Interpreting phenotypes:
The flea's earing organ and other issues

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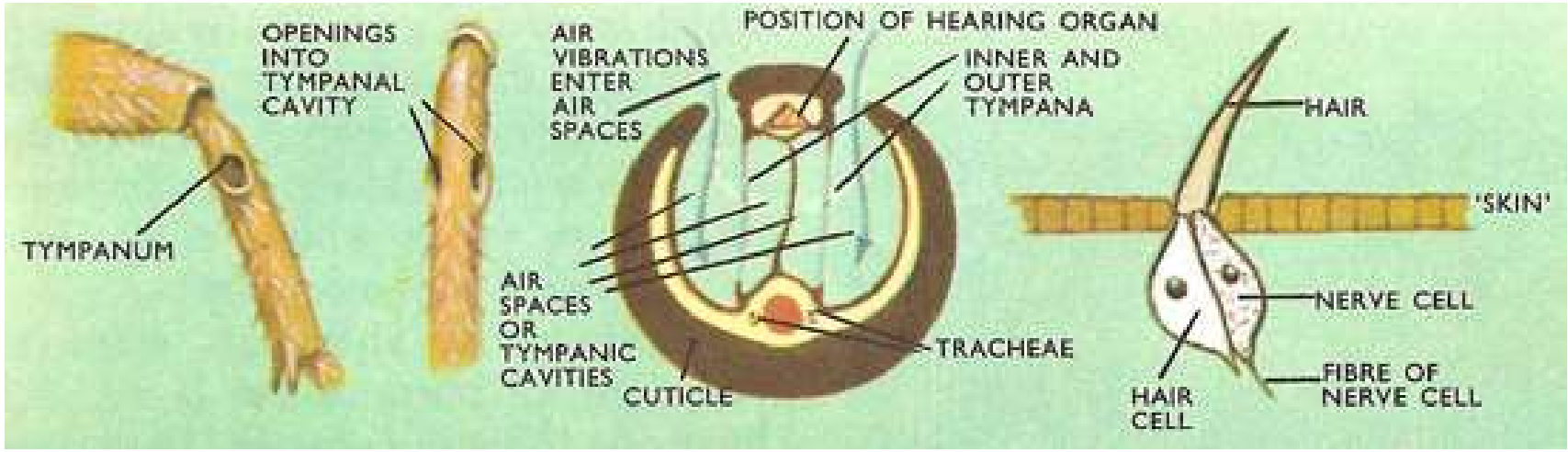
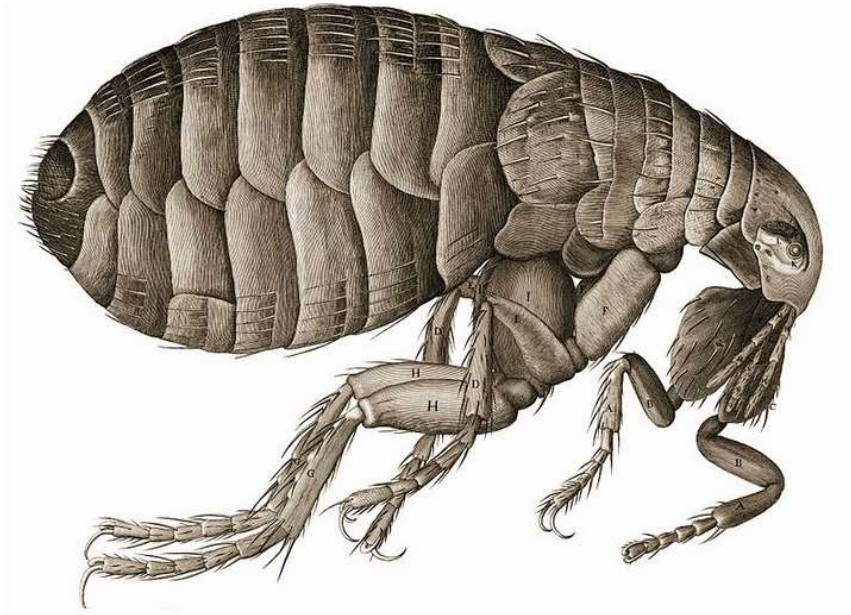
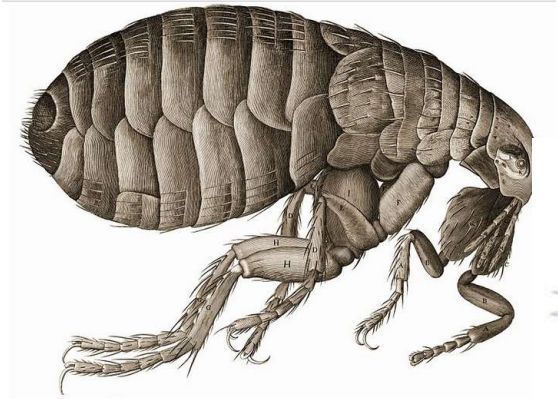


Earing organ in crickets

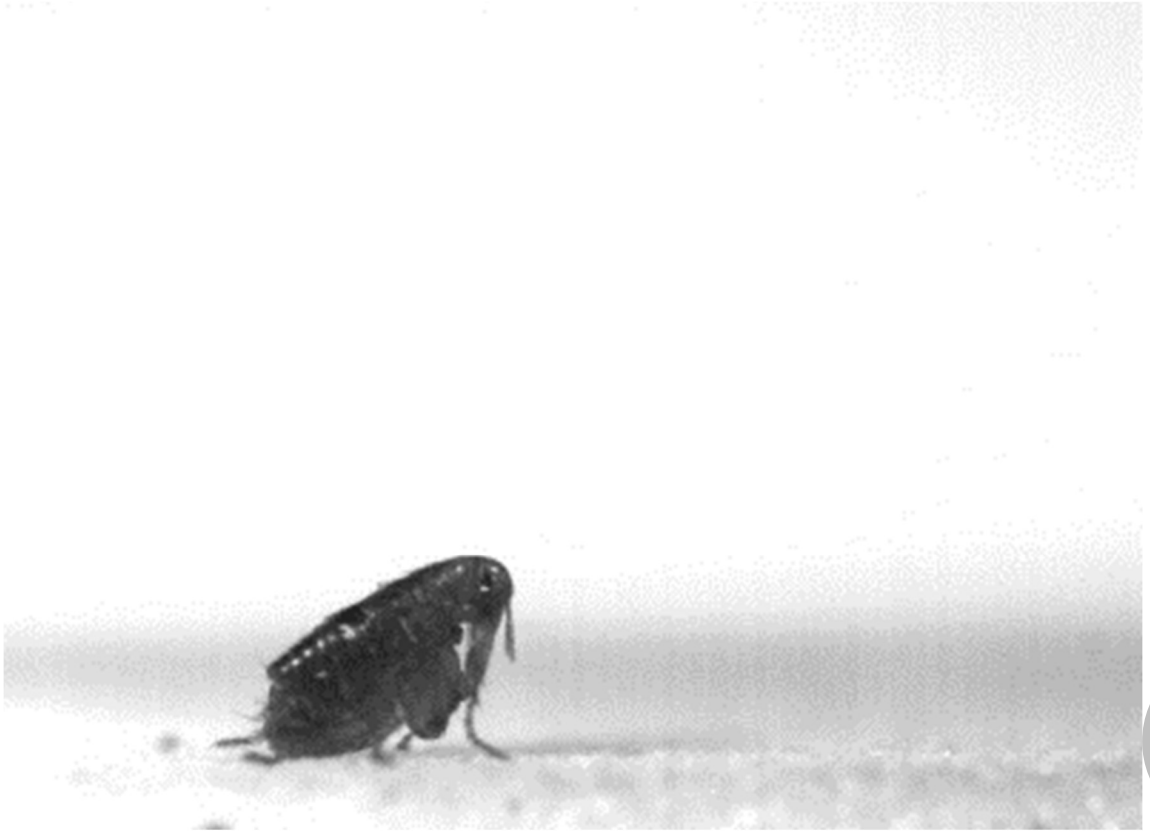


?

Localization of flea’s earing organ

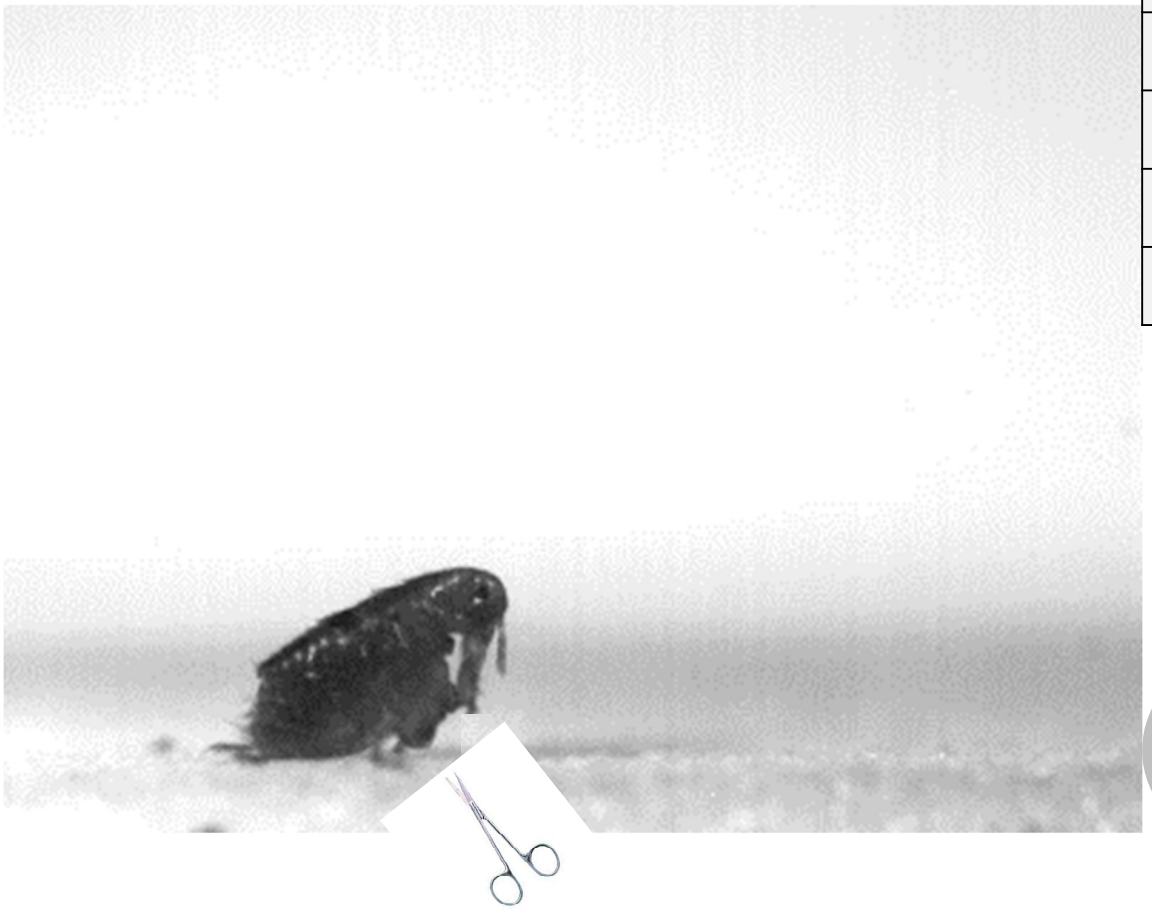


Localization of flea’s earing organ



Localization of flea’s earing organ

Leg pairs	earing
control	√

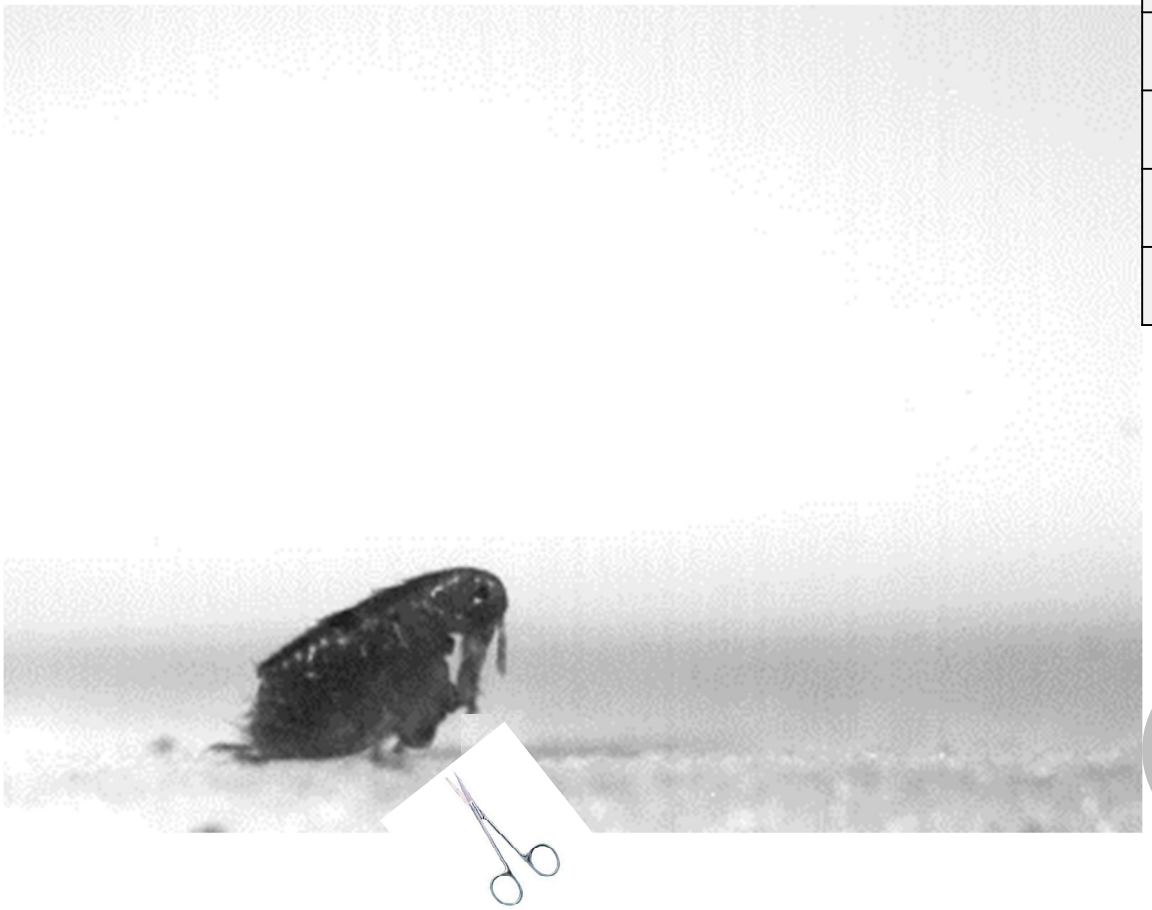


Localization of flea’s earing organ

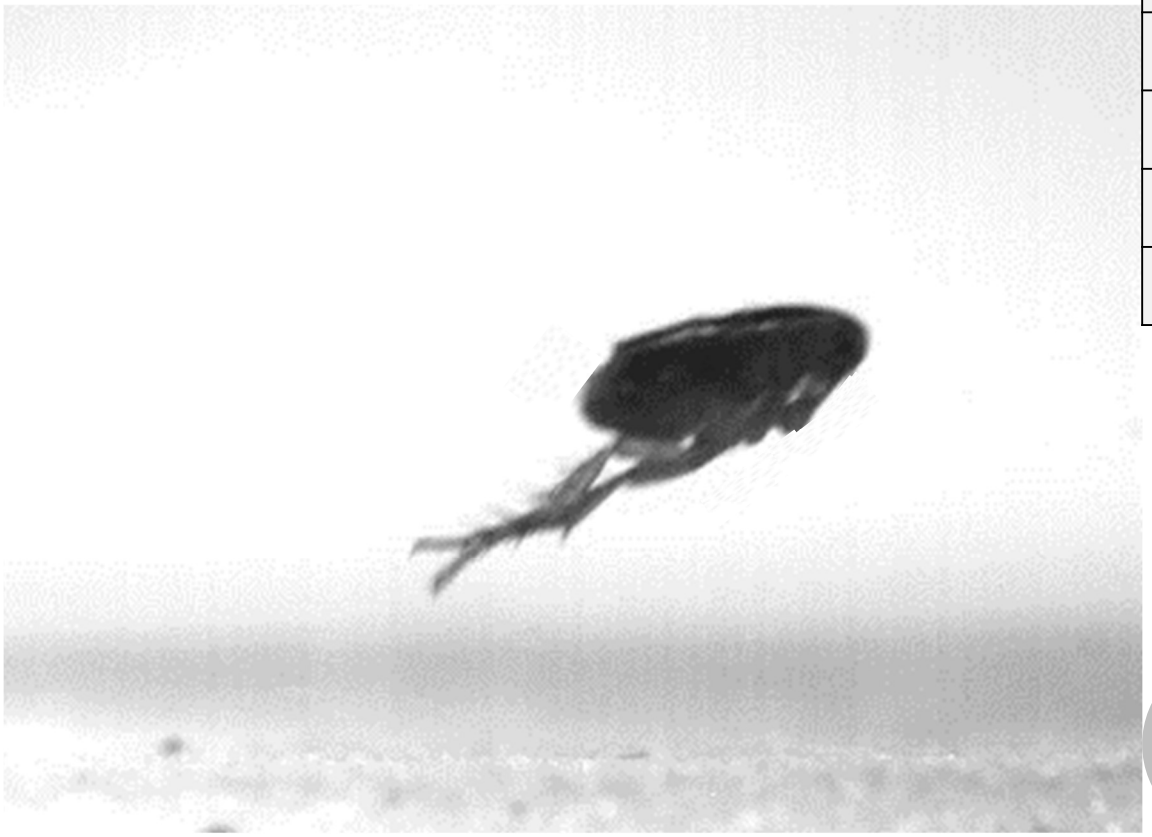
Leg pairs	earing
control	√
-1	√

Localization of flea’s earing organ

Leg pairs	earing
control	√
-1	√
-2	



Localization of flea’s earing organ



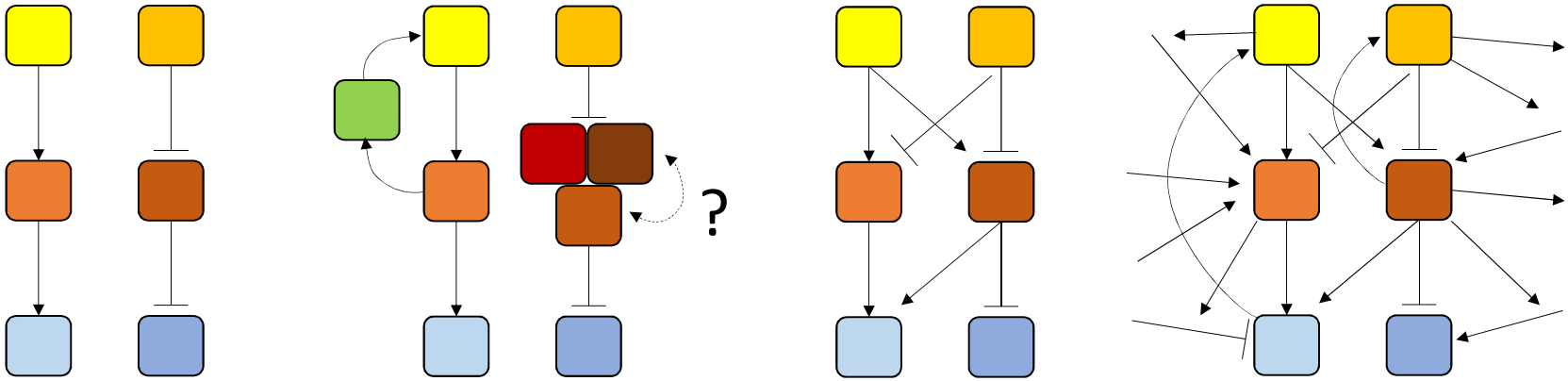
Leg pairs	earing
control	√
-1	√
-2	√

Localization of flea's earing organ

Leg pairs	earing
control	√
-1	√
-2	√
-3	X

Epistasis and increased complexification of genetic/molecular systems

garden of Eden
of geneticists



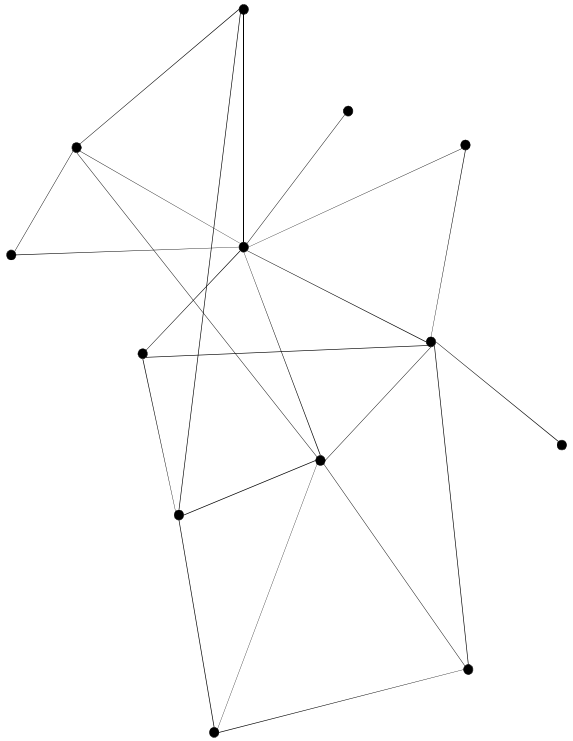
“Systems biology” -> ‘interaction networks’

Who is doing what?

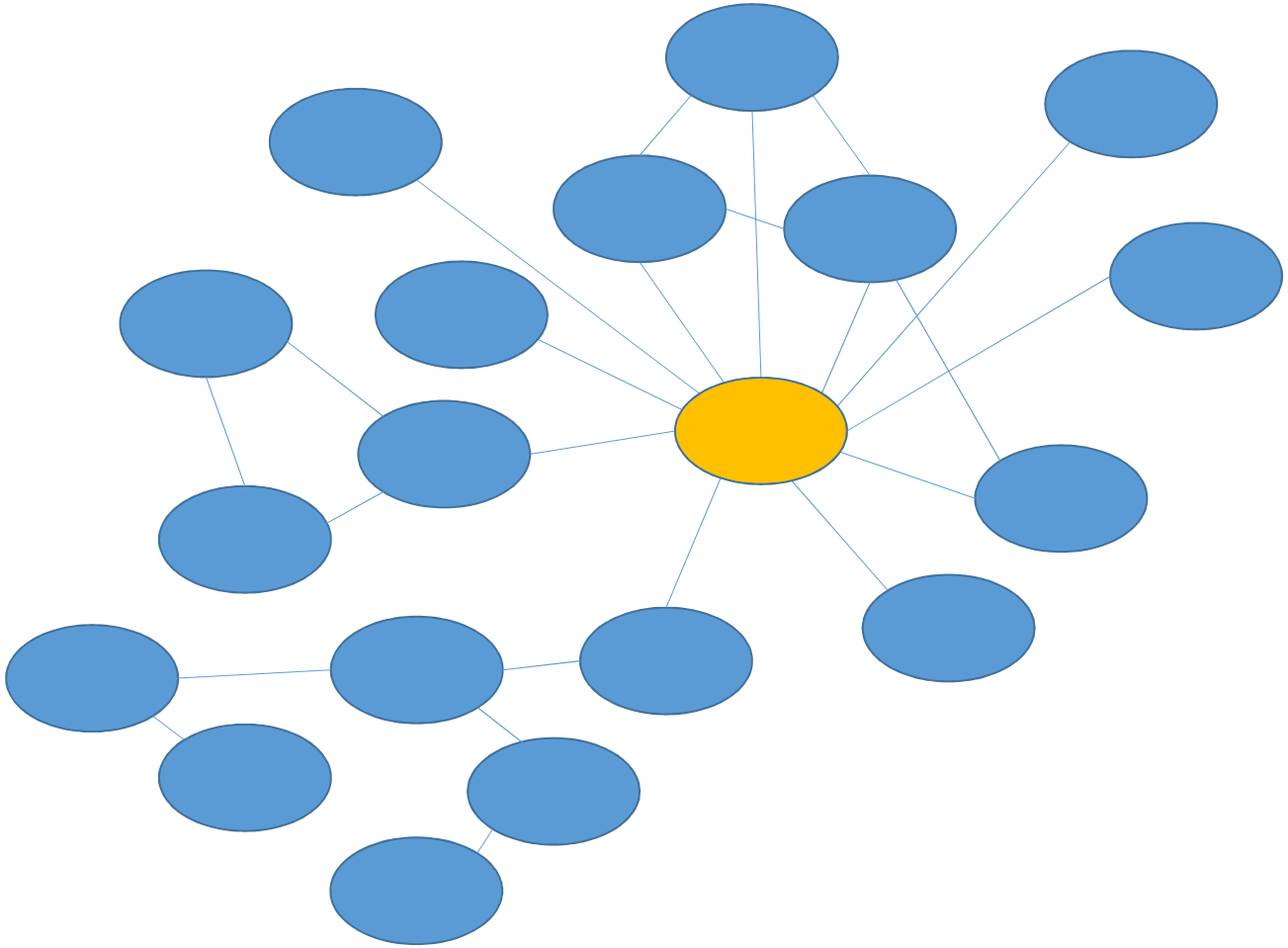
Who is ‘important’?

“Hubs”?

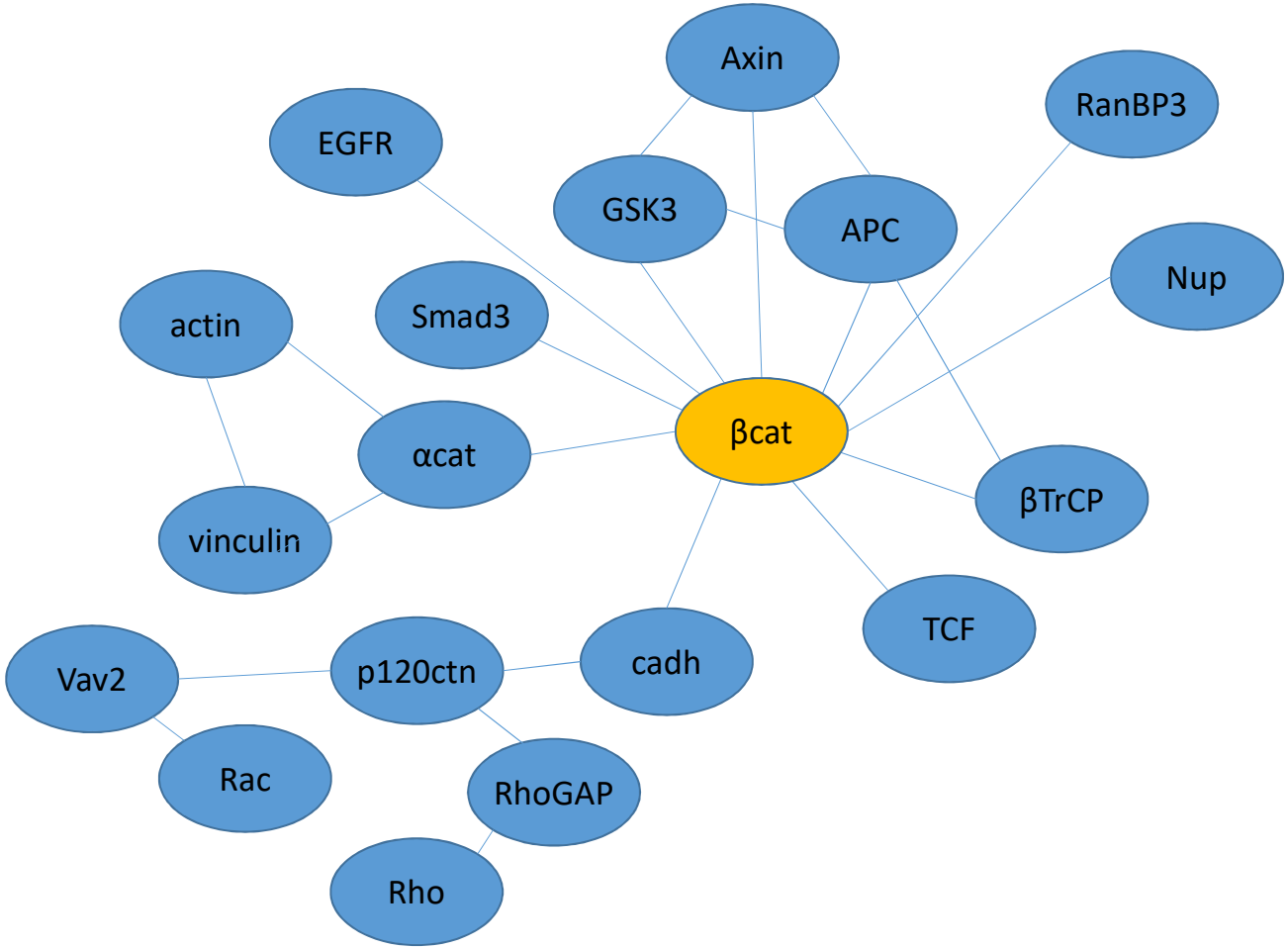
“Master regulators”?



“Systems biology” -> ‘interaction networks’



“Systems biology” -> ‘interaction networks’



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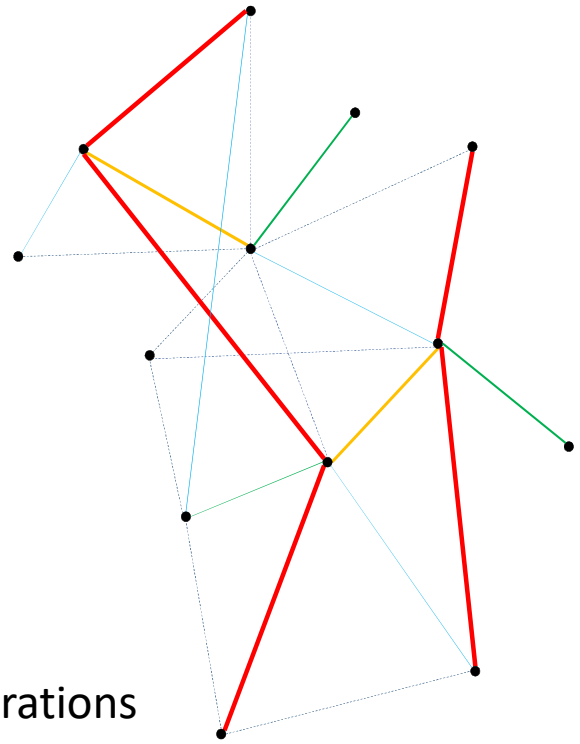


Absolute and relative concentrations

Affinities, dynamics (K_{off})

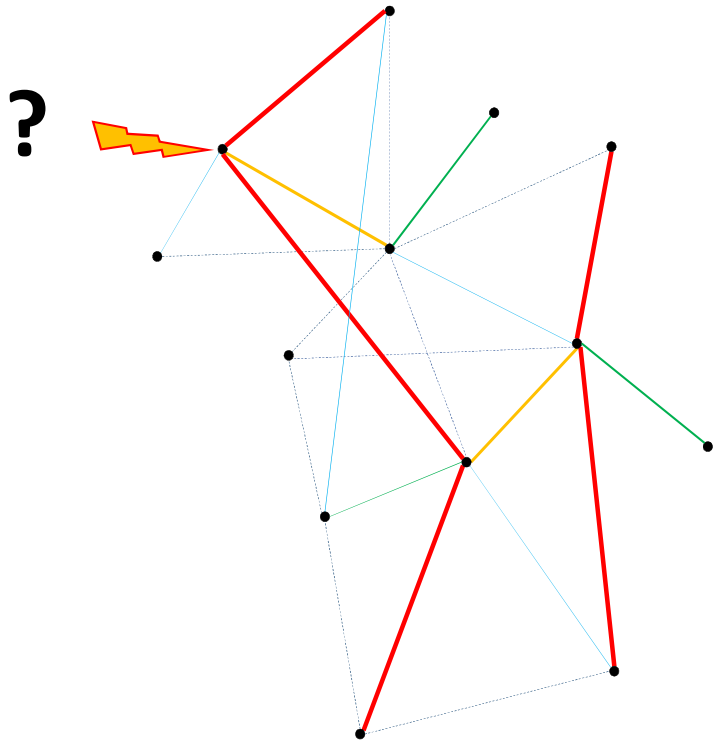
Regulations/posttranscriptional modifications

Competition (mutually exclusive binding)/synergy (cooperative binding, stabilization)

Localization

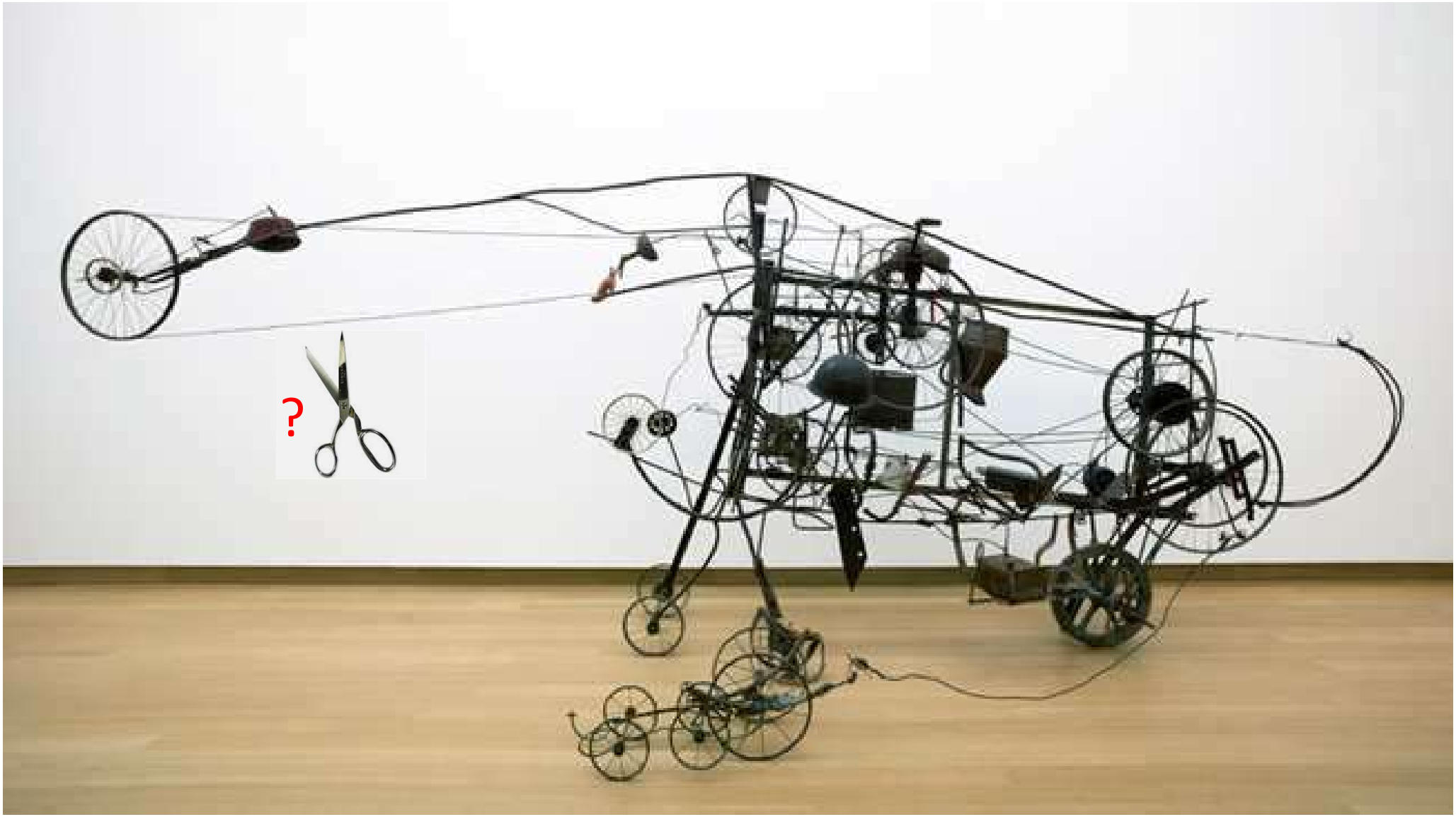
Time-dependent changes (fluctuations, regulations, cell cycle,…) and their magnitude

We ‘still’ need to manipulate single gene/molecule functions!!



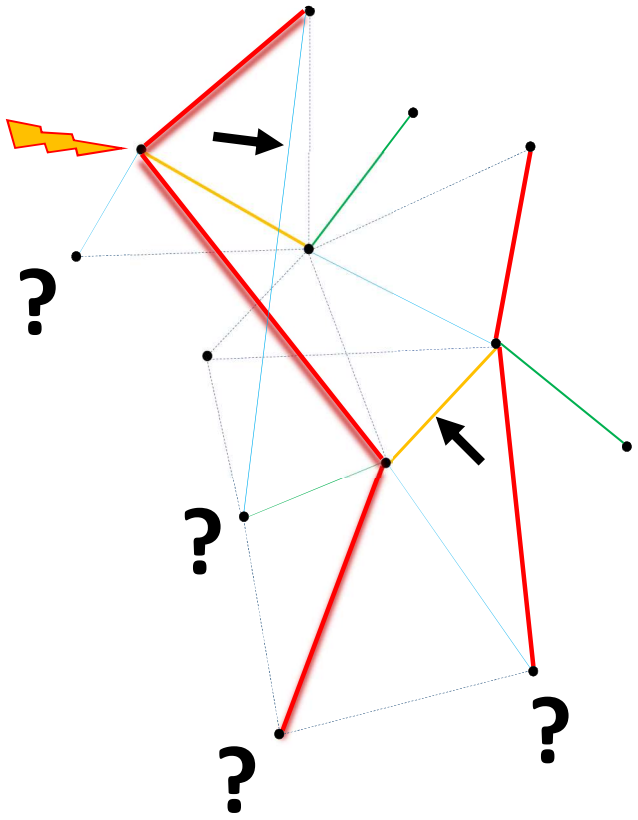
… but interpretation is getting complicated!!

What will be the effect of disrupting this thread?



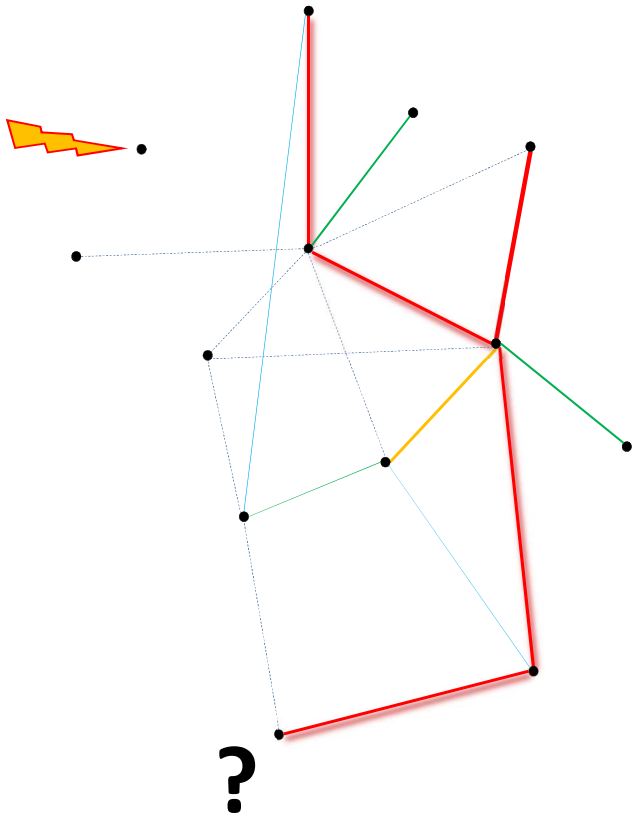
Jean Tinguely, Gismo, 1960

Complications in interpretation of phenotypes



1) Multiple/pleiotropic functions

Complications in interpretation of phenotypes



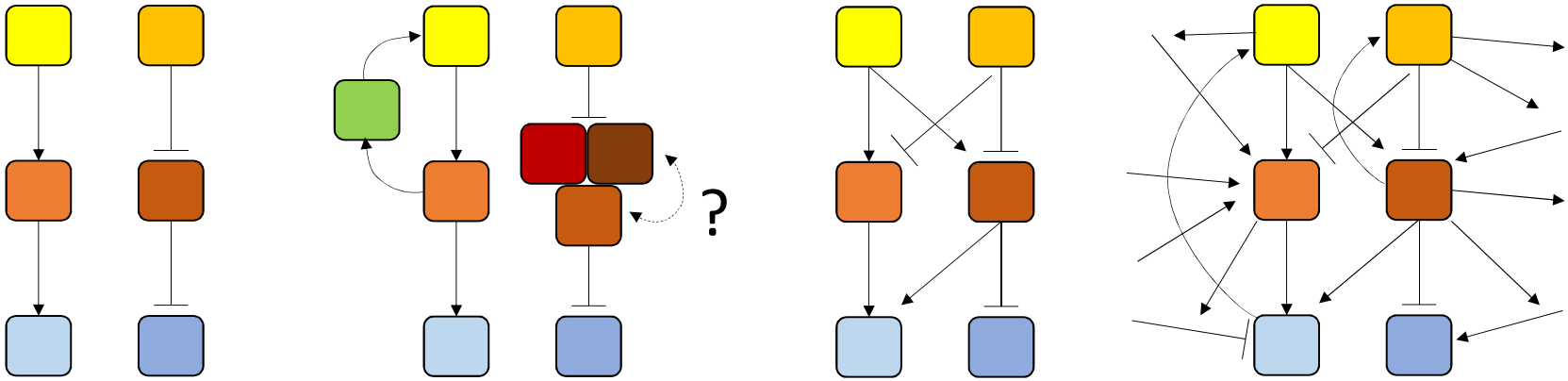
1) Multiple/pleiotropic functions

2) Redundancy (partial/complete)

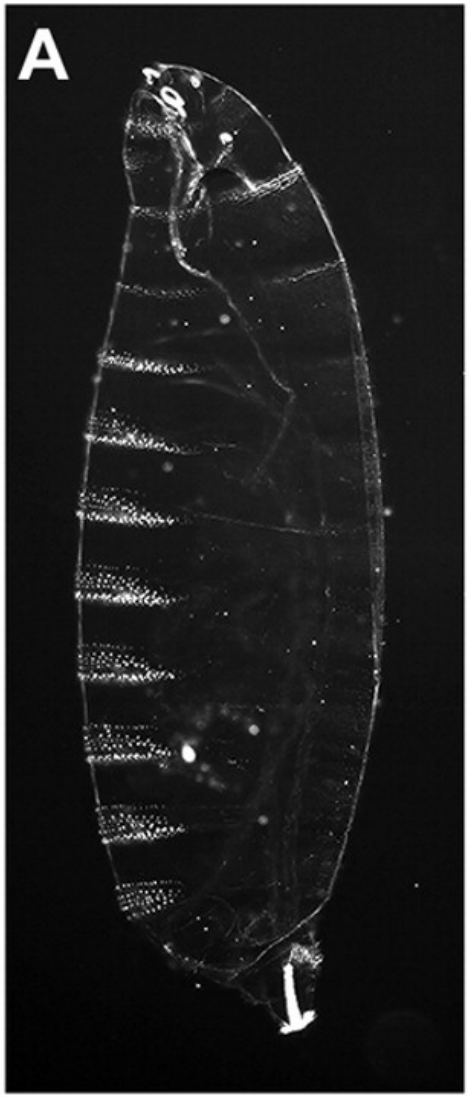
3) Compensation/Adaptation

Epistasis and increased complexification of genetic/molecular systems

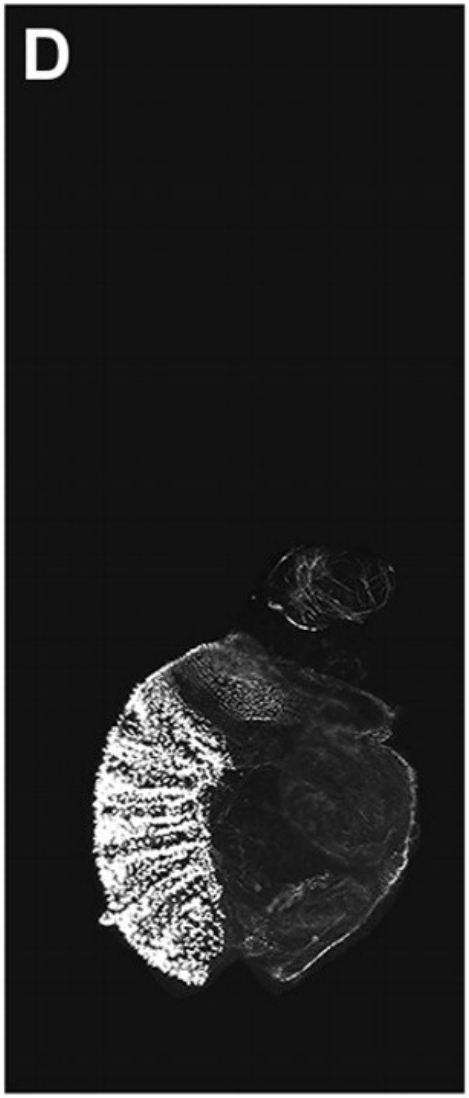
garden of Eden
of geneticists



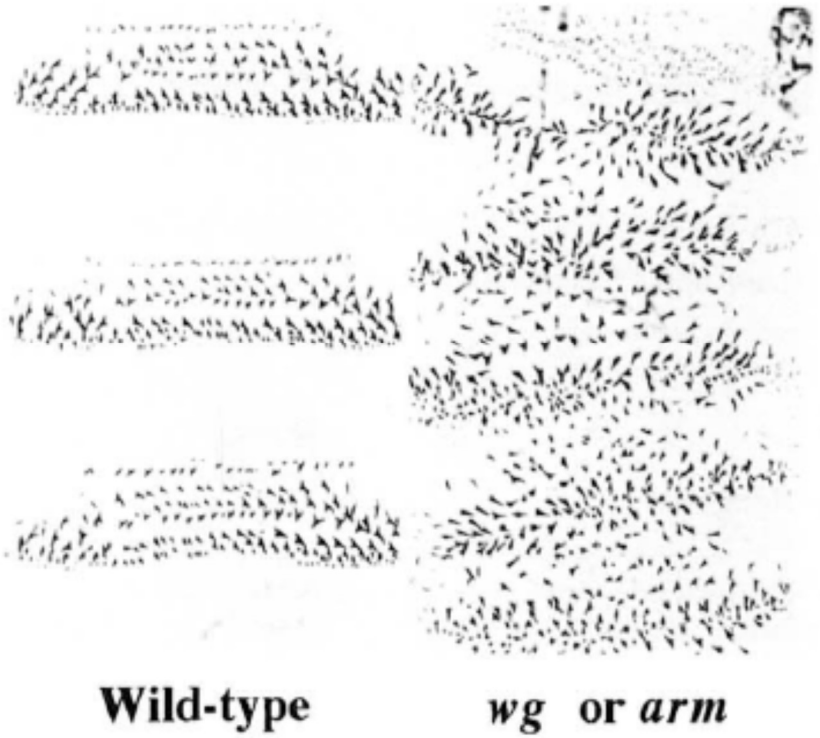
Discovery of the Wnt pathway: genetic identification of the first components



wt



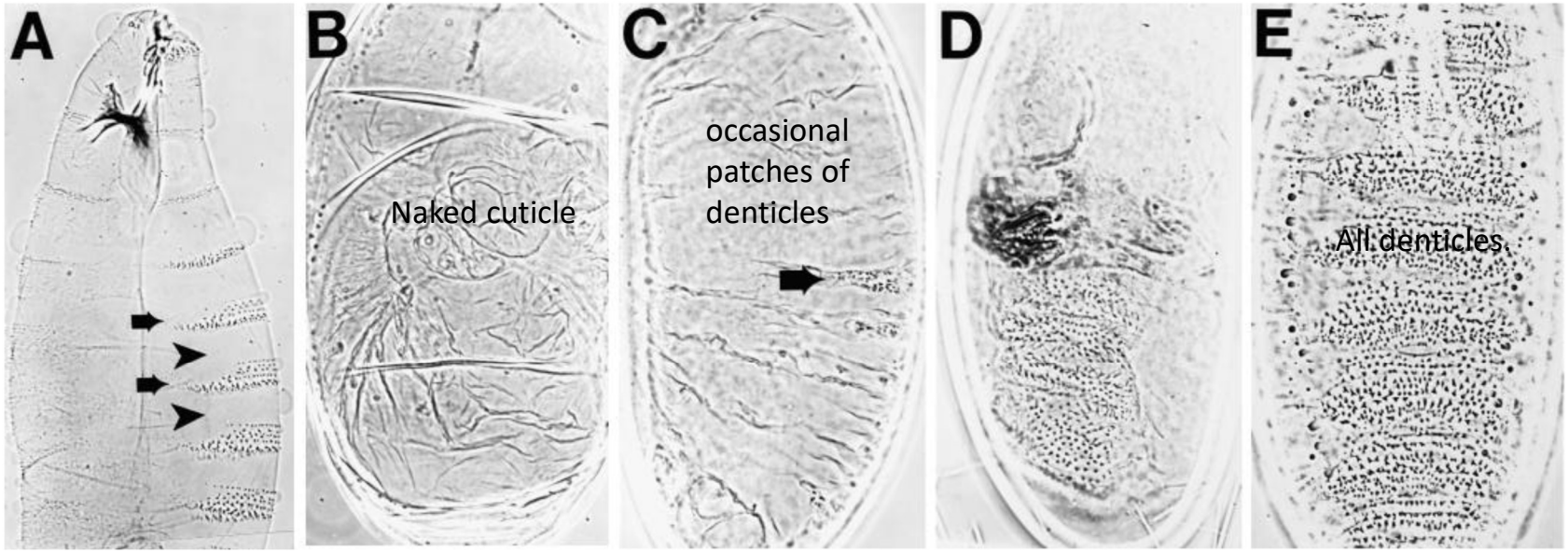
arm⁻



wg = wingless

arm = armadillo -> sequence homology = β-catenin

Discovery of the Wnt pathway: genetic identification of the first components

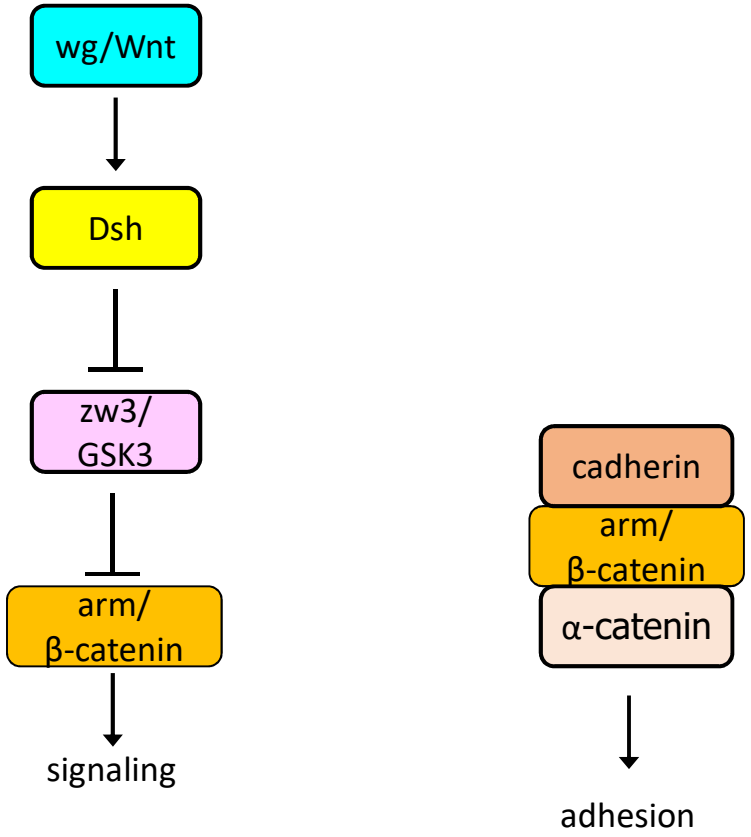


Development 120, 369-380 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994

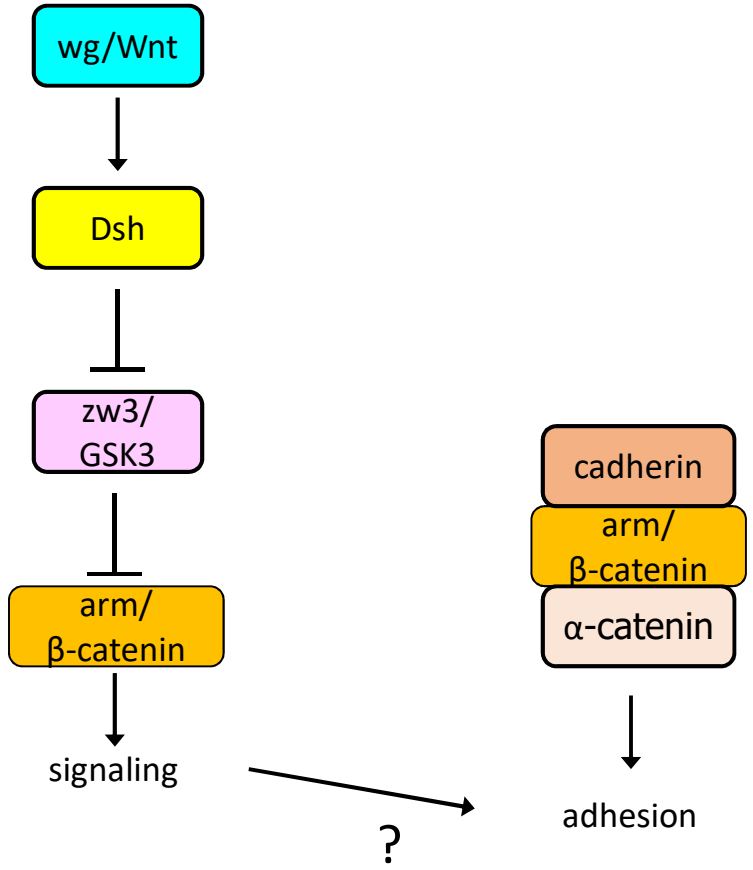
wingless signal and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo

Mark Peifer[1,*], Dari Sweeton[2], Michael Casey[1] and Eric Wieschaus[2]

Parallel discovery of the Wnt pathway and of the cadherin-catenin-complex



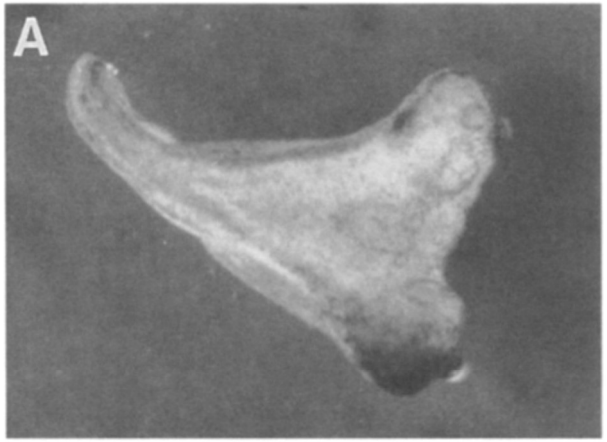
Discovery of the Wnt pathway: β-catenin -> signaling and/or adhesion?



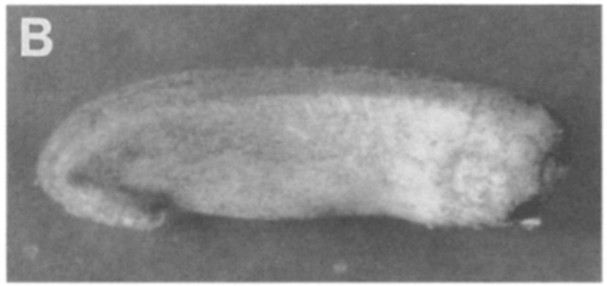
Discovery of the Wnt pathway: β-catenin -> signaling and/or adhesion?

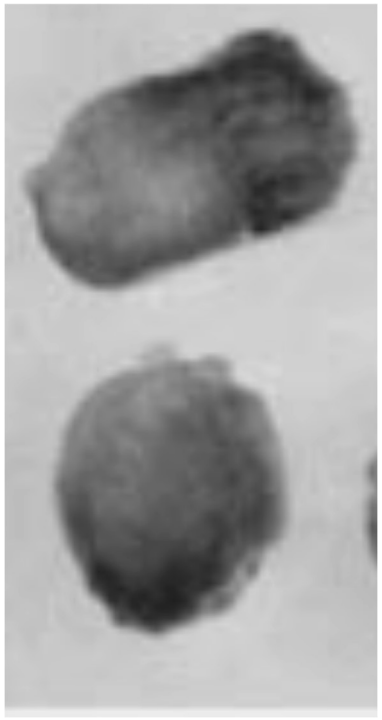
Xenopus axis determination

Wnt or β-catenin:
axis duplication



Normal control tadpole

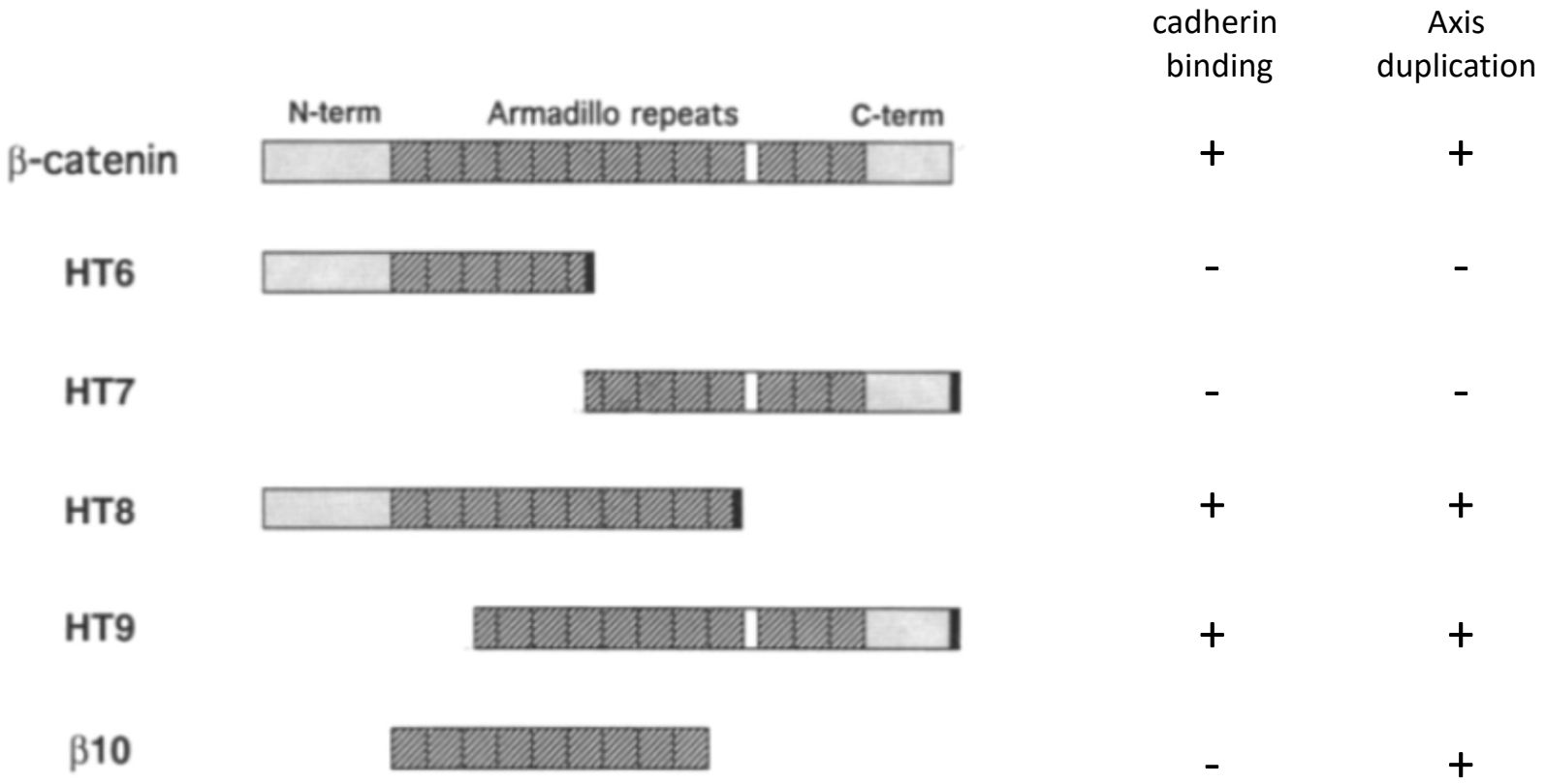




Inhibition of Wnt
pathway: no axis

Binding to cadherins antagonizes the signaling activity of beta-catenin during axis formation in Xenopus.
Fagotto F, Funayama N, Gluck U, Gumbiner BM.
J Cell Biol. 1996 Mar;132(6):1105-14.

Discovery of the Wnt pathway: β-catenin -> signaling and/or adhesion? Use of separation of function mutant!



Conclusion ?

Binding to cadherins antagonizes the signaling activity of beta-catenin during axis formation in Xenopus.
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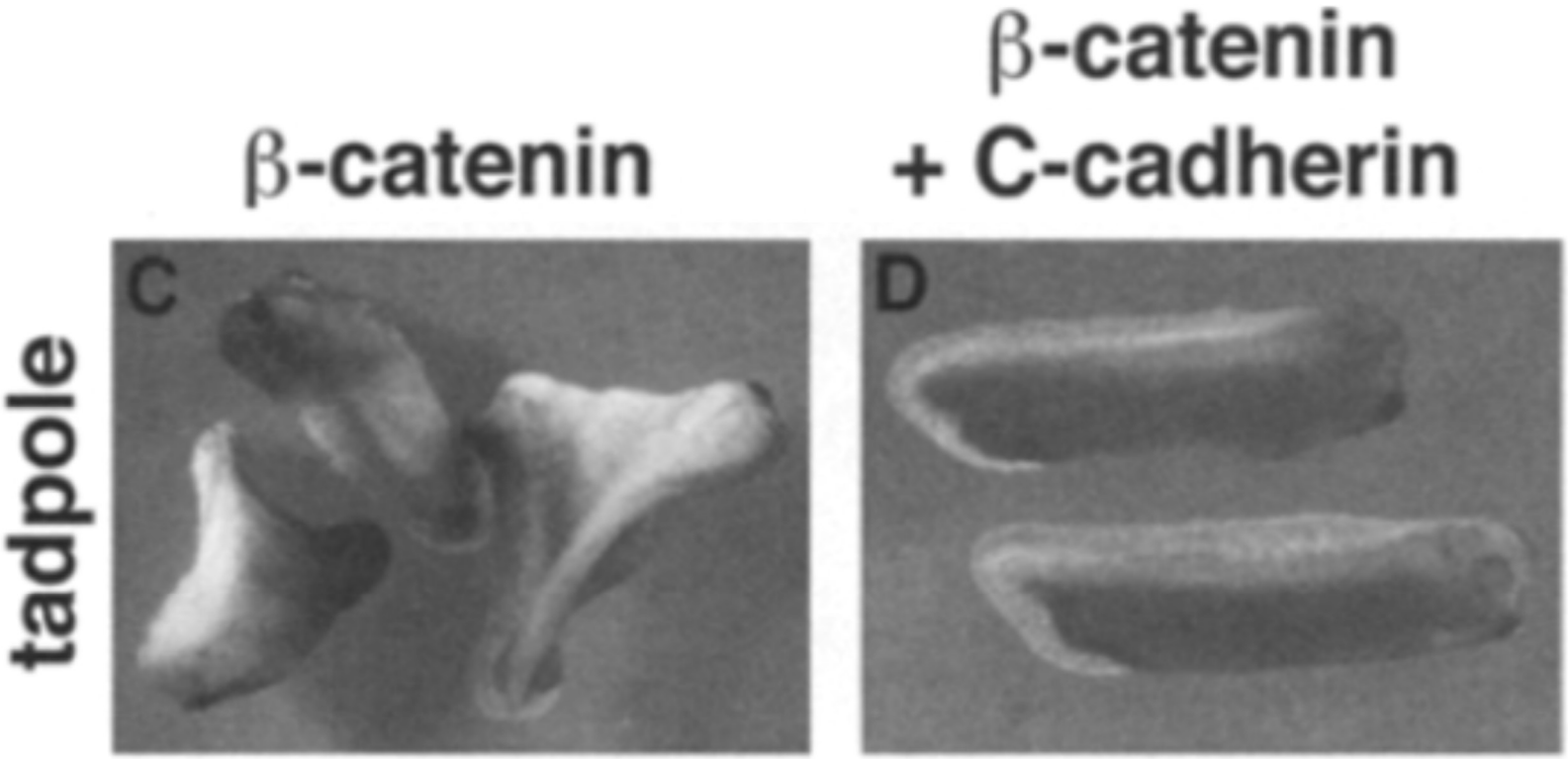

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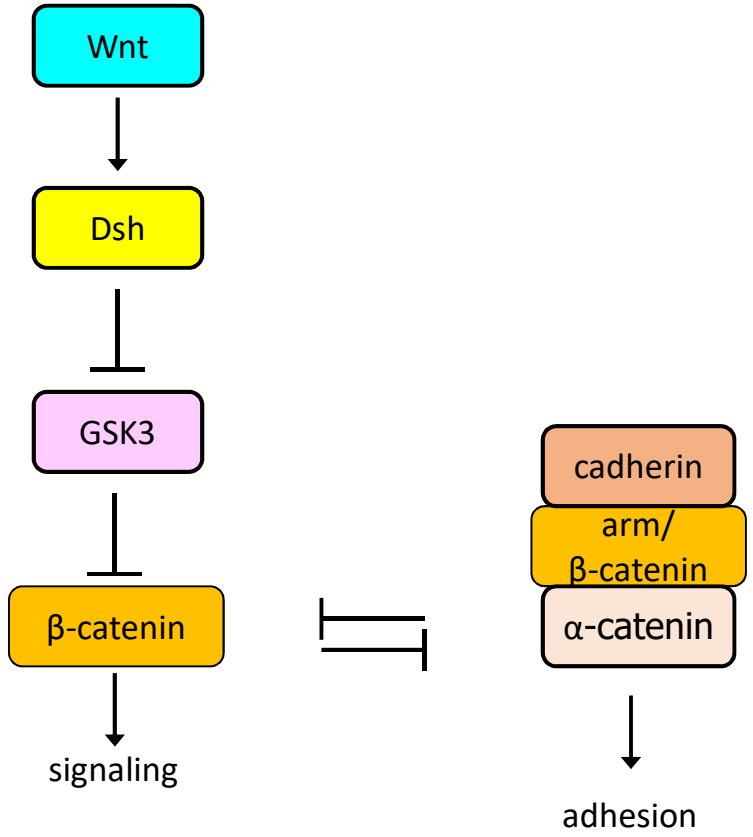
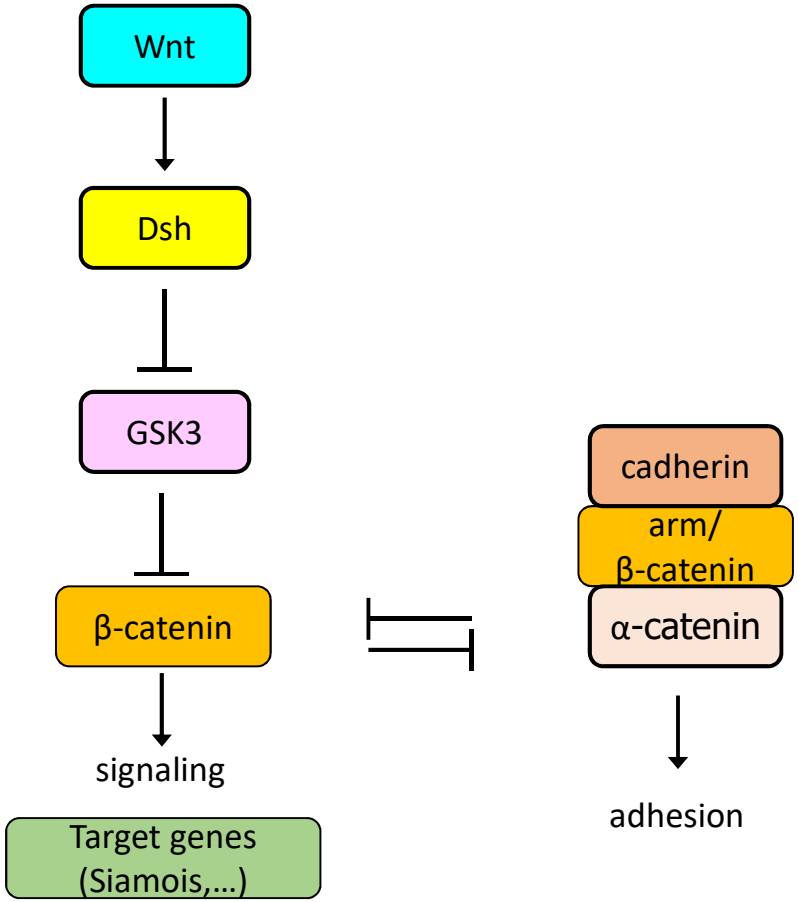
Binding to cadherins antagonizes the signaling activity of beta-catenin during axis formation in Xenopus.
Fagotto F, Funayama N, Gluck U, Gumbiner BM.
J Cell Biol. 1996 Mar;132(6):1105-14.

Discovery of the Wnt pathway:
β-catenin signaling and adhesion functions are antagonistic



Discovery of the Wnt pathway: Targets of β-catenin signaling

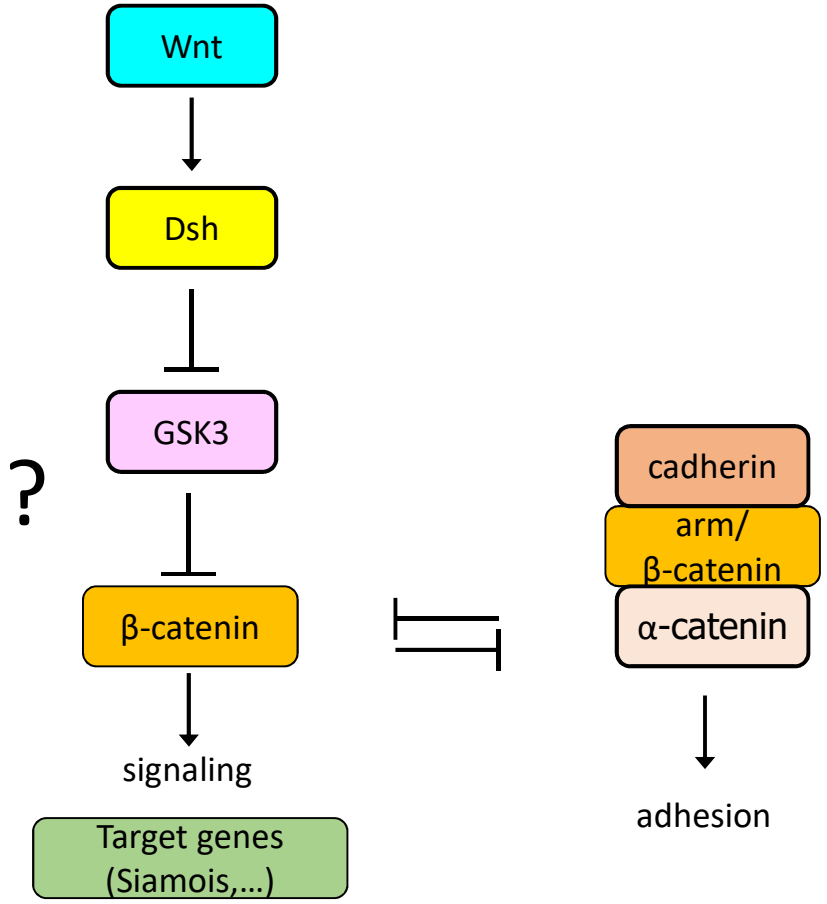


Expression cloning of Siamois, a Xenopus homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis.
Lemaire P, Garrett N, Gurdon JB. Cell. 1995, 81: 85-94.

The homeobox gene Siamois is a target of the Wnt dorsalisation pathway and triggers organiser activity in the absence of mesoderm.
Carnac G, Kodjabachian L, Gurdon JB, Lemaire P.
Development. 1996, 122:3055-65.

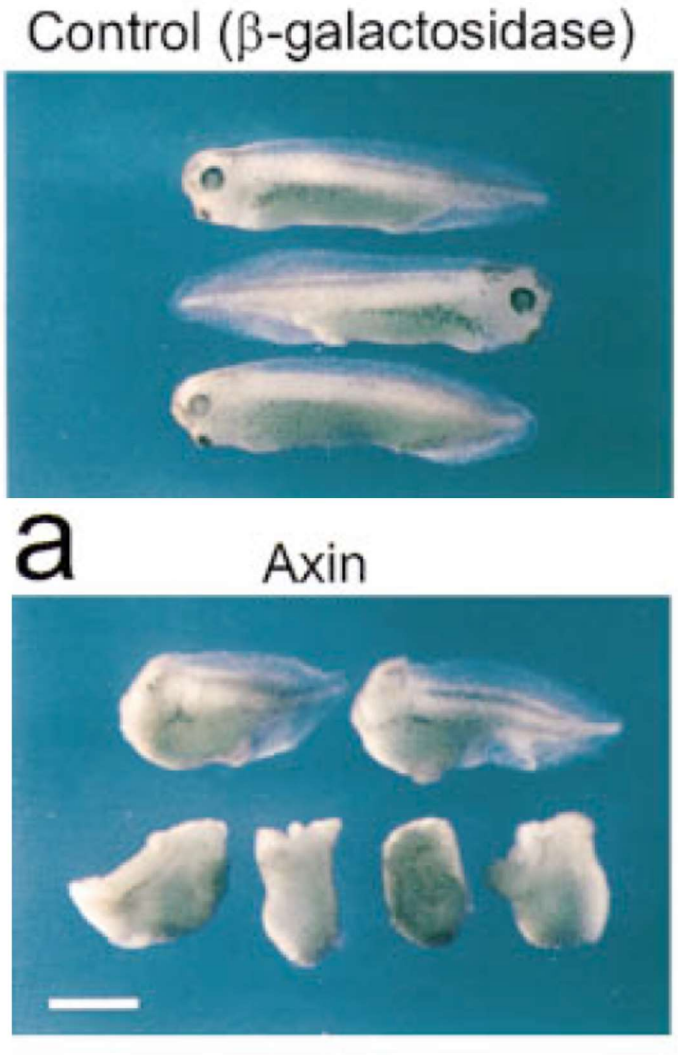
Induction of the primary dorsalizing center in Xenopus by the Wnt/GSK/beta-catenin signaling pathway, but not by Vg1, Activin or Noggin.
Fagotto F, Guger K, Gumbiner BM. Development. 1997, 124: 453-60.

Discovery of the Wnt pathway: How is β-catenin regulated?



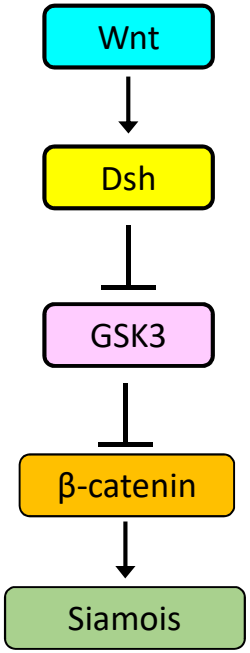
Discovery of the Wnt pathway: Discovery of Axin

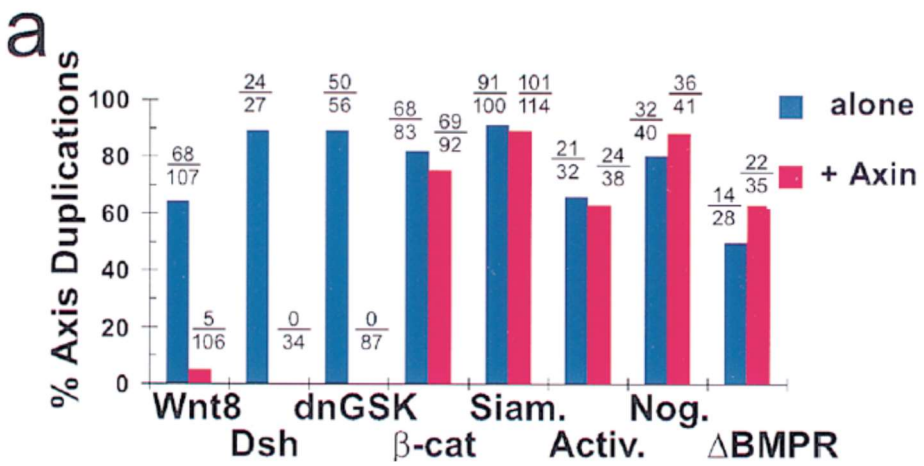
Axin = Axin inhibitor

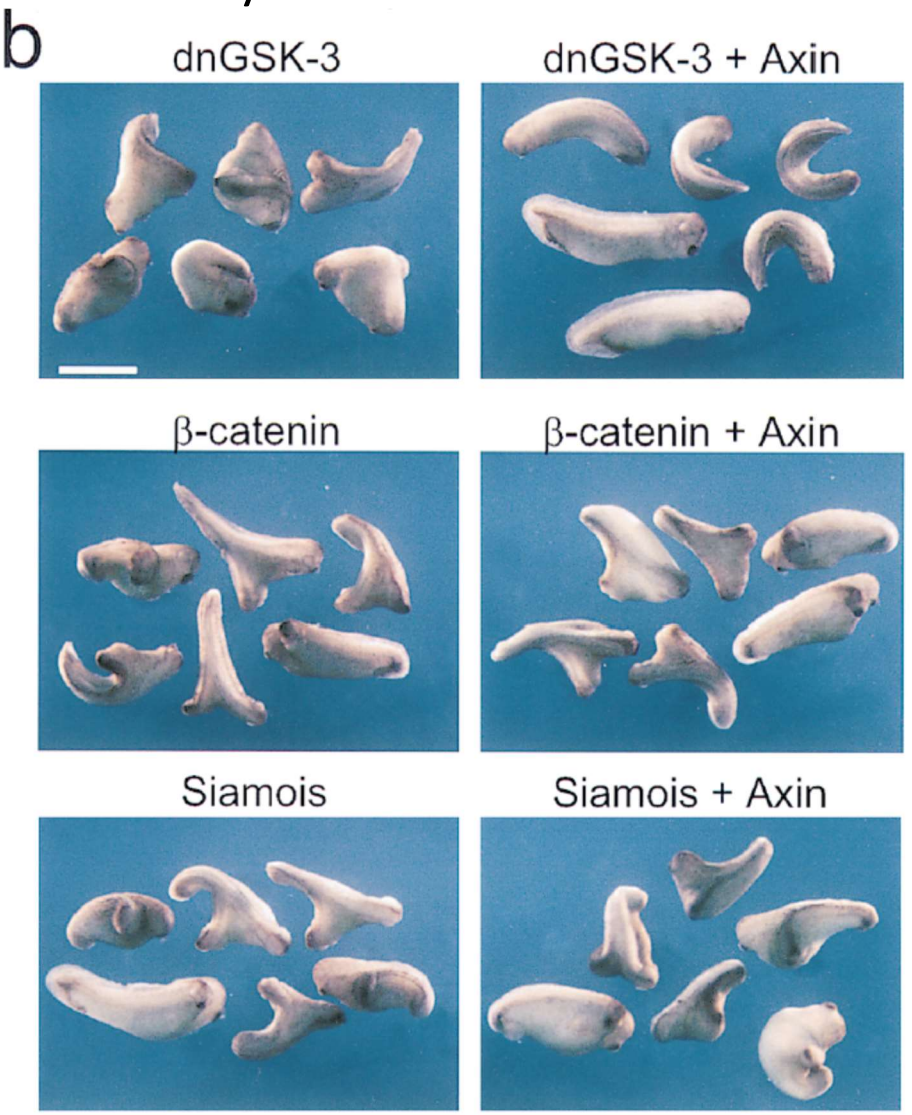


The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation.
Zeng L*, Fagotto F*, Zhang T, Hsu W, Vasicek TJ, Perry WL 3rd, Lee JJ, Tilghman SM, Gumbiner BM, Costantini F.
Cell. 1997 Jul 11;90(1):181-92. *Equal authors

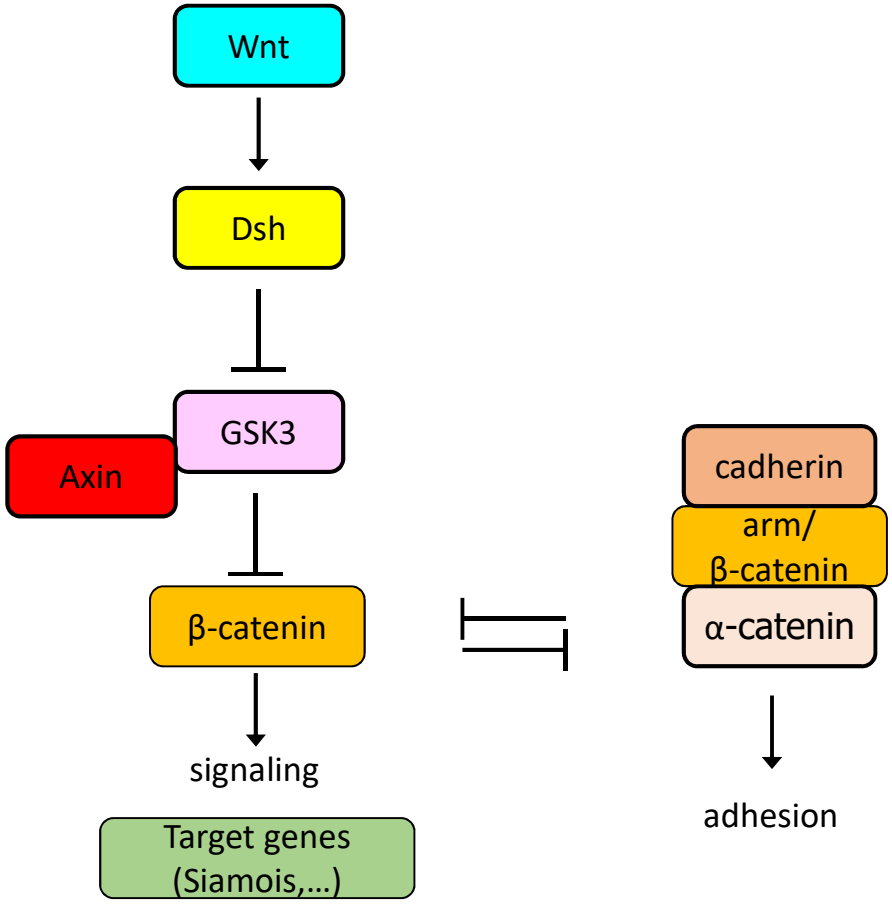
Discovery of the Wnt pathway: Discovery of Axin







Discovery of the Wnt pathway: Axin at the core of β-catenin regulation



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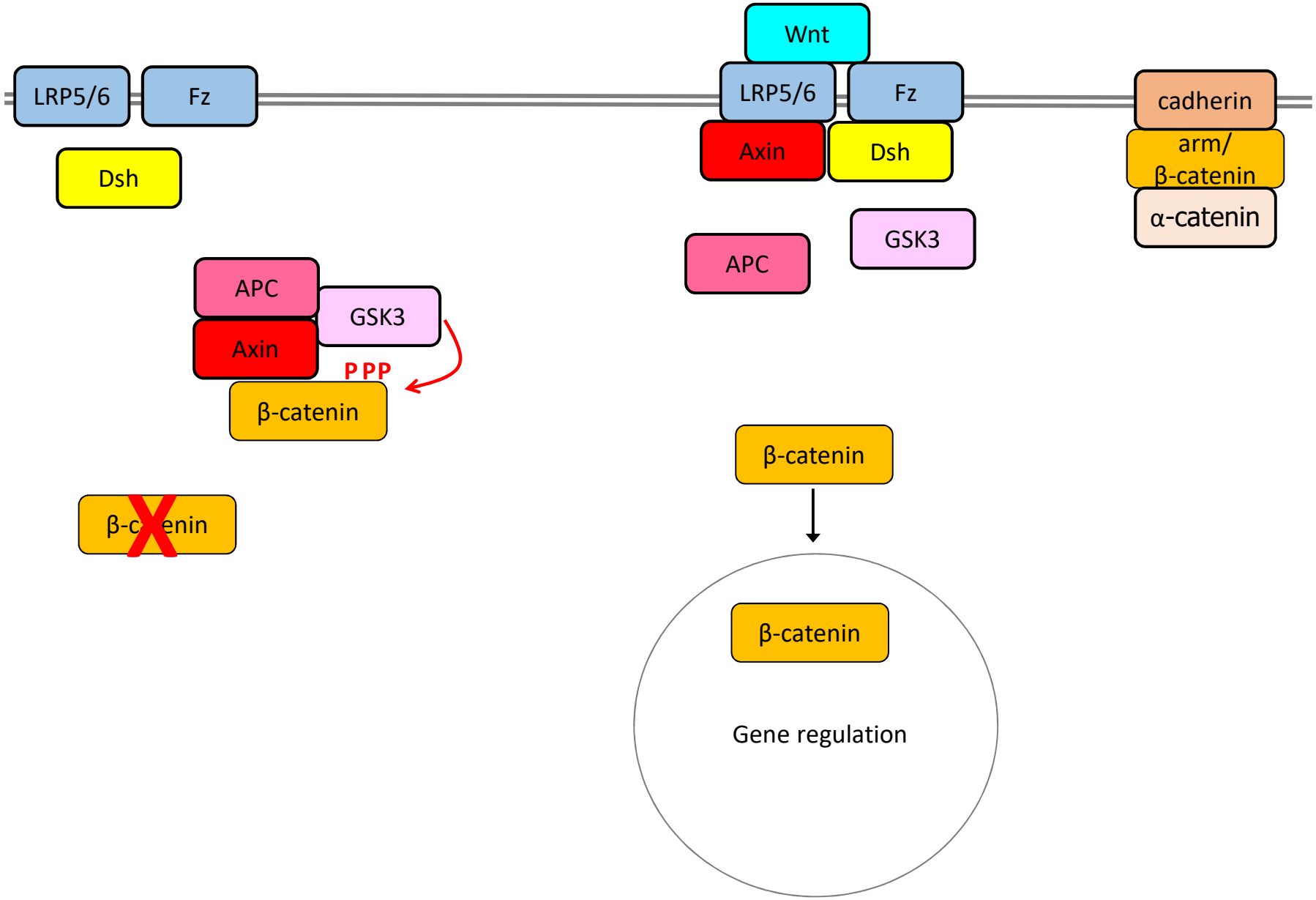


Discovery of the Wnt pathway: one more kinase, casein kinase 1 (CK1)
-> a case where simple epistasis is getting into trouble

Axin-mediated CKI phosphorylation of β-catenin at Ser 45: a molecular switch for the Wnt pathway

Sharon Amit,[1] Ada Hatzubai,[1] Yaara Birman,[1] Jens S. Andersen,[2] Etti Ben-Shushan,[1] Matthias Mann,[2] Yinon Ben-Neriah,[1,3] and Irit Alkalay[1]

Cell, Vol. 108, 837–847, March 22, 2002, Copyright ©2002 by Cell Press

Control of β-Catenin Phosphorylation/Degradation by a Dual-Kinase Mechanism

Casein kinase 1 = negative regulators of the Wnt pathway!

Casein kinase I phosphorylates and destabilizes the β-catenin degradation complex

Zhong-Hua Gao*†, Joni M. Seeling*‡, Virginia Hill*, April Yochum*, and David M. Virshup*§

A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation

Xin Zeng[1], Keiko Tamai[1], Brad Doble[3], Shitao Li[1], He Huang[1], Raymond Habas[1]†, Heidi Okamura[2]†, Jim Woodgett[3] & Xi He[1]

Casein kinase Iε in the Wnt pathway: Regulation of β-catenin function

Chie Sakanaka*†‡§, Peng Leong*‡, Licen Xu*‡¶, Stephen D. Harrison*, and Lewis T. Williams*†§‖

articles

Casein kinase I transduces Wnt signals

John M. Peters*†, Renée M. McKay*†, James P. McKay & Jonathan M. Graff*

Casein kinase 1 = positive regulators of the Wnt pathway!

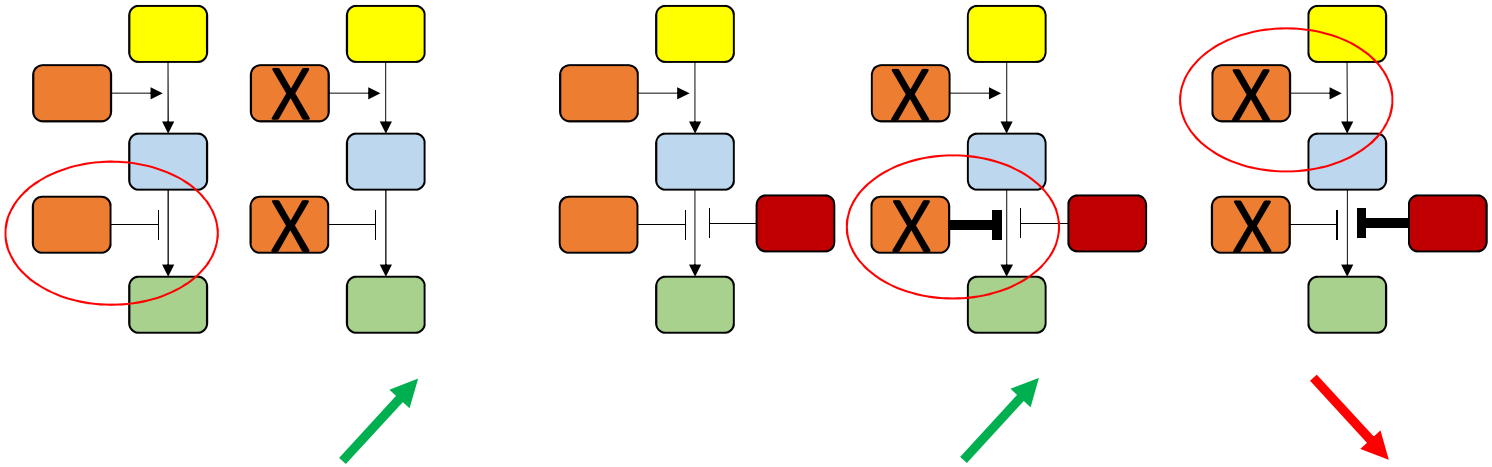
?

Perhaps CK1 is playing more than one function??

A ‘specific’ phenotype may hide multiple (antagonistic) functions

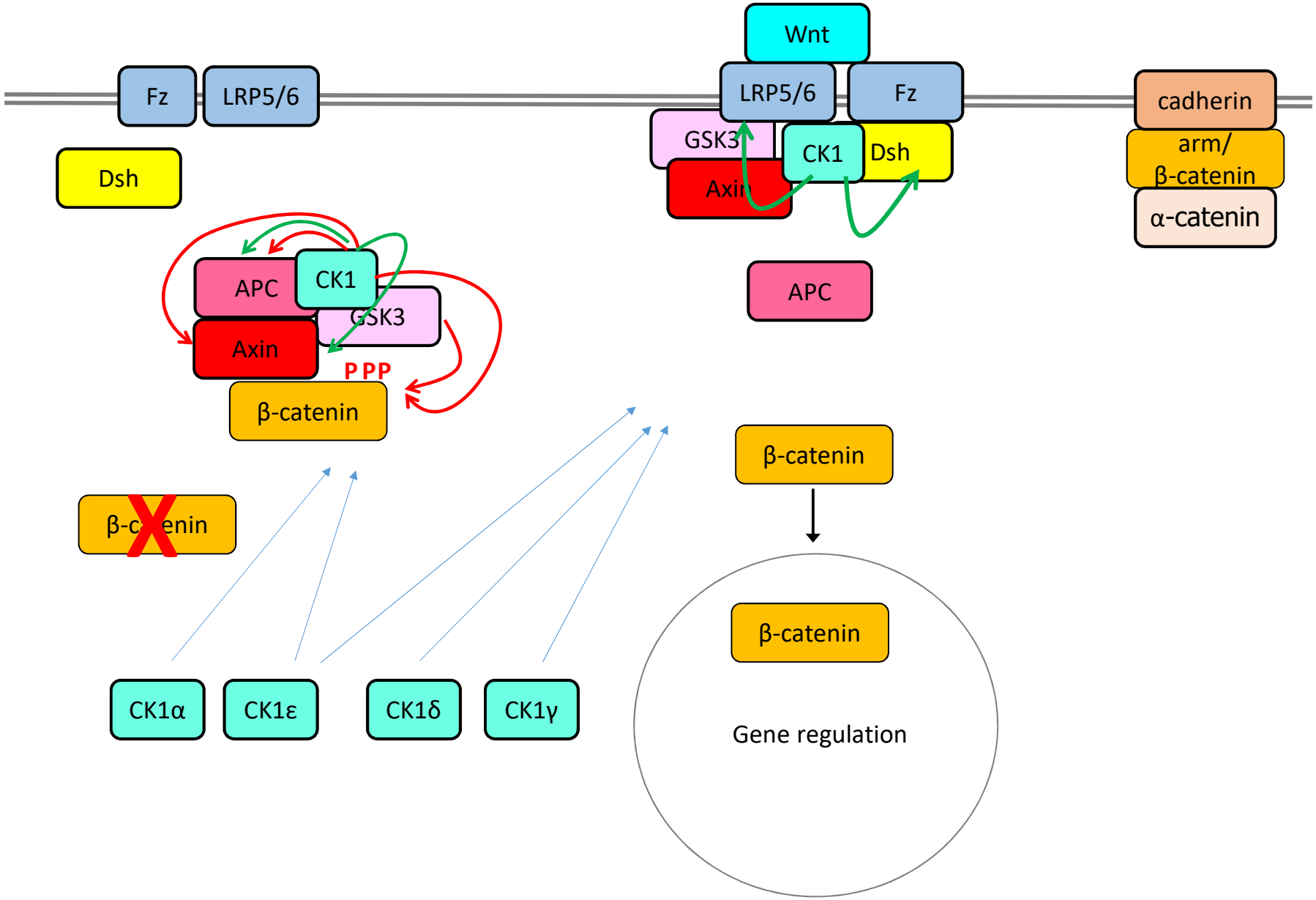
The (apparently) easy case

Now getting harder…



Let’s remove

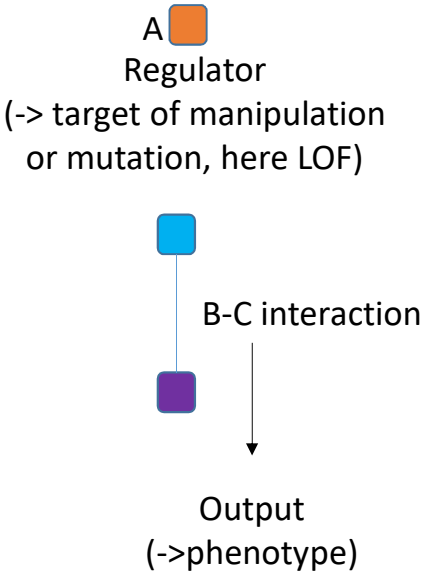
Discovery of the Wnt pathway: CK1 has multiple functions (and multiple isoforms)

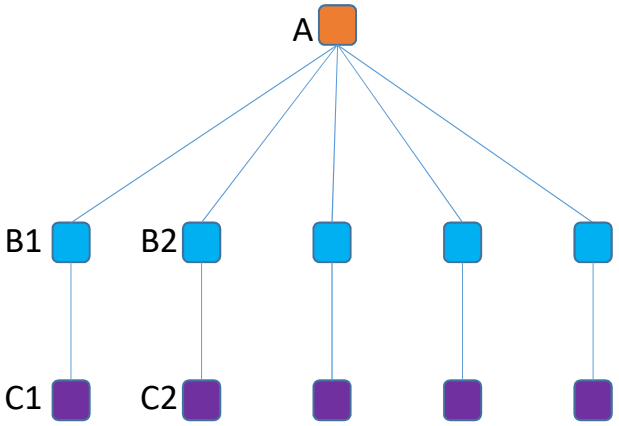


A ‘specific’ phenotype can hide pleiotropic functions

Multiple targets with different sensitivities to levels/activities

Output signal depends on B-C interaction, which is regulated by A

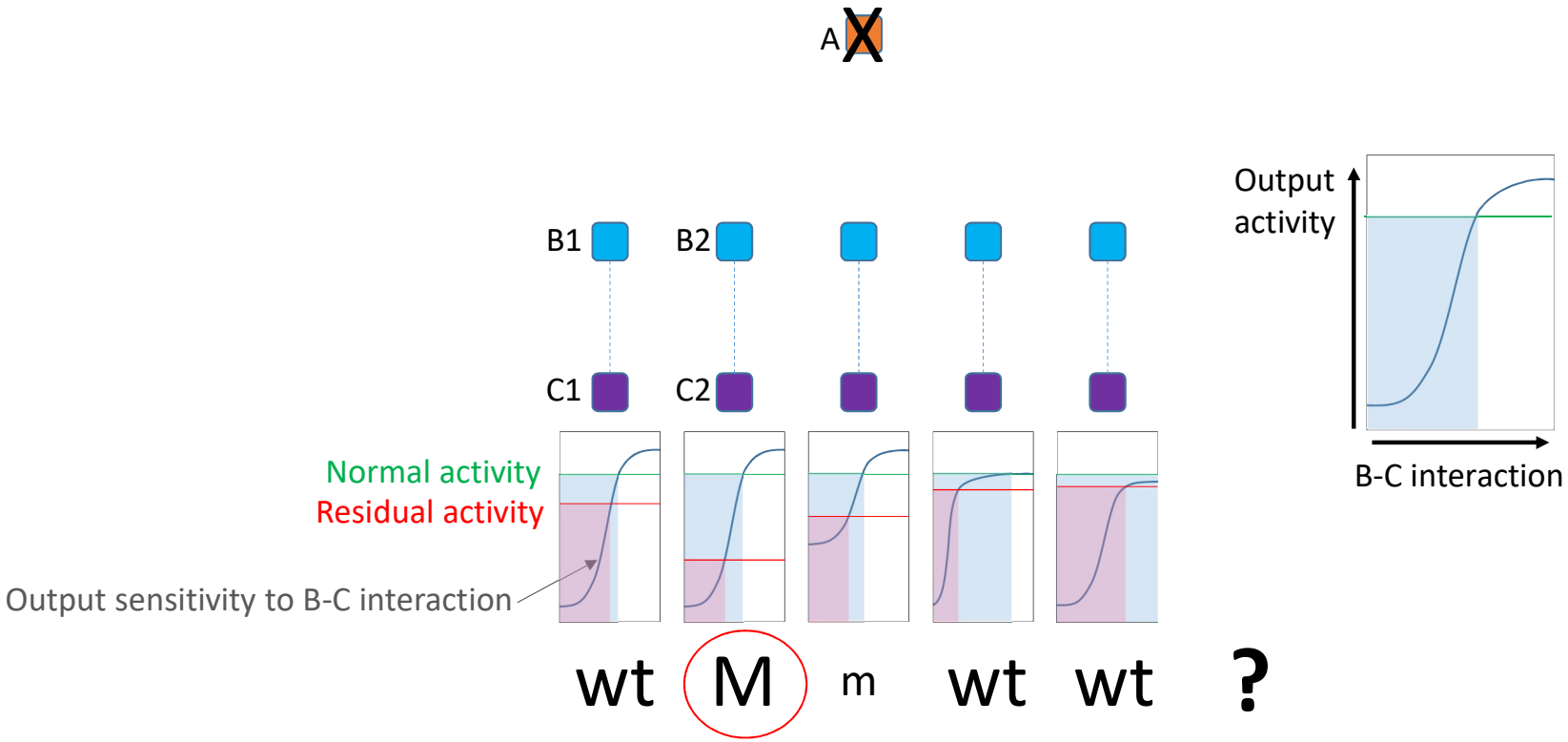




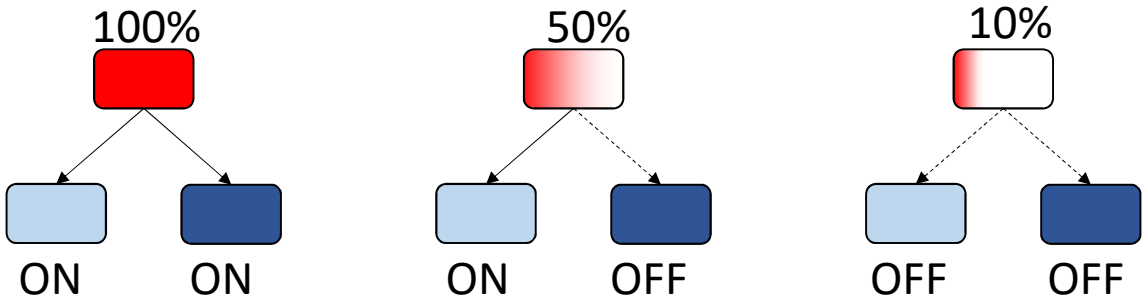
A ‘specific’ phenotype can hide pleiotropic functions

Multiple targets with different sensitivities to levels/activities

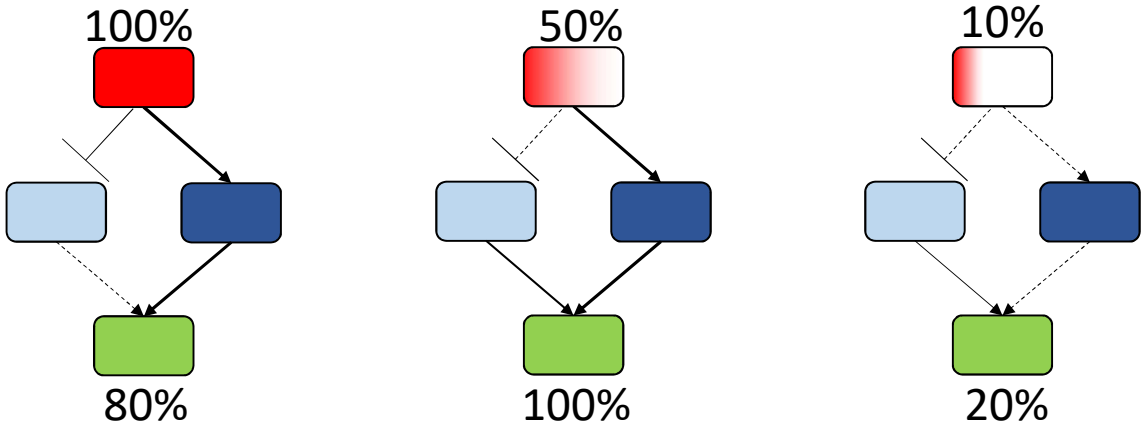
Effect of A LOF



Complex effects of partial LOF

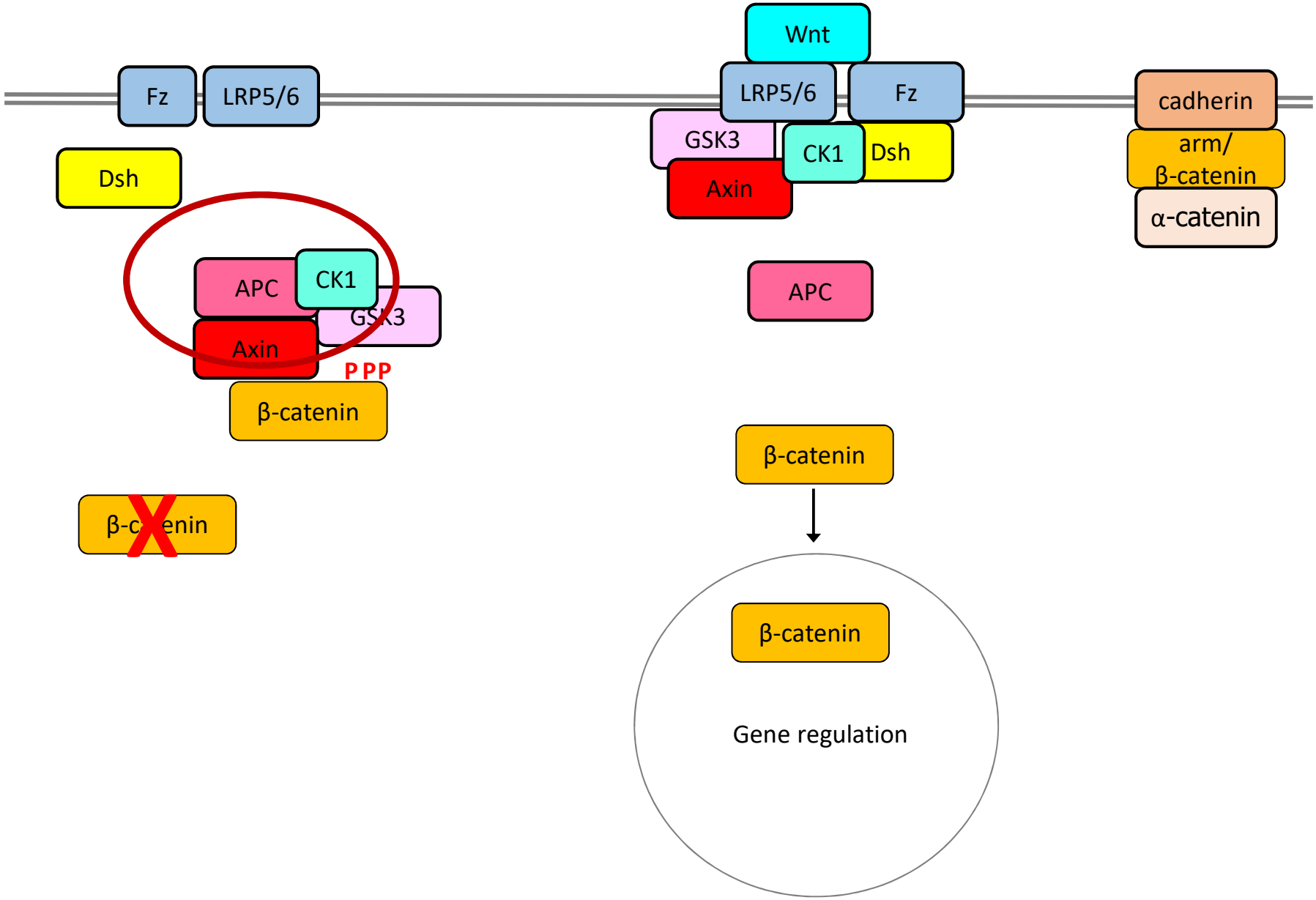


Why?

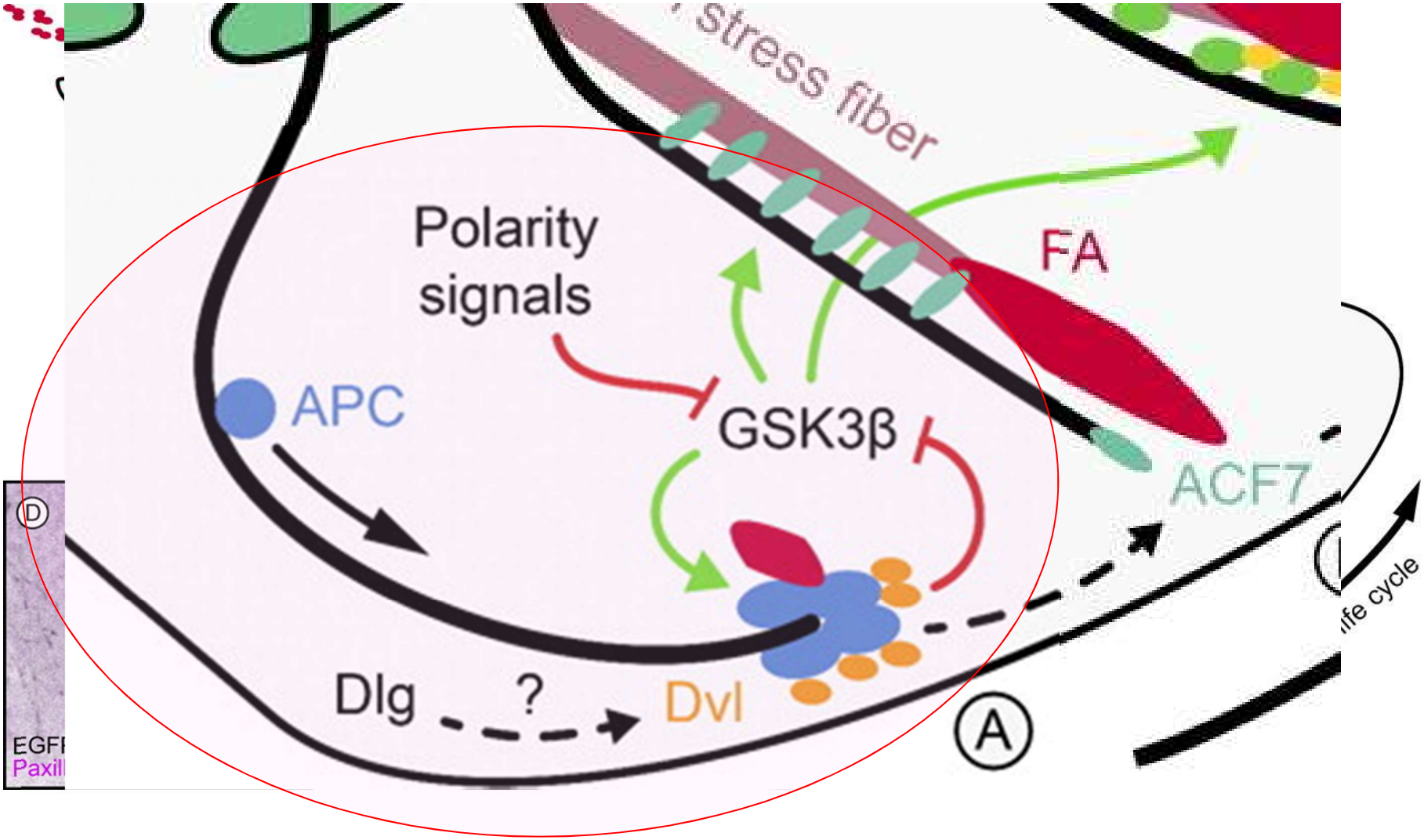


Which phenotype(s) would you predict?

Back to the Wnt pathway with an even more complex case: **APC**



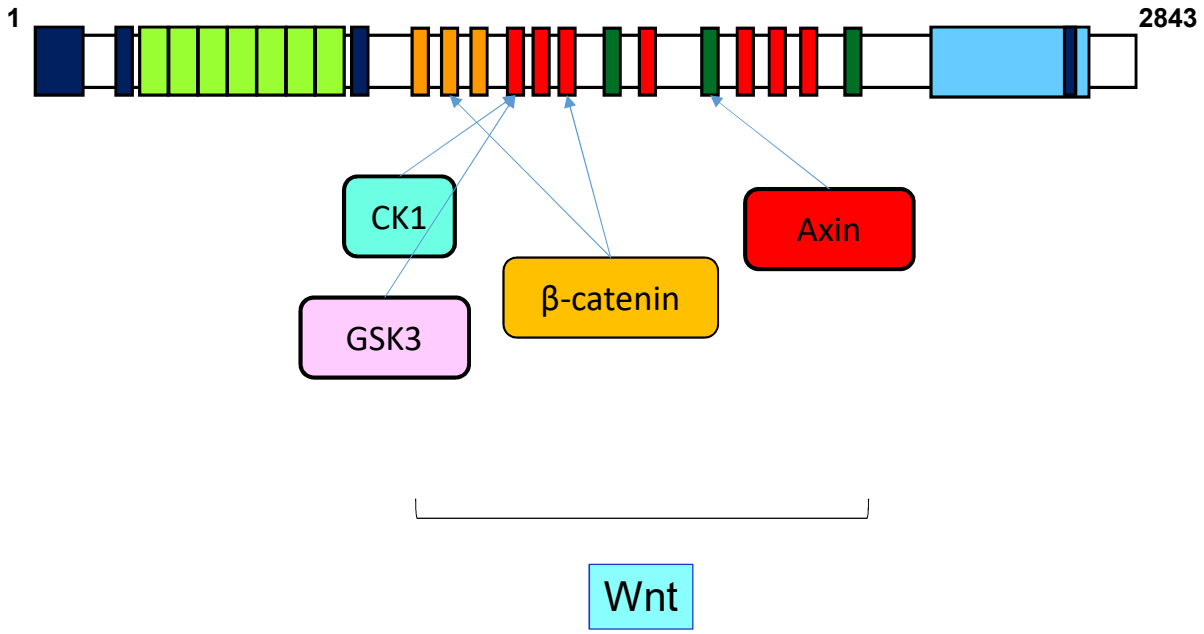
+TIP-mediated microtubule–FA interactions in the front of a migrating cell.



Samantha Stehbens, and Torsten Wittmann J Cell Biol
2012;198:481-489

APC is a huge scaffold protein with multiple interaction domains

(350kDa, ~ 3000 amino acids!!!)



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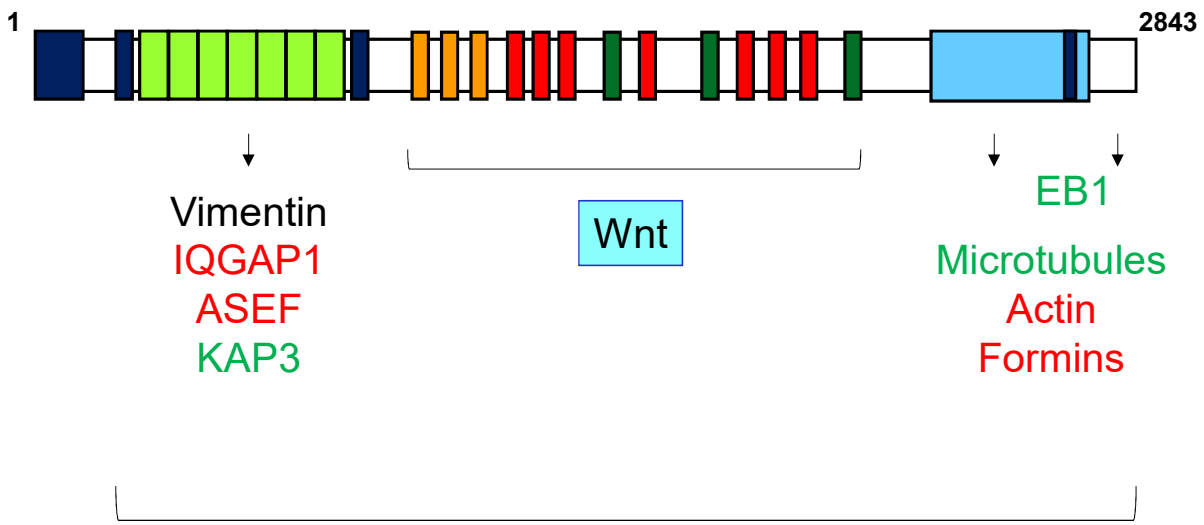
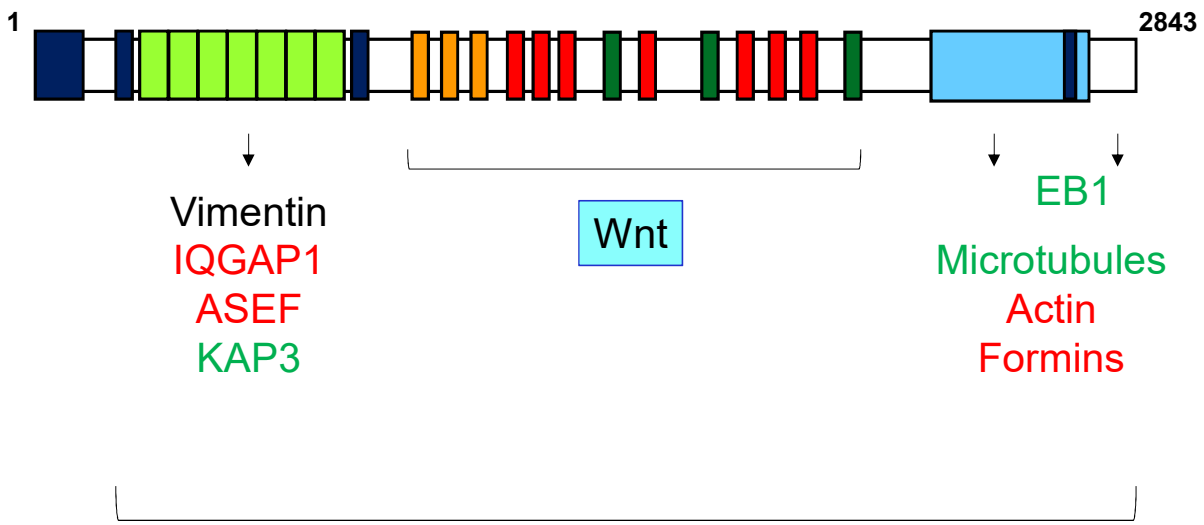


???

What is the role of APC-cytoskeleton interactions?

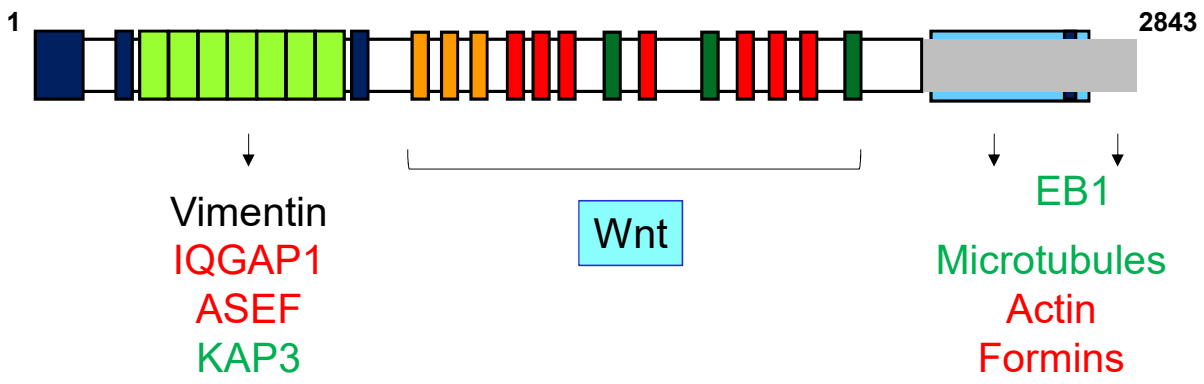


???

What will a knock-out tell you?

Any other strategy?

APC truncations?

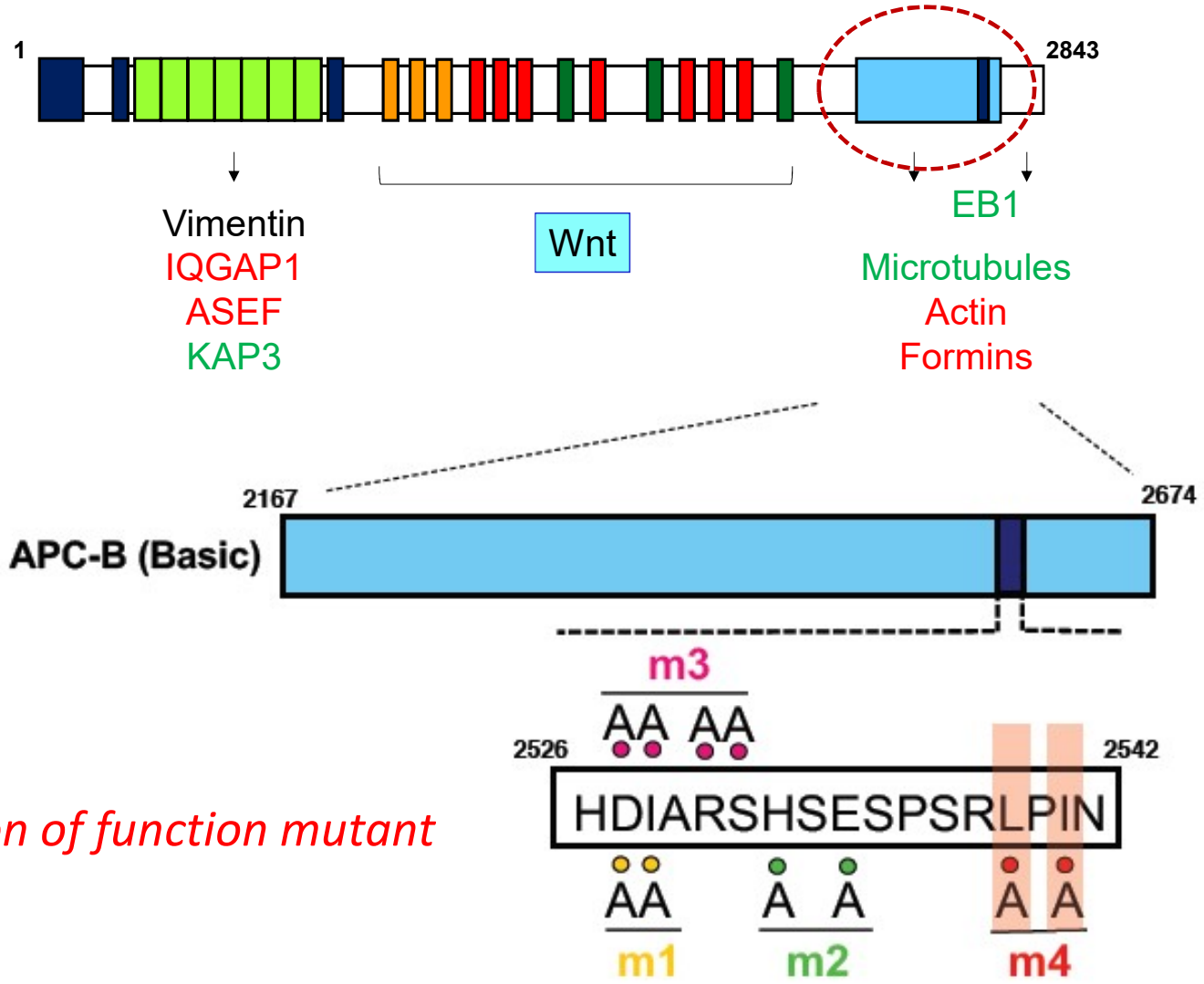


Wnt signalling OK

Defects in cell migration

Are the defects due to interaction with microtubules or actin cytoskeleton?

Let's see if we can take these interactions apart!



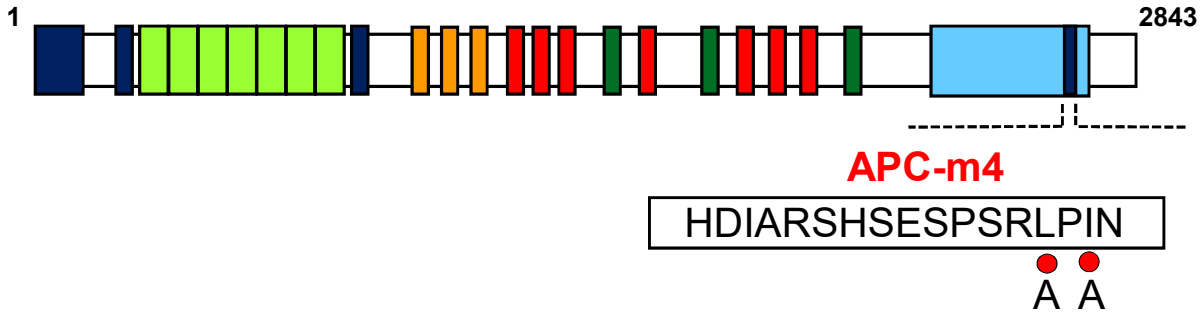
Separation of function mutant

Adenomatous polyposis coli nucleates actin assembly to drive cell migration and microtubule-induced focal adhesion turnover.

Juanes MA, Bouguenina H, Eskin JA, Jaiswal R, Badache A, **Goode** BL.

J Cell Biol. 2017 Sep 4;216(9):2859-2875

Dissecting APC functions:
specific abolishment of actin-nucleating activity



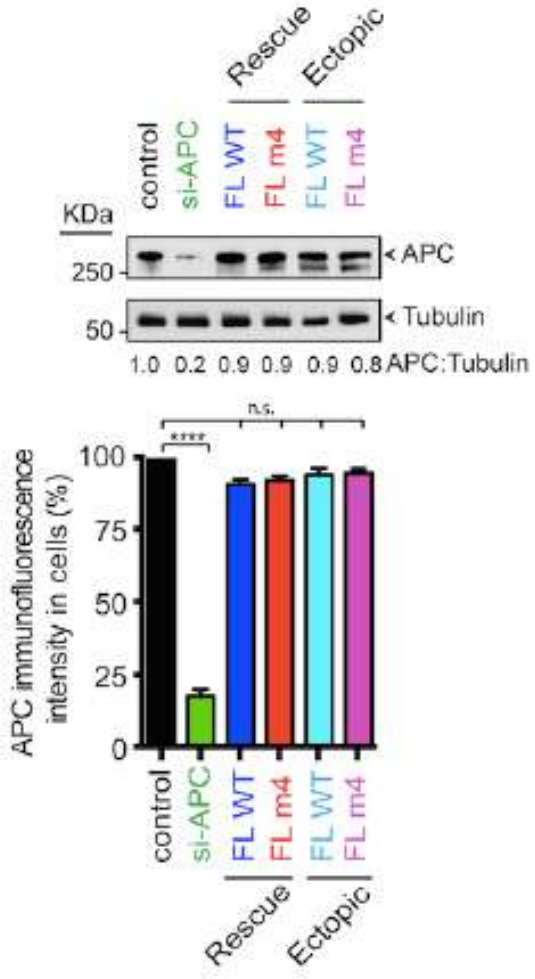
Microtubule organization

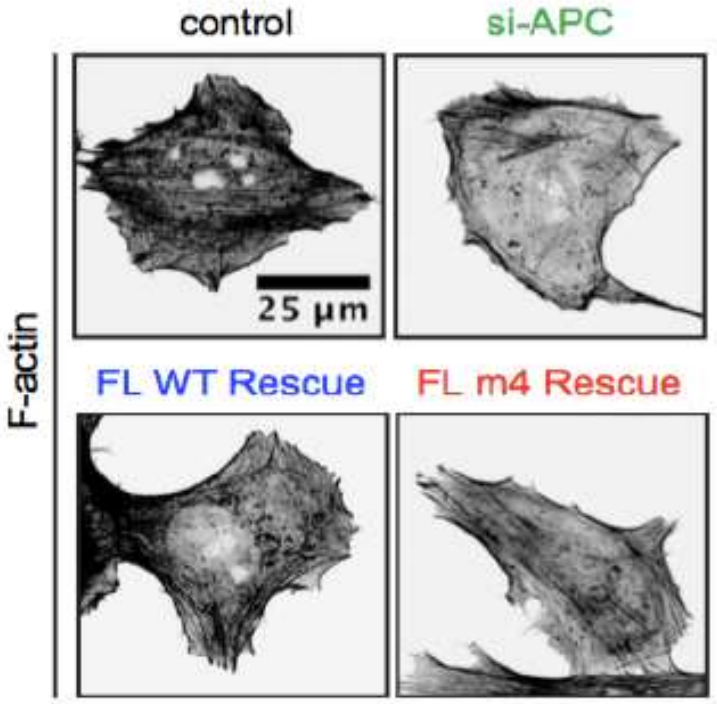
Actin nucleation

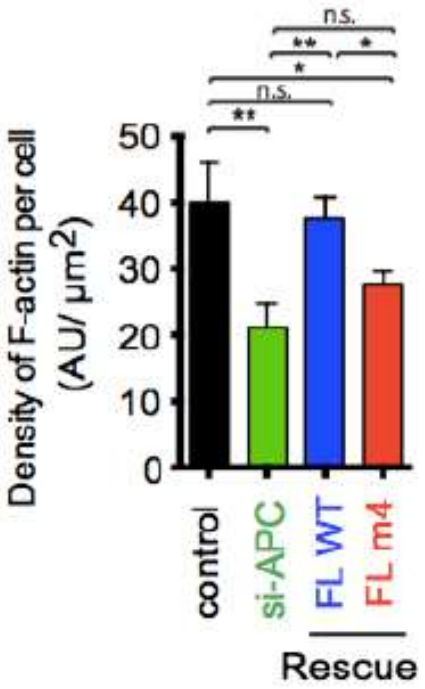
[Adenomatous polyposis coli nucleates actin assembly to drive cell migration and microtubule-induced focal adhesion turnover.]
Juanes MA, Bouguenina H, Eskin JA, Jaiswal R, Badache A, **Goode** BL.
J Cell Biol. 2017 Sep 4;216(9):2859-2875

Dissecting APC functions:
specific abolishment of actin-nucleating activity

APC depletion (siRNA) -> rescue with wild type or m4 mutant







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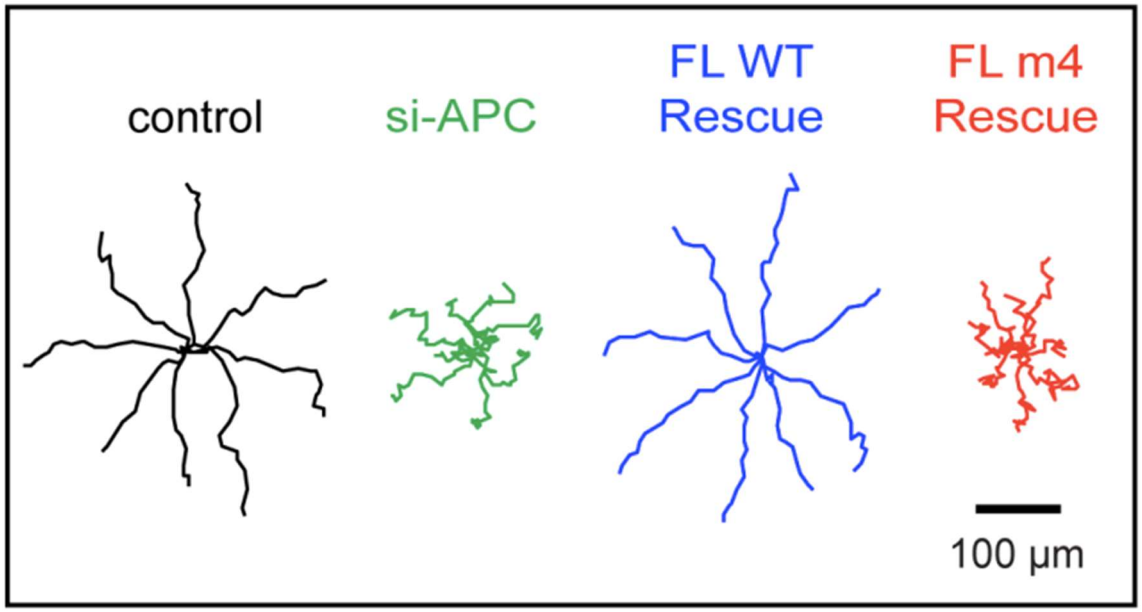

Directed cell migration

Adenomatous polyposis coli nucleates actin assembly to drive cell migration and microtubule-induced focal adhesion turnover.
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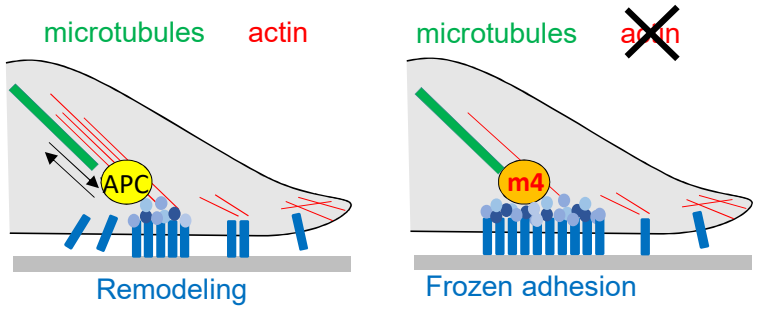
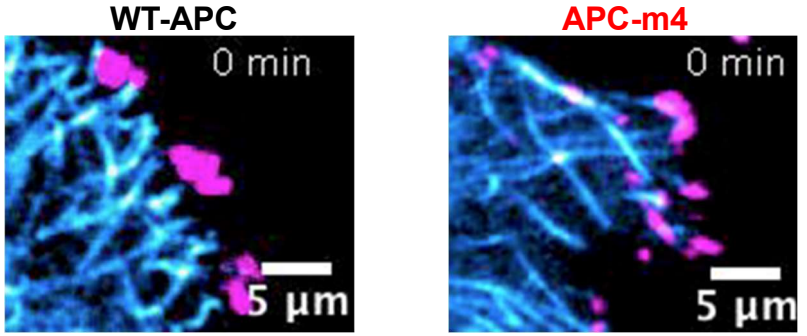
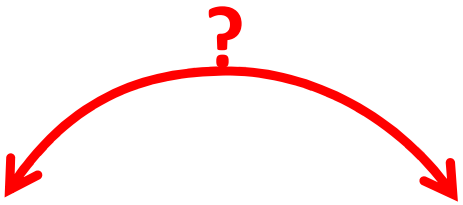




Microtubules
Focal adhesions

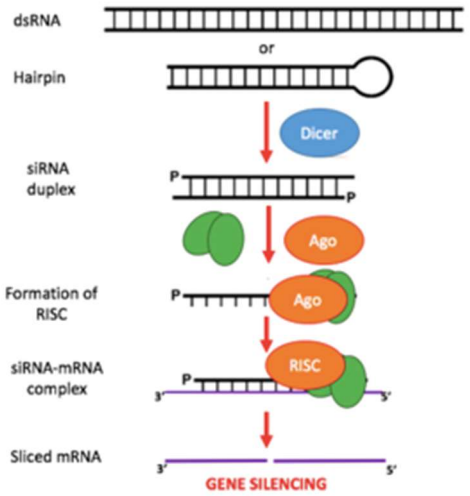


APC m4 -> decreased actin nucleation -> MTs more stable -> impaired adhesion turnover

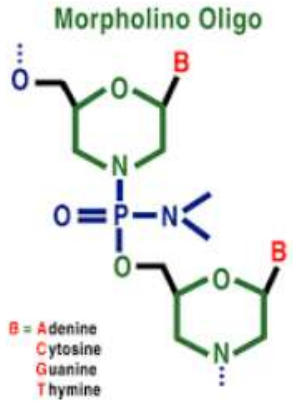
Adenomatous polyposis coli nucleates actin assembly to drive cell migration and microtubule-induced focal adhesion turnover.
Juanes MA, Bouguenina H, Eskin JA, Jaiswal R, Badache A, **Goode** BL.
J Cell Biol. 2017 Sep 4;216(9):2859-2875

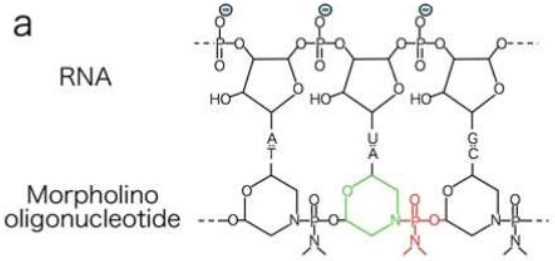
Non-genetic approaches to produce loss-of-functions:

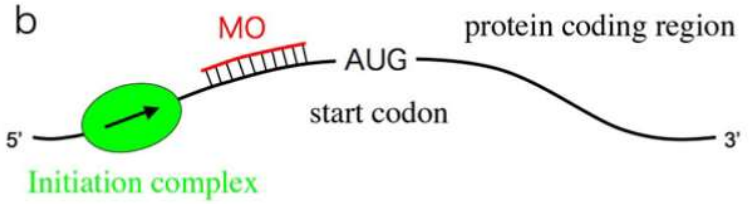
RNA interference



Antisense morpholinos: Rapid specific gene inactivation by blocking translation







Dissecting complex systems: picking the right model helps!

E.g. yeast (see Dom Hemlinger lecture)
Most of molecular and cellular processes:
-> transcription
-> chromatin structure
-> cell cycle
-> cytoskeleton
-> vesicle trafficking
-> nuclear transport
……

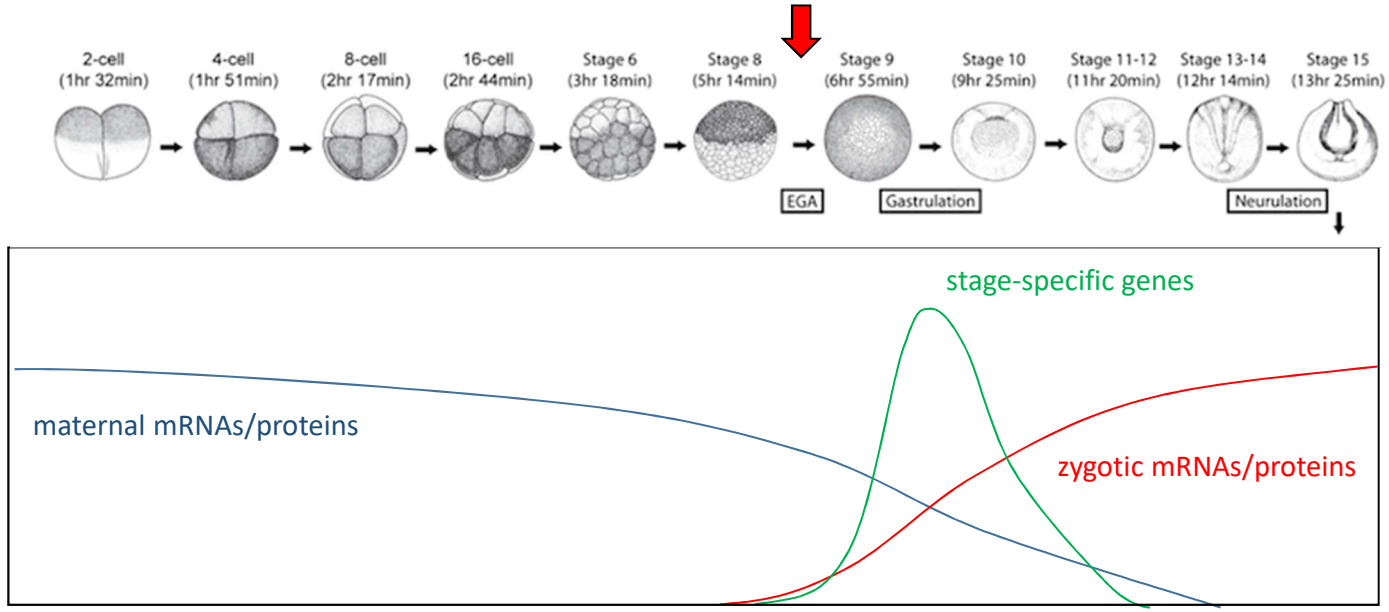
But obviously not for multicellular processes!!!

Example of versatile multicellular model: Xenopus early gastrula

Pick the right situation:

Ex: Early gastrulation, ideal stage to study gene function in embryo

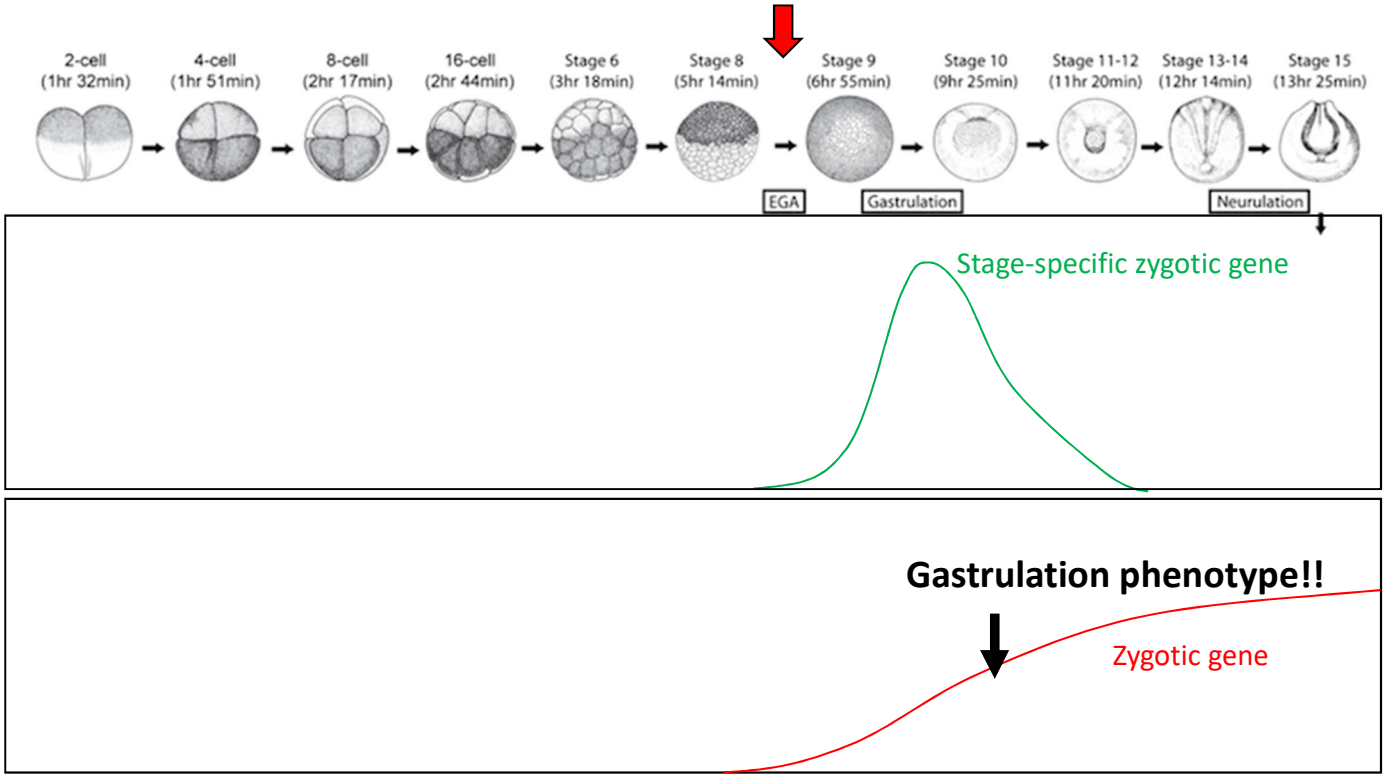
(including for essential genes!)



Early development depend only on maternal genes. Zygotic transcription starts just before gastrulation (red arrow).
Strikingly, many genes are expressed specifically for the period of gastrulation, indicating that this is a crucial phase of development, and also a good stage to test the function of many different regulators.

Pick the right situation:
Ex: Early gastrulation, ideal stage to study gene function in embryo

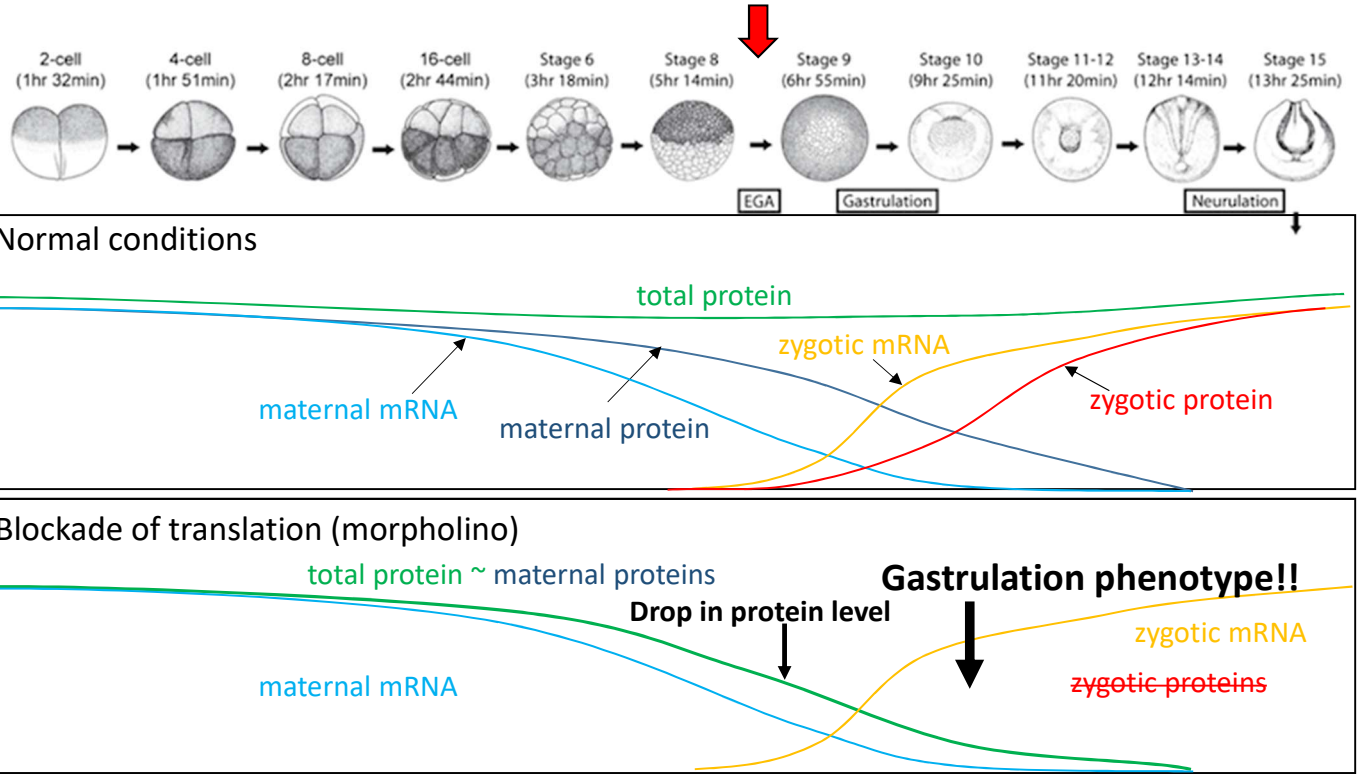
stage-specific genes



Many zygotic genes are specifically activated just before gastrulation. Some remain active later, others are very specific for gastrulation stages. Morpholino-depletion can readily block completely translation of all these gene products, and we can look at the immediate effect on gastrulation

Pick the right situation:
Ex: Early gastrulation, ideal stage to study gene function in embryo

constitutively expressed genes



Maternal and zygotic contribution to the total levels for a protein expressed constantly during development.

Changes in mRNA and protein levels (maternal + zygotic = total) after injection of a specific morpholino oligonucleotide.

A) For most genes, maternal proteins accumulated in egg are sufficient for all the first phase of development (cleavage and early patterning, until stage 10).
B) Thus by simply injecting morpholino oligonucleotides, we block translation of both maternal (light blue) and zygotic (orange) mRNAs, and this results in protein depletion (compare green lines) just at the right stage, i.e. just at the start of gastrulation, without affecting any earlier process. This gives us the chance to test the role of most components, even of components that are required for many functions, such as RhoGTPases or other regulators of the cytoskeleton.
In other systems, one would need to set complicated conditional knock-out strategies to obtain the same situation.

Is “classical” genetics always “right”?

Is CRISPR-Cas the solution to every question?

Is knocking out every gene the holy grail?

Additional complications: Compensation/Adaptation

Reverse Genetic Screening Reveals Poor Correlation between Morpholino-Induced and Mutant Phenotypes in Zebrafish

Fatma O. Kok,[1,7] Masahiro Shin,[1,7] Chih-Wen Ni,[1,7,8] Ankit Gupta,[1] Ann S. Grosse,[1] Andreas van Impel,[2] Bettina C. Kirchmaier,[2,9] Josi Peterson-Maduro,[2] George Kourkoulis,[1] Ira Male,[1] Dana F. DeSantis,[1] Sarah Sheppard-Tindell,[1] Lwaki Ebarasi,[3,4] Christer Betsholtz,[3,4] Stefan Schulte-Merker,[2,5] Scot A. Wolfe,[1,6] and Nathan D. Lawson[1,*]

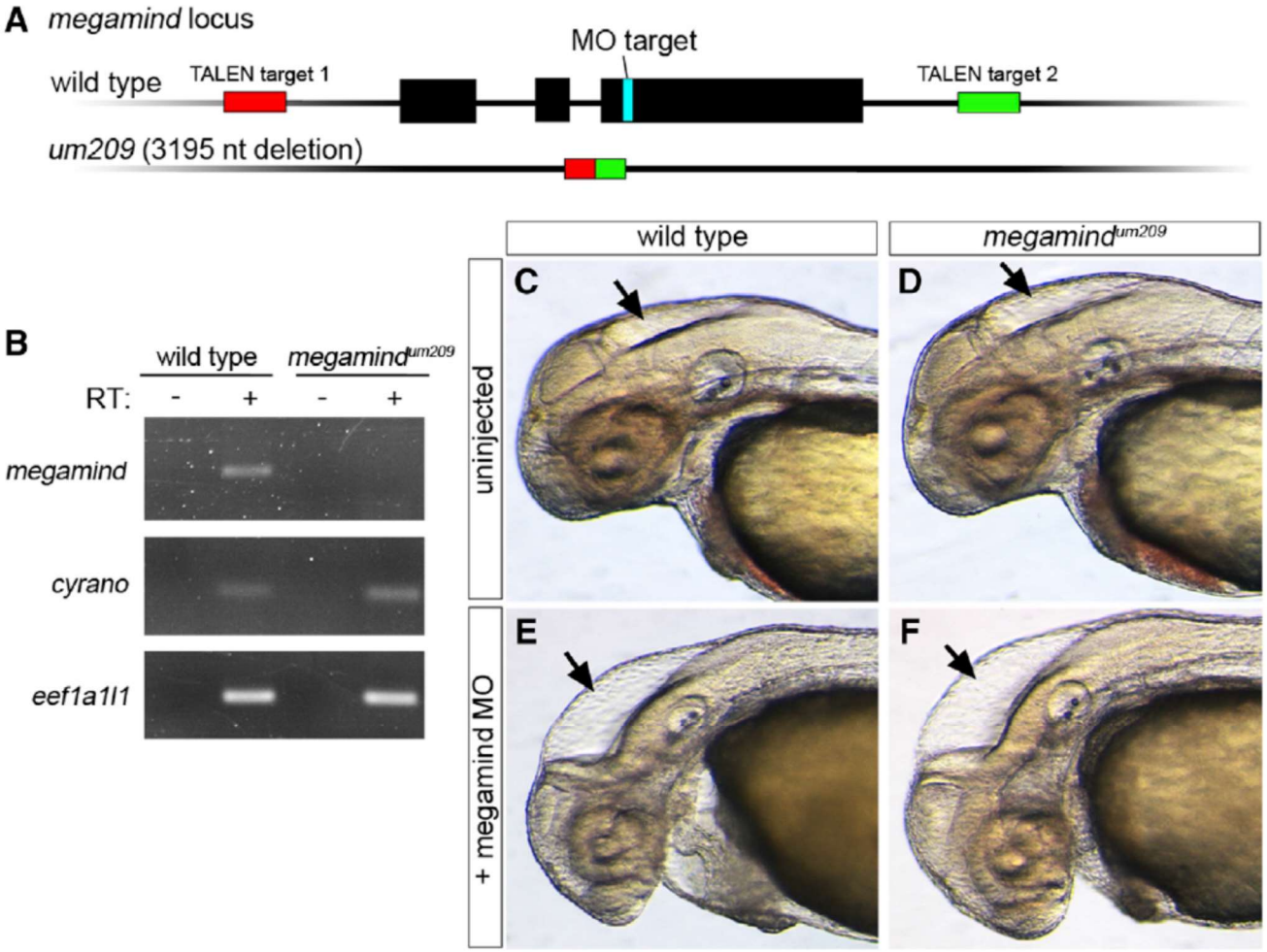


Figure 5. Normal Hindbrain Development in *megamind* Mutant Embryos

(A) Schematic of the *megamind* locus in wild-type and *megamind*^{um209} mutants. The red and green boxes indicate relative position of the TALEN target sequences, and the MO target sequence is shown.

(B) RT-PCR for the *megamind* and *cyrano* lincRNAs as well as *eef1al1* in wild-type and *megamind*^{um209} mutant embryos. RT refers to RNA template without (–) or with (+) reverse transcription.

(C–F) Transmitted light images of the head region in embryos at 48 hrpf. The hindbrain ventricle is indicated with an arrow in each image. Lateral views, anterior to the left, and dorsal is up. (C) Wild-type, uninjected; (D) *megamind*^{um209} mutant; (E) wild-type injected with 20 ng *megamind* conserved site MO; and (F) *megamind*^{um209} mutant injected with *megamind* conserved site MO.

Table 1. Inconsistencies between Morphant and Mutant Phenotypes

	Morphant			Mutant	
Gene	Phenotype	Rescue	Reference	Zygotic Phenotype	Source
Morphants with Specific Phenotypes					
flt4	loss of lymphatics, stalled ISVs	ND	(Covassin et al., 2006)	loss of lymphatics	um
ccbe1	loss of lymphatics	ND	(Hogan et al., 2009a)	loss of lymphatics	hu
gata2a	lack of trunk circulation at 48 hrpf	ND	(Fiedler et al., 2011)	lack of trunk circulation	um
amot	stalled ISVs	yes	(Aase et al., 2007)	normal	um
elmo1	stalled ISVs	yes	(Epting et al., 2010)	normal	um
ets1	stalled ISVs, no circulation at 48 hrpf	ND	(Pham et al., 2007)	normal	um
fmnl3	stalled ISVs	yes	(Hetheridge et al., 2012)	normal	um
mmp2	loss of lymphatics	ND	(Detry et al., 2012)	normal	hu
nrp1a	ISV stalling and mispatterning	ND	(Martyn and Schulte-Merker, 2004)	normal[a]	hu
pdgfrb	ISV stalling	yes	(Wiens et al., 2010)	normal	hu
Morphants with Overt Phenotypes					
bmp7a	dorsalized	ND	(Lele et al., 2001)	dorsalized	sa
ttna	decreased heartbeat, edema	ND	(Seeley et al., 2007)	loss of heartbeat	sa
dag1	dystrophic	ND	(Parsons et al., 2002a)	dystrophic[a]	sa

So? Does it mean that we can throw to the garbage all data from non-genetic techniques, such as siRNA, antisense morpholinos,…?

linc:birc6	hindbrain ventricle inflation	yes	(Ulitsky et al., 2011)	normal	um
appl1	necrosis, short yolk extension	yes	(Schenck et al., 2008)	normal[a]	sa
arrb1	severe delay and necrosis	yes	(Yue et al., 2009)	normal	sa
bmper	severe trunk malformation	ND	(Moser et al., 2007)	normal	sa
ccdc78	curved and shortened trunk	ND	(Majczenko et al., 2012)	normal	sa
cep290	cystic and slight curvature	ND	(Sayer et al., 2006)	normal	sa
col2a1a	curved trunk	ND	(Mangos et al., 2010)	normal	sa
hsp90ab1	shortened axis	ND	(Pei et al., 2007)	normal[a]	sa
glcci1	curved trunk	yes	(Nishibori et al., 2011)	normal	sa
hspg2	curved and shortened trunk	yes	(Zoeller et al., 2008)	normal	sa
htra1a	dorsalized	ND	(Kim et al., 2012a)	normal	sa
lratb	shortened and necrotic	yes	(Isken et al., 2007)	normal[a]	sa
itga2b	curved trunk	ND	(San Antonio et al., 2009)	normal	sa
nrp1a	curved trunk	ND	(Hillman et al., 2011)	normal[a]	sa
pcm1	curved, cystic	yes	(Stowe et al., 2012)	normal[a]	sa
mfn2	hindbrain ventricle inflation, curved trunk	yes	(Vettori et al., 2011)	normal	sa
ptenb	kinked trunk/tail	ND	(Croushore et al., 2005)	normal	sa
ptger4a	shortened axis	yes	(Cha et al., 2006)	normal	sa
usp33	CNS necrosis, curved trunk	ND	(Tse et al., 2009)	normal[a]	sa
zbtb4	dorsalized	yes	(Yao et al., 2010)	normal[a]	sa

ND, not determined; um, UMass Medical School; hu, Hubrecht Institute; and sa, Sanger ZMP.
[a]Possible maternal contribution.

Developmental Cell
Article

Reverse Genetic Screening Reveals Poor Correlation between Morpholino-Induced and Mutant Phenotypes in Zebrafish

Fatma O. Kok,[1,7] Masahiro Shin,[1,7] Chih-Wen Ni,[1,7,8] Ankit Gupta,[1] Ann S. Grosse,[1] Andreas van Impel,[2] Bettina C. Kirchmaier,[2,9] Josi Peterson-Maduro,[2] George Kourkoulis,[1] Ira Male,[1] Dana F. DeSantis,[1] Sarah Sheppard-Tindell,[1] Lwaki Ebarasi,[3,4] Christer Betsholtz,[3,4] Stefan Schulte-Merker,[2,5] Scot A. Wolfe,[1,6] and Nathan D. Lawson[1,*]

LETTER

-> Not quite….!!

doi:10.1038/nature14580

Genetic compensation induced by deleterious mutations but not gene knockdowns

Andrea Rossi[1]*, Zacharias Kontarakis[1]*, Claudia Gerri[1], Hendrik Nolte[1]†, Soraya Hölper[1], Marcus Krüger[1]† & Didier Y. R. Stainier[1]

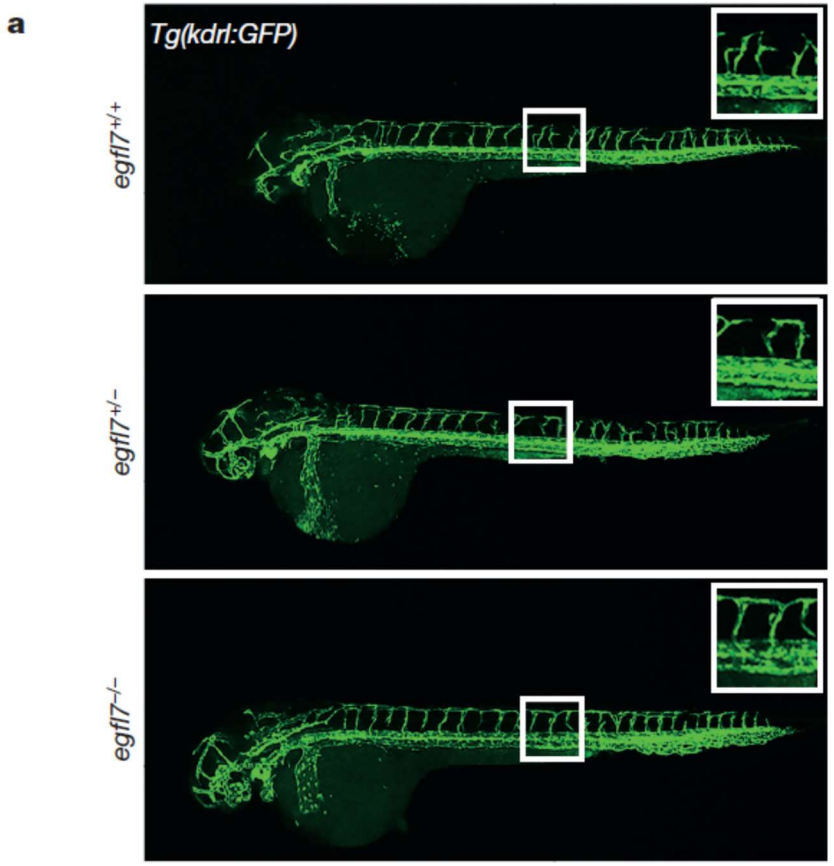


Figure 2 | Zebrafish egfl7 mutant embryos are less sensitive to egfl7 morpholino injections.

Hypothesis?

Emilin3a is upregulated in mutant but not in morphant or CRISPRi embryos.

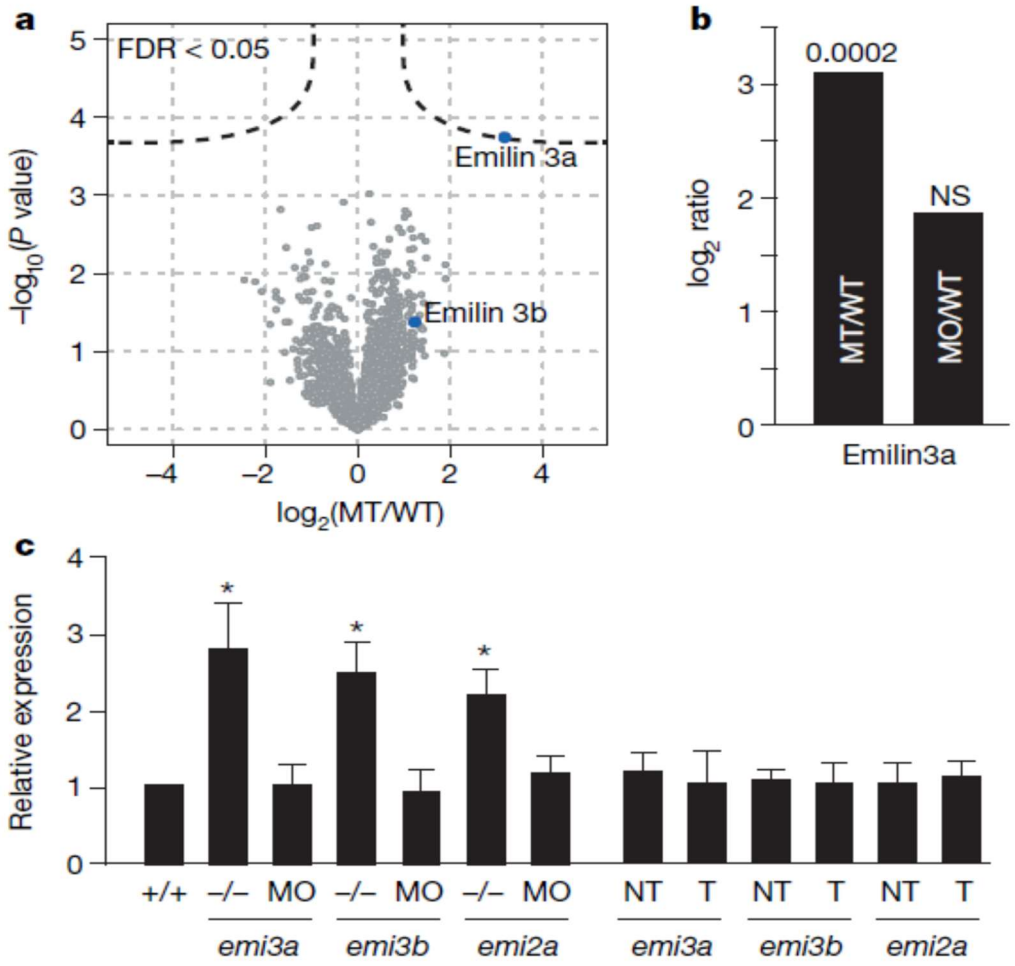


Figure 3 | Emilin3a is upregulated in mutant but not in morphant or CRISPRi embryos. **a**, Volcano plot showing significantly dysregulated proteins between 24 hpf *egfl7* WT and *egfl7*^{s981} mutant embryos using label-free quantification. Emilin3a and Emilin3b are highlighted in blue. **b**, Morphants did not show a significant upregulation of Emilin3a in unbiased mass-spectrometry-based proteomics comparing mutants, WT and morphants. A two-sided *t*-test was used to assess *P* values and FDR was controlled by a randomization-based SAM approach. **c**, mRNA expression of *emilin3a*, *emilin3b* and *emilin2a* in *egfl7* WT, mutant, morphant and CRISPRi (template and non-template strand) embryos at 20 hpf; qPCR data, pools of 20–30 embryos each, expression normalized to *gapdh* (WT expression set at 1 for each gene). The *emilin* genes were upregulated in *egfl7*^{s981} mutants but not after translational or transcriptional inhibition. *$P \leq 0.05$.

Emilin2 andEmilin3 can rescue egfl7 morphants

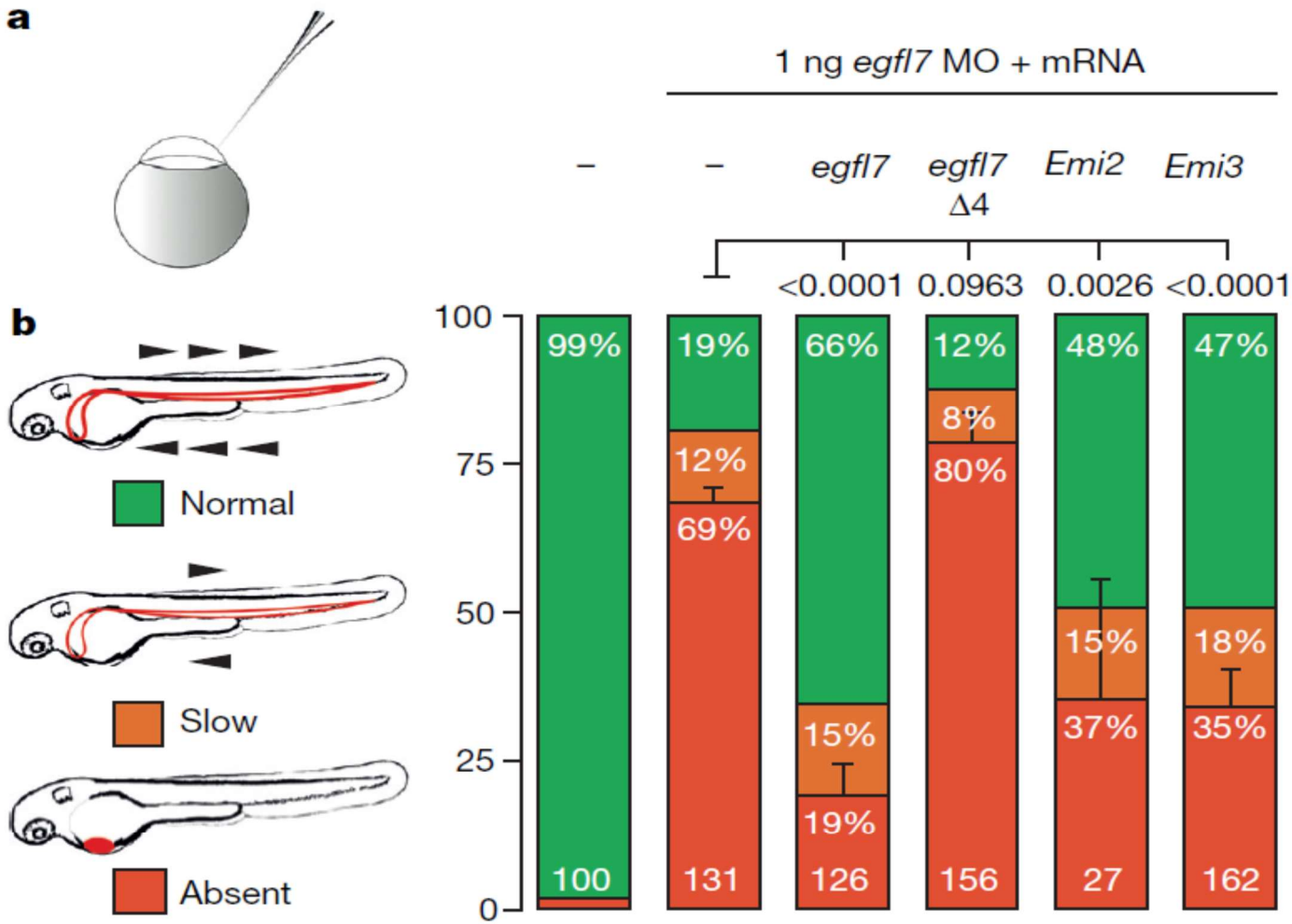


Figure 4 | *Emilin2* and *Emilin3* can rescue *egfl7* morphants. **a**, Design of the rescue experiment. One nanogram of *egfl7* MO was injected in WT embryos, alone or together with 400 pg of mRNA (*egfl7* WT, *egfl7 Δ4*, *Emilin2* or *Emilin3*). **b**, Injected embryos were sorted according to their circulatory loop phenotype into three classes: normal, slow and absent circulation. Injection of *egfl7* MO resulted in 69% of embryos lacking circulation at 48 hpf. This percentage was reduced to 19% when co-injecting *egfl7* mRNA, and to 37% and 35% when co-injecting *Emilin2* and *Emilin3* mRNA, respectively. In contrast, mRNA from the *egfl7 Δ4* mutant allele did not rescue (80% of embryos lacked circulation). Uninjected siblings are shown for comparison (99% normal). Number at the bottom of each bar is the total number of embryos from two independent experiments. Error bars, s.e.m. for the 'absent circulation' class. *P* value represents two-tailed value for Fisher's exact test.

specific effects. The mechanisms underlying the compensation observed in mutants but not in morphants are likely to be complex and so will their investigation. Interestingly, we observed no upregulation of the *emilin* genes in the *Δ3* (*s980*) allele, suggesting that a non-deleterious genomic lesion is not sufficient to trigger this response. On the other hand, we observed *emilin* gene upregulation in embryos injected with *egfl7* TALENs, indicating that a deleterious mutation does not need to go through the germline to trigger this response. We also detected *emilin* gene upregulation in embryos carrying only one $egfl7^{s981}$ mutant allele (data not shown). This observation might explain the partial protection of heterozygous embryos from *egfl7* MO injections (Fig. 2).

Be critical! Don’t shout with the crowd!

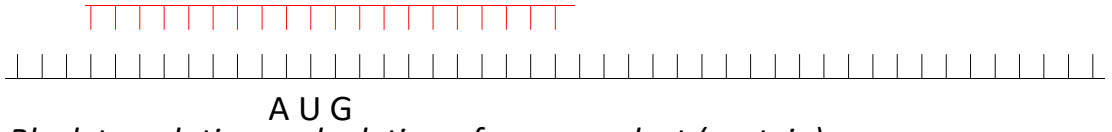
Be critical! Don’t shout with the crowd!

Let’s consider carefully the issue of specificity for loss-of-function / gene-manipulation techniques:

Possible issues with techniques based on hybridization:

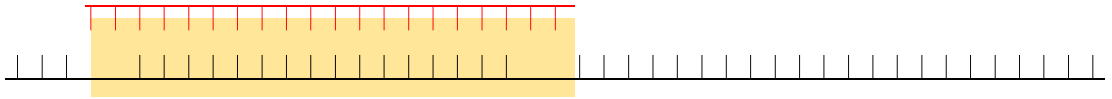
Targeted gene of interest:

Antisense Morpholinos (MO)



Block translation -> depletion of gene product (protein)

Non-specific hybridization with another mRNA -> interference



How to control for specificity of phenotype?

Possible issues with techniques based on hybridization:

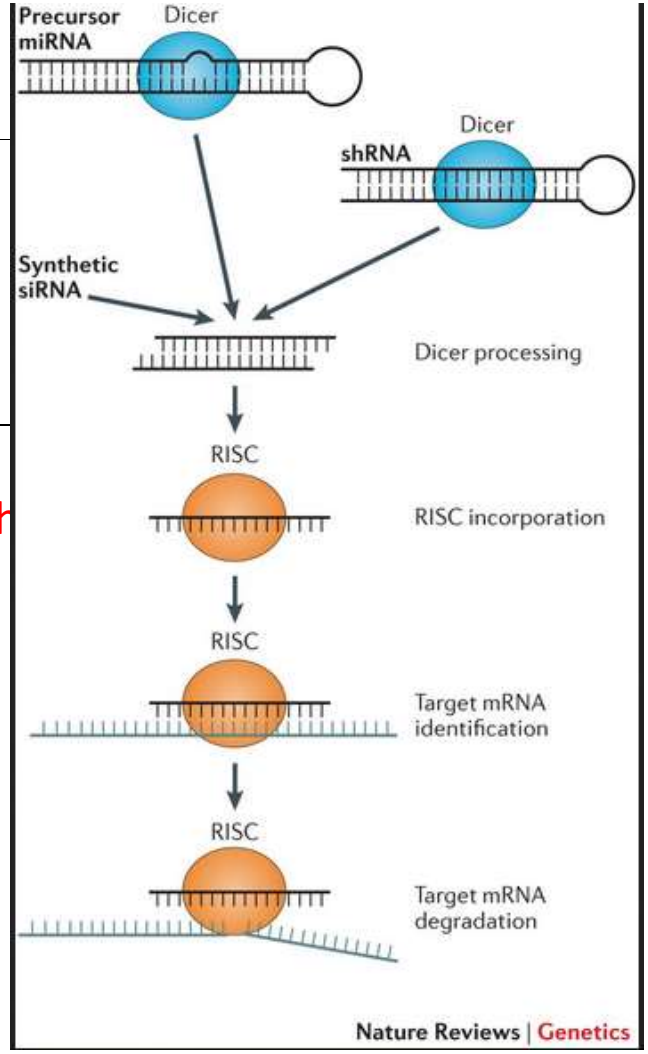
Antisense Morpholinos (MO)

mRNA

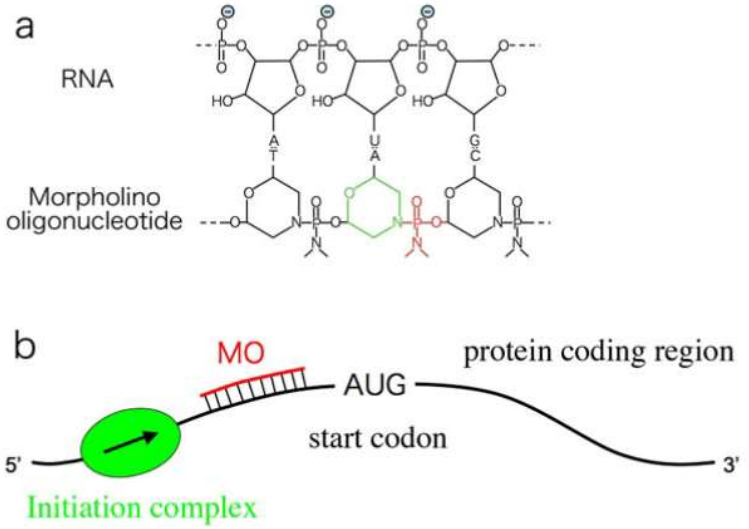
A U G

Block translation -> depletion of gene product (protein)

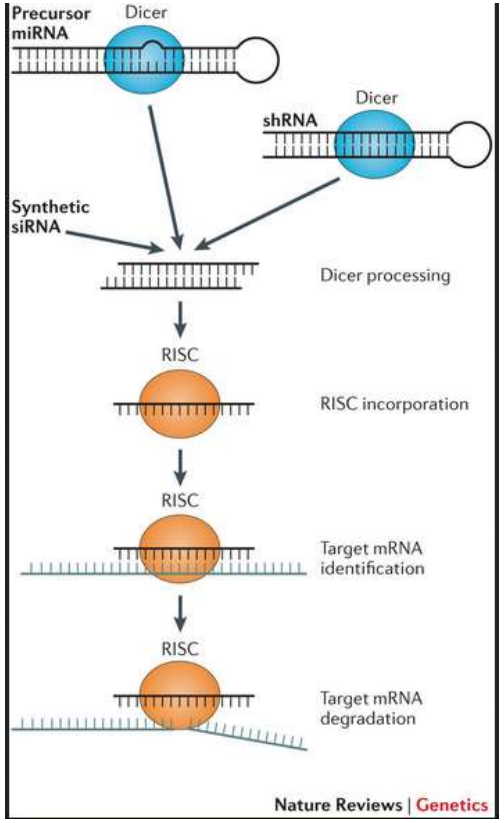
Is the situation different with RNAi (siRNA, sh



Possible issues with techniques based on hybridization:



So now what about CRISPR-Cas9?



Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements

Michael Kosicki, Kärt Tomberg & Allan Bradley

Nature Biotechnology **36**, 765–771 (2018)

GENOME EDITING

Not so CRISP(R)

The CRISPR–Cas9 system is thought to be a reasonably specific method for genome editing. But Bradley and colleagues now report that CRISPR–Cas9 induces extensive on-target mutagenesis in mouse and human cells, calling for greater caution when using it in clinical contexts and stressing the need for comprehensive genomic analyses before edited cells can be used in patients.

The authors analysed the allelic diversity induced by the introduction of Cas9 and single-guide RNAs that target introns and exons of *PigA* (an X-linked locus) in mouse embryonic stem cells (ESCs), as targeting the introns also led to the loss of PigA. Sequencing of PigA-deficient single-cell clones revealed that, in addition to creating the expected insertions and deletions (indels) of <50 bp, CRISPR–Cas9 generated deletions of >250 bp to 6 kb in more than 20% of alleles. Moreover, single-nucleotide polymorphisms, indels and large deletions and insertions that were non-contiguous with the Cas9 cut site were identified; such mutations would be missed if the analysis was limited to the vicinity of the cleavage site, as is often the case. In some cases, changes in parts of the genome distant from the target site were also found.

By editing and analysing other loci in different cell lines — immortalized human retinal pigment epithelial cells and progenitor cells isolated from mouse bone marrow — the authors confirmed that the extensive on-target damage, which is associated with DNA repair, is not an intrinsic property of mouse ESCs.

Thus, CRISPR–Cas9 can induce extensive on-target genomic rearrangements that could potentially lead to pathogenic lesions in clinically relevant cell types.

Kim Baumann

ORIGINAL ARTICLE Kosicki, M. et al. Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. *Nat. Biotechnol.* **36**, 765–771 (2018)
FURTHER READING Dominguez, A. A. et al. Beyond editing: repurposing CRISPR–Cas9 for precision genome regulation and interrogation. *Nat. Rev. Mol. Cell Biol.* **17**, 5–15 (2016)

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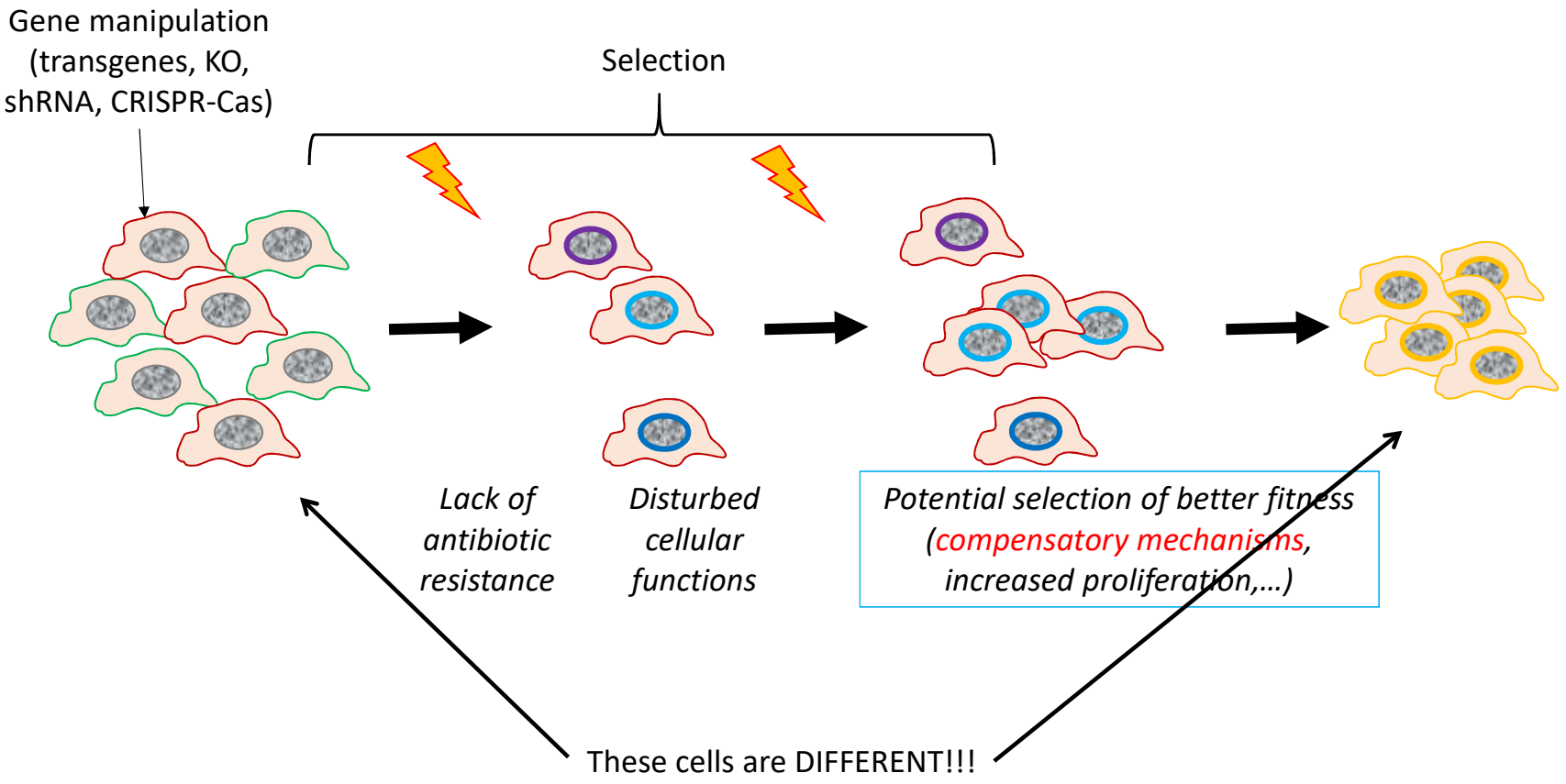
Back to compensation and adaptation:

A very general issue with gene manipulation!

Compensation/Adaptation

Potential issue when attempting stable gene manipulations

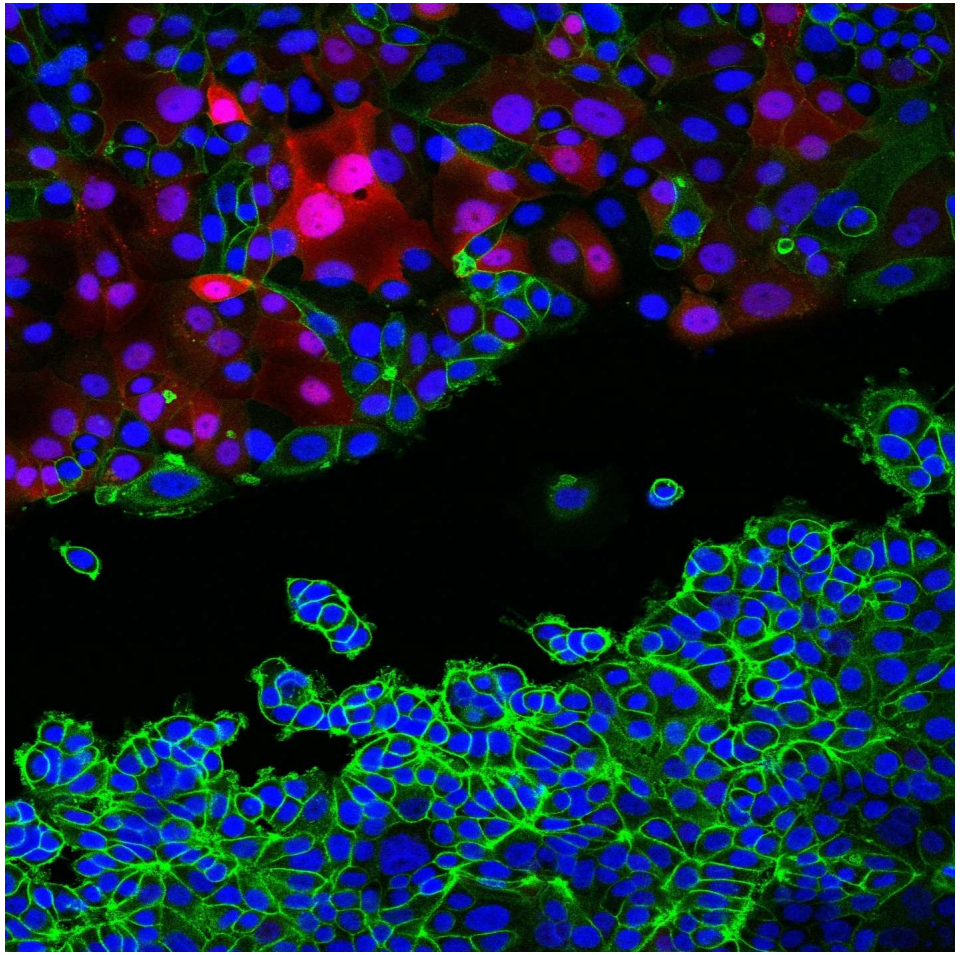


Example of adaptation: stable cell lines with EpCAM shRNA

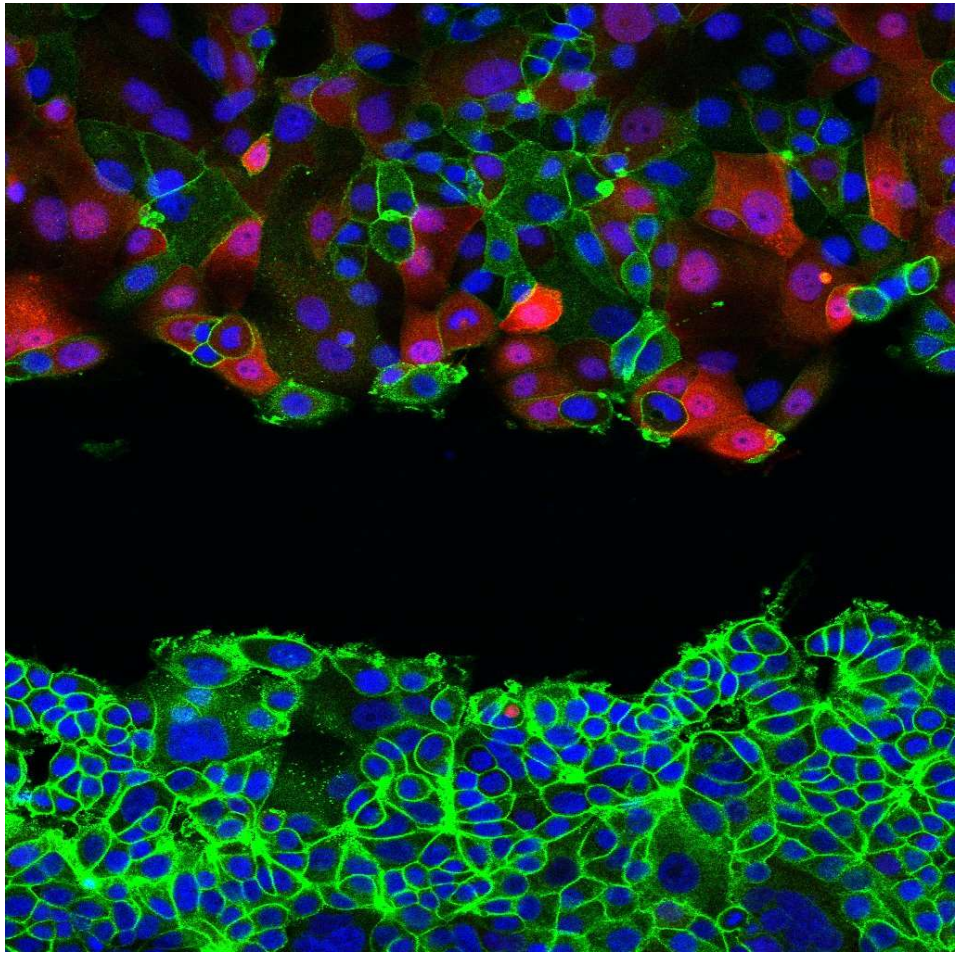
(Aslemarz and Fagotto, unpublished)

EpCAM
Nuclei
Reporter for sh plasmid

Stable EpCAM siRNA line (early passages) → Stable EpCAM siRNA line (later passage)



Wild type cells

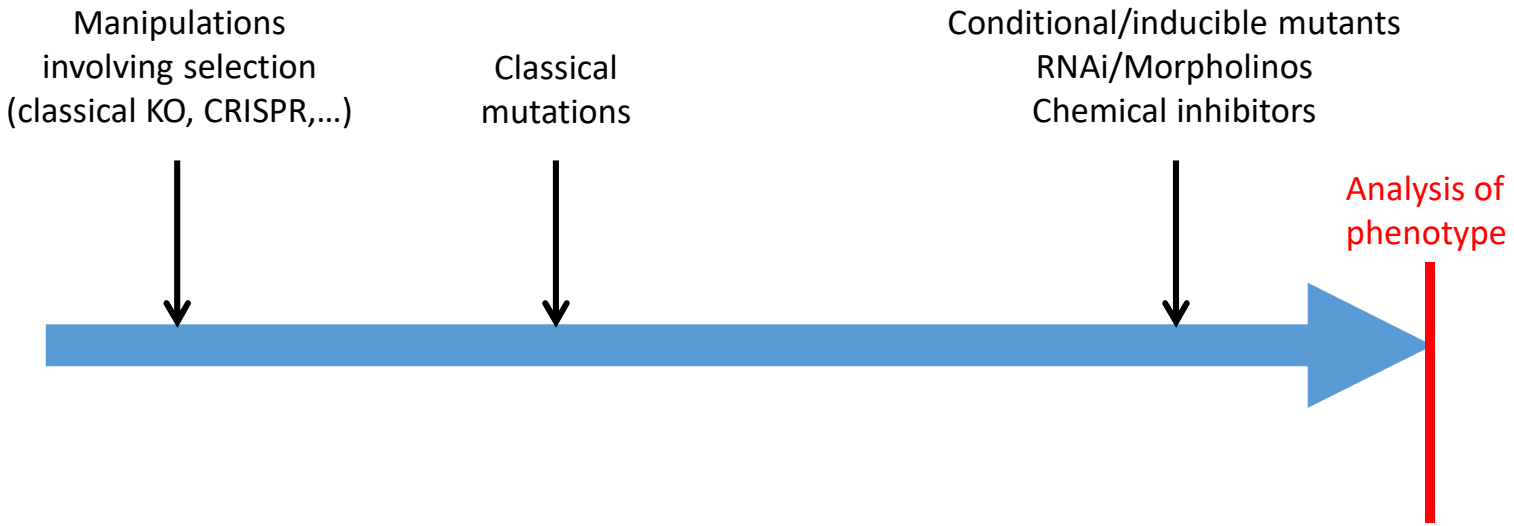


Wild type cells

Solutions?

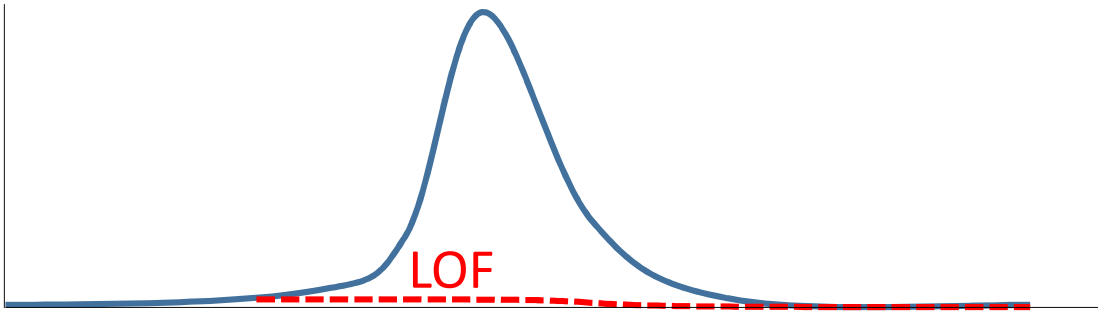
- Minimize chances for compensation/adaptation
  (+ pleiotropic/collateral/pervasive effects)

TIME!!

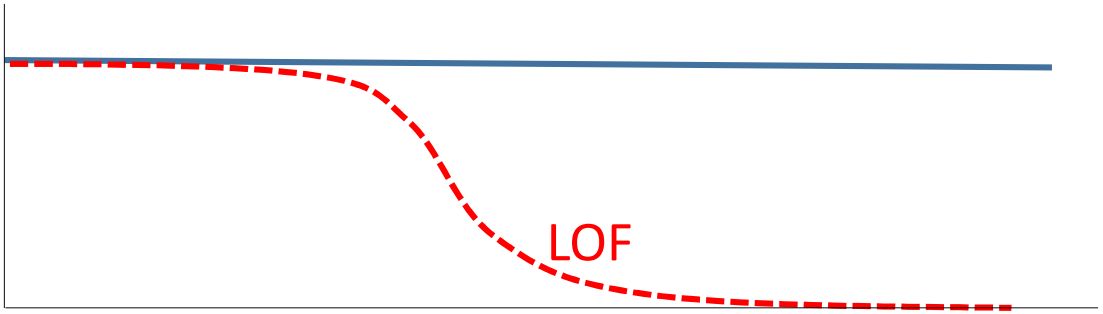


Some situations may be more favourable for interpretation of phenotype

Transient expression and/or activity



Constitutive expression and/or activity



Solutions?

- Refinement -> Maximize specificity of manipulation

-> when possible, try target a specific subcellular pool (e.g. chimeras, optogenetics)

SPACE!!!

Interpreting phenotypes in a complex world

Multiple parameters influence the relationship between a gene product and its partners

Need for precise definitions Regulators, integrators,…

Phenotypes and function:

1) The easy cases

2) Complex cases: Hidden functions

Redundancy, compensation, strength of LOF, limiting/not limiting, more or less sensitive

3) Pleiotropic effects

Affecting general functions

Affecting a molecular balance

Affecting a network of functions

Approaches for refined analysis of function

1) multilevel analysis (molecules to multicellular/organismal level)
2) Using the right systems: e.g. easy manipulation of multiple genes/functions (-> redundancy, …)
3) Minimizing the odds for compensation: Short term gene interference (avoid adaptation/compensation), temp sens mutants, conditional mutants, siRNA, morpholinos
4) Refining experimental manipulations:
    - Complement classical LOF/GOF with more specific interference approaches -> optogenetics, separation-of-function mutants
    - Fine manipulation of gene (activity): weak alleles, inducible/tunable LOF/GOF, titration (morpholinos,…)

None of these approaches are perfect, can still affect multiple functions, but significant improvement over classical LOF/GOF