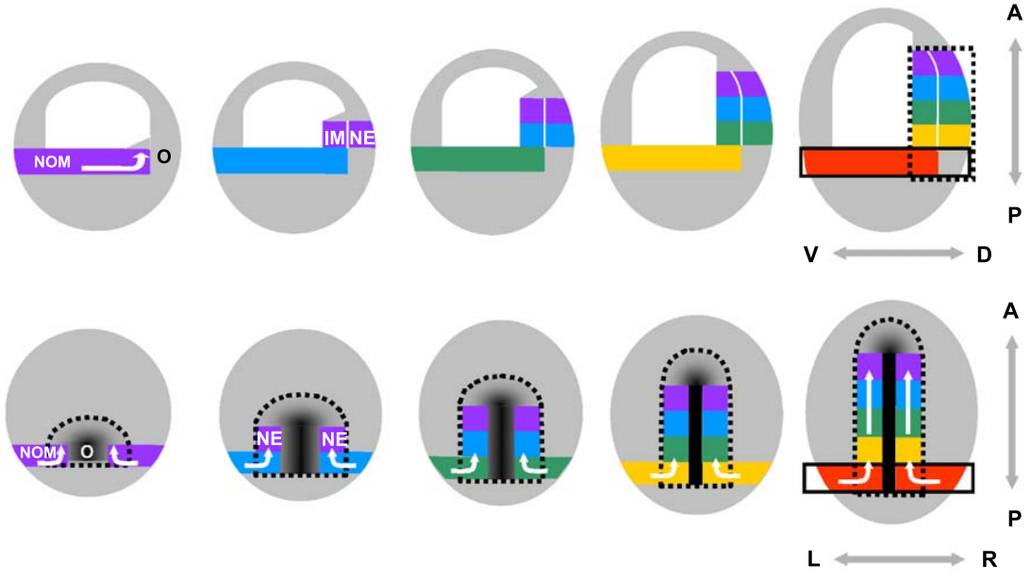
Somite clock FGF, Wnt, RA (Pourquie..)

Neural tube D-V Shh (Briscoe)

Digits Hh, Hox

Images of time dep morpho

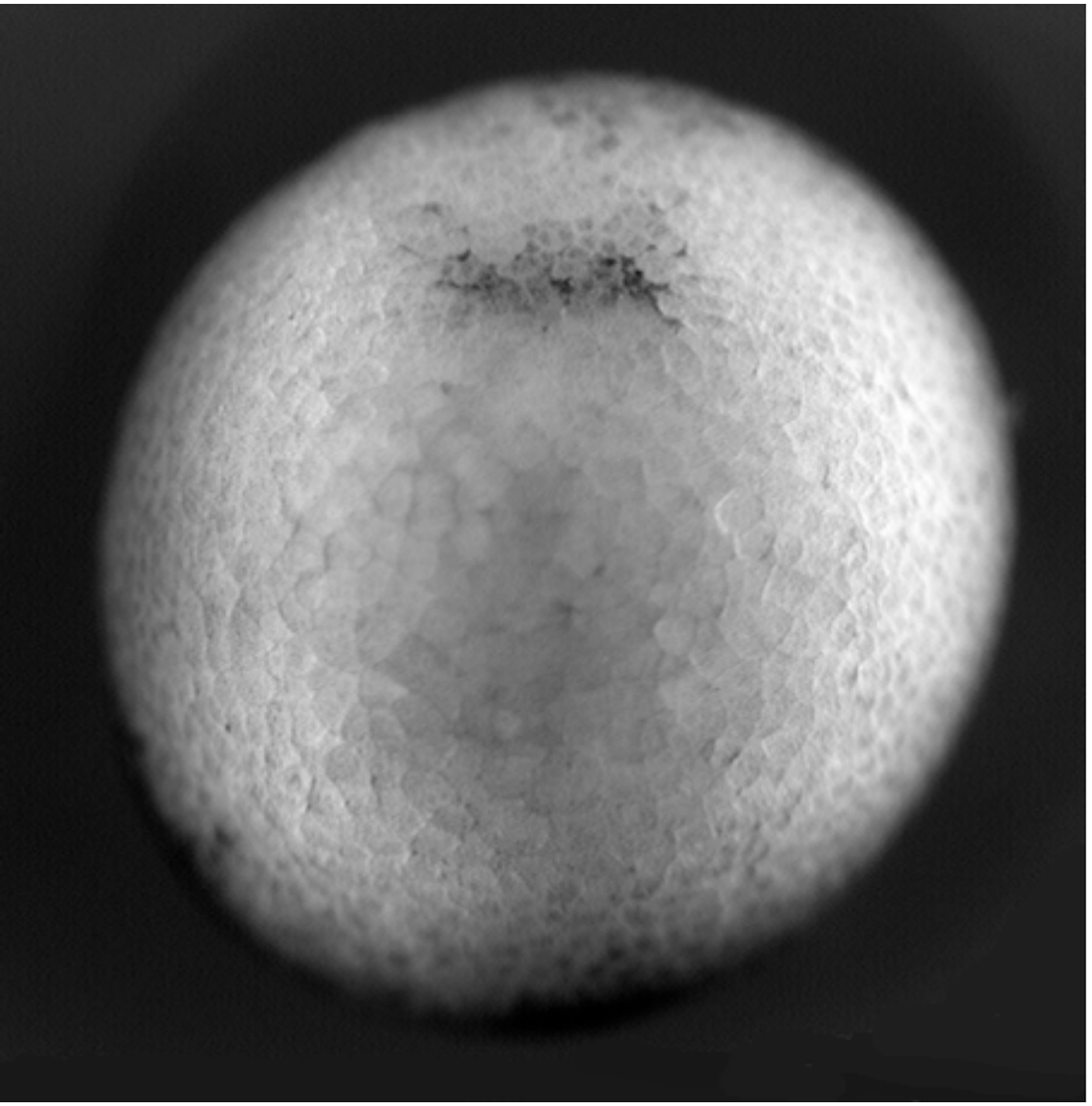


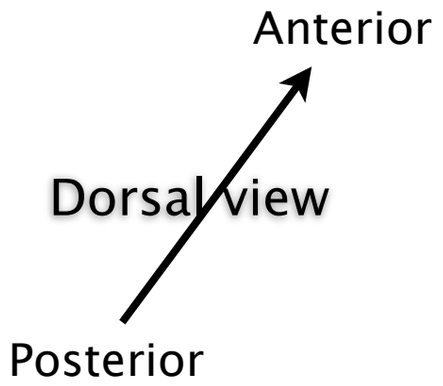


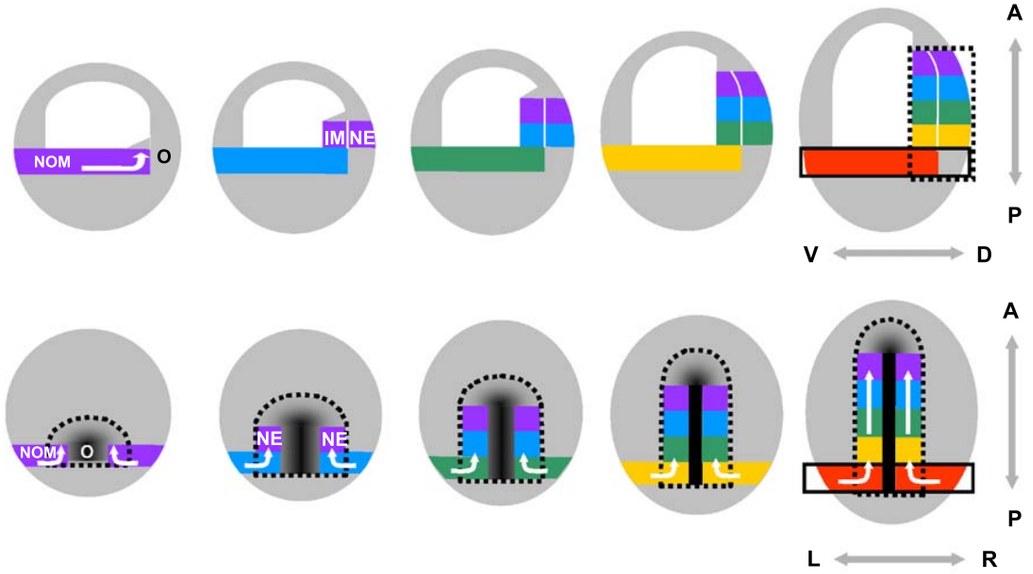
1.2mm egg, movie begins 5 hrs post fertilization, lasts 17 hrs at 23C

Hox genes pattern the A-P axis as it is formed.
(Wacker Durston 2004)

Images of time dep morpho







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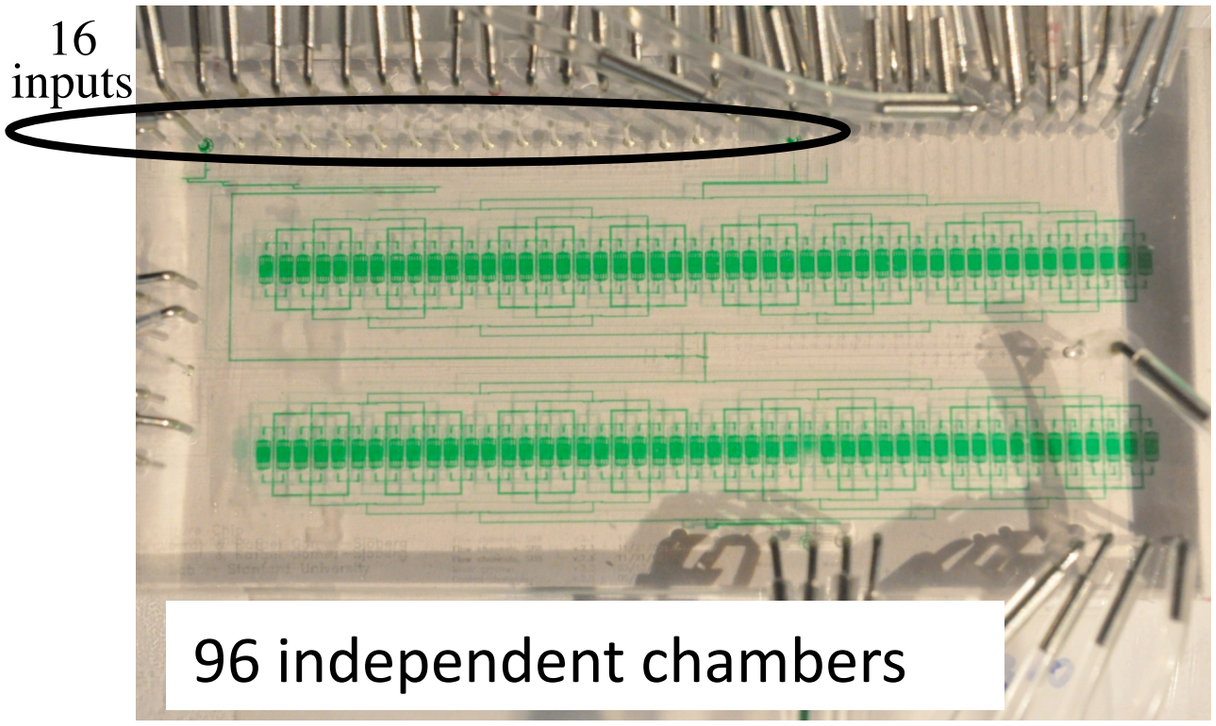
Morphogen patterning in an embryo?

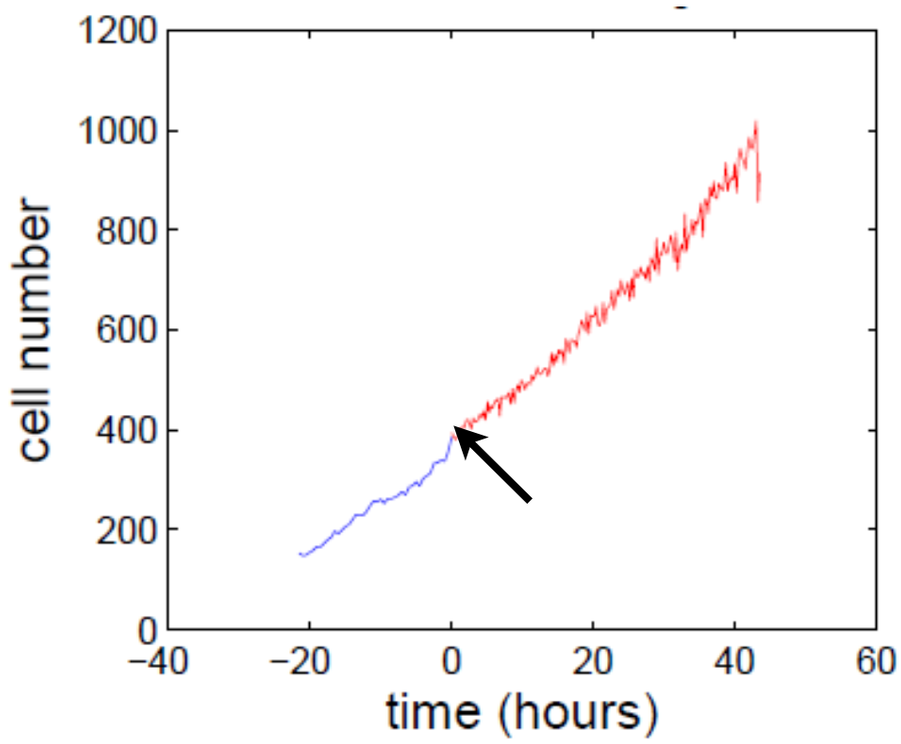
How does an adaptive pathway facilitate dynamic patterning?

Can not control or directly measure morphogen in embryo -->
measure response to complex stimuli

Control the ligands



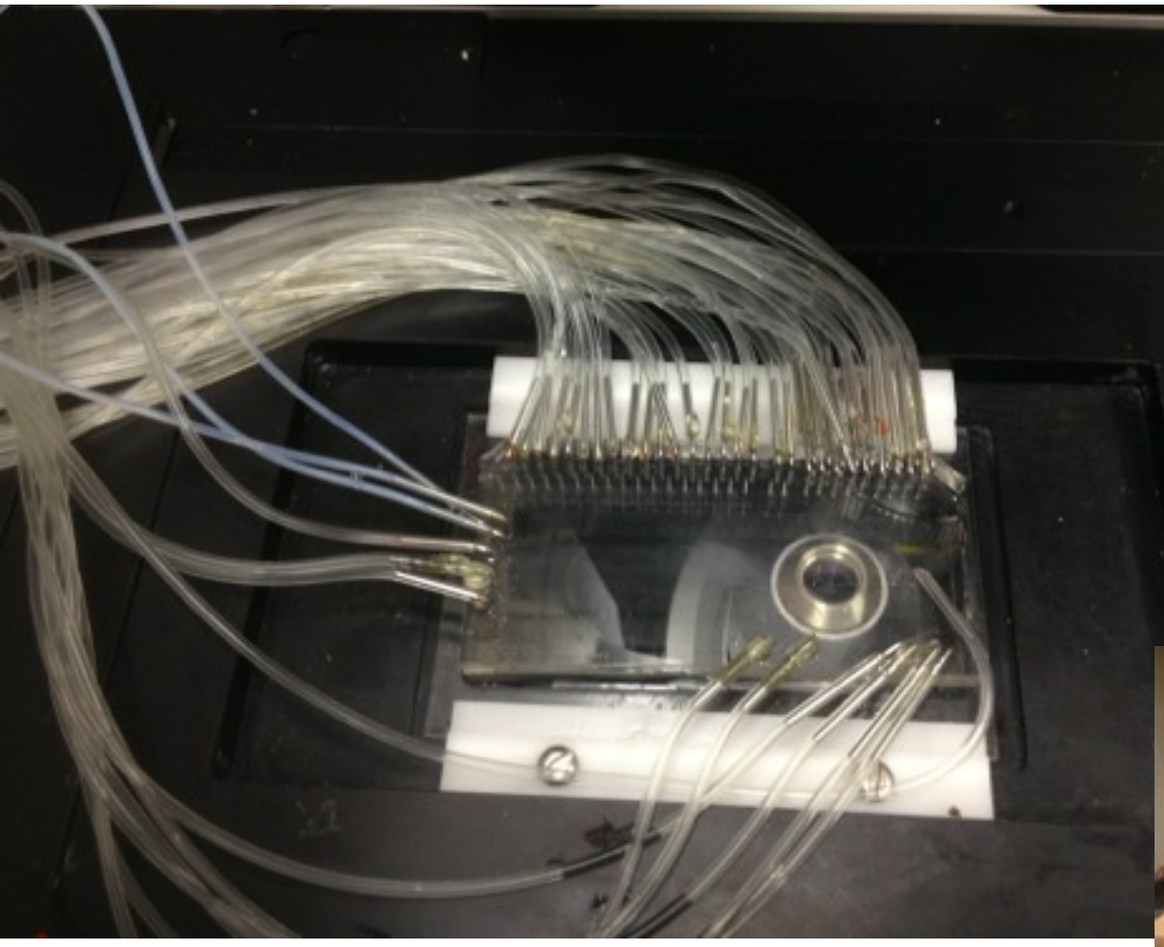
2 level PDMS**, multiplexed addressing.
Chambers: 1mm² x 40µ, cells on PDMS +FN
(Image phase + 2 colors)/15 min + feedings
Leica + matlab (microscope + fluidics)



Cells grow normally (TGFβ + imaging start at t=0)

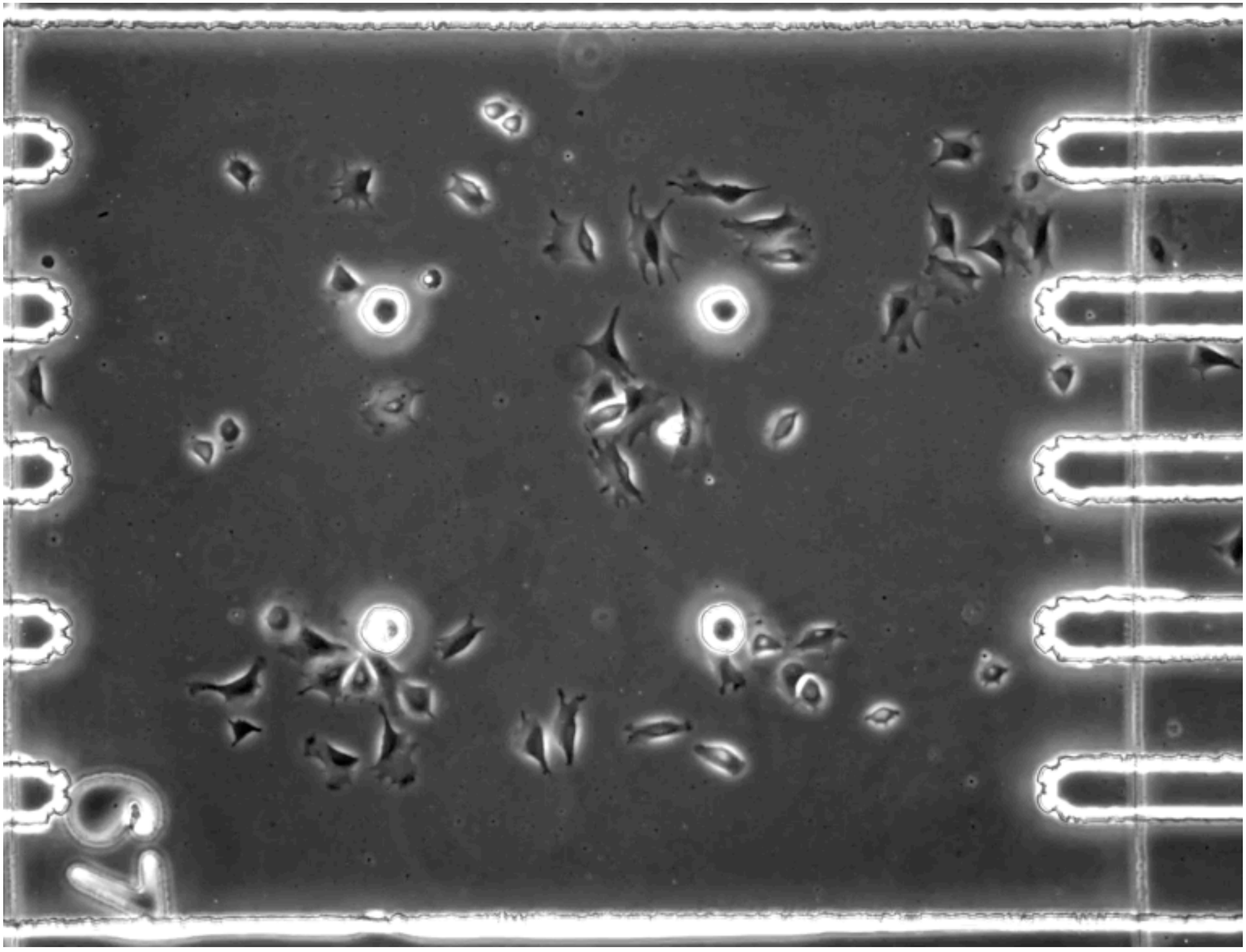
**Quake Lab www.stanford.edu/group/foundry/
Gómez-Sjöberg, et al. (2007) *Anal Chem.*
Tay, S.et al (2010). *Nature*

Lots of plumbing

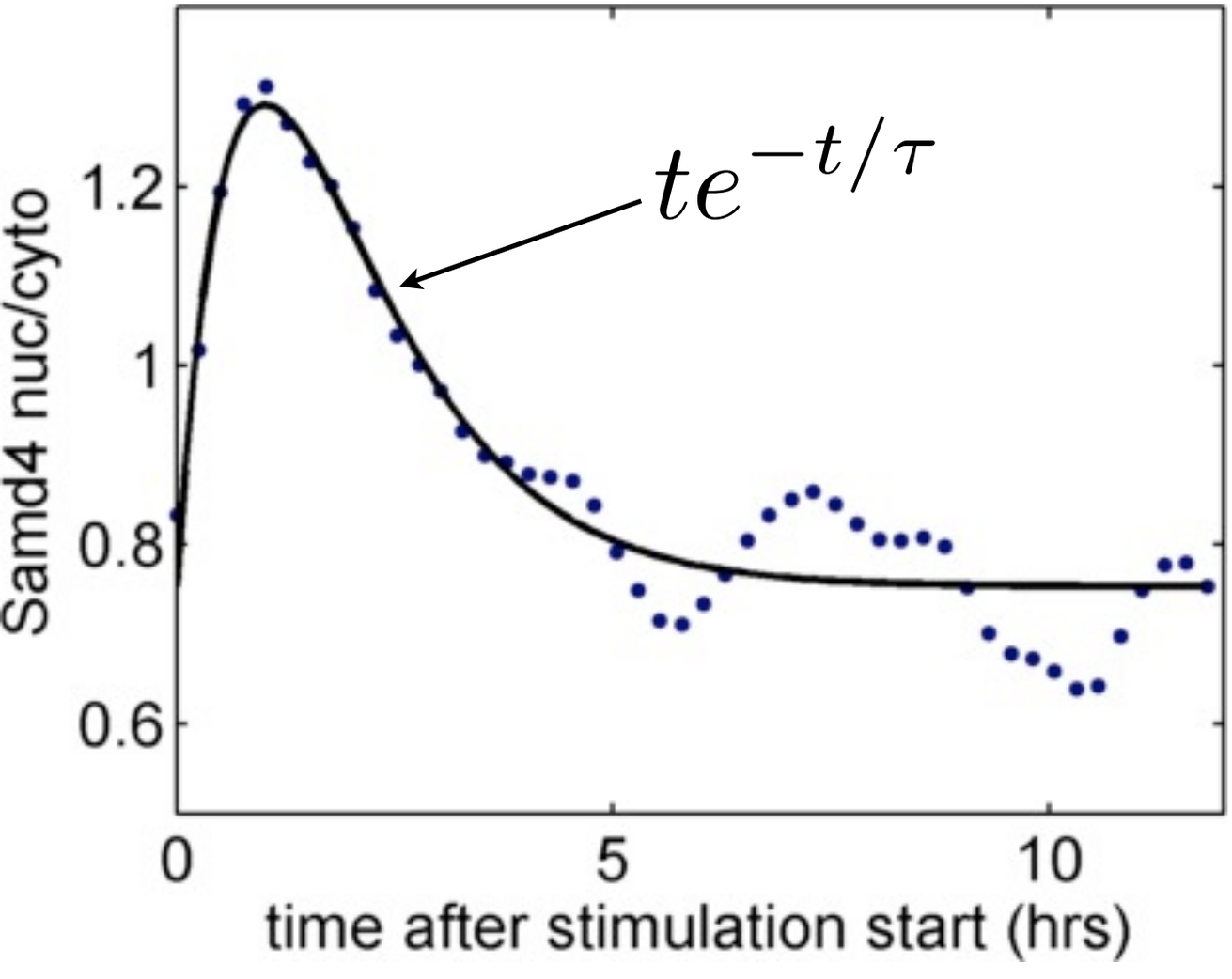


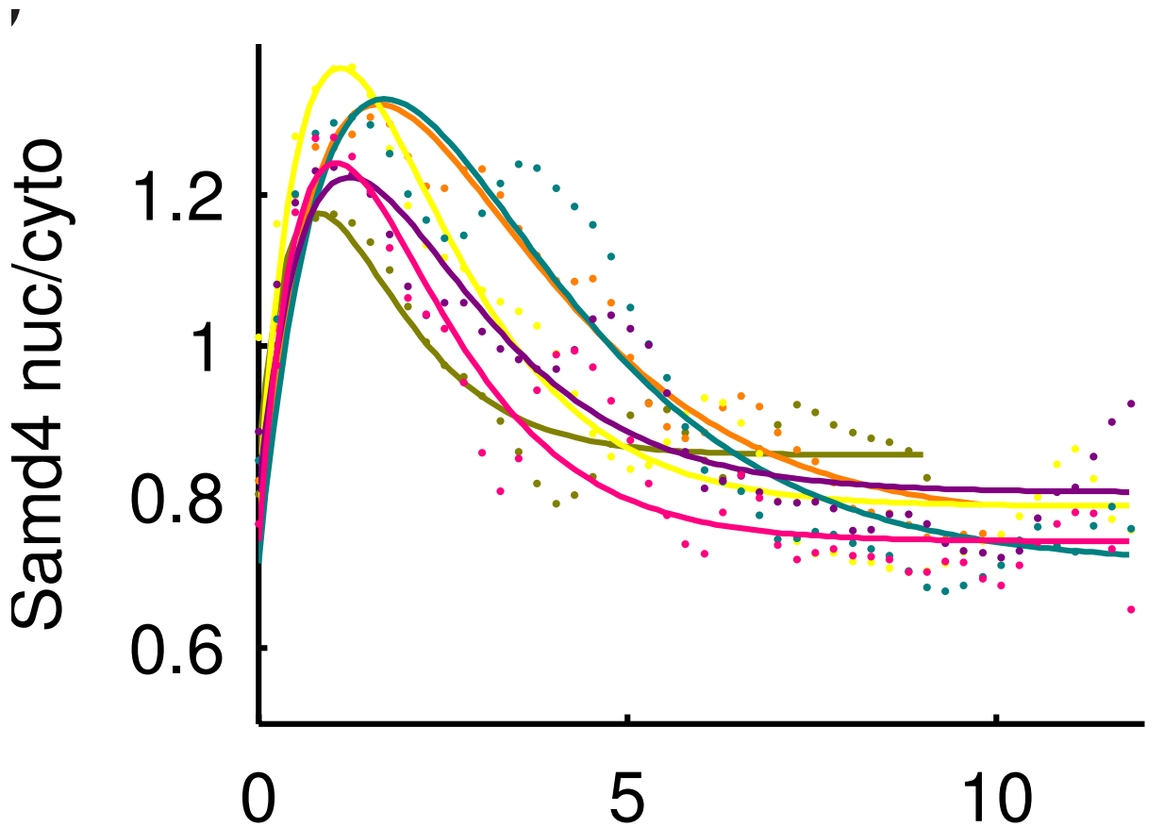
cell culture chip (46 hrs)

cell culture chip (46 hrs)



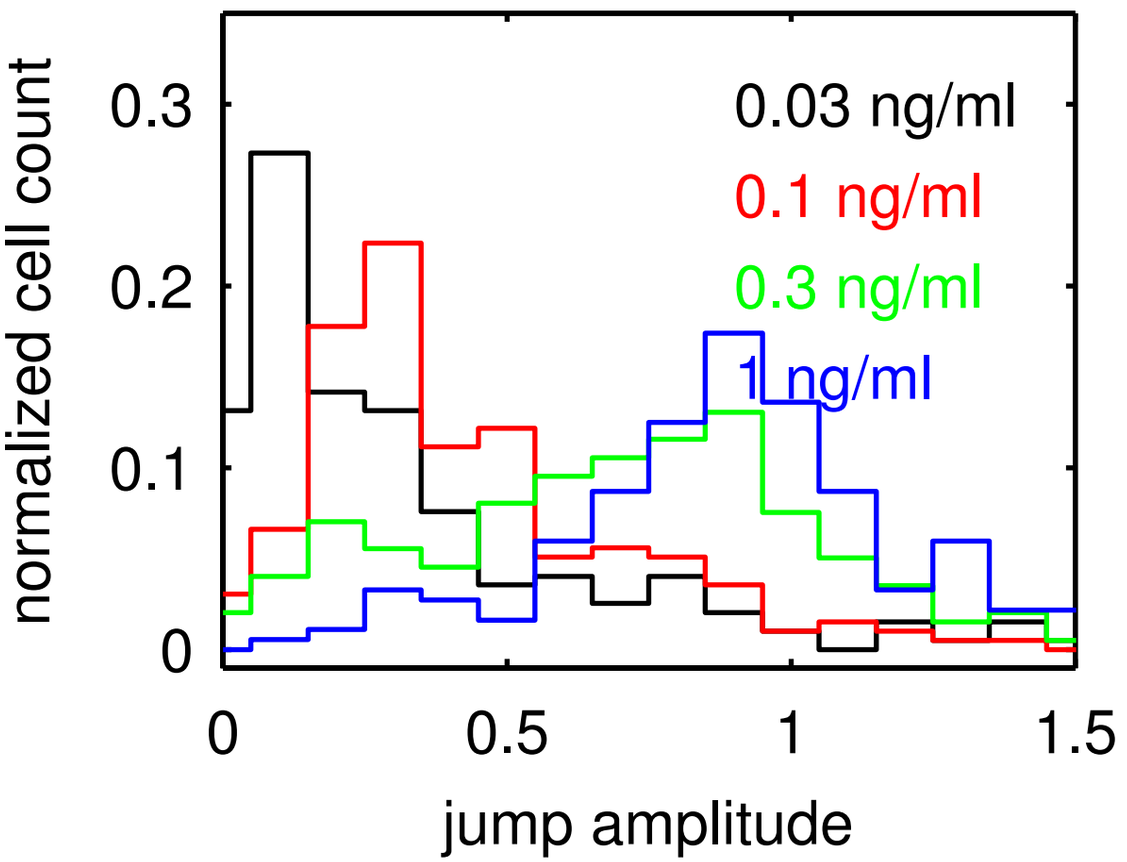
Single cell response to a ligand step at t=0

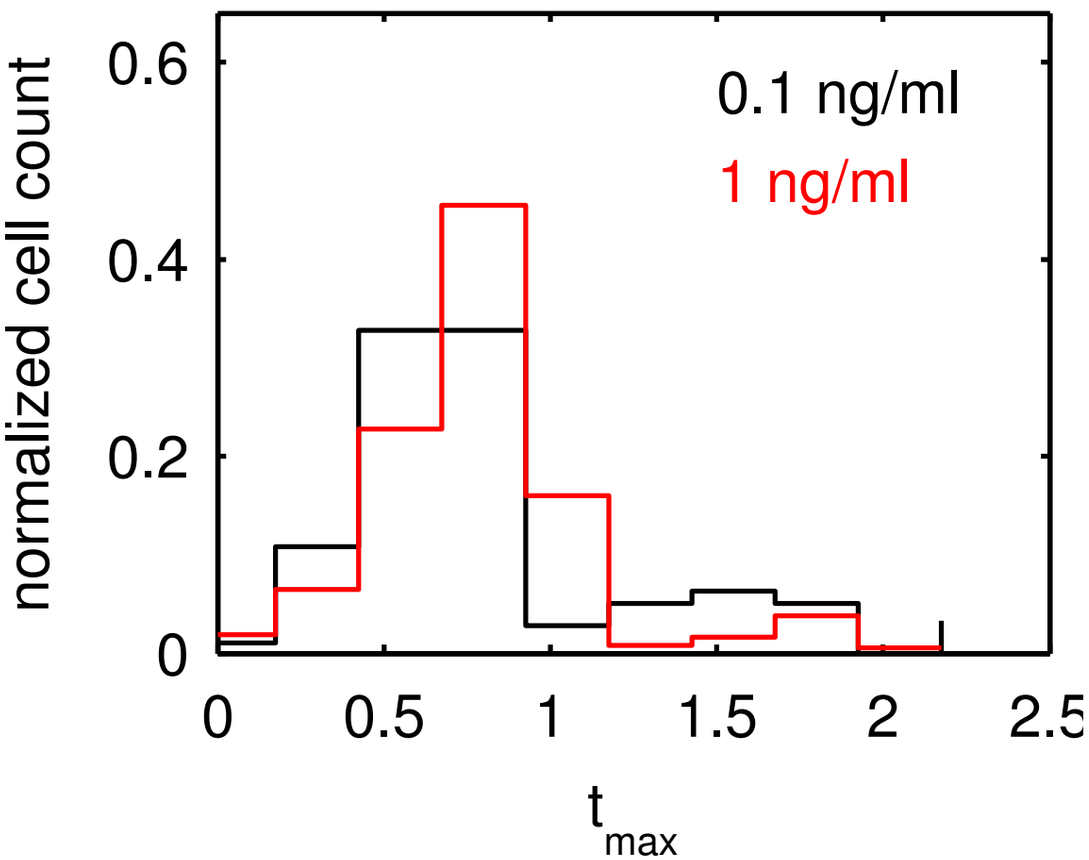




$\tau \sim 1\text{hr}$

All cells respond in graded manner to ligand step

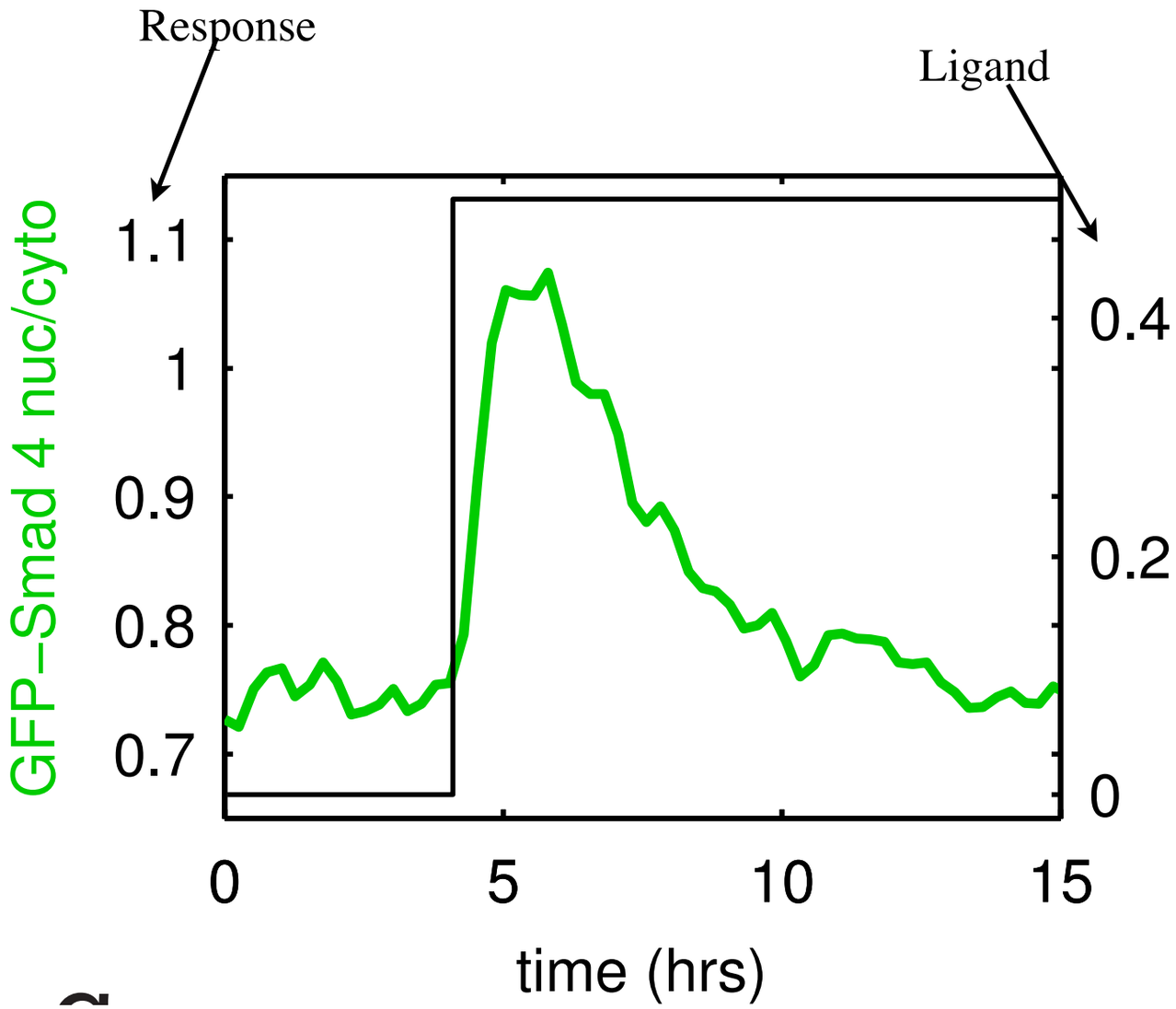




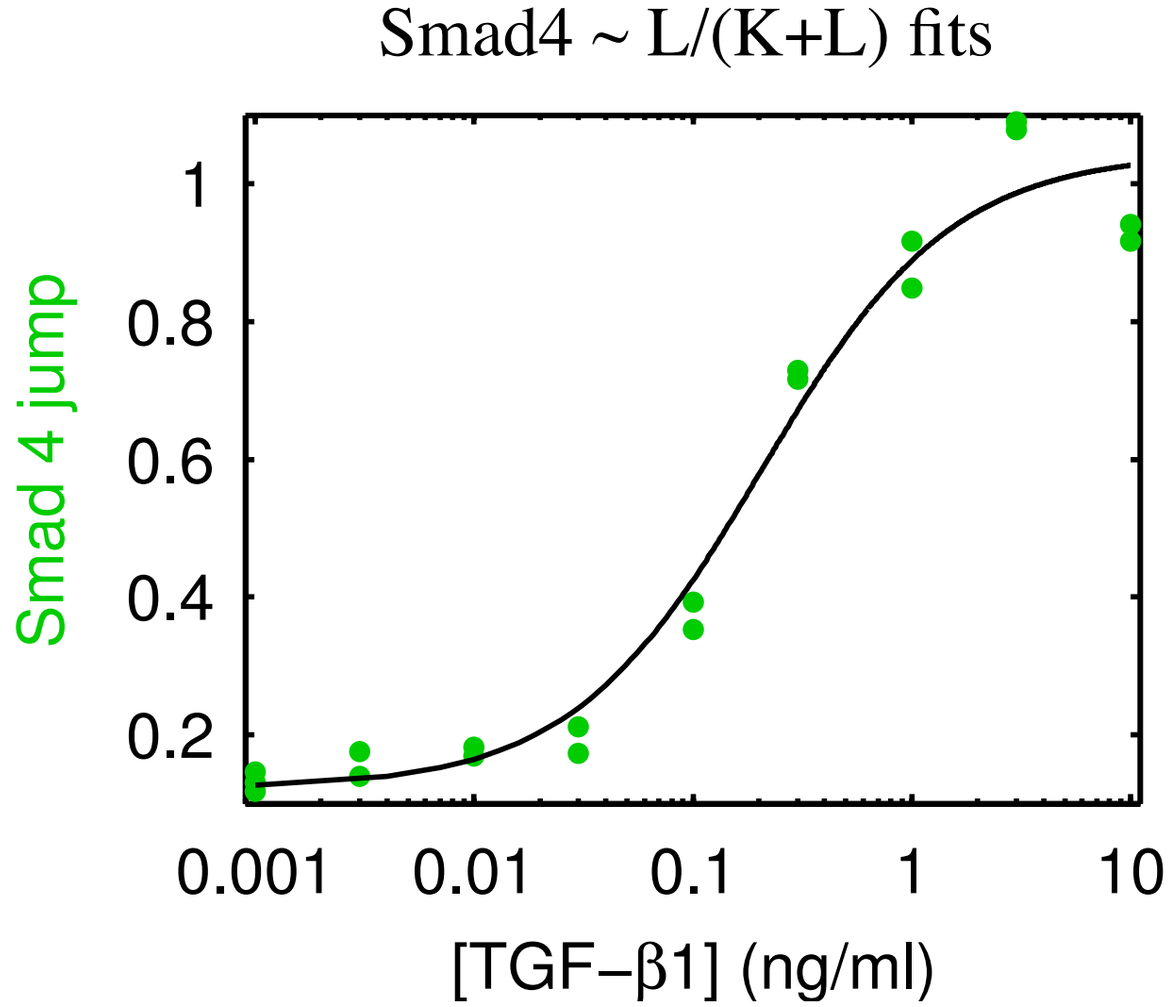
Histograms for amplitude of Smad4 jump and time of max response as fit:

$$cst + ampl*t*\exp(-t/t_{max})$$

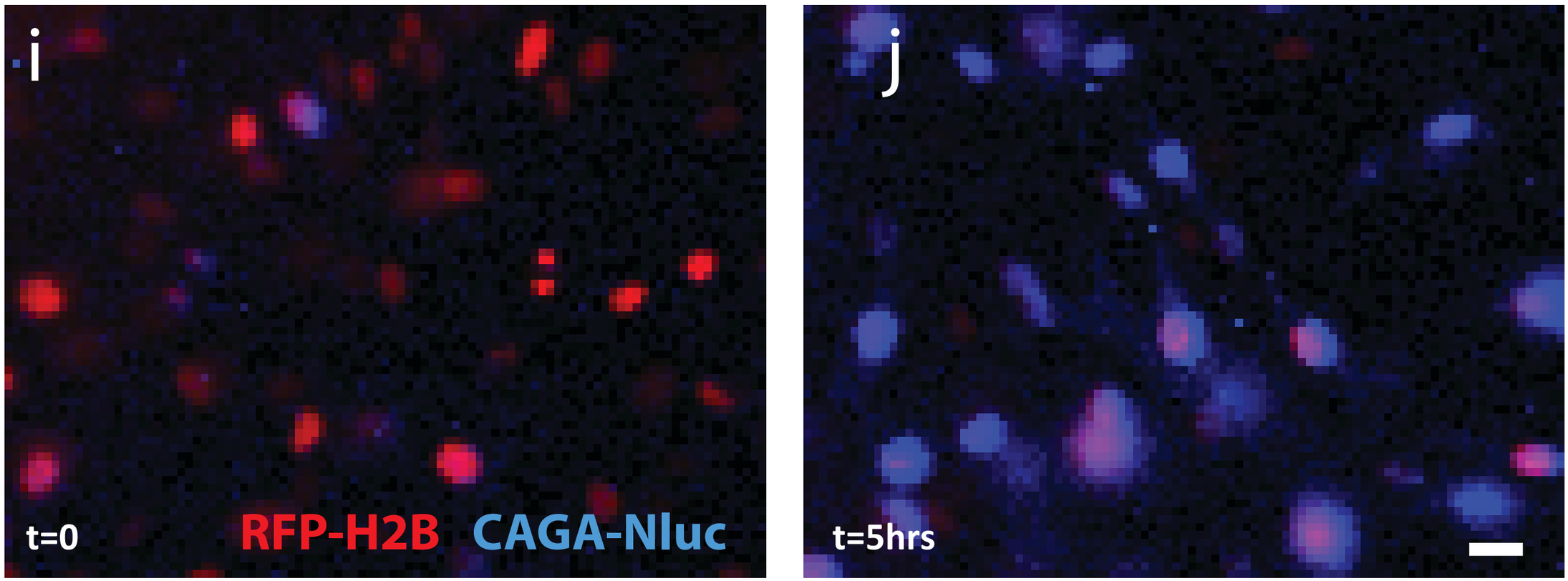
Population response nuc-Smad4 response ~ dish



Ligand Step



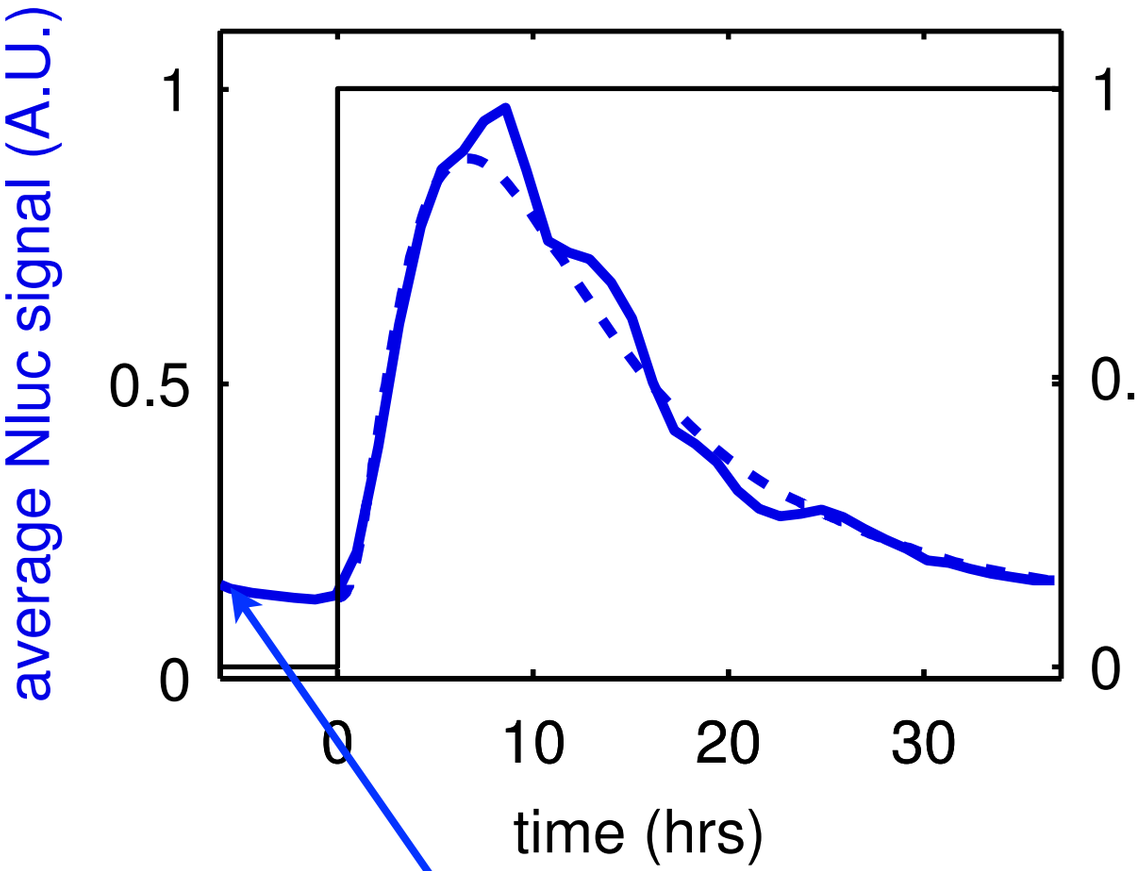
Nano-luc: live transcriptional reporter



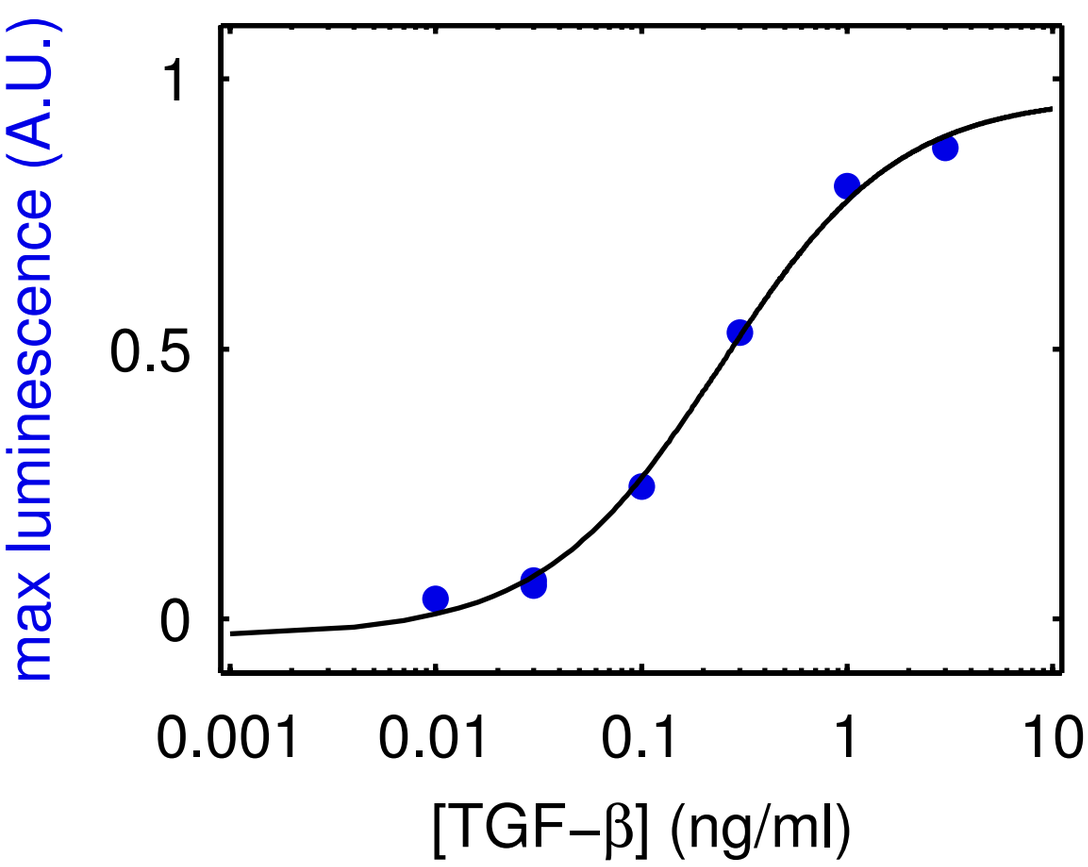
Nluc ~ 100x brighter than firefly/Renilla
Luminescence not fluorescence, lower backgrounds
Substrate unstable in cell culture (microfluidics solves)
Enzyme life time activity dependent (lowers background)

$CAGA_{12}$-Nluc = synthetic TGFβ reporter (with H2B-RFP), (invisible with GFP)

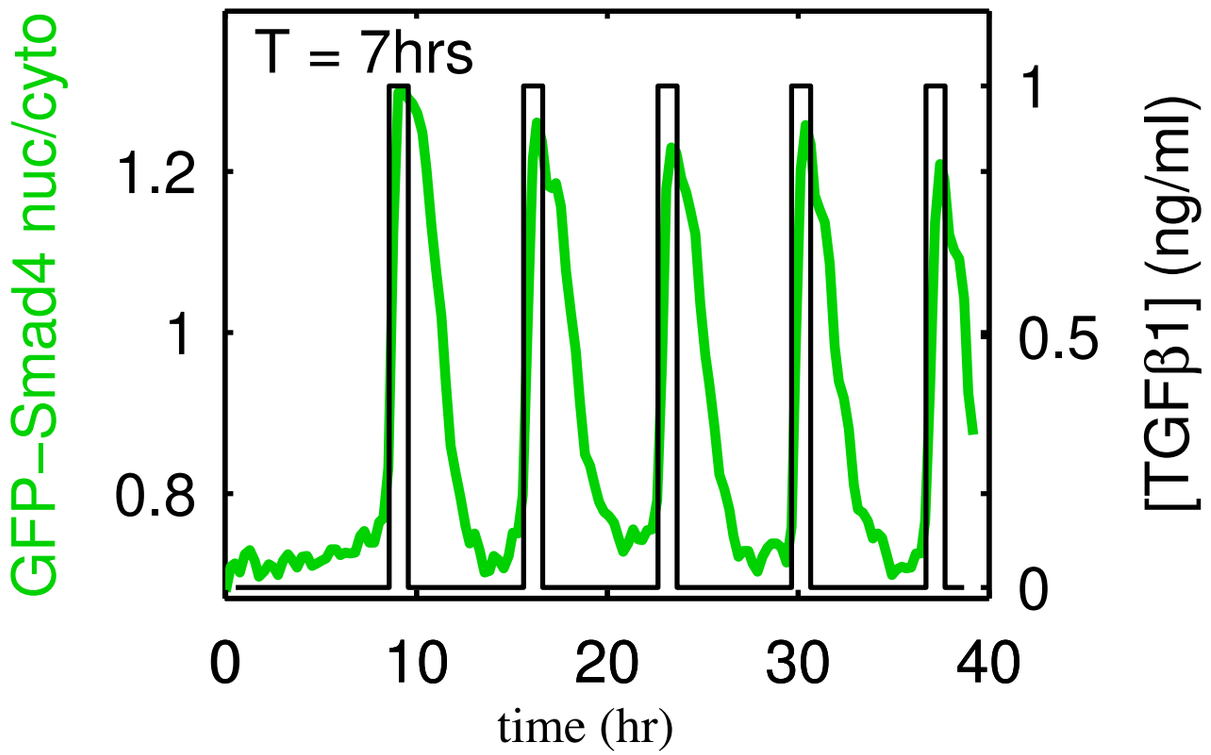
Nluc: Population response



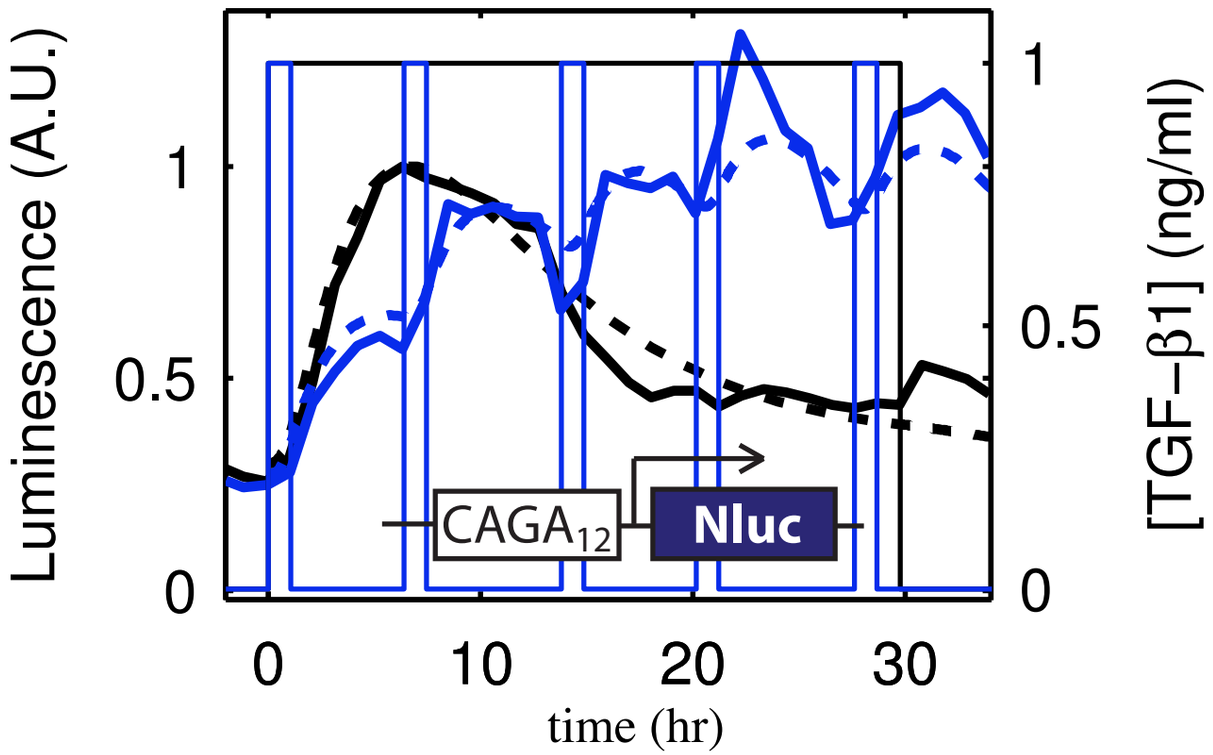
NB fold change! vs Smad4



Response to pulses



Complete response to pulses (1 hr on, 6 off), period > adaptation time
Cells uncorrelated

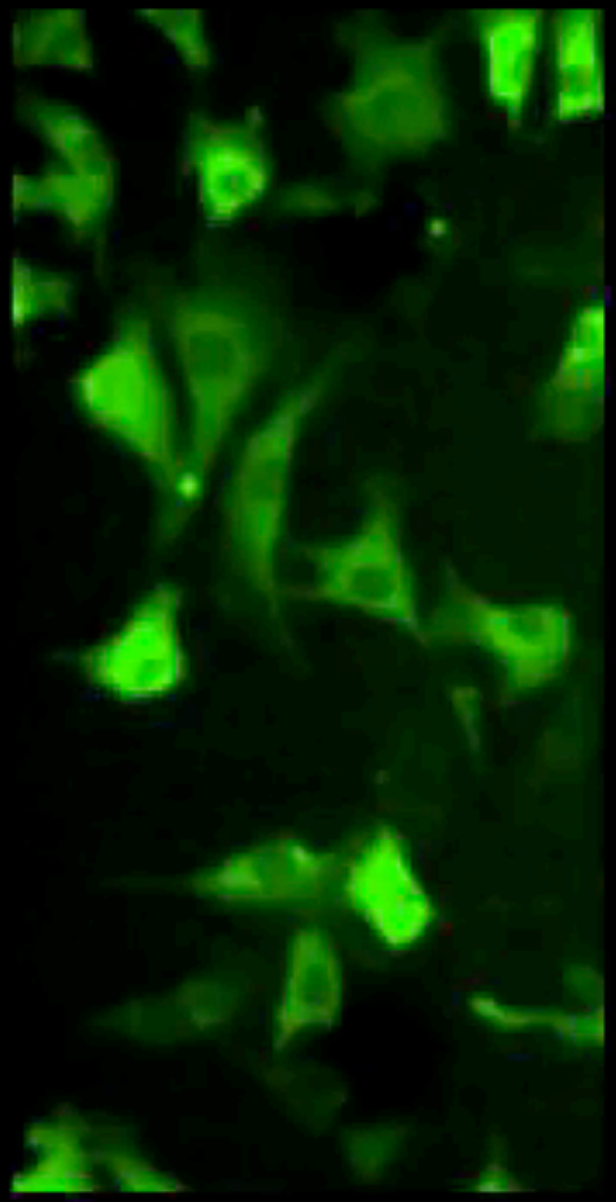


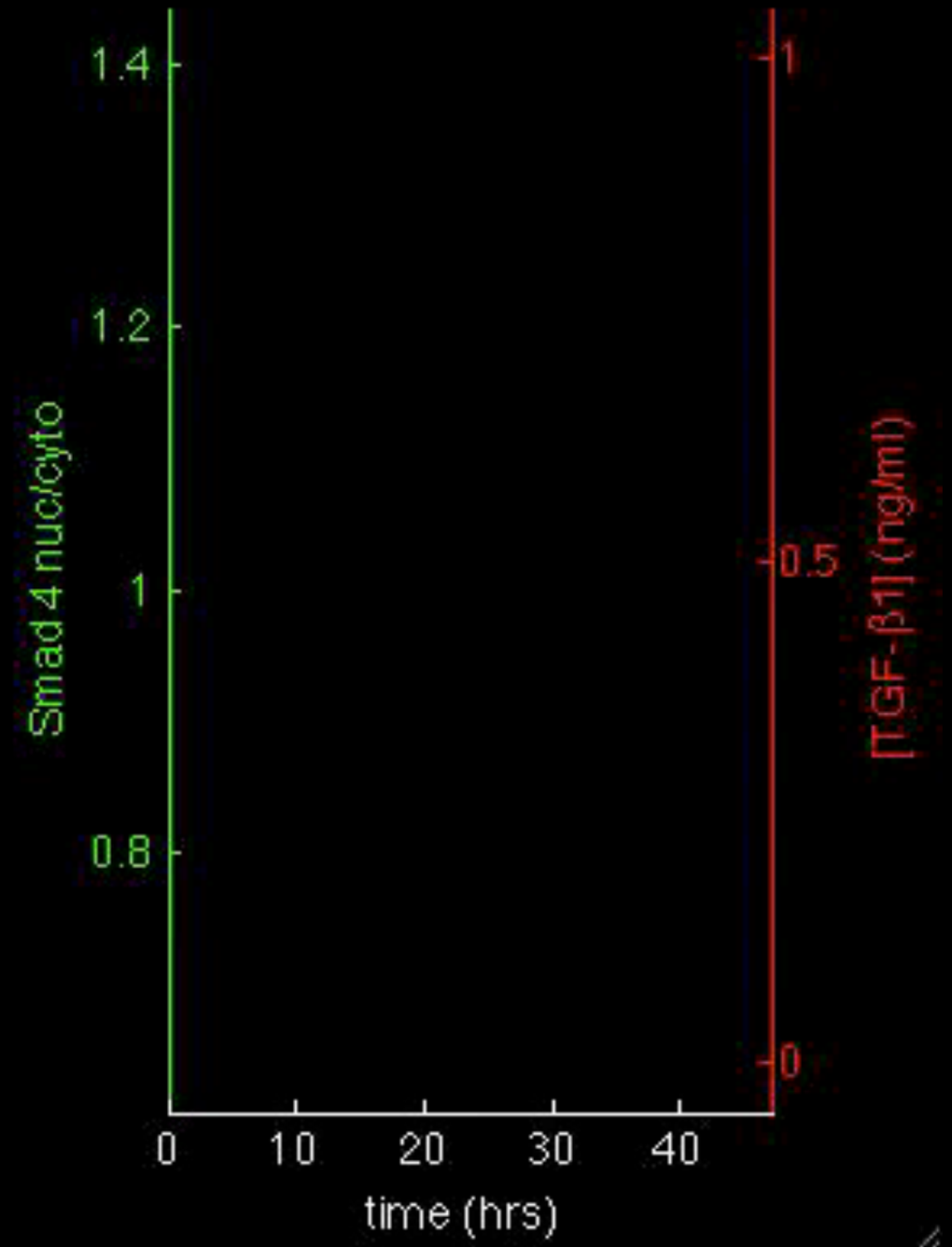
Nluc: response to pulses -->
life time vs production of enzyme

response to step -->
internal response time

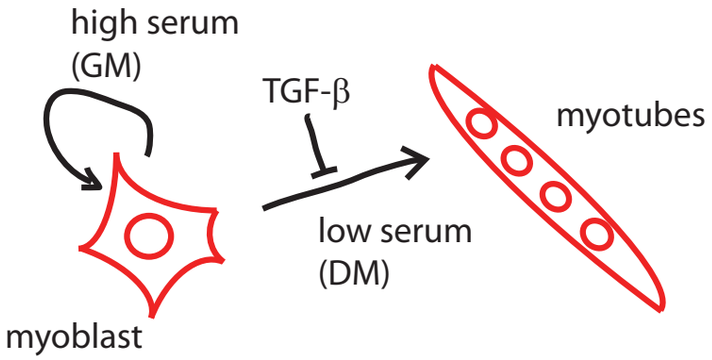
----- fits (below)

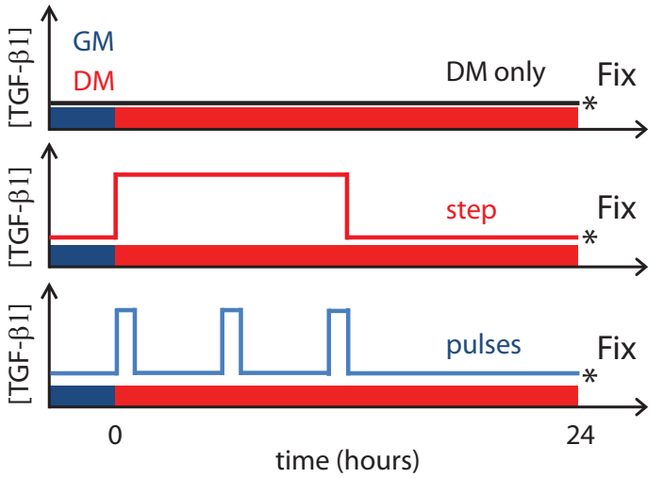
At the level of Smad4/transcription pathway is not a ratchet -- responds independently to each pulse

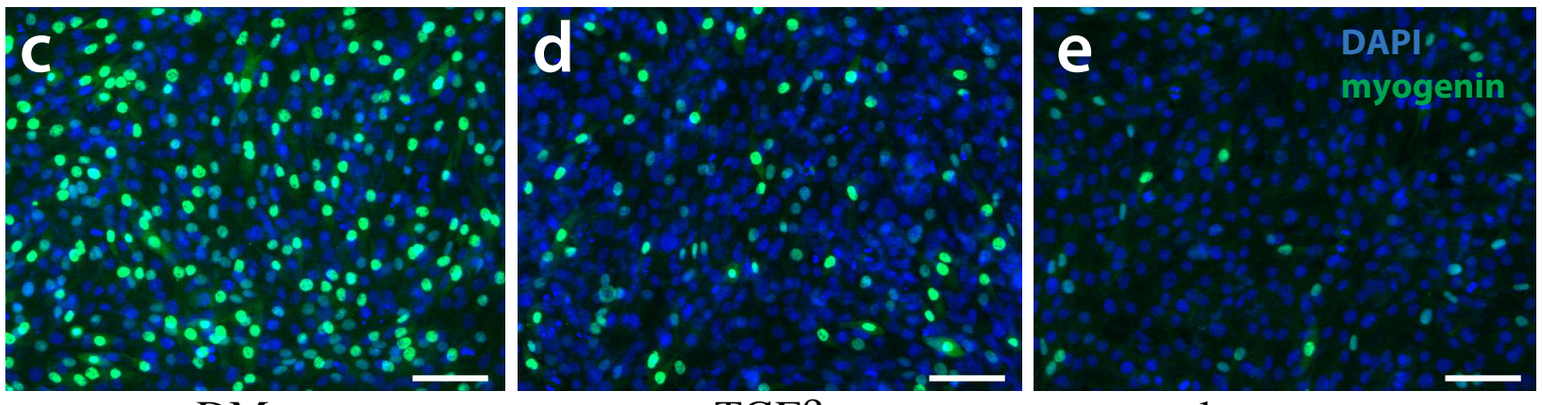




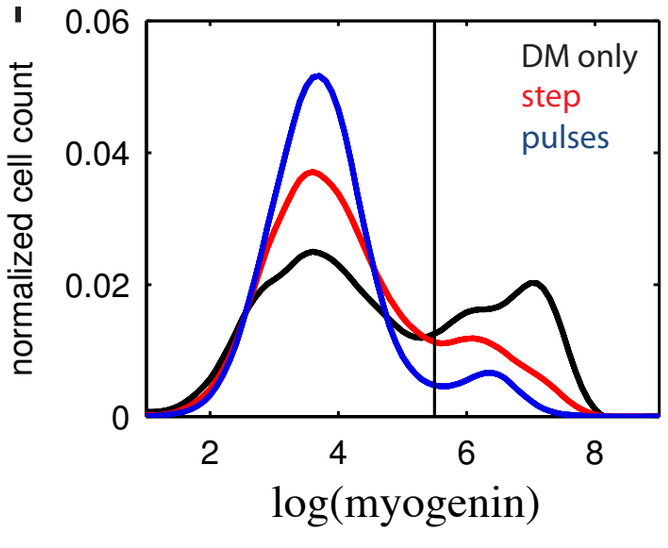
Pulses block differentiation more effectively than continuous

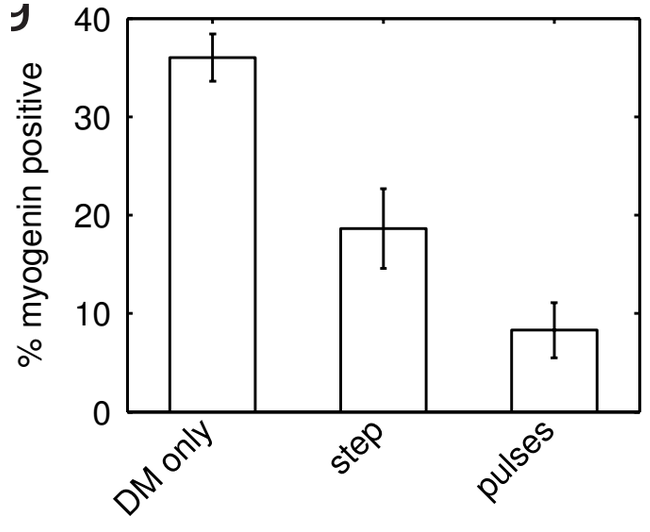




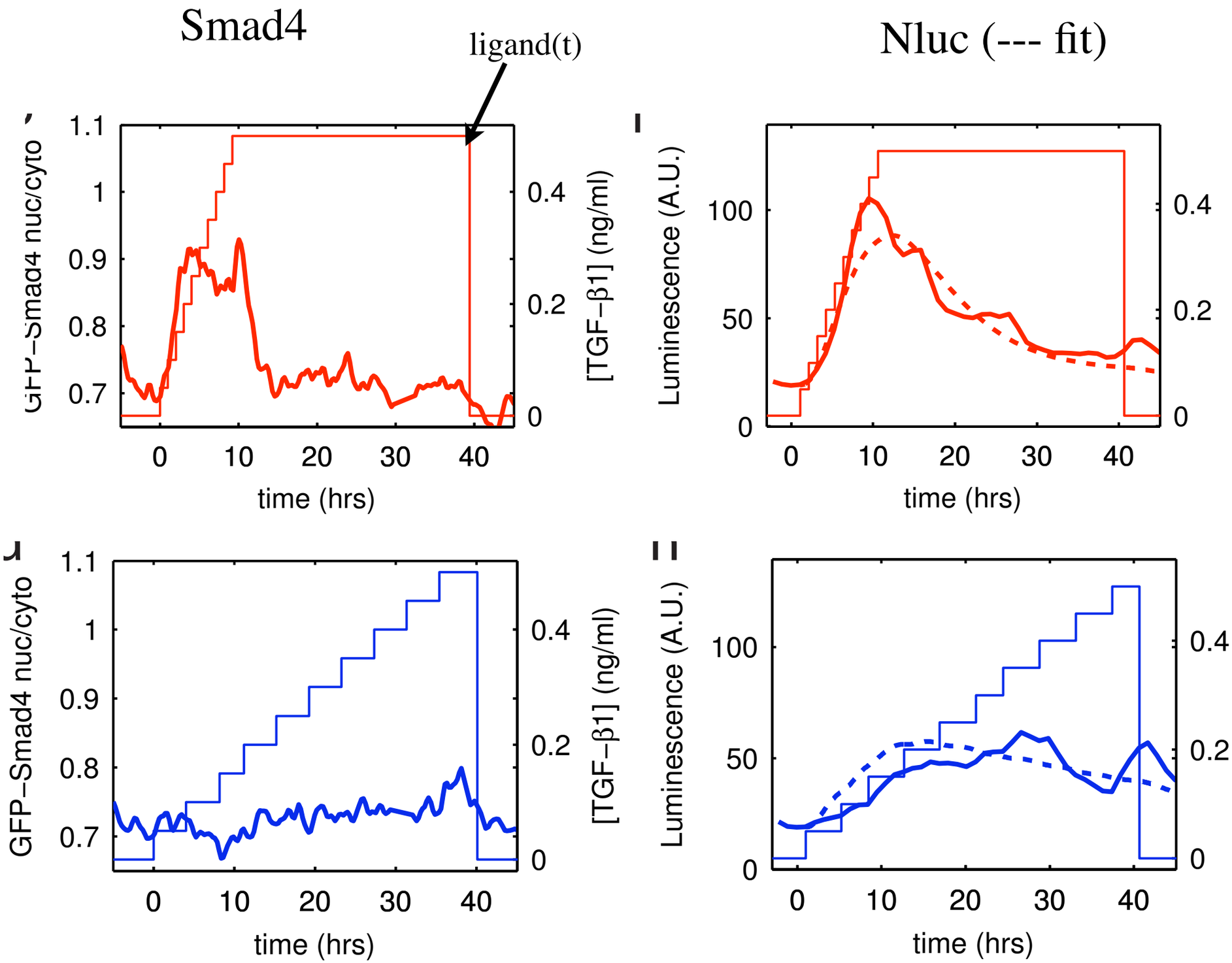


DM step TGFβ pulses





Response to ramps (transcription ~ d signal/dt)



Is any useful modeling to be done?

Have nuclear Smad2, Smad4 fluorescent proteins(time),

Transcription

All for various ligand time courses.

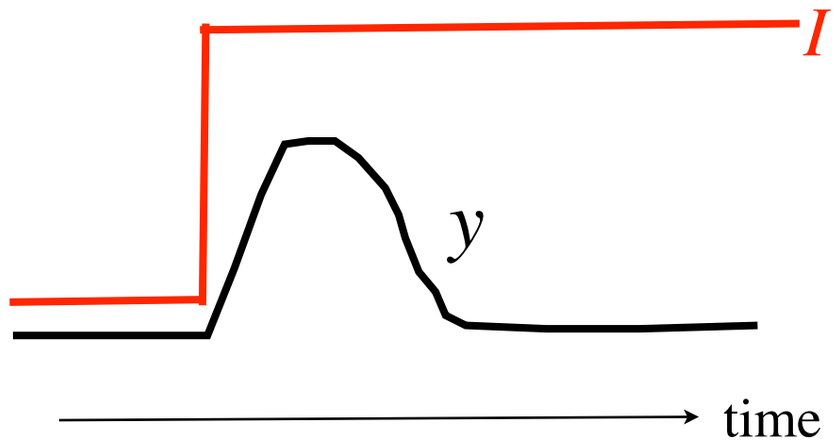
???Many missing steps ???

Phenomenology!

Minimal model of adaption

$$\begin{aligned} \dot{x} &= y \\ \dot{y} &= -bx - cy + I \end{aligned}$$

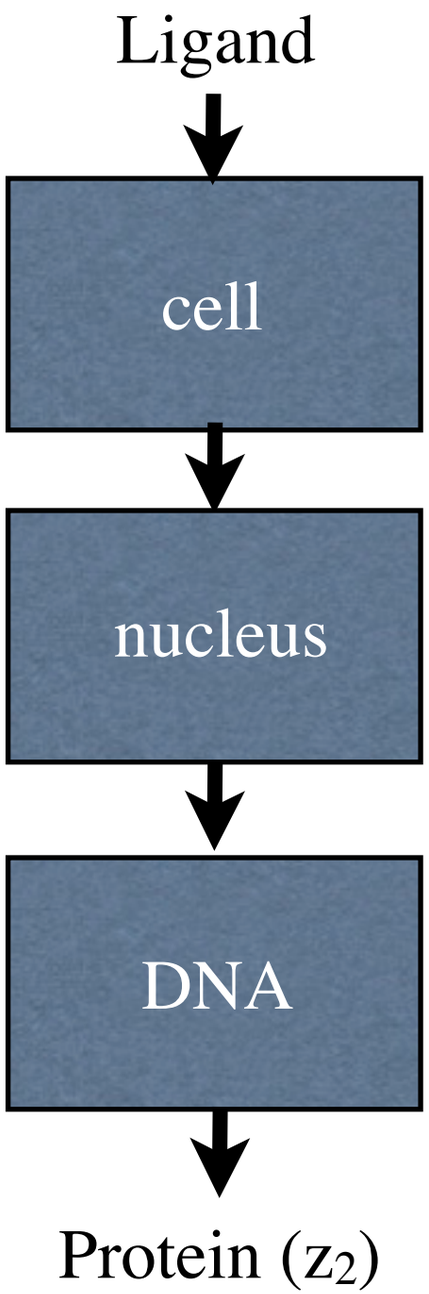
Generic linear adaption system:
y returns to baseline after step in I(nput)



$$\begin{aligned} y &= 0 \qquad t < 0 \\ y &= Ite^{-ct/2} \qquad t > 0 \end{aligned}$$

fastest response if two eigenvalues equal:
$4b = c^2$, one time scale to fit.

Transcription from ligand ramp



$$I(t) = \frac{L(t)}{K_I + L(t)}$$

Some limiting component upstream of adaption ->I(Ligand)

$$\dot{x} = y$$

$$\dot{y} = c^2 x/4 - cy + I(t)$$

Adaption module between receptor and DNA

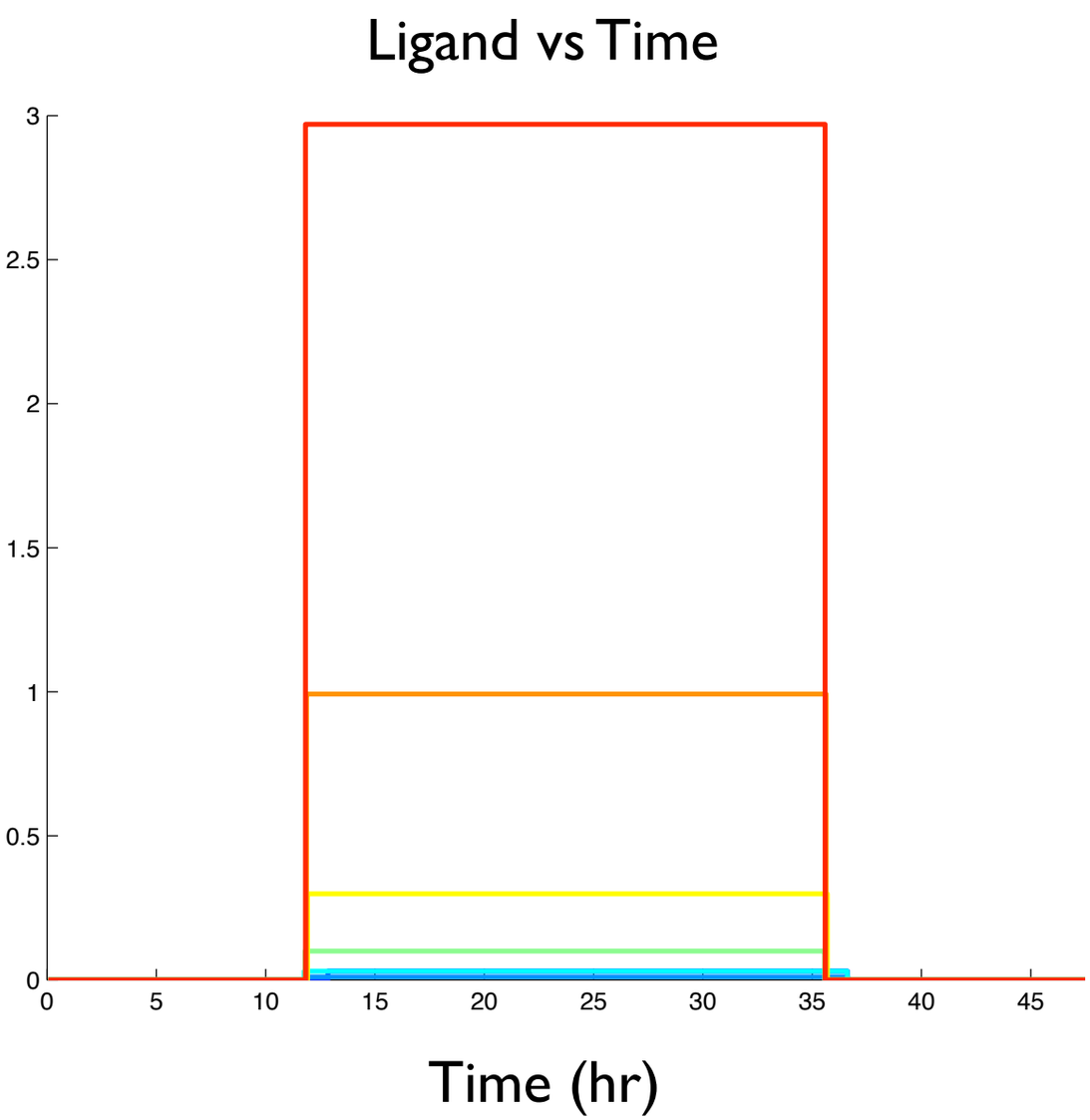
$$\dot{z}_1 = max(0, y) - z_1/\tau$$

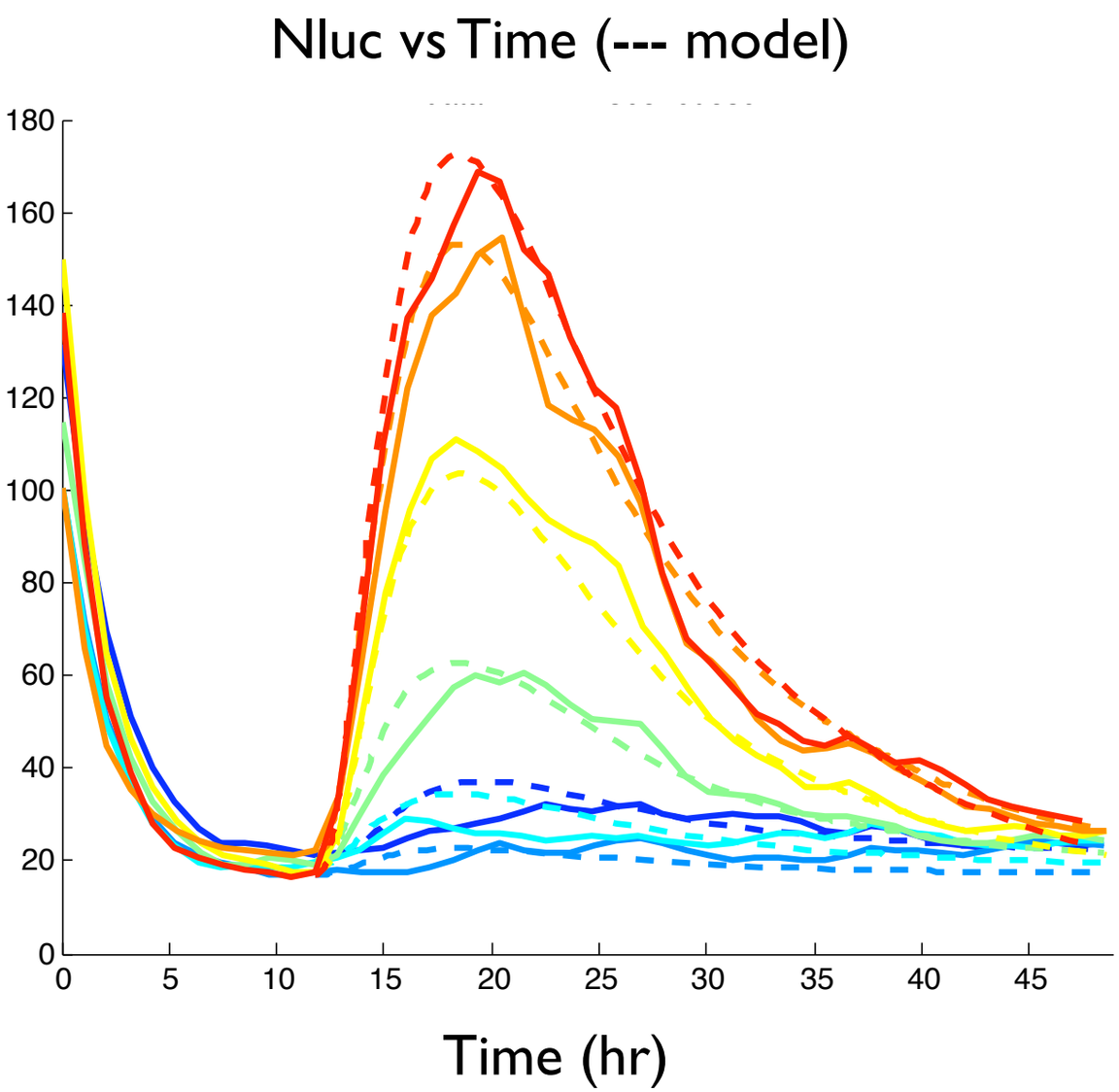
$$\dot{z}_2 = z_1 - z_2/\tau$$

y registers ~ d I/dt, keep positive
one τ since $z_{1,2}$ in series.
need 2 z's for shape

3 parameters + scale !!

Model fits variable height steps vs time

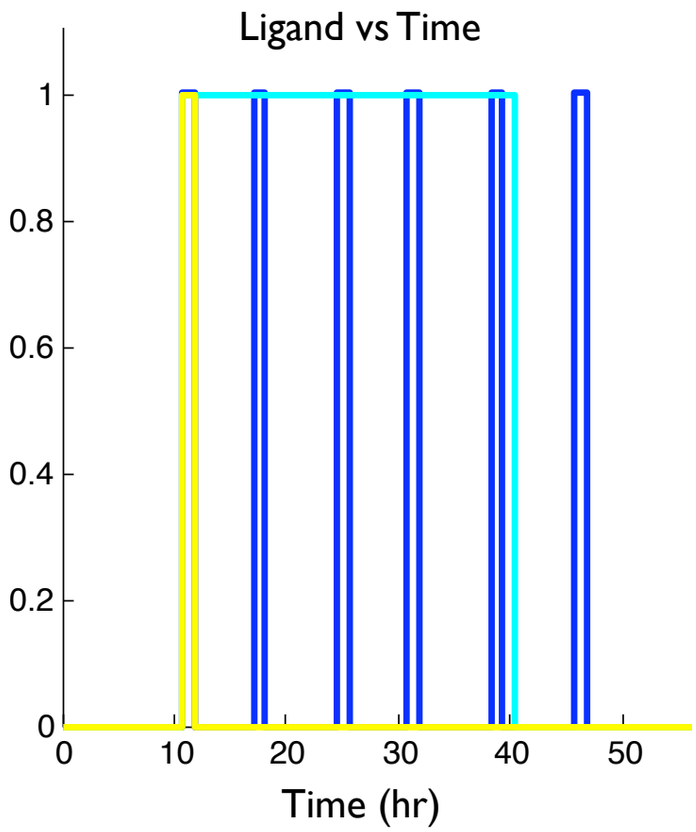


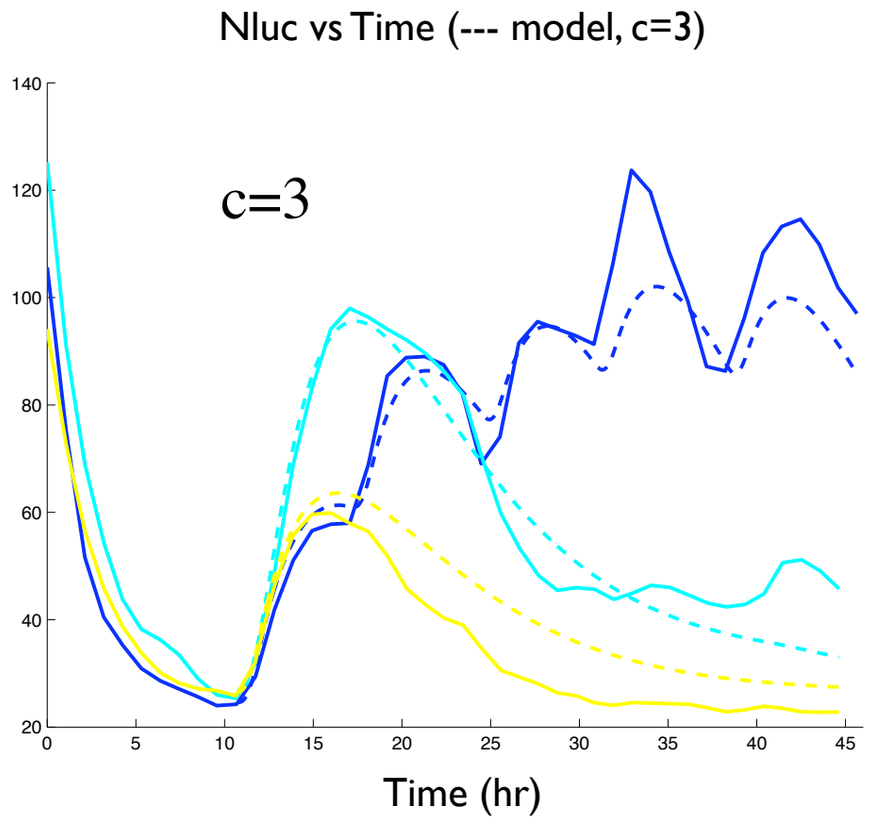


Determines $\mathbf{K_I}$ (ligand-receptor MM) and

$\boldsymbol{\tau}$ transcription-transl time

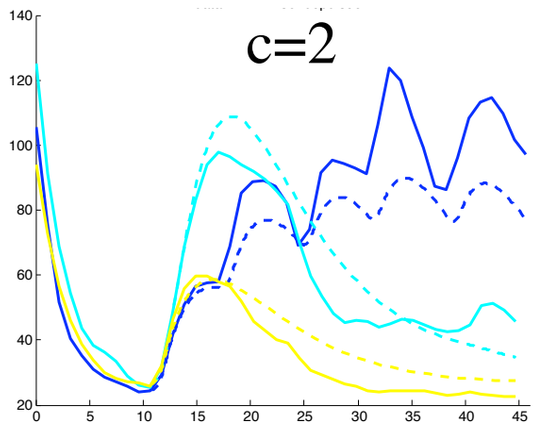
1 Pulse vs N pulses vs step fits **c, τ**

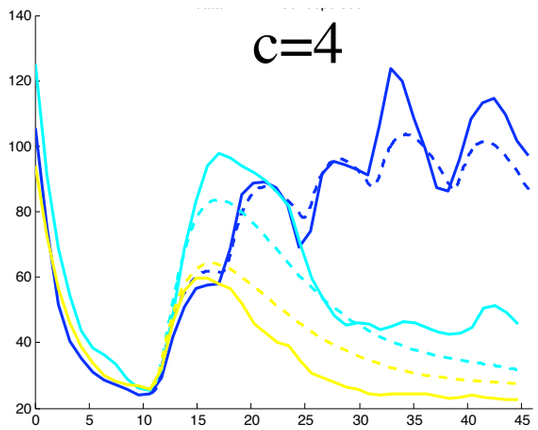




y ~ t exp(-1.5*t) ~ Smad1

microfluidics + slow adder -->
fast internal time scales



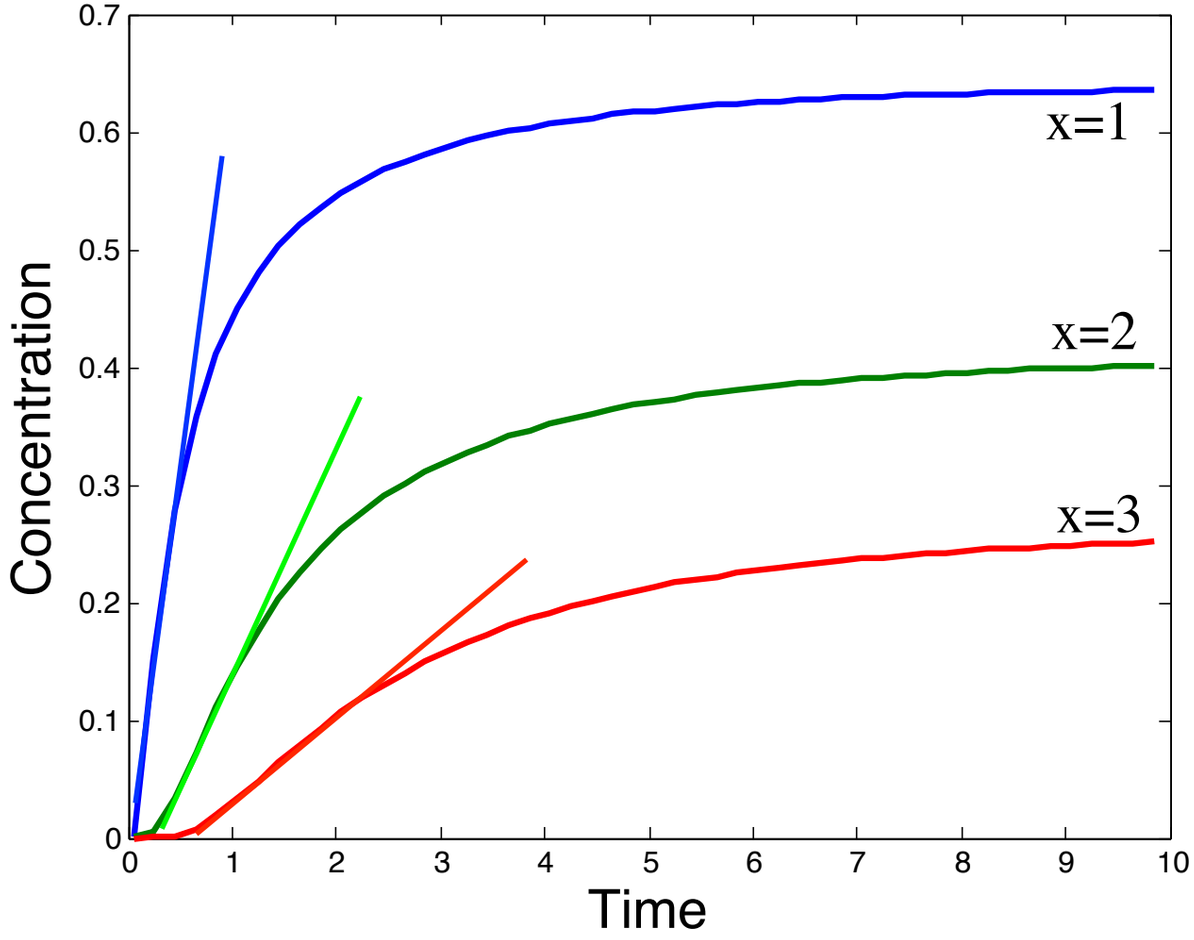


Model of spreading ligand

Ligand spreads to x>0 from a fixed source==1 at x=0

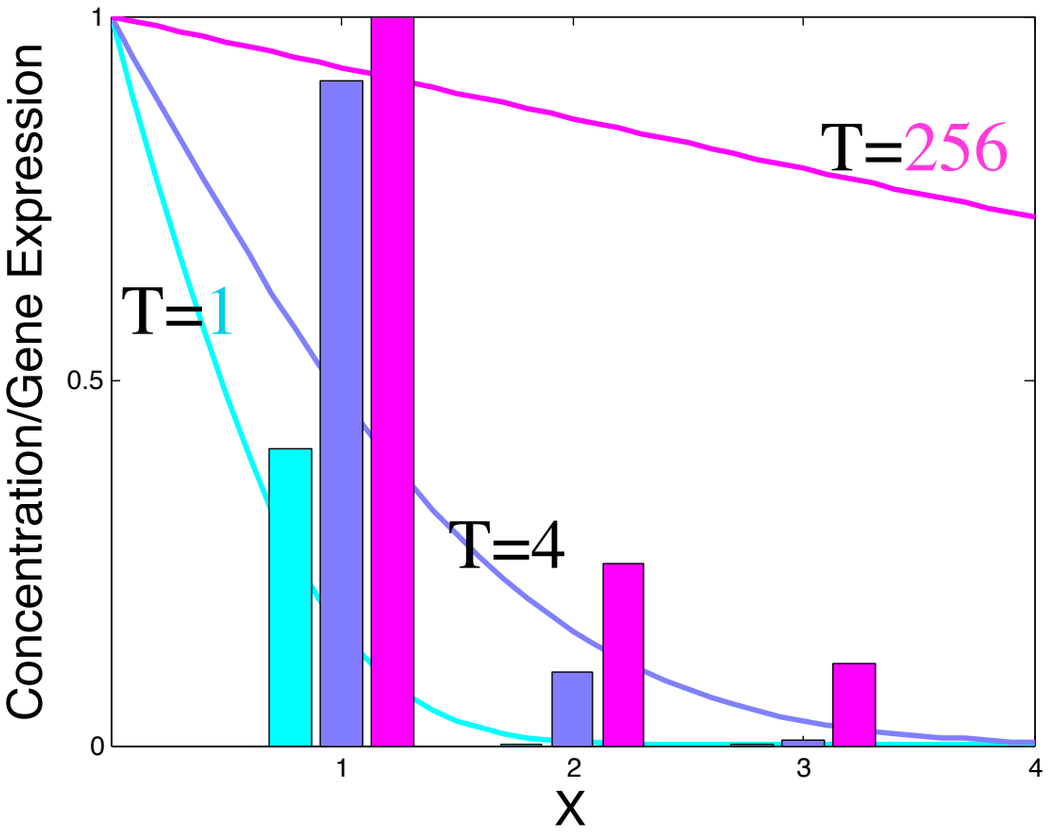
Adaptive pathway will register initial slope and not final value.
Infer ‘x’ from slope

$$\partial_t c(x,t) = D\partial_x^2 c(x,t) - kc(x,t)$$

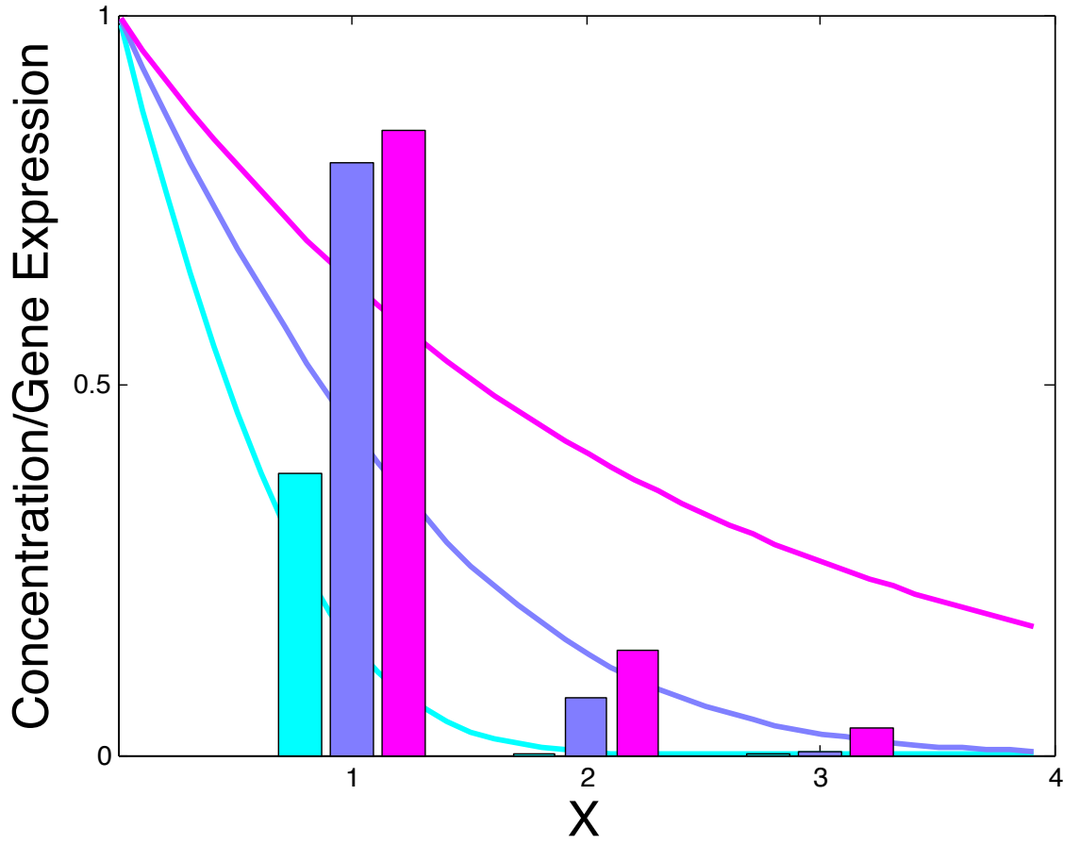


$$c(x,\infty) \sim e^{-\sqrt{D/k}x}$$

Ligand & Gene Expression(bars) vs X (T= 1, 4, 256)



k=0, conserved ligand



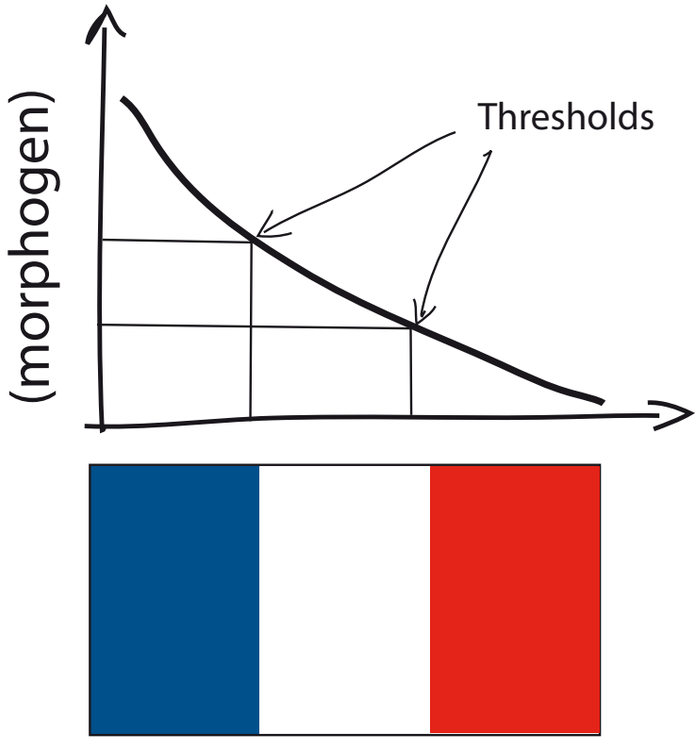
k>0, decaying ligand

Model for adaptive signaling:

$$\text{transcription} \sim \int_0^\infty (\partial_t c(x,t))^2 dt \sim x^{-2}$$

$$\sim \int_0^\infty (\partial_t c(x,t))^1 dt \quad \text{(static morphogen)}$$

Embryo (*Xenopus*)??



evidence for TGFβ as static morphogen:
in-vitro: ligand step/pulse

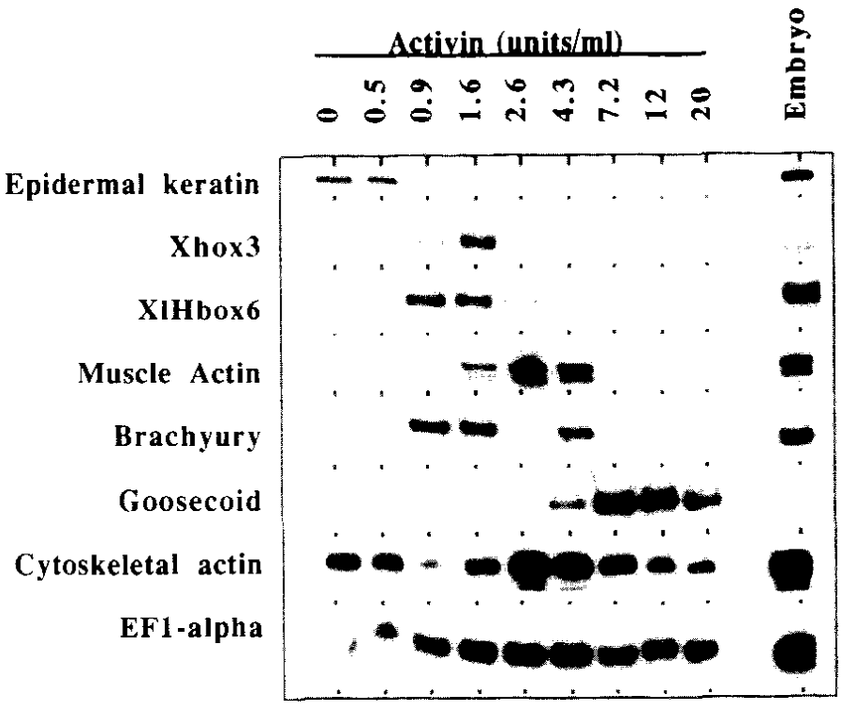
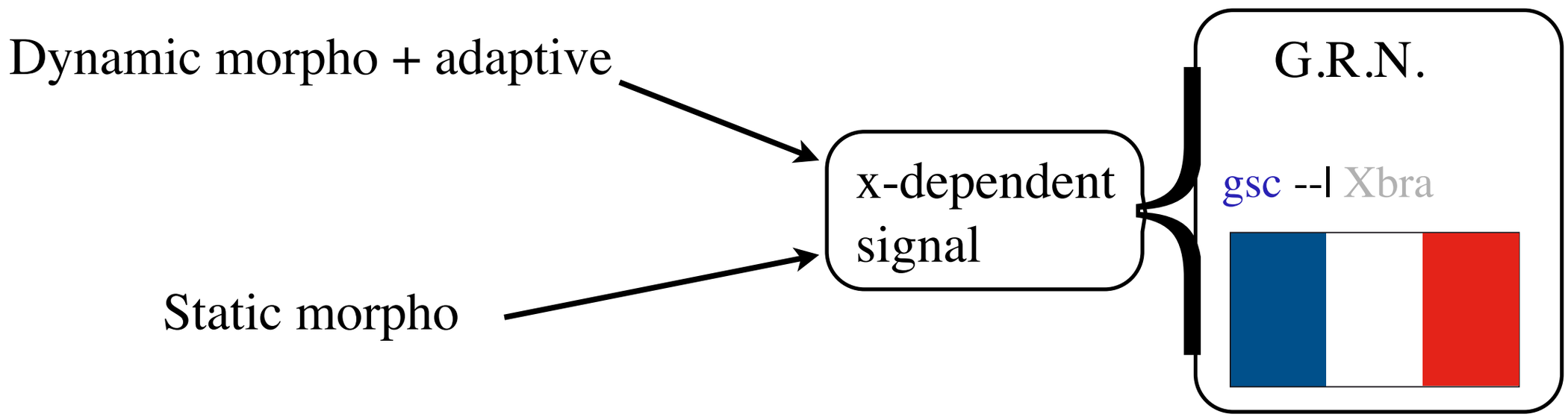


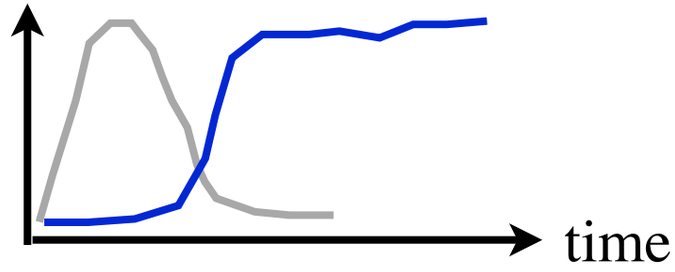
Figure 1. Induction by Activin of an Organizer-to-Posterior Spectrum of Genes Showing Multiple Dose Thresholds

Green-Smith 1990,2: dispersed animal cap cells RNA probe ~ much later: discrete fates

in-vivo: Whitman, Fagotto (2000-2)
α pSmad2, Dorsal to ventral gradient + wave

Embryos ?? II





for x~organizer, first Xbra then gsc

The transcriptional gene regulatory network takes x-dependent activation and converts to mutually exclusive gene expression territories. Evolution can tune x-dependent signal and leave GRN alone.

Conclusions

Transcriptional response to step in TGFβ ligand adapts:

- Response graded (development) vs NFκB binary (immuno)?

- Smad4 reporter for transcriptional adaptation
  (R-Smads reports receptor activation by ligand ≠ transcription)

- Ligand pulses elicit more response than continuous stimulation in context of differentiation of C2C12 to myotubes.

- Transcription depends on rate of ligand presentation (ramp/step)

Embryos may read positional information from rate of change in response to spreading ligand.

- Development is quicker if cells acquire fates while signals are changing

Embryos

"To anyone with his normal quota of curiosity, developing embryos are perhaps the most intriguing objects that nature has to offer. If you look at one quite simply .... and without preconceptions .... what you see is a simple lump of jelly that .... begins changing in shape and texture, developing new parts, sticking out processes, folding up in some regions and spreading out in others, until it eventually turns into a recognizable small plant or worm or insect...

Nothing else that one can see puts on a performance which is both so apparently simple and spontaneous and yet, when you think about it, so mysterious."

C.H.Waddington 1966 *Principles of Devel. Differentiation*
(Current Concepts in Bio. Series)

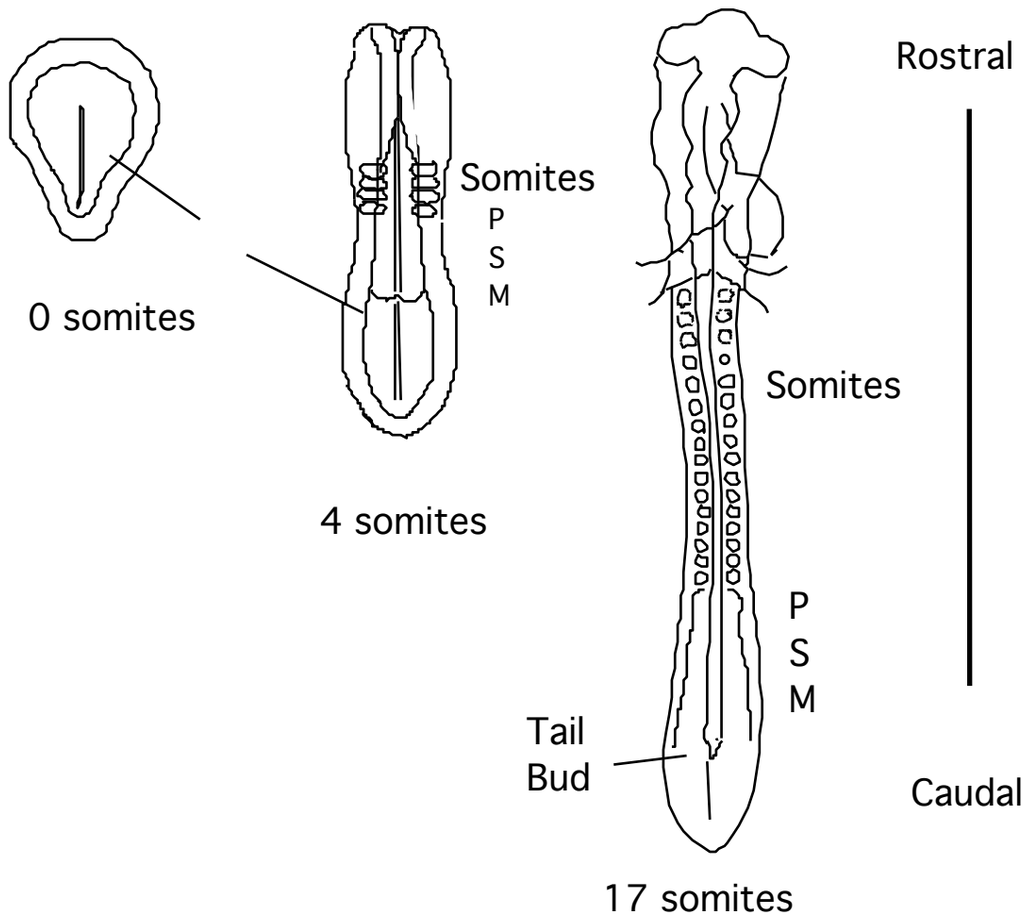
"Perfect Adaptation in the TGF-beta Pathway and its Implications for Embryonic Patterning"

>

> Genetics and biochemistry have defined the components and wiring of the signaling pathways that pattern the embryo. Many of these pathways have the potential to behave as morphogens: in vitro experiments have clearly established that these molecules can dictate cell fate in a concentration dependent manner. How morphogens convey positional information in a developing embryo, where signal levels are changing with time, is less understood. New data shows that the evolutionarily conserved TGF-b pathway responds transiently and adaptively to a step in ligand stimulation. As a consequence it is shown using integrated microfluidic cell culture and time-lapse microscopy that the speed of ligand presentation has a key and unexpected influence on signaling outcomes. Slowly increasing the ligand concentration diminishes the response while well-spaced pulses of ligand combine additively resulting in greater pathway output than is possible with constant stimulation. Our results suggest that in an embryonic context, an adaptive pathway can extract positional information as ligand spreads dynamically from a source, thereby providing an alternative to the static morphogen model. Thus the rate of change of ligand concentration, rather than its level, is the instructive signal for patterning.

>

Phenotype of somitogenesis invariant, genes drift



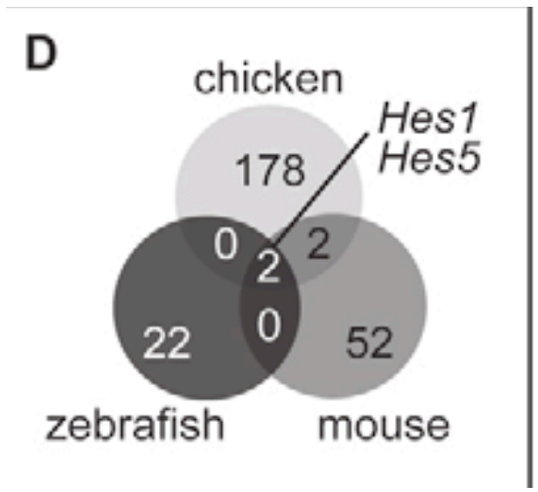
clock + growth velocity ->
spatial period

MODEL PHENOTYPE
(Francois & EDS Curr Opin G&D)

Evolutionary plasticity of segmentation clock networks

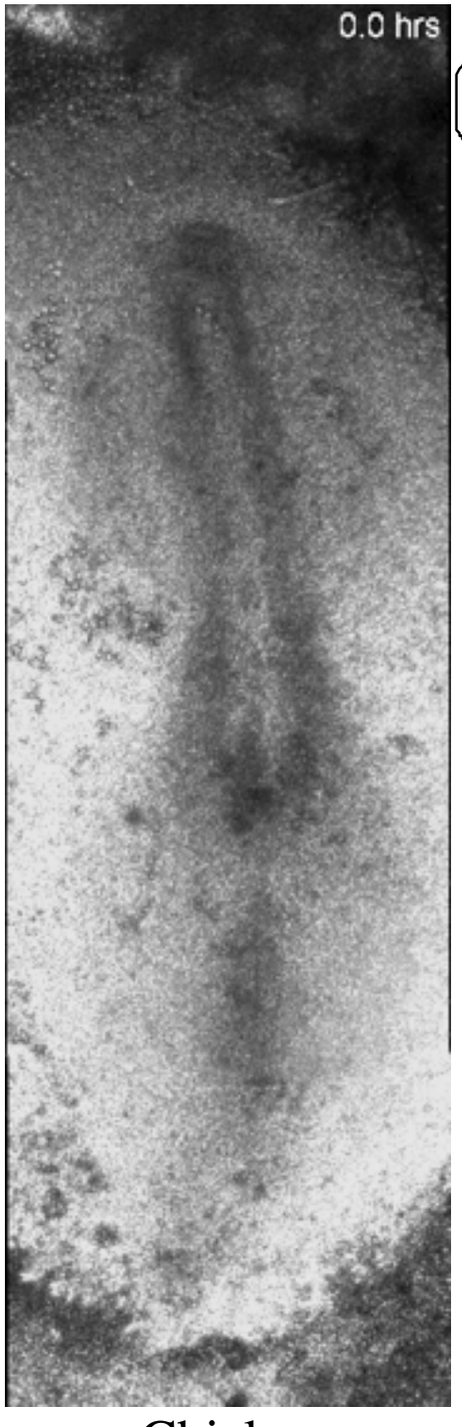
Aurélie J. Krol[1,2,*], Daniela Roellig[3,*], Mary-Lee Dequéant[1,*], Olivier Tassy[1,2,4,*], Earl Glynn[1], Gaye Hattem[1], Arcady Mushegian[1], Andrew C. Oates[3] and Olivier Pourquié[1,2,4,†]

Overlap of oscillating genes among three vertebrates is nil.
Among Wnt, FGF, N, RA pathways many ways to make oscillators



Chick
O. Pourquie lab

Phenotype of somitogenesis invariant, genes drift



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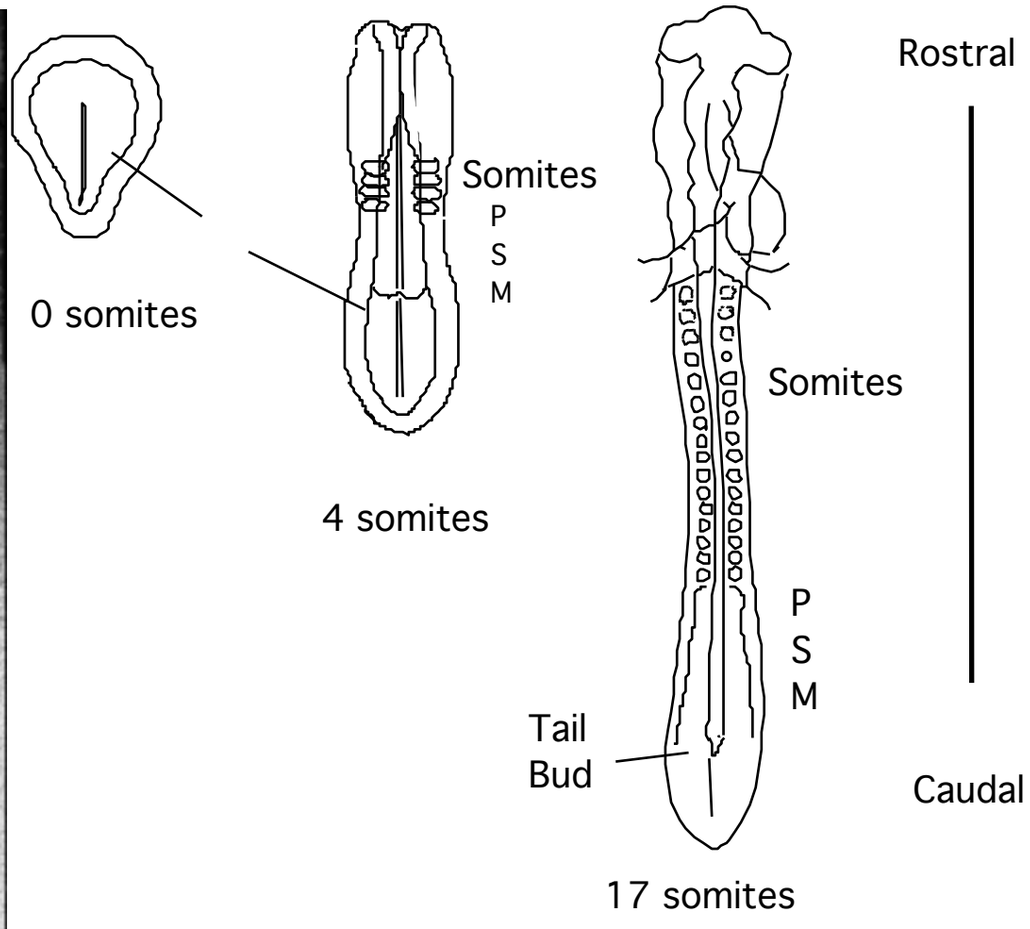

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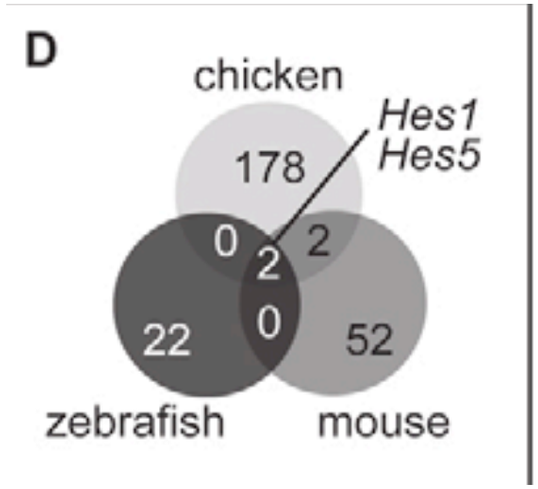
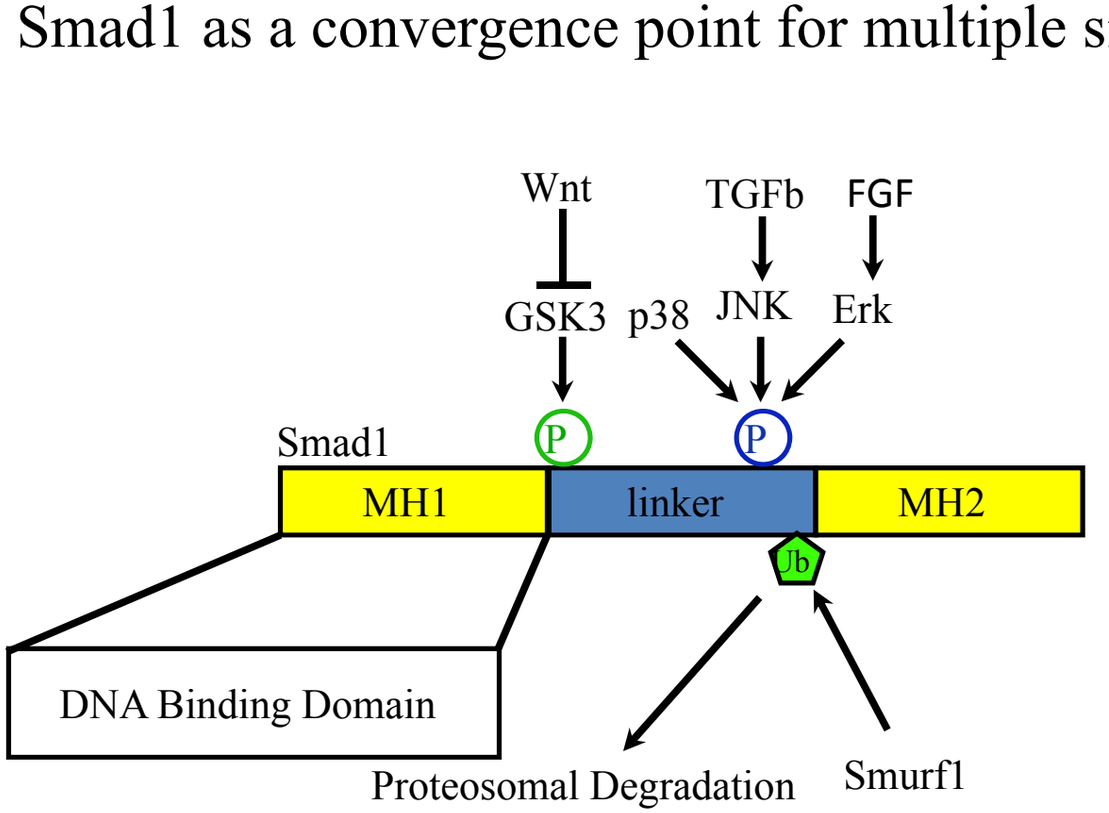


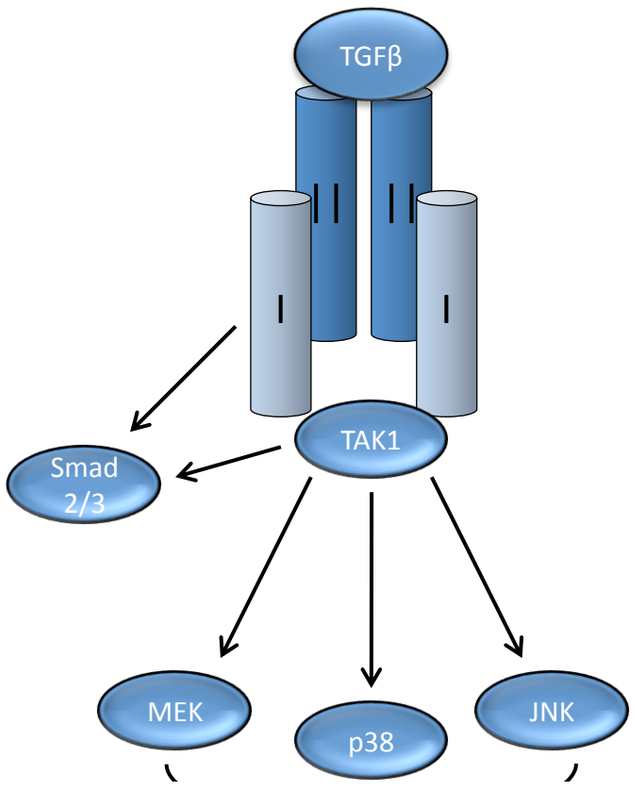
Biology is not simple

+ 33 TGFβ ligands in mammals.

non-canonical TGFβ--> MAPK

Smad1 as a convergence point for multiple signaling pathwa

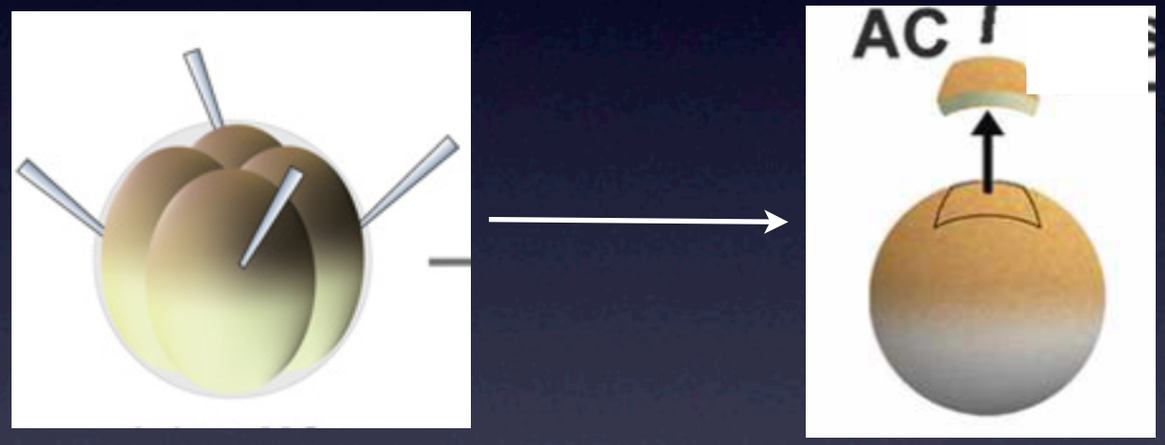




Experimental Strategy

At 2-4 cell state: inject animal caps with mRNA encoding fluorescent proteins

At late blastula stage: dissect the cap at late blastula stage and image



mRNAs encoding fluorescent proteins: GFP-XSmad1, GFP-XSmad2, Venus-XSmad2, mCherry-XH2B

Misc conclusions

Mention R. Young paper, assumes Smad2 nuc == expression,
Schier diffusion is enough for activity, community effect etc.

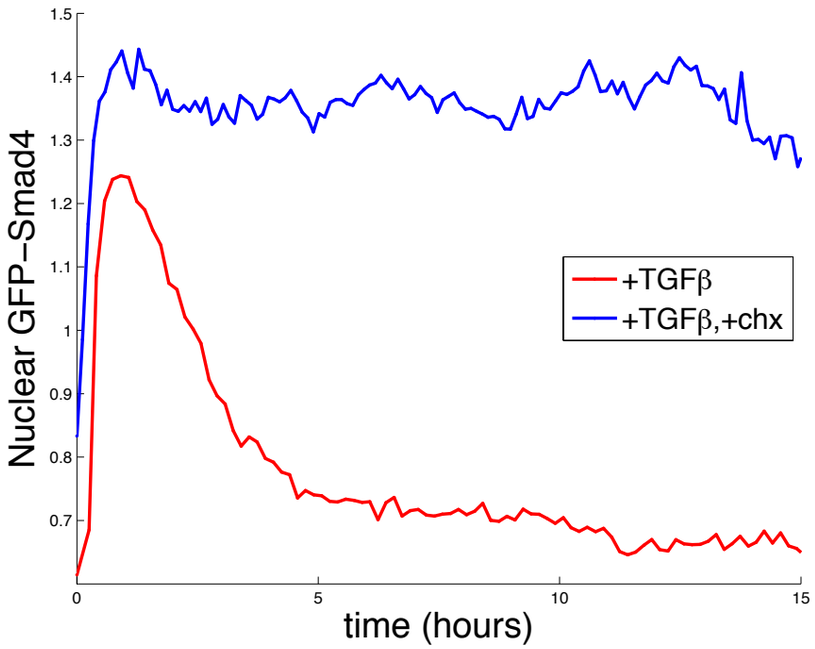
Get new AW movies, more details on frog, note separated cells no smad4 flashes and no fates, but mixed up with no smad1

Questions:

activin on isolated frog cells, oscillates? yes pulse
CHX on animal caps? ND
arguments against endog ligands are inhibit BMP add activin (uniform)
and against moving lefty or smad7 etc, are uniform smad2 response

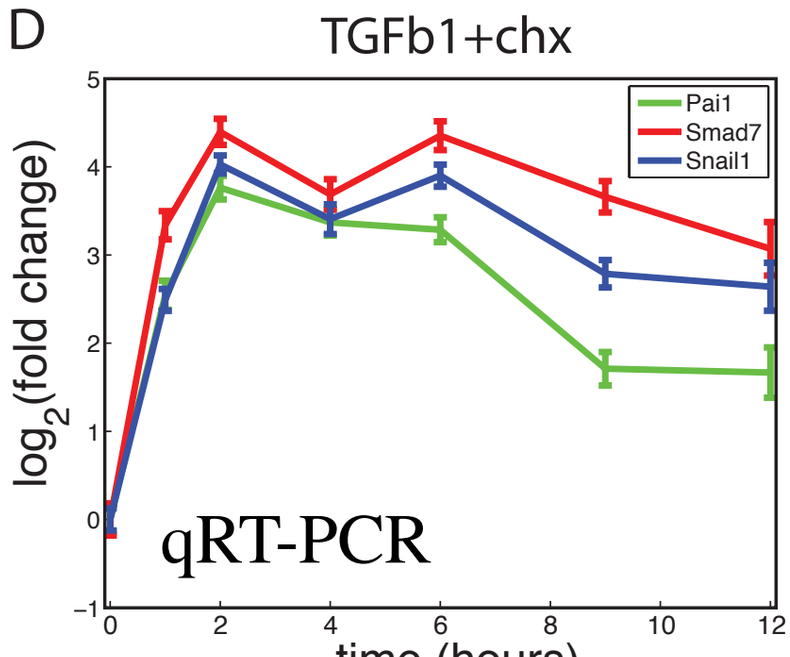
CCC movie with step stimulation and smad 4 or 2 + nuclear

Adaptation requires transcription

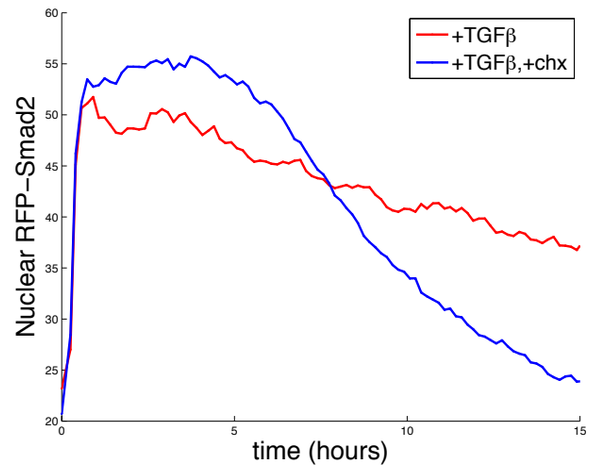


with CycloHex Smad4

D



Transcription lasts
longer, follows Smad4



--> Adaption not due to inhibitory ligands (eg *lefty*)
or inhibitory Smads, since would affect pSmad2