Embryonic Left-Right Asymmetry:

A fundamental problem of patterning
at the intersection of cell, developmental, and evolutionary biology

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Outline for this session:

1) introduction to the meaning of asymmetry

2) basic facts which need explanation

3) clinical significance of asymmetry

4) history of the field and classical studies

5) in depth: the asymmetric gene pathway

6) midline

7) physiological early mechanisms

8) major open issues

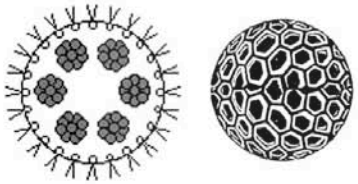
9) what's next?

Main points:

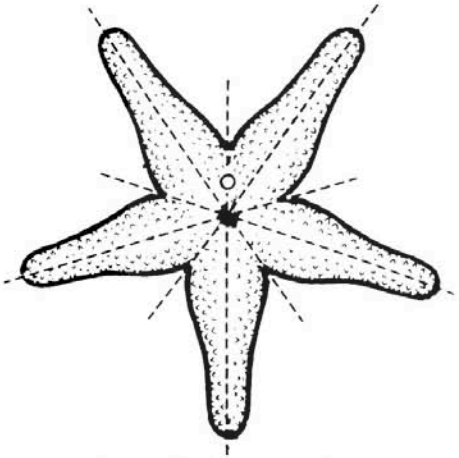
logic of addressing developmental questions, techniques,

evolutionary comparison of LR mechanisms

Types of Symmetry in the Animal Kingdom



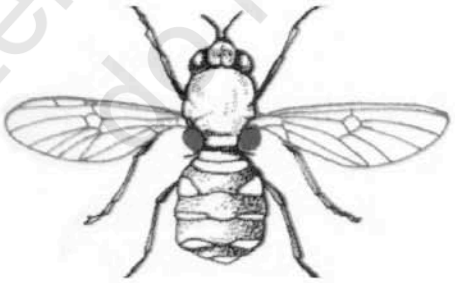
Spherical



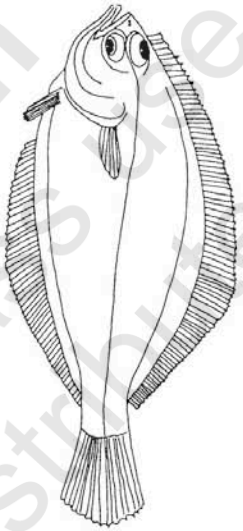
Five-fold radial

radial

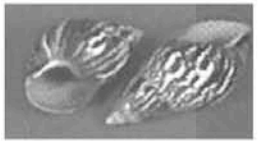
Pseudo-bilateral

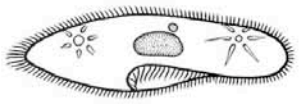


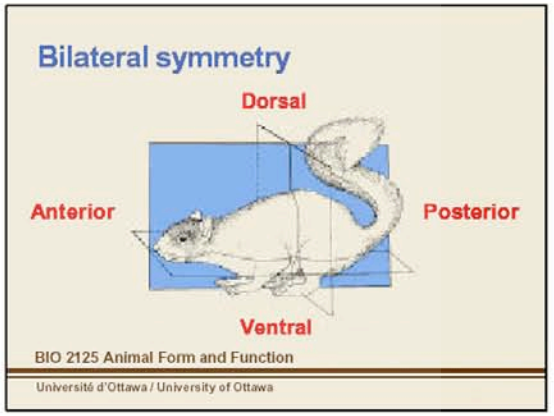
Bilateral



Chiral





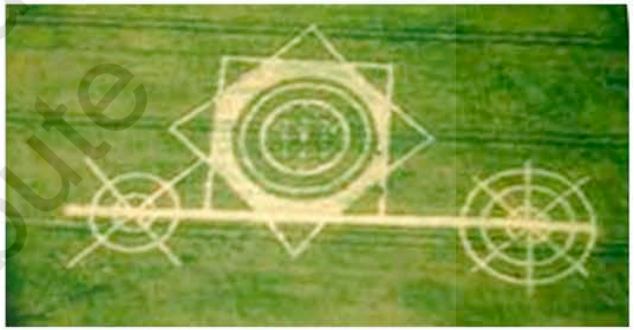


Left-right asymmetry is defined as a consistent difference in reflection across midline axis (not developmental noise).

How much, and what kind of information is needed to specify the LR axis?

Your alien friend sends you photos of his lawn. You realize you don't know whether you're seeing them correctly, or mirror image! You decide to settle the meanings of the words "left" and "right".

looking forward; feet pointing to center of planet; your left hand is the one that ???

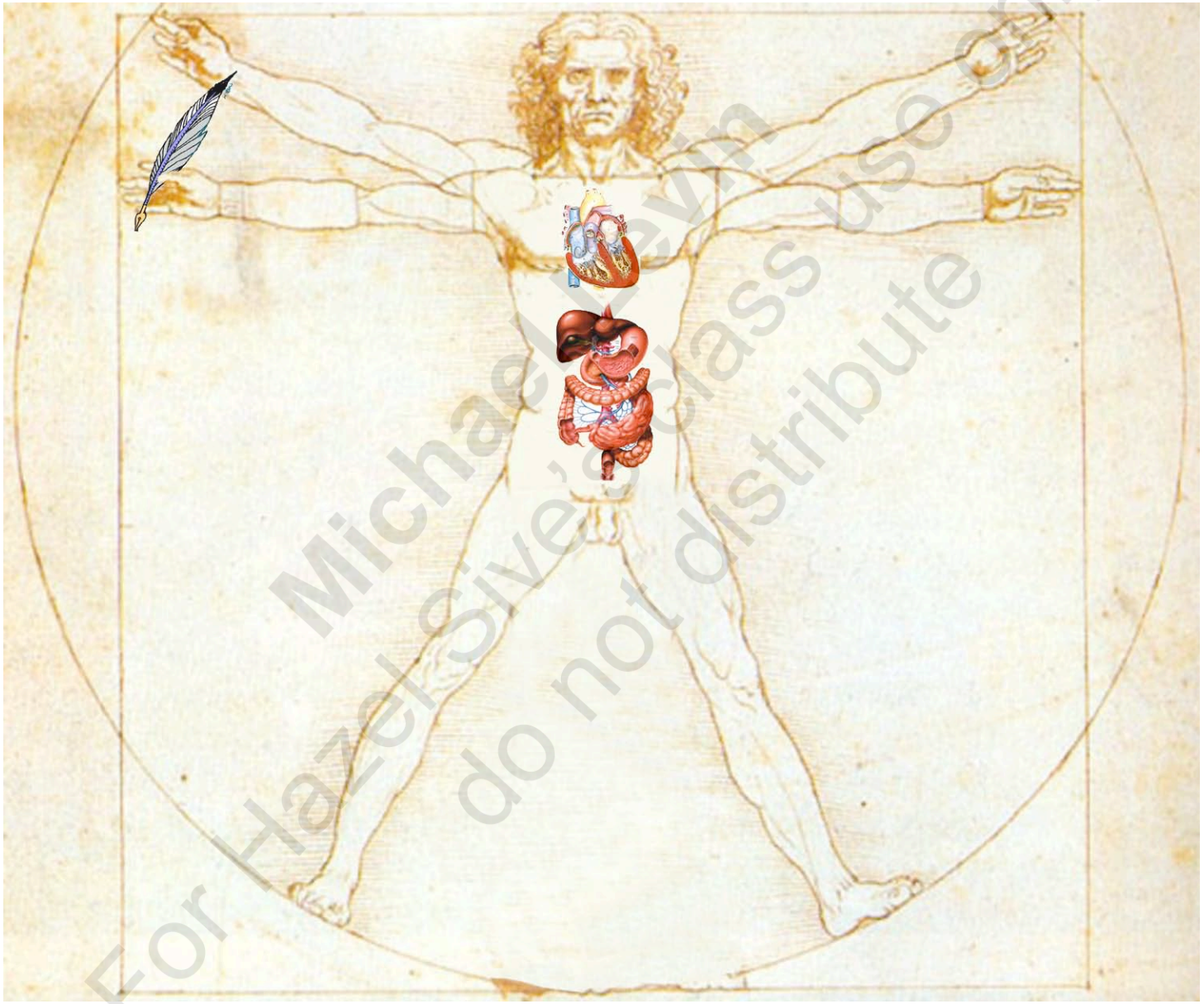


if your communication is purely verbal (no shared asymmetric objects in common), it's a very difficult problem.

(1) is chirality the same thing as asymmetry?

(2) is the LR axis a true graded axis or is it binary? L vs. R or degrees of medial vs. lateral positional information?

Human Laterality



Some basic facts about normal human asymmetry:

1) Heart, brain, viscera are consistently asymmetric in all normal individuals.

2) Human language is in left hemisphere, 90% right-handed preference (mice have random paw preference, great apes and parrots can approach >60% right-handers; primitive man left tools showing right-handedness as well. Likely link to evolution of language).

3) Brain asymmetry does not correlate with visceral asymmetry (people with full *situs inversus* or with primary ciliary diskinesia still language in L hemisphere, 90% right handed).

4) Immune sensitivity is higher on the left side.

5) Dermatoglyphics have consistent asymmetries (ridge-counts, etc.).

6) Left foot is larger in women, right foot larger in men.

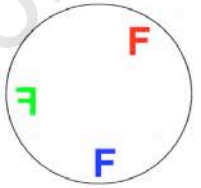
7) Conjoined twins exhibit visceral randomization.

9) Non-conjoined twins exhibit normal viscera but conservation of chirality in bookending - opposite sidedness of hair whorl, unilateral eye/tooth defects, etc.

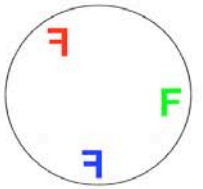
Ways Asymmetry can go Wrong

Asymmetry phenotypes:

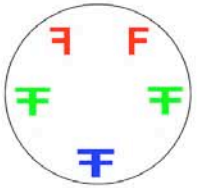
1. Situs solitus - normal situs - all organs on their correct side.



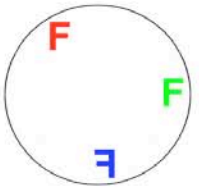
2. Situs inversus - complete mirror image reversal - all organs on the opposite side.



3. Isomerism - a loss of asymmetry - left and right sides identical.



4. Heterotaxia - loss of concordance - each organ makes independent decision; thus, phenotype is a statistical spectrum.



Keep in mind difference between an individual's phenotype and phenotype of population resulting from some condition!

Clinical applications of LR asymmetry:

1) Primary laterality syndromes - *situs inversus totalis,* heterotaxia, isomerism affect about >1 in 8,000 babies born to term. *Situs inversus totalis* has no serious medical problems. Others do.

2) Laterality defects accompany some other syndromes (holoprosencephaly, short-rib polydactily and renal-hepatic-pancreatic dysplasia syndromes).

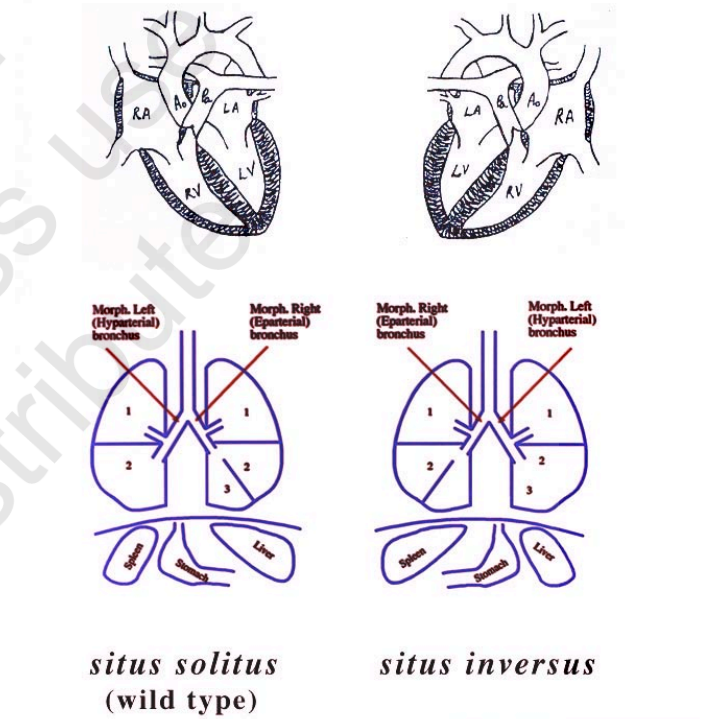
3) Some syndromes have a unilateral presentation in tissues which normally have no asymmetric characteristics (e.g., cleft lip, and Holt-Oram syndrome, which results in left-sided upper limb malformations).

4) Reversed cerebral asymmetry is associated with breast cancer.

5) In hermaphrodites with only 1 cell line, ovaries are on the left and testes are on the right.

situs inversus: mirror-image reversal of all LR-asymmetric structures

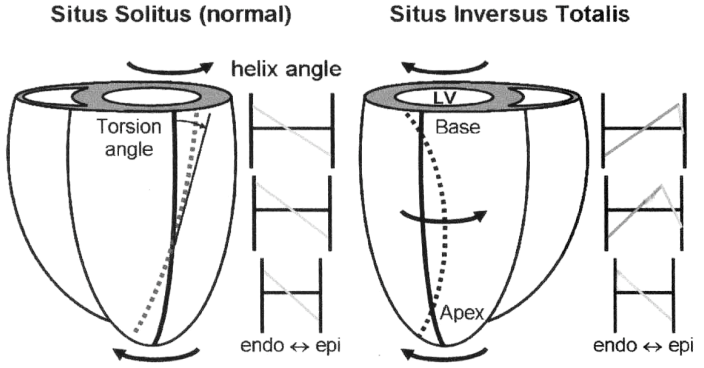
- 1/10 000 individuals



situs solitus (wild type)

situs inversus

(Courtesy of Cliff Tabin)



(Delhaas et al., 2004)

Laterality presents us with several profound mysteries in basic biology:

What are the implications of asymmetry for the normal structure and physiology of the heart, gut, and brain?

Why are all normal individuals not only asymmetric, but asymmetric to the same direction (i.e., why a consistent bias and not a 50%/50% mix)?

When, during evolution, did handed asymmetry appear?

Is it connected to chirality in lower forms (such as snail shell coiling and chirality in plants)?

At what developmental stages is asymmetry initiated in vertebrate embryos?

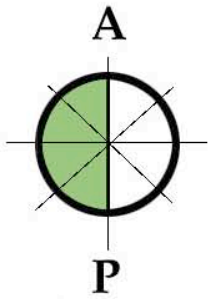
How conserved are the molecular mechanisms establishing correct asymmetry in animals with drastically different modes of gastrulation?

And, how can the left-right axis be consistently oriented with respect to the anterior-posterior and dorso-ventral axes in the absence of any macroscopic feature of chemistry or physics which distinguishes left from right?

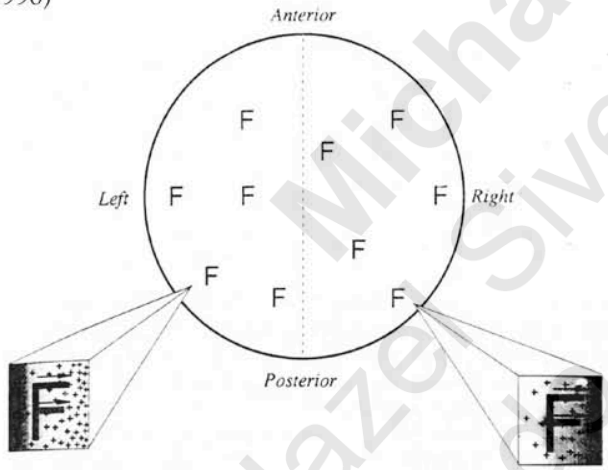
Theoretical candidates for mechanisms to align LR axis:

1) External influence
- **but most organisms develop the LR axis properly even when cultured separately from mother, or gravity, or other influences**

2) Pre-pattern in egg
- **this can work for C. elegans, maybe frog.**
- **but mouse blastomeres can be scrambled, added to, etc., and result in LR-normal mice**



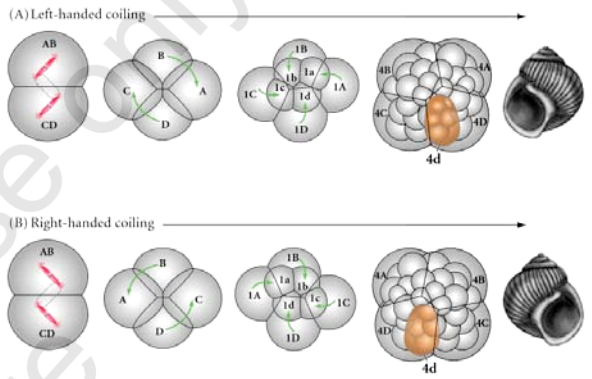
3) F-shaped molecule
(Brown & Wolpert, 1990)



4) Physics of electromagnetic fields
- **(Huxley & DeBeer, 1930's)**
- **no evidence for such a mechanism**

State of the field before molecular markers (< mid-1990's):

1. **Catalog of Morphological Asymmetries ("Animal Asymmetry" by A. C. Neville, 1976).**
2. **Genetics of snail chirality - maternal cytoplasmic product** →



3. **Mutants:**

Name	Species	Phenotype	Reference
Mgat1 k.o.	mouse	randomized turning and heart	Metzler et al., 1994
Fused toes	mouse	randomized turning, heart ok	Hoeven et al., 1994
inv	mouse	100%, all organs inverted	Yokoyama et al., 1993
iv	mouse	50%, all organs inverted	Brueckner et al., 1989
legless	mouse	right limb defects	Singh et al., 1991
heterotaxia	man	independent situs of all organs	Afzelius, 1976
Dh	mouse	high incidence of organ reversal	Carter, 1954
Hyd	rat	total situs inversus	Koto, 1987
Py	mouse	right limb defects	Kochar, 1977

4. **Drug Effects:**

Drug	Drug type	Species	Phenotype	Reference
Cadmium		rat	left limb deformities (!)	Barr, 1973
Cadmium		mouse	right limb deformities	Layton, 1979
Acetazolamide	carbonic anhydrase inhibitor	rat	right limb deformities	Layton and Hallesy, 1965
MNNG	alkylating agent	mouse	left ectodactyly	Inouye et al., 1978
Xyloside	proteoglycan synthesis inhibitor	frog	no cardiac looping	Yost, 1990
Nitrous oxide	anesthetic	rat	inverted organs	Fujinaga et al., 1990
Phenylephrine	adrenergic agonist	rat	inverted organs	Fujinaga et al., 1991
Nitrofurazone	antimicrobial	rat	right-sided hypoplasia	Greenaway et al., 1986

5. **Manipulation of cortical rotation and ECM migration in Xenopus randomizes LR (Yost, 1990-1992)**

Progress of the LR Asymmetry field over the last century

This field is unique in that very little had been known before 1990

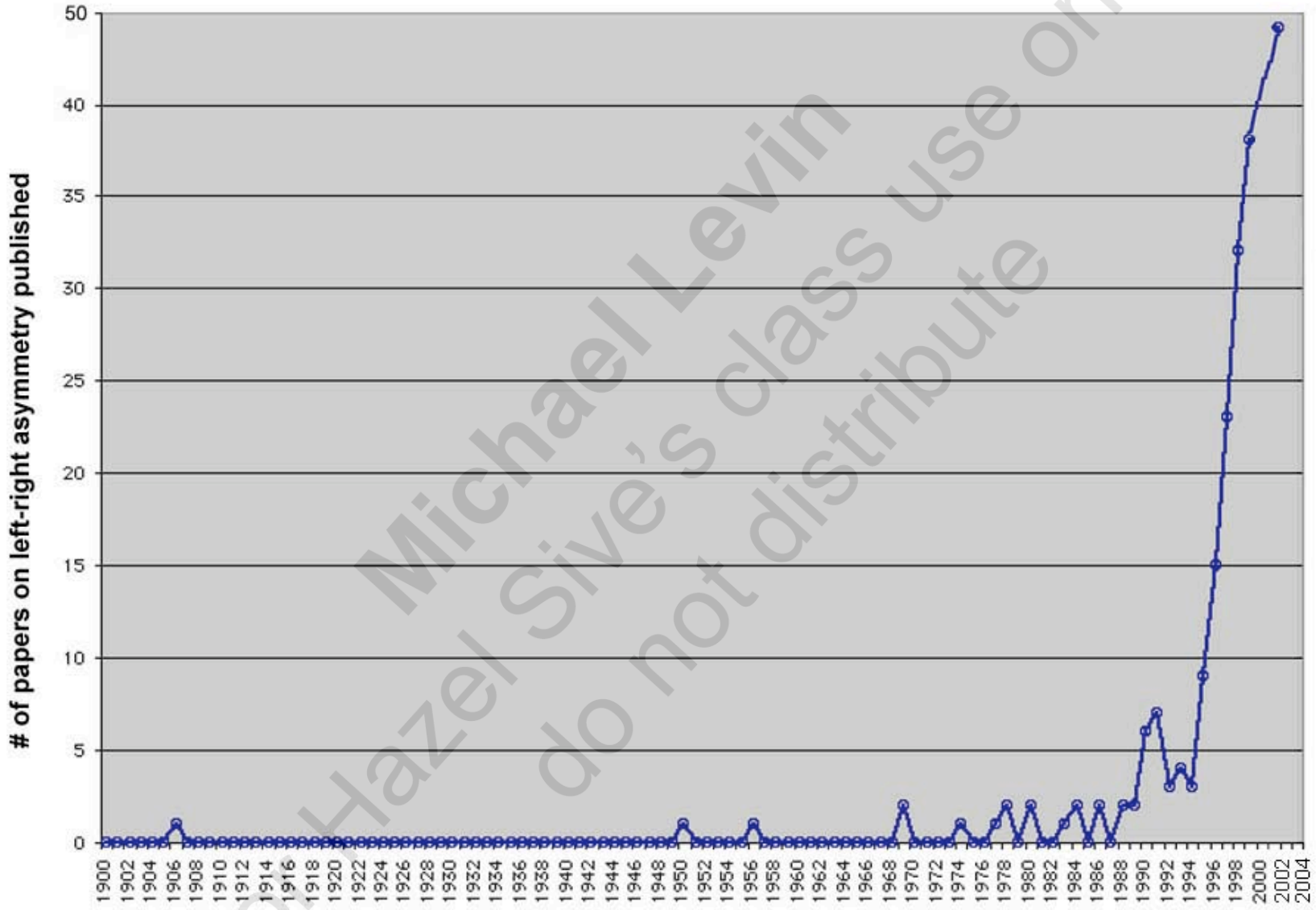


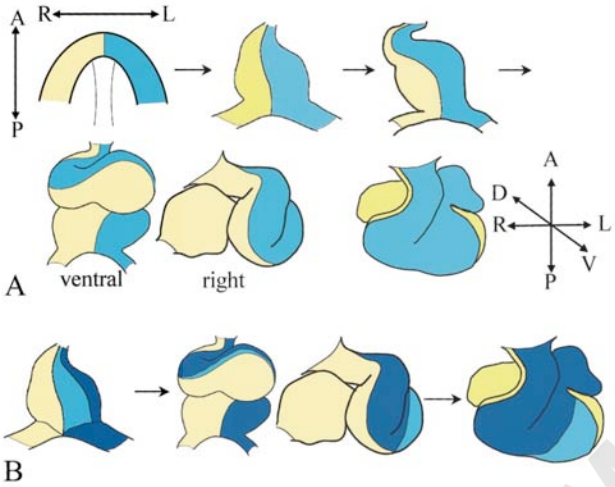
Table 1: Asymmetrically expressed genes in embryos which have been the focus of a paper on LR asymmetry

Gene	Species	Product/Role	Side	Reference
lefty	Mouse, chick, frog	TGF-β-family signaling molecule	Left	(Meno et al., 1996; Meno et al., 1998; Branford et al., 2000; Cheng et al., 2000; Essner et al., 2000)
Activin–βB	chick	TGF-β-family signaling molecule	Right	(Levin et al., 1997)
cAct-RIIa	chick	Activin receptor	Right	(Levin et al., 1995)
Shh	chick	Signaling molecule	Left	(Levin, 1995)
cSnR	chick	Zinc finger protein	Right	(Isaac et al., 1997)
Nodal	chick, mouse, frog	TGF-β-family signaling molecule	Left	(Levin et al., 1995; Collignon et al., 1996; Lowe et al., 1996a; Lohr et al., 1997; Morokuma et al., 2002)
cPTC	chick	Shh Receptor	Left	(Levin, 1998b; Pagan-Westphal and Tabin, 1998)
Cerberus	chick	Signaling molecule	Left	(Zhu et al., 1999)
BMP-4	Zebrafish, chick	BMP family signaling molecule	Left	(Chen et al., 1997; Monsoro-Burq and LeDouarin, 2000)
Pitx-2	chick, frog, mouse	Transcription factor	Left	(Logan et al., 1998; Ryan et al., 1998; Morokuma et al., 2002)
NKX3.2	chick, mouse	Transcription factor	Left in chick, Right in mice!	(Schneider et al., 1999)
Follistatin	chick	Signaling molecule	Right	(Levin, 1998a)
FGF-8	chick	Growth factor	Right	(Boettger et al., 1999)
flectin[†]	chick	Extra-cellular matrix molecule	Left	(Tsuda et al., 1996)
dHAND	chick, mouse, frog	bHLH transcription factor	Right	(Srivastava, 1995; Angelo et al., 2000)
eHAND	chick, mouse, frog	bHLH transcription factor	Left	(Cserjesi et al., 1995; Srivastava, 1995; Biben and Harvey, 1997; Sparrow et al., 1998; Angelo et al., 2000)
Caronte	Chick	Cerberus/DAN family member	Left	(Yokouchi et al., 1999)
N-Cadherin	Chick	Adhesion molecule	Right node, Left groove	(Garcia-Castro et al., 2000)
Cx43	Chick	Gap junction protein	Right	(Levin and Mercola, 1999)
Islet-1	Chick	LIM homeobox gene	Left	(Yuan and Schoenwolf, 2000)
H^+/K^+-ATPase	Frog, chick	H^+ and K^+ ion pump	Right	(Levin et al., 2002)
PKI–α	Chick	PKA inhibitor	Right	(Kawakami and Nakanishi, 2001; Rodriguez-Esteban et al., 2001)
NCX-1	Chick, mouse	Sodium-Calcium exchanger	Right	(Linask et al., 2001)
HoxC-8	Frog	Transcription factor	Left	(Thickett and Morgan, 2002)
Xin	Mouse	?	Right	(Wang et al., 1999)
Southpaw	Zebrafish	TGF-β family	Left	(Long et al., 2003)
cMid-1	Chick	Microtubule-associated protein	Right	(Granata and Quaderi, 2003)

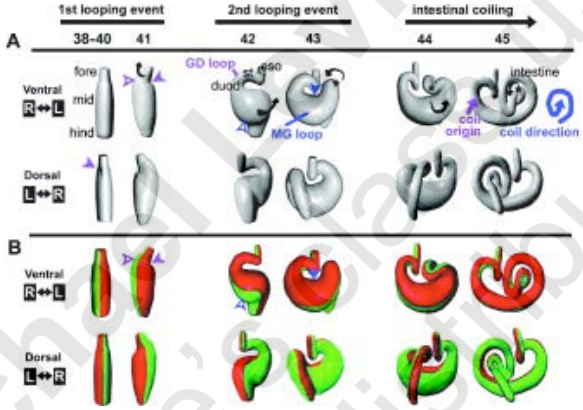
Table 2: genes involved in LR asymmetry but not asymmetrically expressed

Gene	Species	Product/Role	Reference
lv	mouse	Dynein (cytoplasmic transport or ciliary motor)	(Lowe et al., 1996b; Supp et al., 1997; Supp et al., 1999; Supp et al., 2000)
Inv	mouse	?	(Mochizuki et al., 1998; Morgan et al., 1998; Morgan et al., 2002)
Vg-1	frog	TGF–β–family signaling molecule	(Hyatt et al., 1996; Hyatt and Yost, 1998)
Connexins	frog, chick, man	System of gap-junctional cell-cell signaling	(Britz-Cunningham et al., 1995; Levin and Mercola, 1998; Levin and Mercola, 1999)
No turning	mouse	Midline patterning	(Melloy et al., 1998)
SIL	mouse	Midline patterning	(Izraeli et al., 1999)
KIF-3	mouse	Component of ciliary motor	(Nonaka et al., 1998; Takeda et al., 1999)
Polaris	Mouse	?	(Murcia et al., 2000)
HFH-4	Mouse	Transcription factor	(Chen et al., 1998; Brody et al., 2000)
Lin-12	C. Elegans	Notch signaling molecule	(Hermann et al., 2000)
Delta-1	Mouse	Notch signaling molecule	(Przemeck et al., 2003)
Notch	Mouse, zebrafish	Notch signaling molecule	(Krebs et al., 2003; Raya et al., 2003)
Smo	Mouse	Membrane protein involved in *hedgehog* signaling	(Zhang et al., 2001)
Ihh	Mouse	Member of *hedgehog* signaling proteins	(Zhang et al., 2001)
GDF-1	Mouse	TGF–β–family signaling molecule	(Rankin et al., 2000)
Lrd	Mouse	Dynein	(Supp et al., 1997; Supp et al., 1999)
DNAH5	Human	Dynein	(Ibanez-Tallon et al., 2002; Olbrich et al., 2002)
PCKD-2	Mouse	Polycistin-2 ion channel	(Pennekamp et al., 2002)
ZIC3	Human, mouse, frog	Zinc-finger protein	(Gebbia et al., 1997; Kitaguchi et al., 2000; Purandare et al., 2002)
EGF-CFC	Mice, fish	Extracellular receptor	(Yan et al., 1999a)
Furin	Mice	Proprotein convertase	(Roebroek et al., 1998; Constam and Robertson, 2000)
Brachyury	Mice	Transcription factor	(King et al., 1998)
Ednrb	Mice	*Piebald* deletion complex	(Welsh and O'Brien, 2000)
Rotatin	Mice	Transmembrane protein	(Faisst et al., 2002)
PDI-P5	Zebrafish	Protein disulfide isomerase	(Hoshijima et al., 2002)
Pol–λ	Mouse	DNA polymerase	(Kobayashi et al., 2002)
PA26	Human	Sestrin-family	(Peeters et al., 2003)
Cryptic	Mouse, human, zebrafish	EGF-CFC gene	(Gaio et al., 1999; Yan et al., 1999b; Bamford et al., 2000)

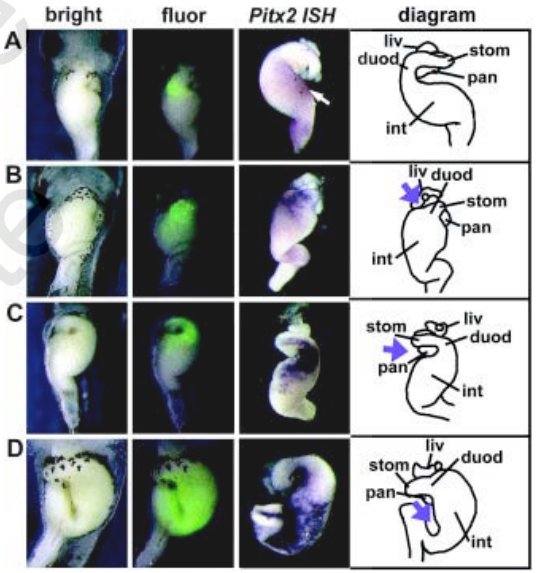
What do we know about the last phase (organogenesis)?



Muller et al., 2003



Campione et al., 2001

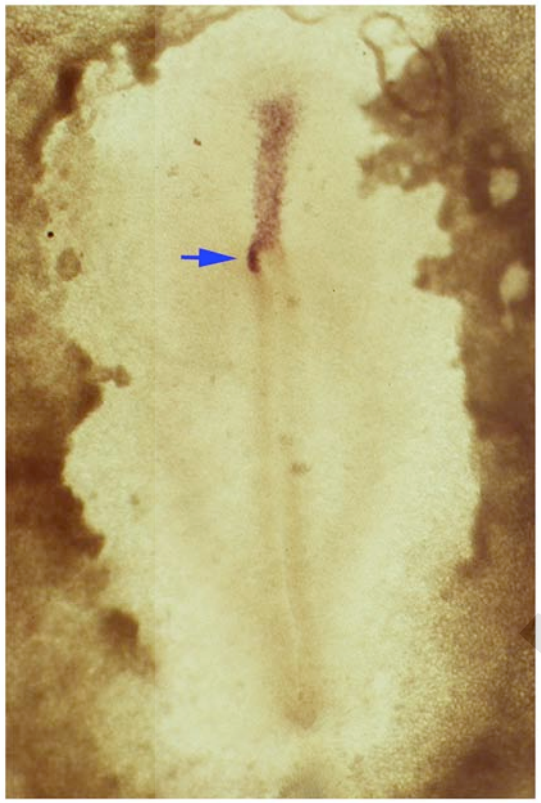


How might asymmetric morphogenesis be achieved?

Differential cues on L vs. R sides give rise to asymmetric organs via different migration, proliferation, and tensile forces

Characterization of the Asymmetric Gene Pathway

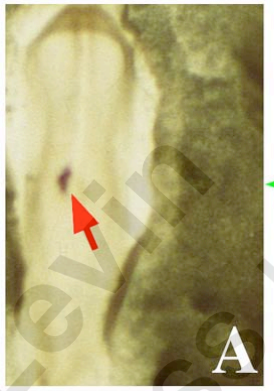
Shh

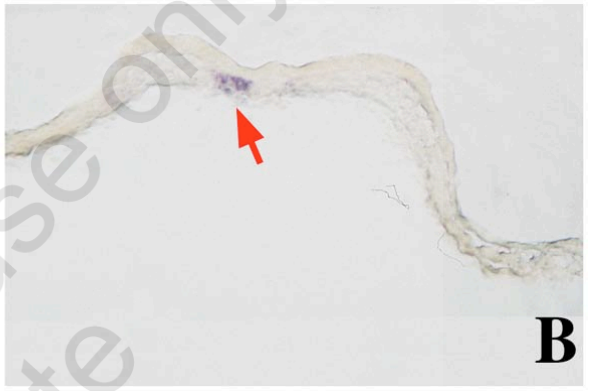


stage 5

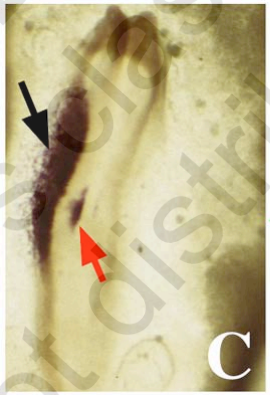
Nodal

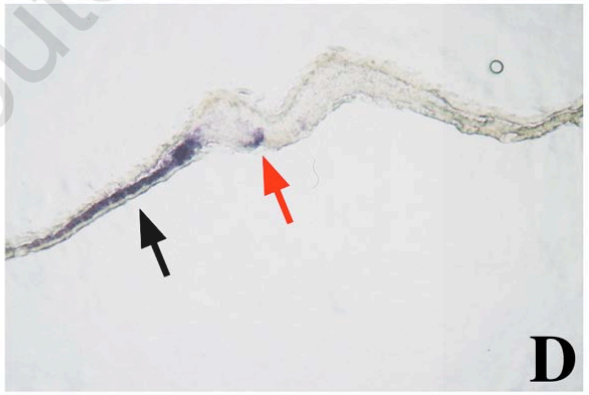
st. 6+



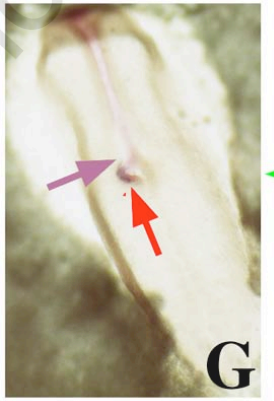


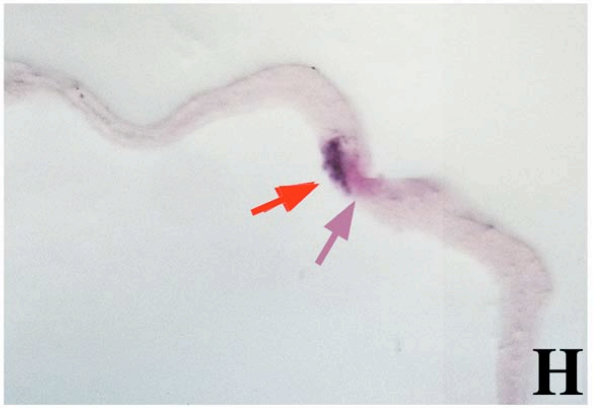
st. 8





st. 6+





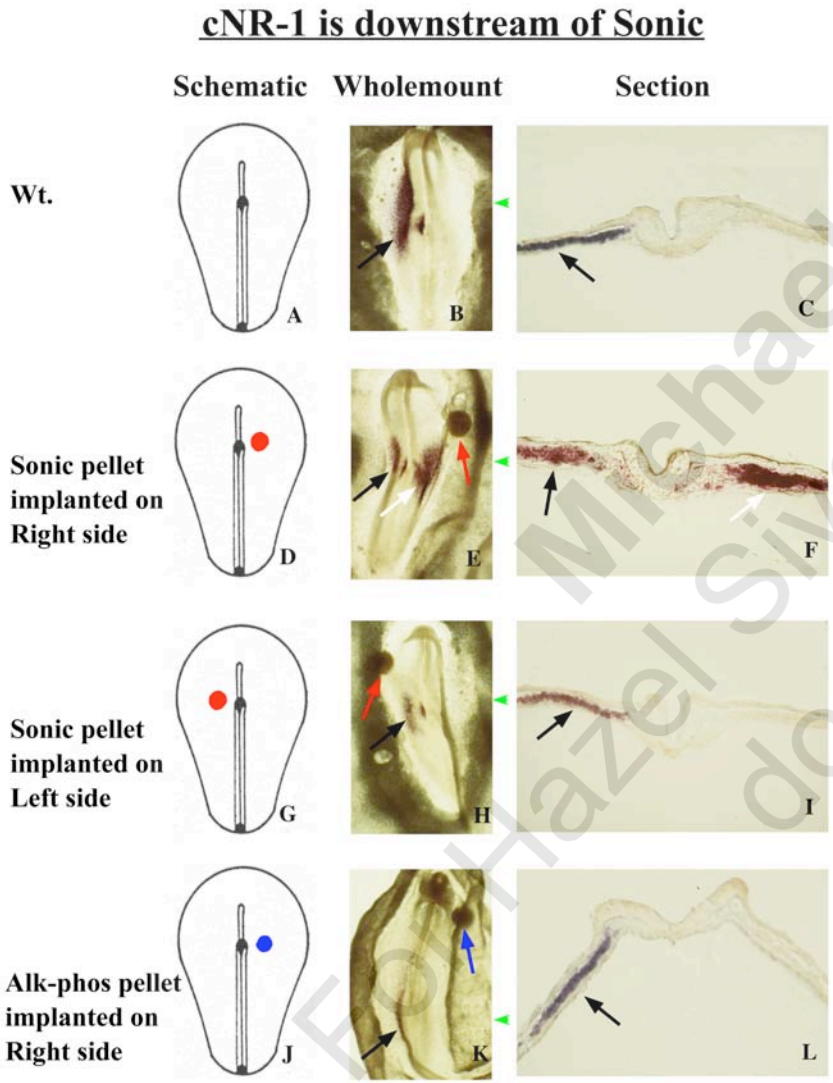
Wholemount

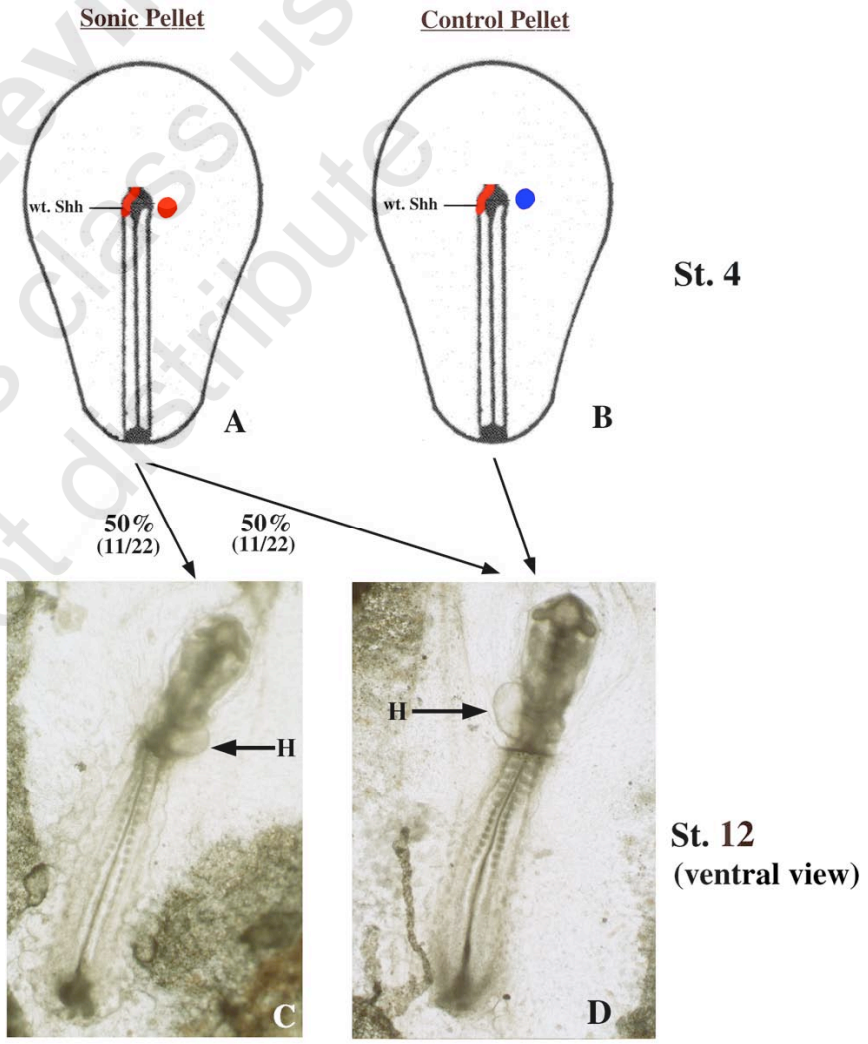
Section

What hypothesis might you formulate?

Gain-of-function experiment to test induction and importance for organ *situs*

(retroviral misexpression)

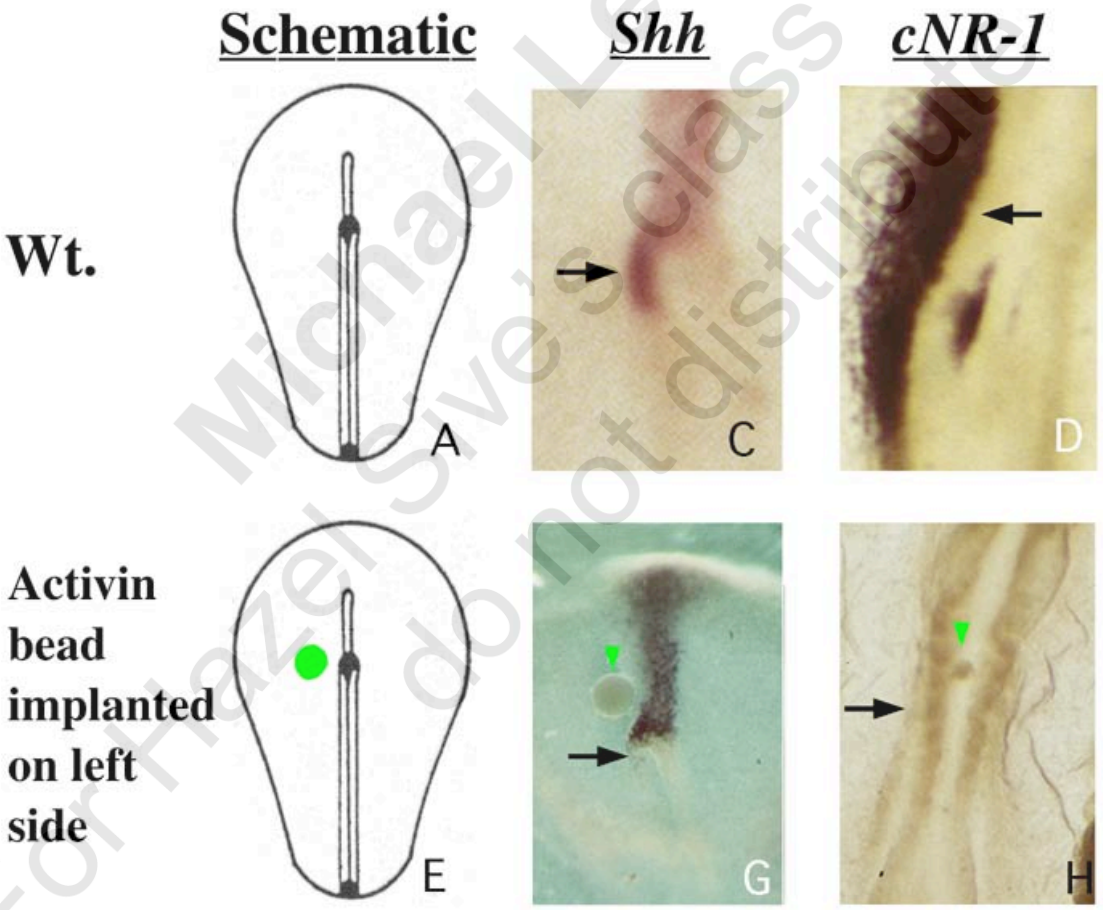


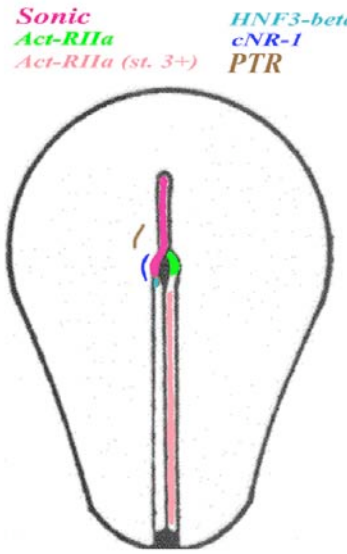


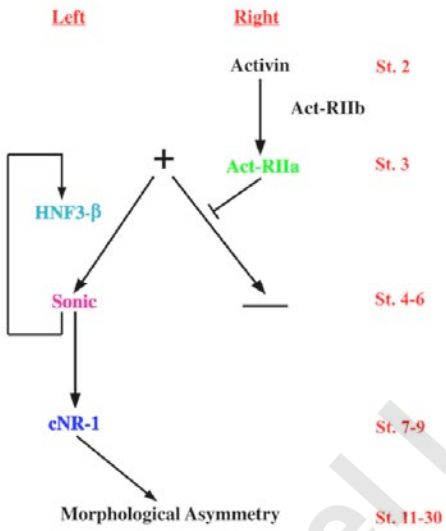
Necessity: is Shh needed for left-sided Nodal expression?

(use activin as a way of shutting off *Shh* on the left)

Shh induces cNR-1 endogenously

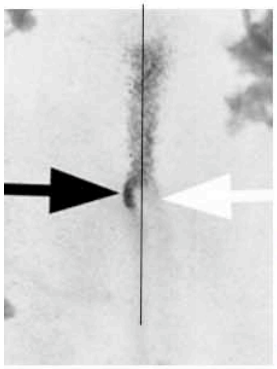


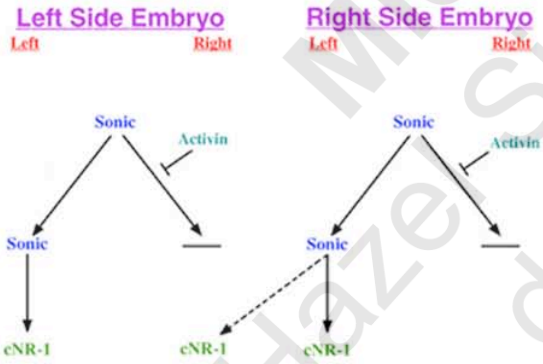


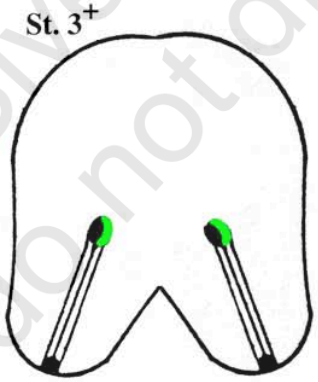


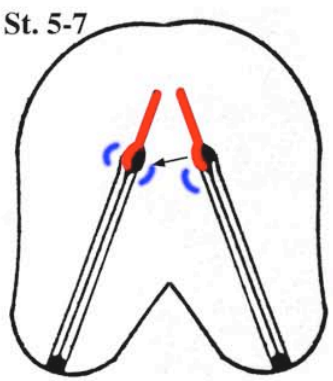
This pathway

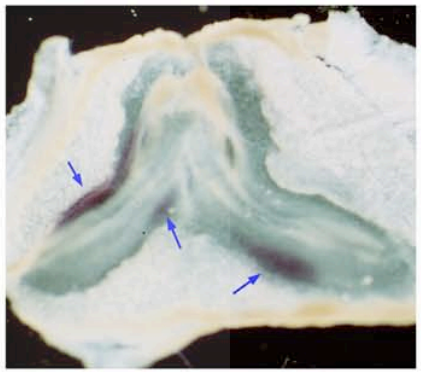
1) Raises issue of midline barrier
2) Explains *situs* inversions in conjoined twins











So, having developed the pathway, what would you like to know now?

Main focus of rest of the talk: early steps

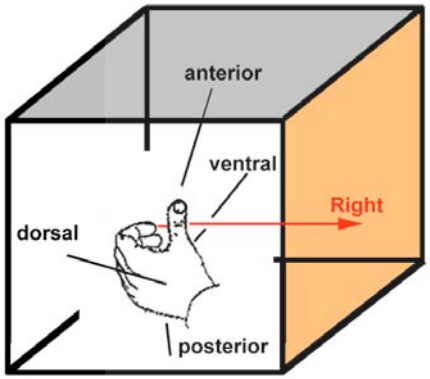
1) Illustrate some unusual approaches in developmental biology involving molecular biology physiology, biophysics, pharmacology, etc.

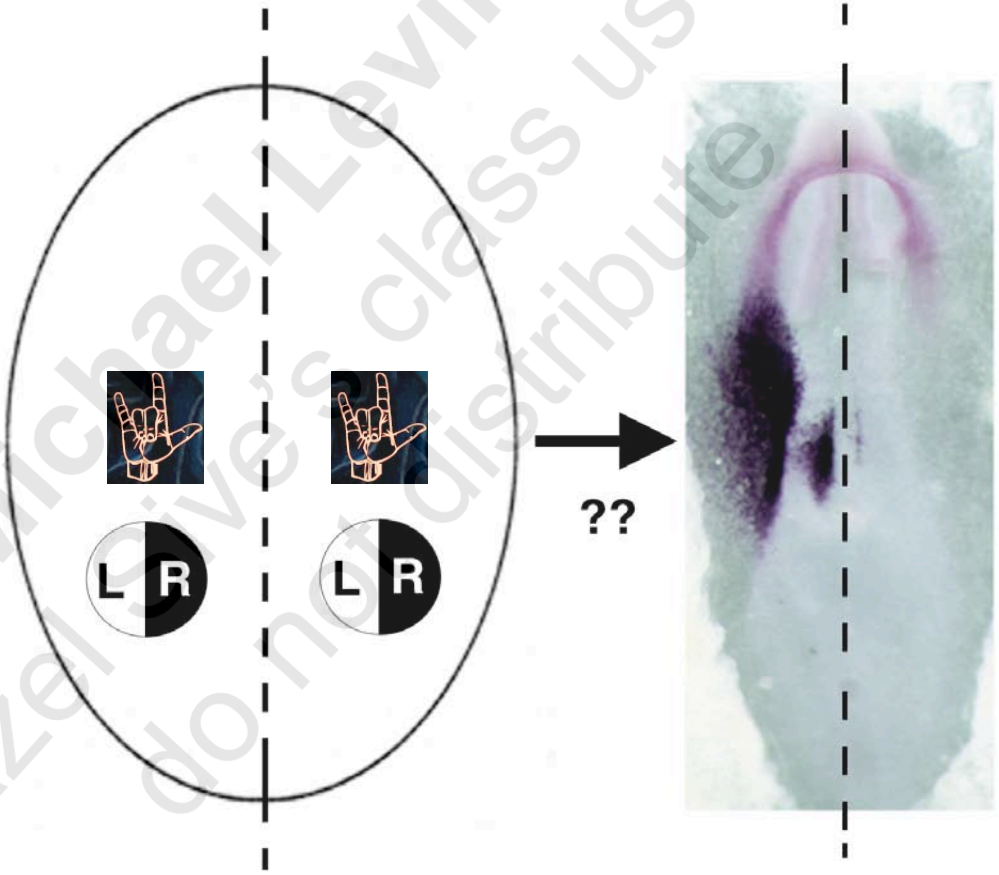
2) In-depth discussion of some specific mechanisms and their evolutionary implications

3) Possibly the most interesting part of the pathway

For earliest steps, different emphasis: think
- epigenetic factors, in addition to transcriptional regulatory networks
- biophysical parameters, not only secreted messengers/receptors

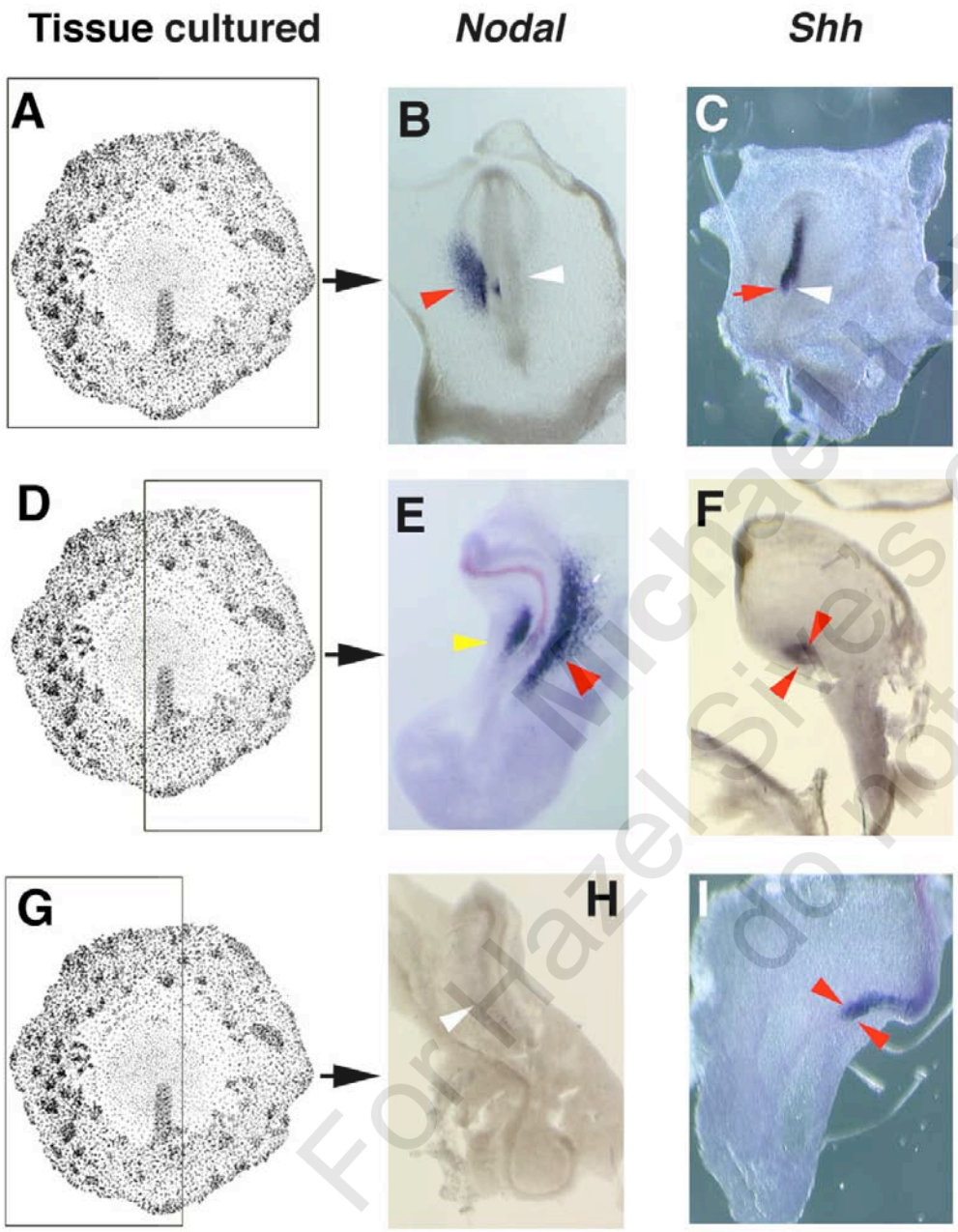
How do we get from cells' knowing which direction is L and which is R, to cells' knowing which side of the midline they are on, in the context of the whole embryo?





Chiral molecule provides direction; asymmetric gene expression needs **global position**.
Are LR decisions local, or do L and R side need to talk? How might this be tested?

Do distant points along LR axis need to communicate for properl LR patterning?



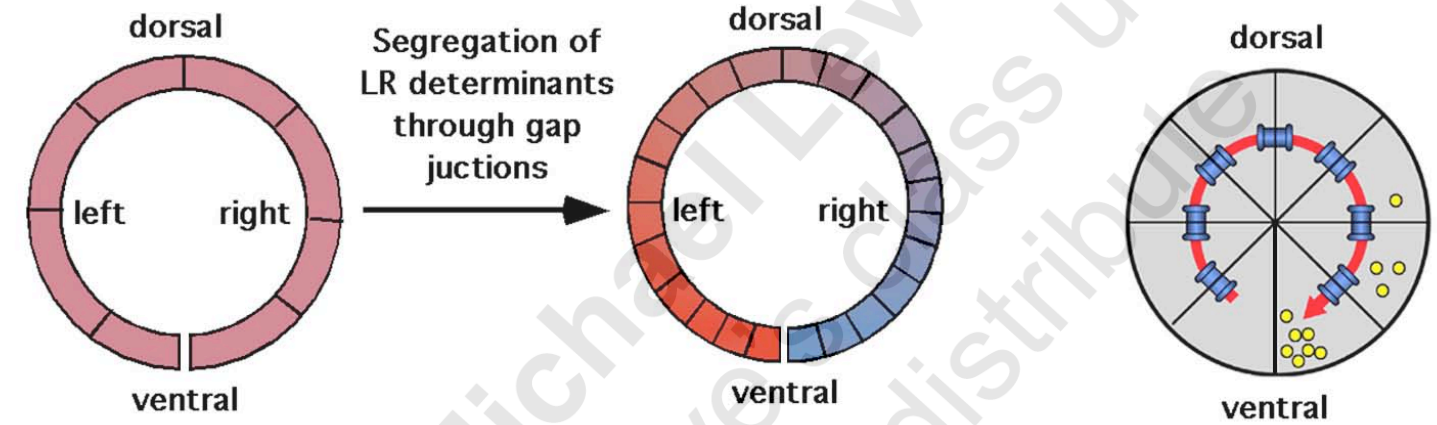
Later - barrier, separate compartments

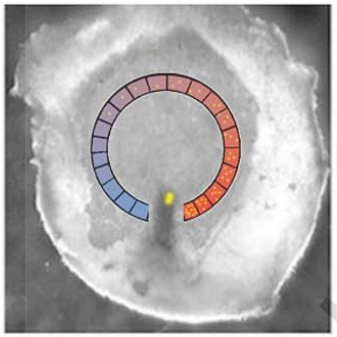
Earlier - long-range communication?

Gap junctions mediate long-range LR signals in 2 species

Data: in chick and frog, a circumferential path of GJC around a zone of isolation is required for normal LR asymmetry. Why?

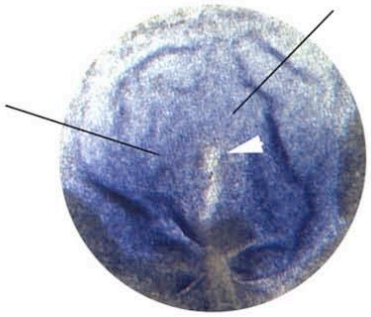
One model:

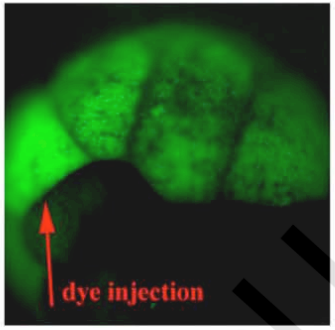


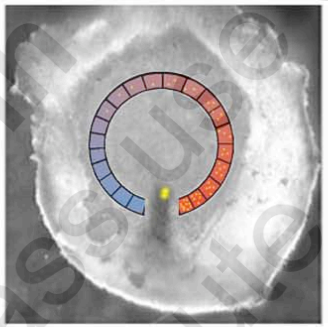


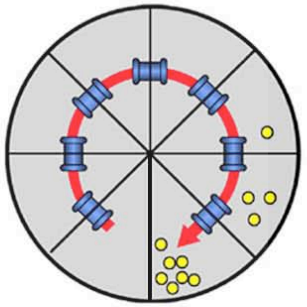
A chiral flow of small molecules traverses the GJC path, resulting in a net gradient which is asymmetric with respect to the LR axis (midline). Once they accumulate preferentially on one side, these determinants are then able to induce asymmetric gene expression in conventional ways.

Developmental Biology, 203(1): 90-105 ; **Development**, 126: 4703-4714



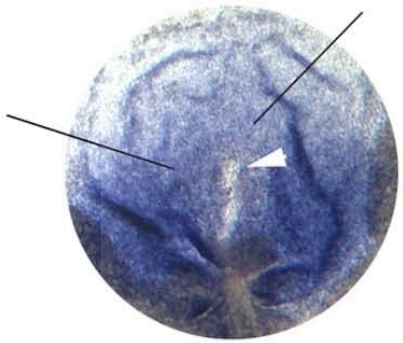


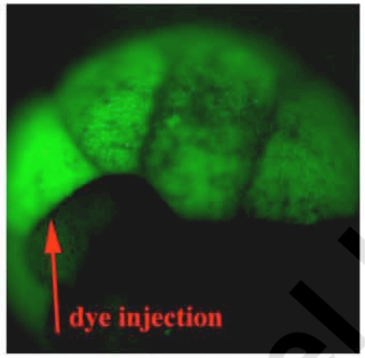


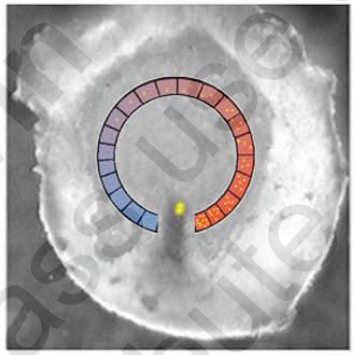


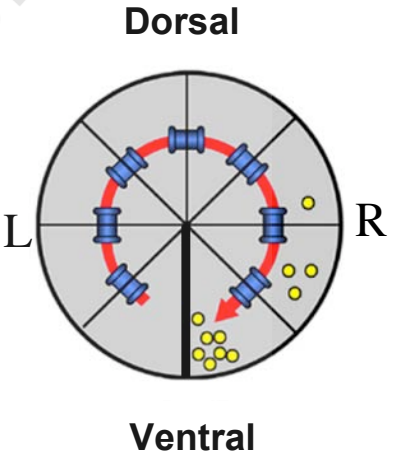
Important questions:

What would you want to know now?









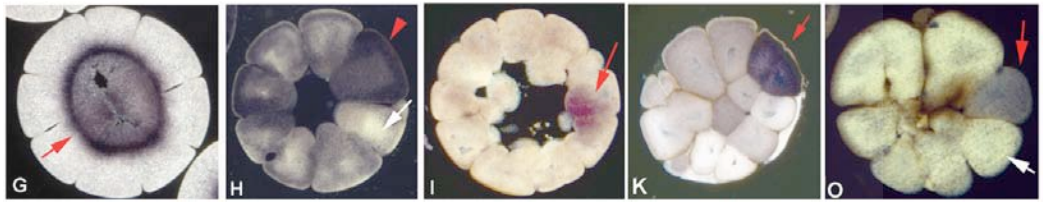
Important questions:

1) What controls the pattern of embryonic GJC (mechanisms upstream)

2) What genes are induced by junctional flow (mechanisms downstream)

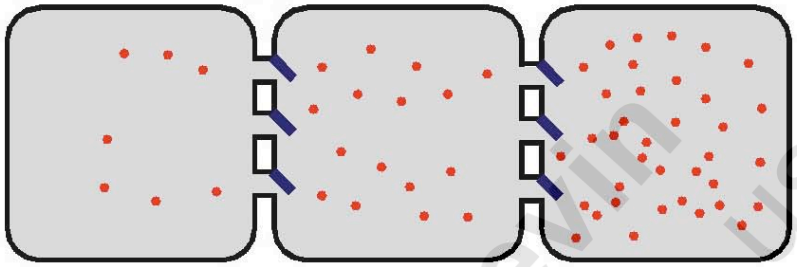
3) What flows through the gap junctions to control pattern?

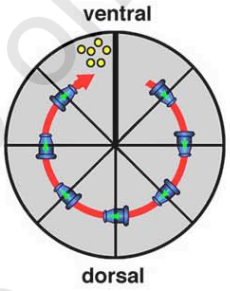
4) Why is the flow chiral (uni-directional)??



How might a gradient be formed across a cell field connected by GJC?

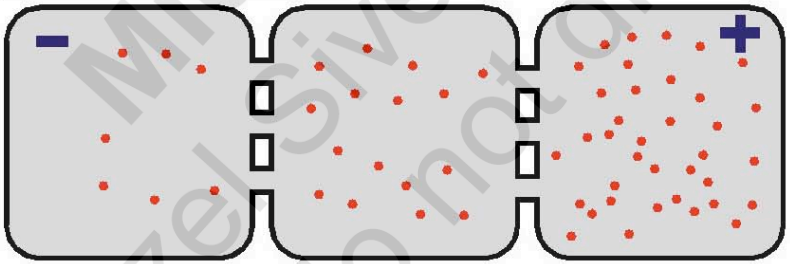
Selective transfer through junctions

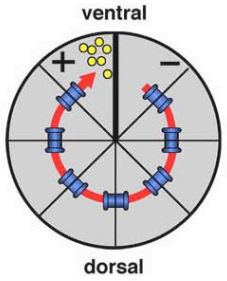




Unidirectional GJC exists in embryos, but thermodynamics requires an energetic process to drive it.

Charge difference across cellular field





Key point: epigenetic mechanisms, biophysics

How to test this model?

A multitude of living systems has been shown to expend significant energy costs (in ATP) to drive strong electric fields. Why?

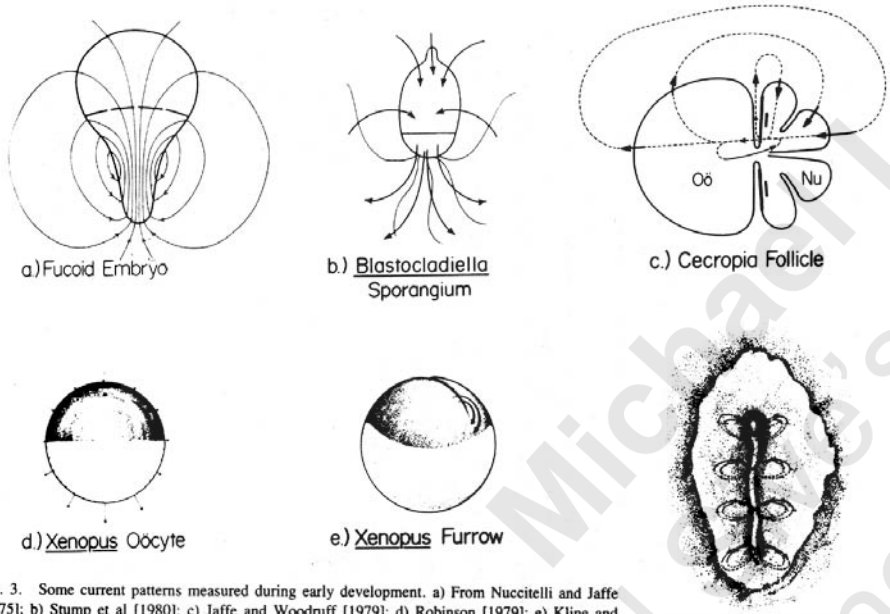
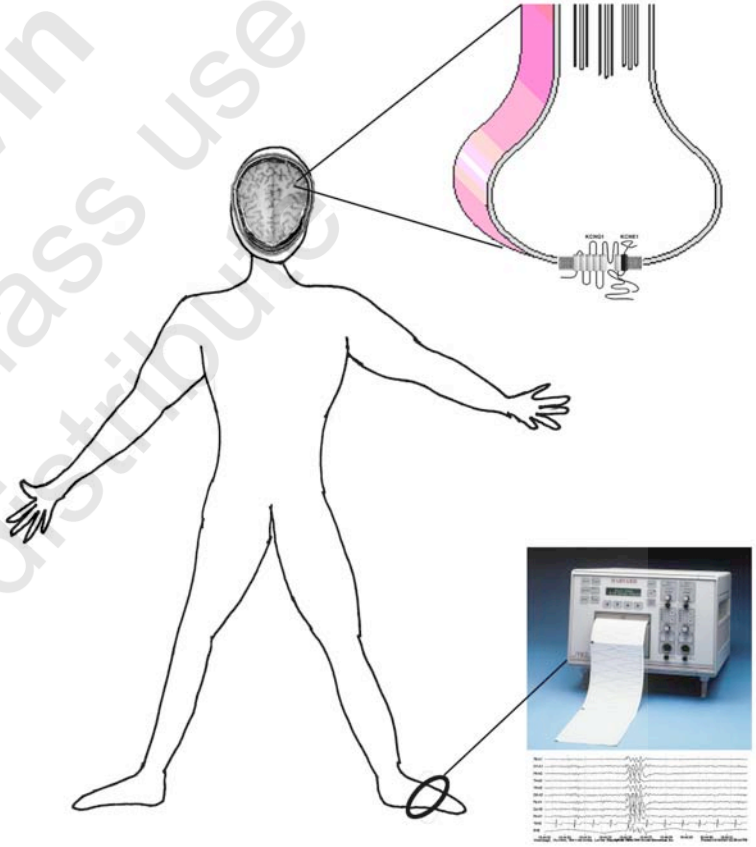


Fig. 3. Some current patterns measured during early development. a) From Nuccitelli and Jaffe [1975]; b) Stump et al [1980]; c) Jaffe and Woodruff [1979]; d) Robinson [1979]; e) Kline and Nuccitelli (unpublished); and f) Jaffe and Stern [1979]. Oö indicates oocyte; Nu, nurse cells. (Composite drawing modified from Jaffe, 1981).

Ion flux events initiate global information

Molecular changes in ion flux genes have effects which can be detected all over the body (EEG, ECG signals can be detected on the hands and feet)

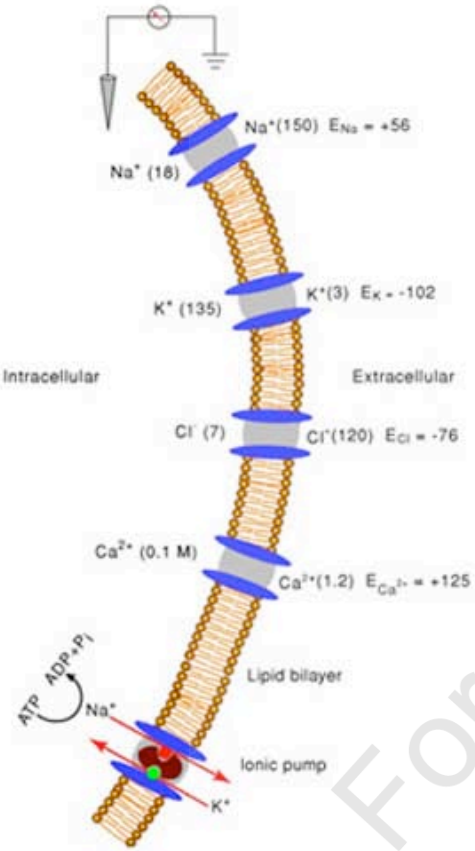


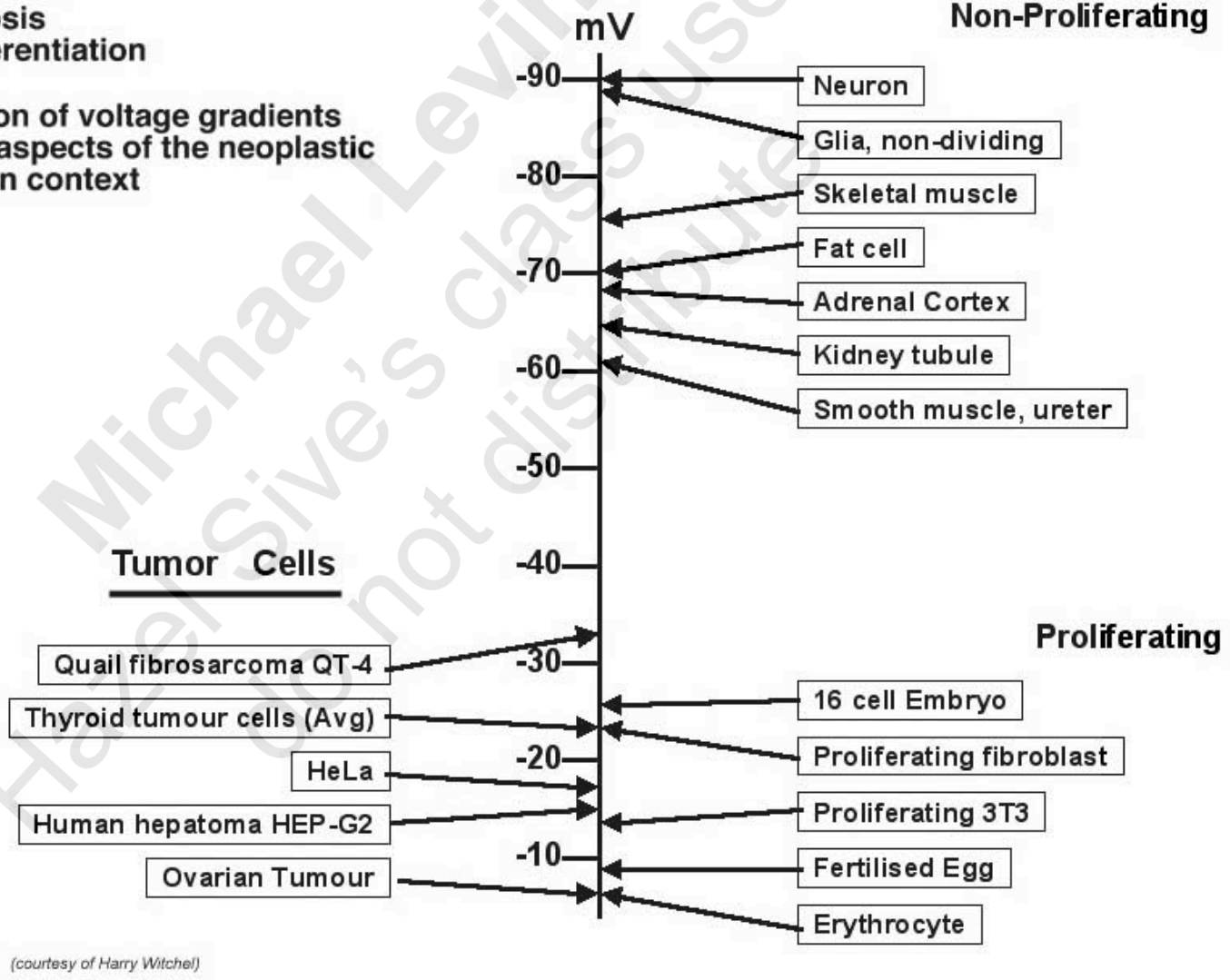
Endogenous electric fields and gradients have been implicated in embryonic development, regeneration, and cancer.

Ion flows as a general cellular control parameter

Voltage Gradients and Neoplasm:

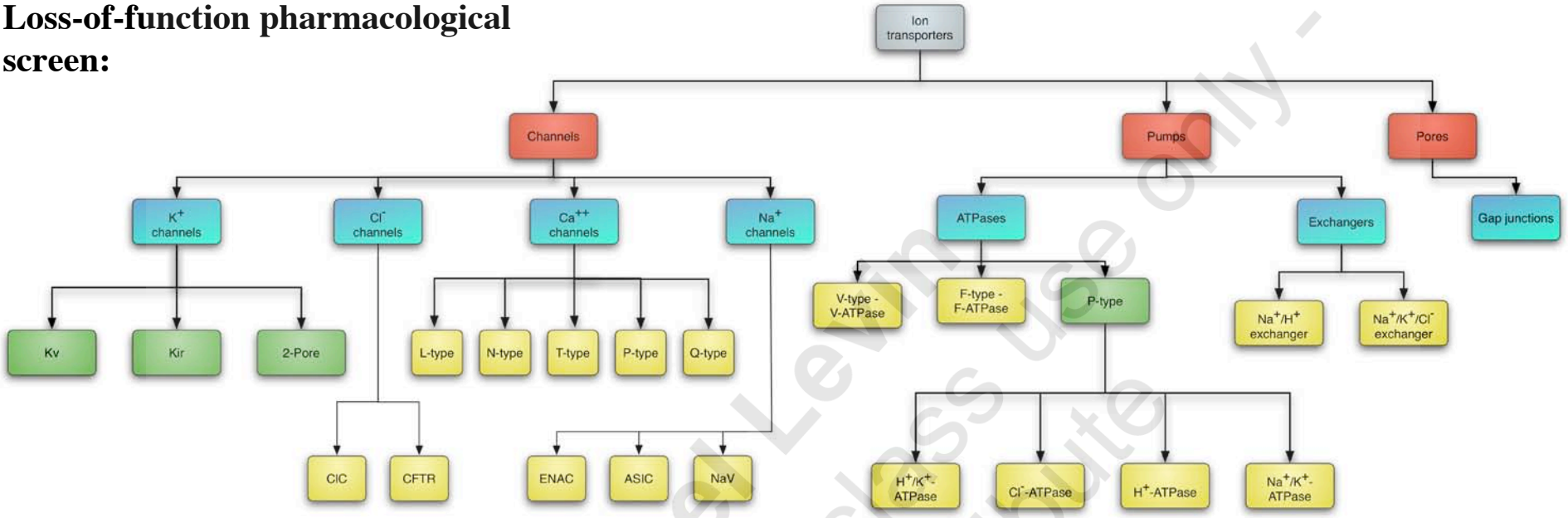
- Tumor tissue can be distinguished from normal tissue by cells' membrane voltage potential
- Endogenous $V_{membrane}$ controls:
  - proliferation
  - cell migration
  - mitosis
  - differentiation
- Experimental manipulation of voltage gradients can induce or diminish aspects of the neoplastic phenotype depending on context

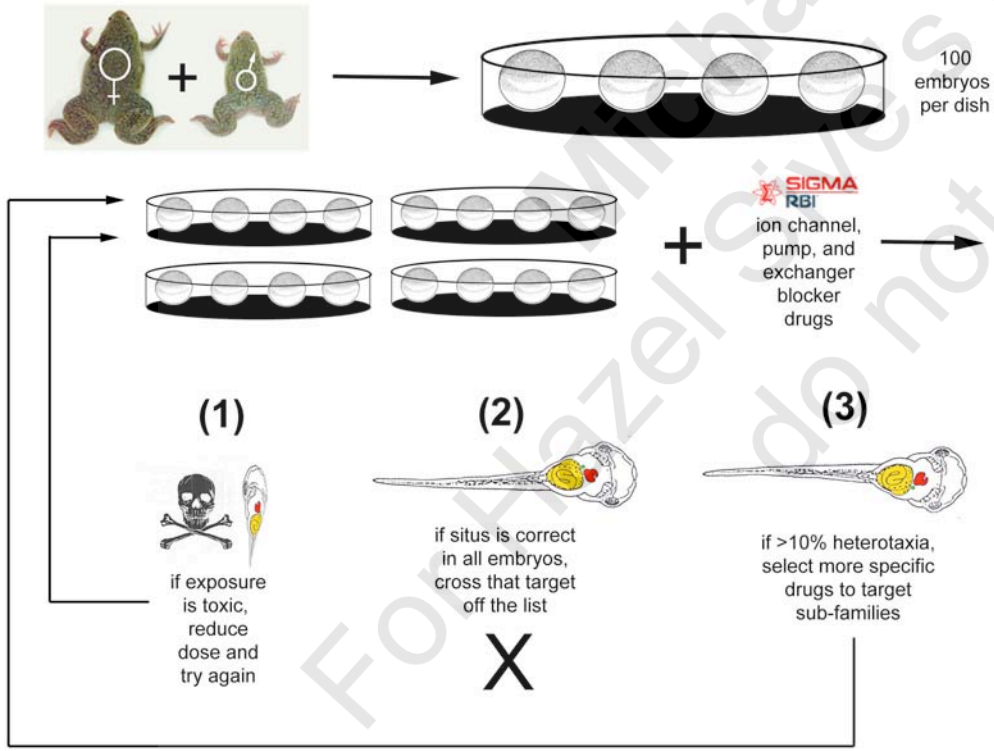




(courtesy of Harry Witchel)

Loss-of-function pharmacological screen:





Purpose:

1) is ion flux involved in LR asymmetry?
2) which genes are implicated in this mechanism?

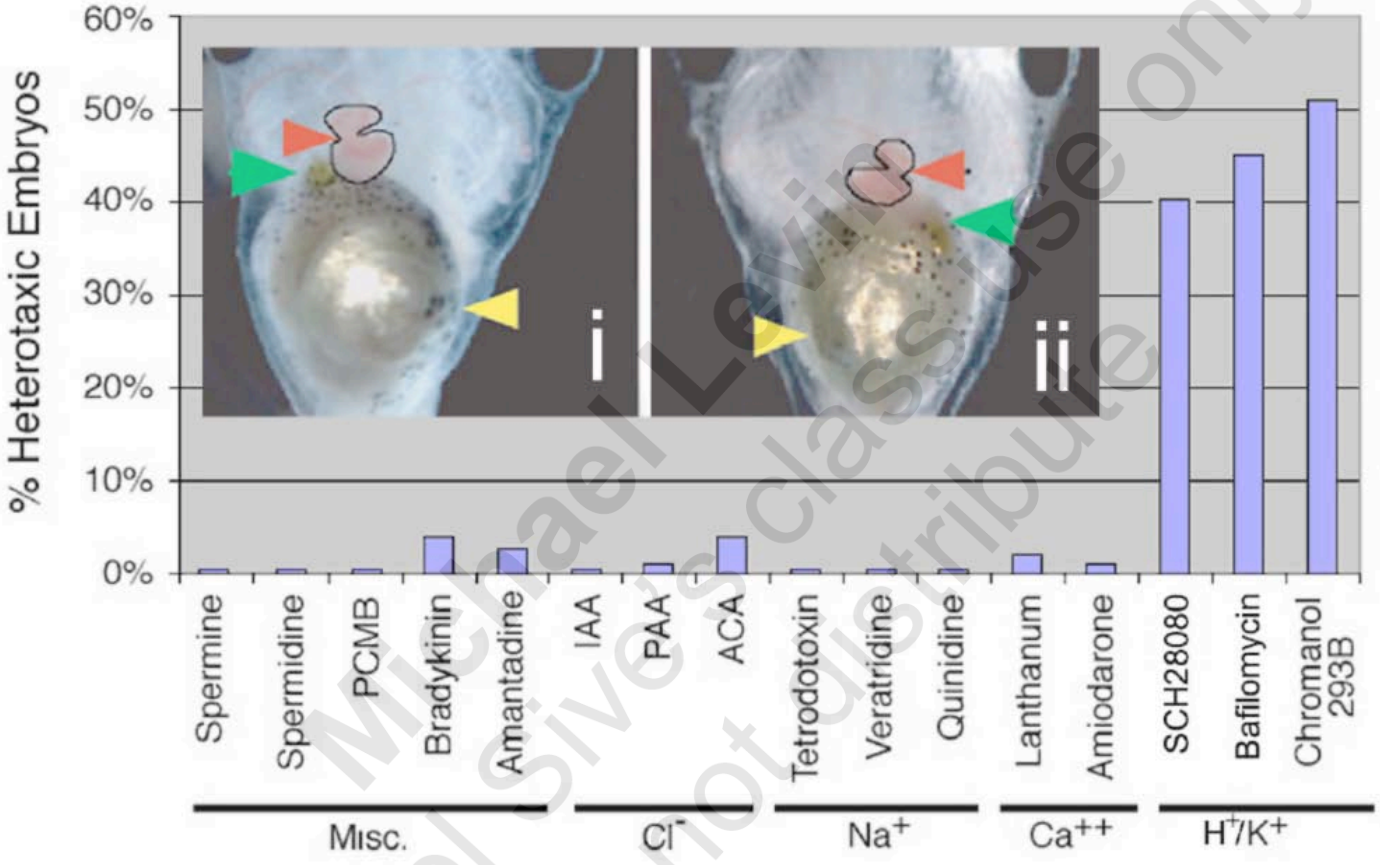
What are the problems/benefits of this approach?

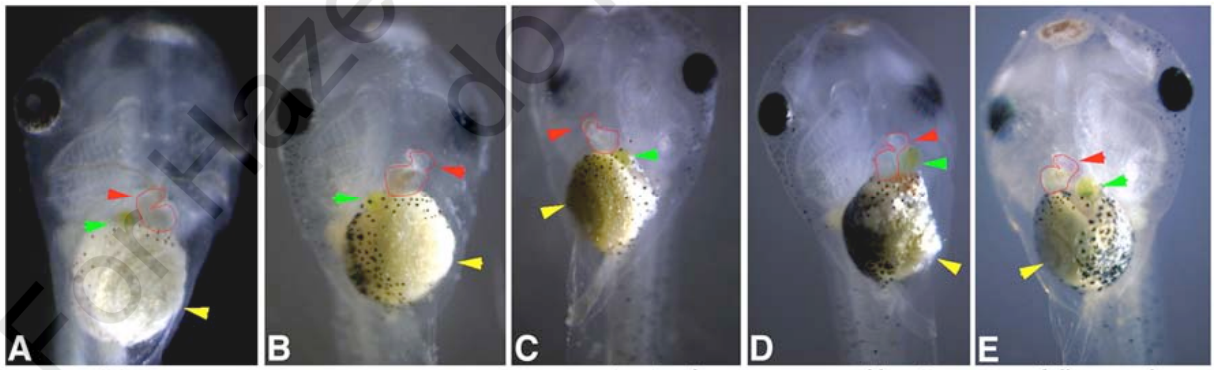
Interpreting heterotaxia %:

1) 87% is maximum, when all three organs are fully randomized
2) all drug doses were low, to escape confounding AP/DV effects
3) control incidence of laterality defects (background) is very low
4) statistical analysis of large number of embryos

Drug experiments cannot prove the involvement of any gene target but they can be used to quickly focus attention on promising candidates for subsequent expensive and time-consuming molecular approaches. This strategy is useful for many other developmental mechanisms, as long as you have a good assay and molecular pharmacology that is hierarchical.

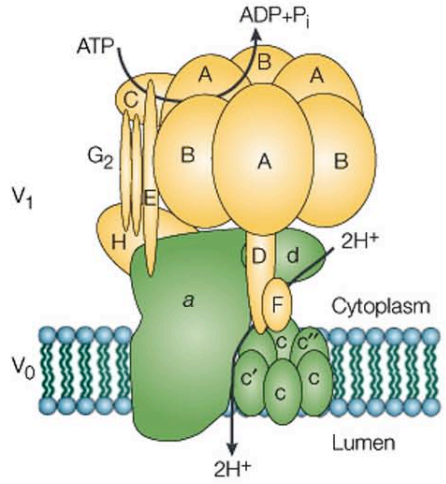
Are ion transporters important in LR asymmetry?





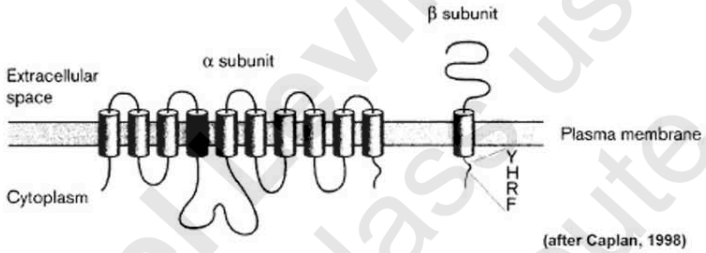
Targets implicated in pharmacological LR screen

V-ATPase



Development, 2006, 133: 1657-1671

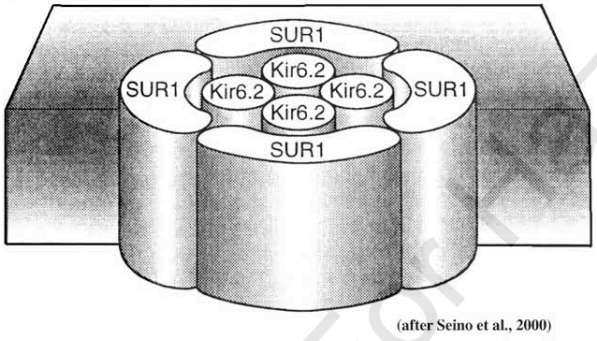
H$^+$/K$^+$-ATPase



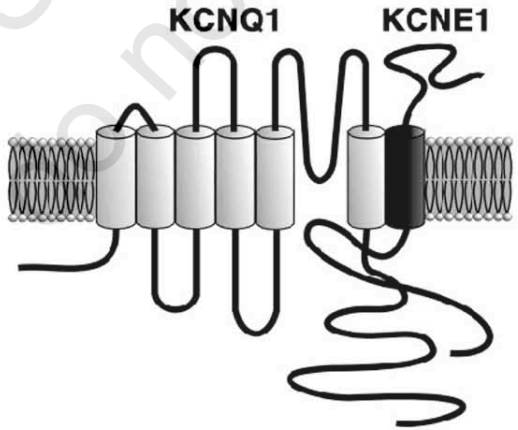
Cell, 2002, 111: 77-89

H$^+$ pumps

K$_{atp}$ channel

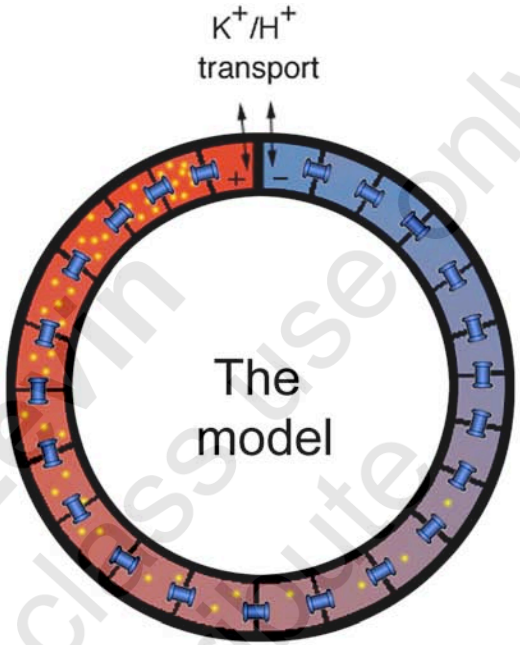


KvLQT-1 channel



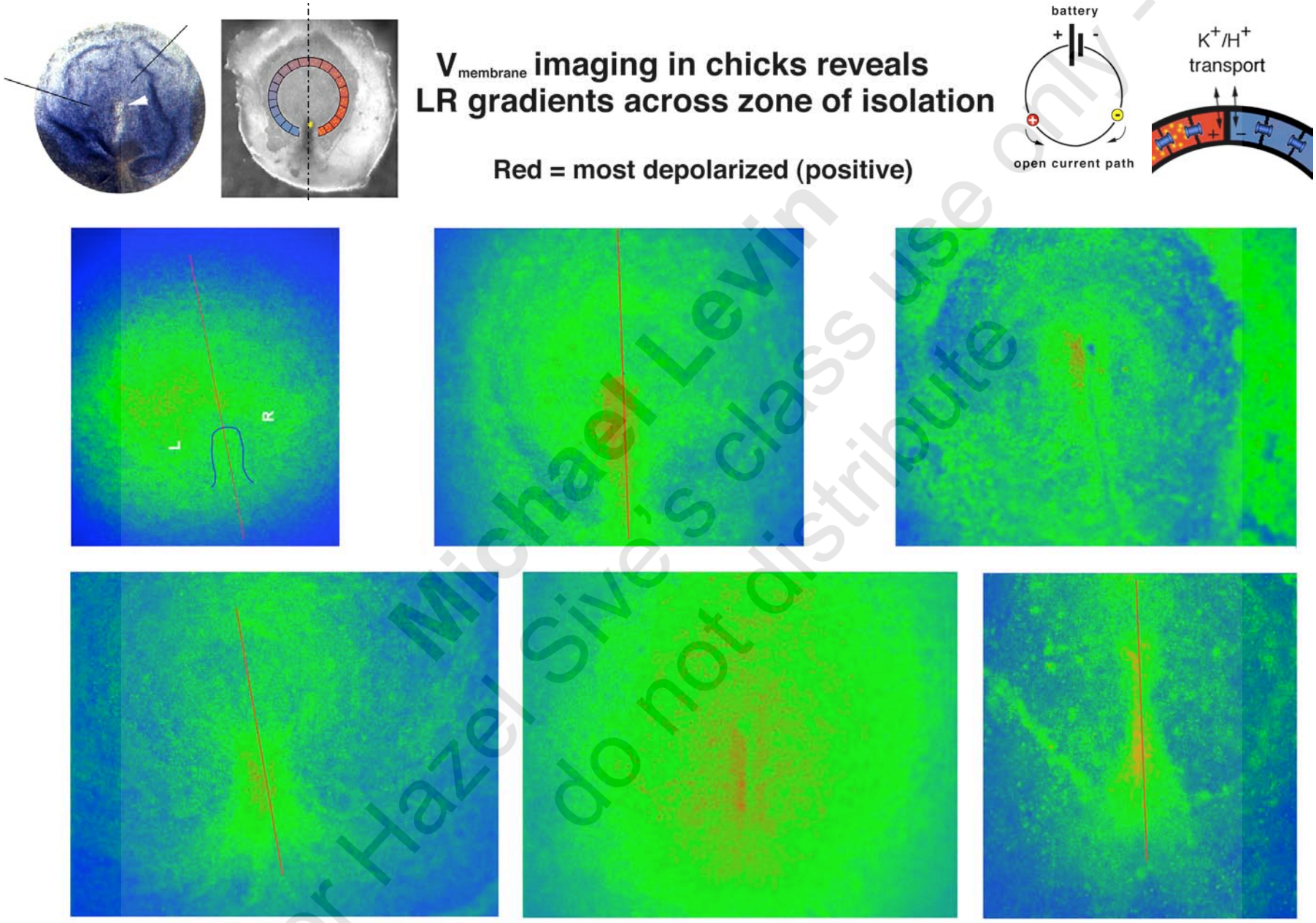
K$^+$ channels

One of the nice features of this model, aside from providing a way for subcellular events to be imposed on large cell fields, is that it makes a number of easily testable specific predictions.



Predictions:

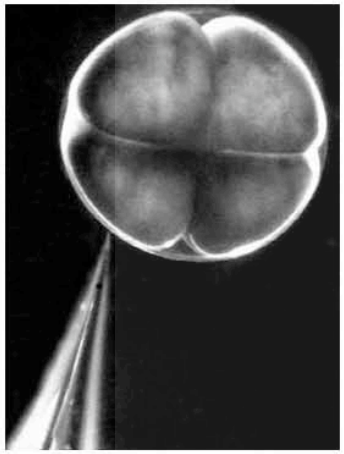
1) **pH and $V_{membrane}$ differences should be detected directly across the zone of isolation**
2) **Physiology should be correlated with function: ion pump and channel blockers which cause heterotaxia should abolish asymmetry in detected gradients**
3) **The ion fluxes should function early (overlapping with GJC stages) and should be upstream of laterality of asymmetric genes**
4) **Gain-of-function experiments (alteration of endogenous voltage or pH asymmetry using misexpression constructs) should cause heterotaxia**
5) **The LR asymmetry of Hensen's node should not be intrinsic but rather should be derived from (communicated to it by) the laterally-adjacent tissues**

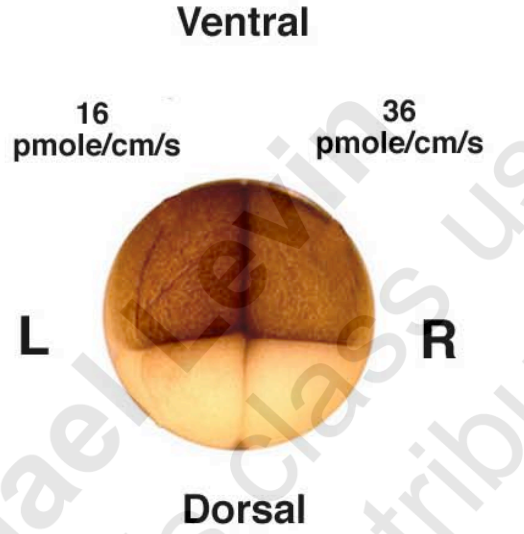


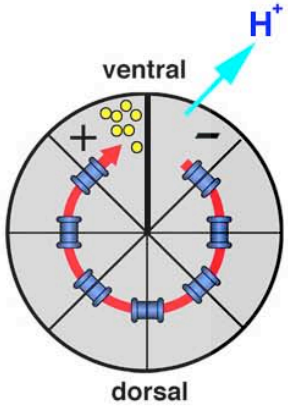
(Dr. Thorleif Thorlin)

Technique: determination of membrane voltage by *in vivo* dye

Vibrating probe measurements reveal differences in H^+ efflux across ventral midline





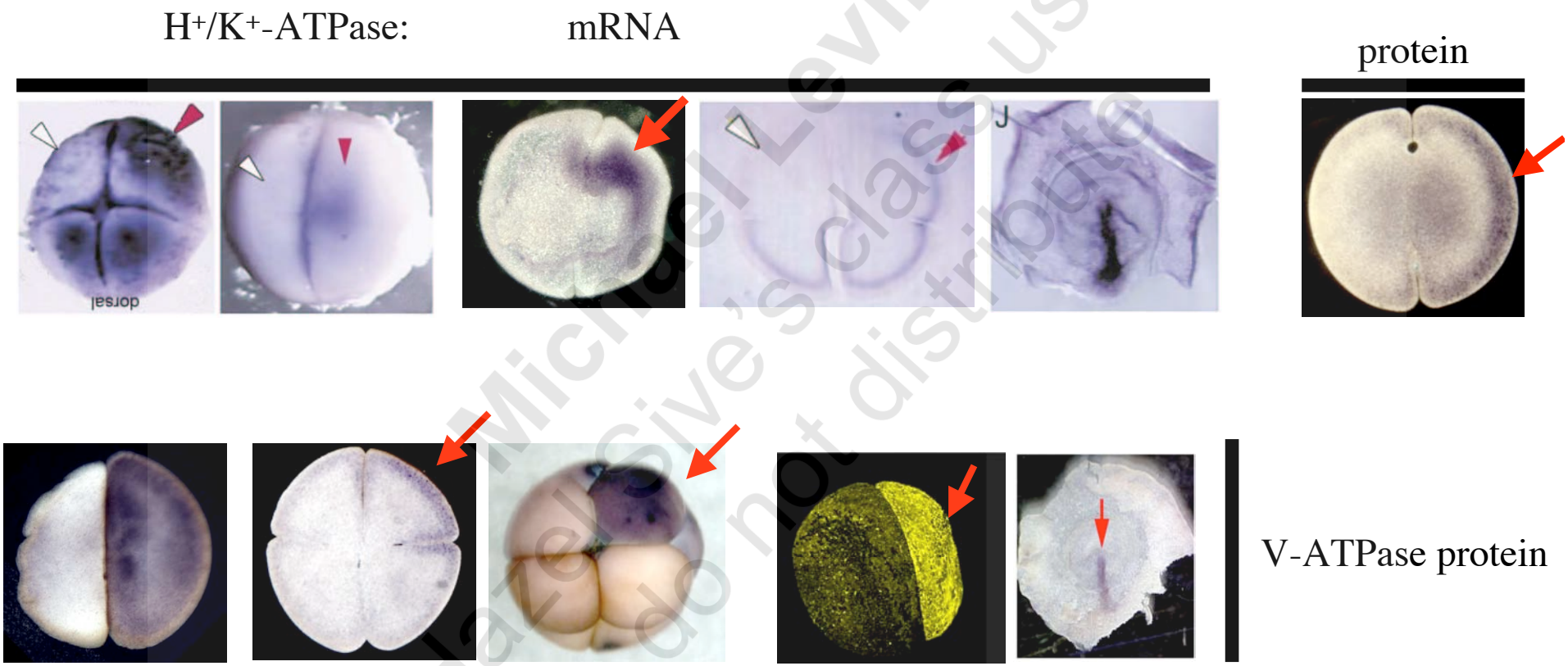


These currents, and the asymmetry in their magnitude, are abolished by the presence of Concanamycin, a blocker of the V-ATPase

(Dr. Ken Robinson)

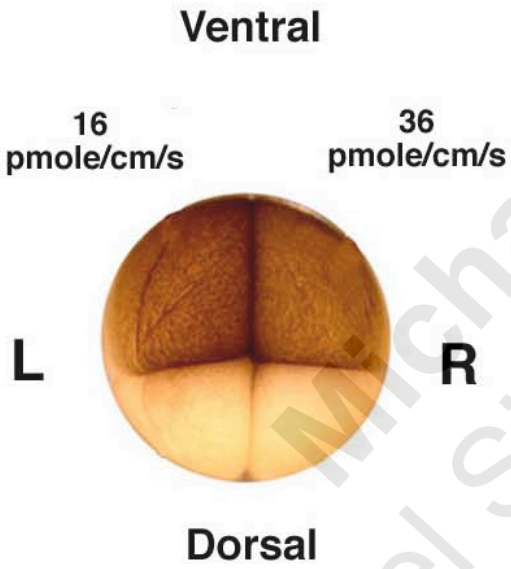
Technique: *in vivo* detection of ion-specific, extracellular flux

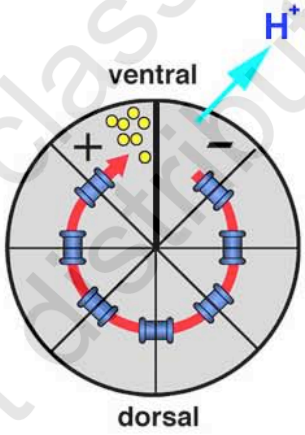
The mRNA and protein for three of the four targets implicated in drug screen reveal asymmetric localizations by the 4-cell stage, setting a new lower bound on "Step 1" and suggesting novel localization mechanisms

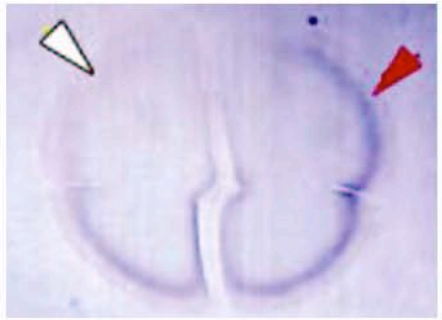


Localization of HK-ATPase mRNA is consistent with the source of H^+ efflux which results in a net loss of positive ions

Proton efflux =



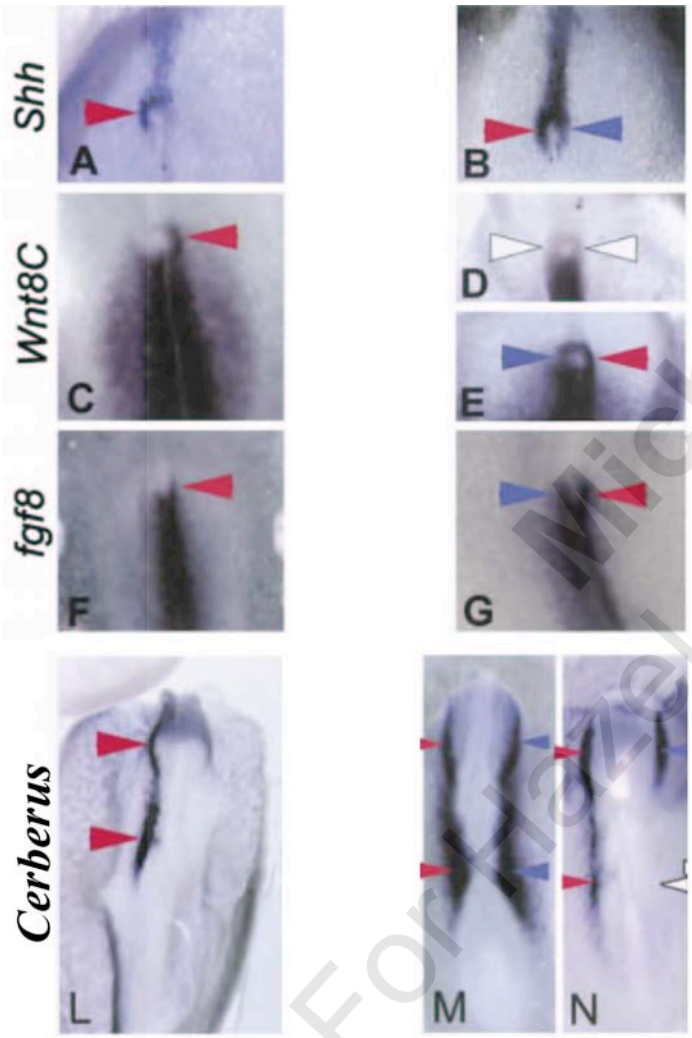




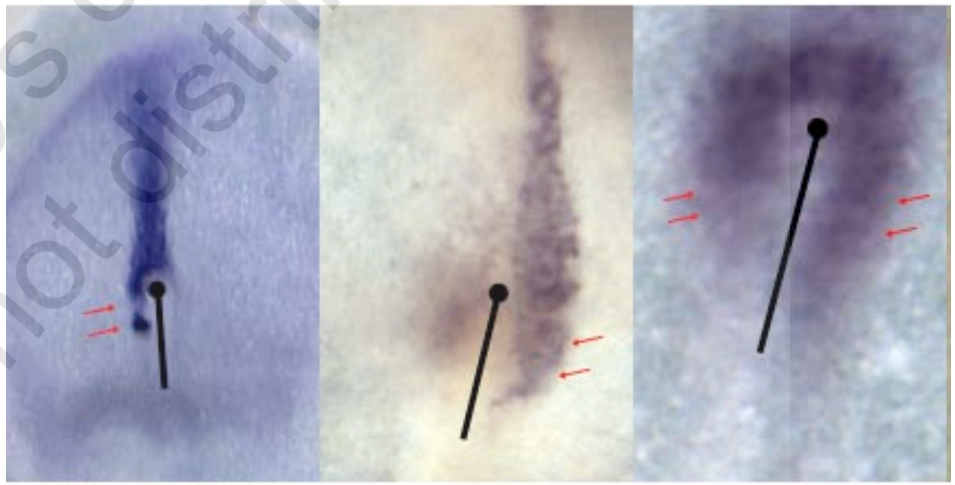
Analyze expression/localization - does it match physiology data?

Epistasis: the V-ATPase and H^+/K^+-ATPase are upstream of left-sided *Sonic* and *Nodal* expression

Controls HK-inhibited



V-ATPase inhibited

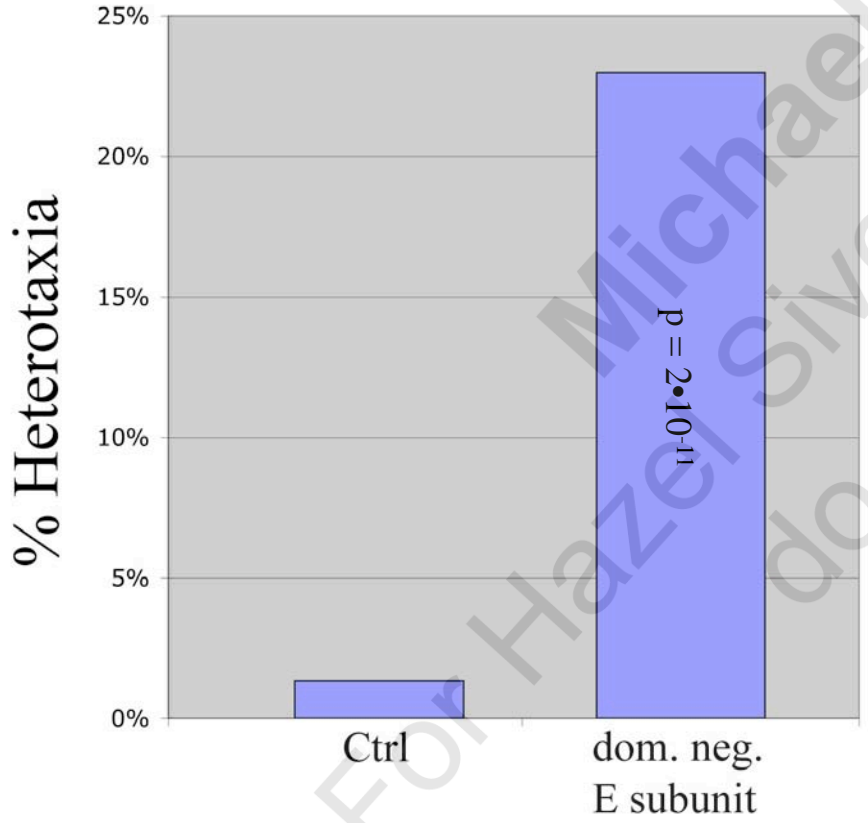


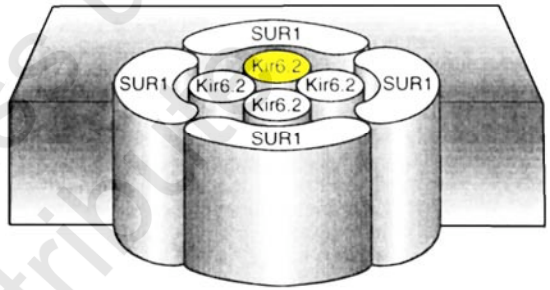
WILDTYPE left-sided Shh expression | right-sided Shh expression | bilateral Shh expression

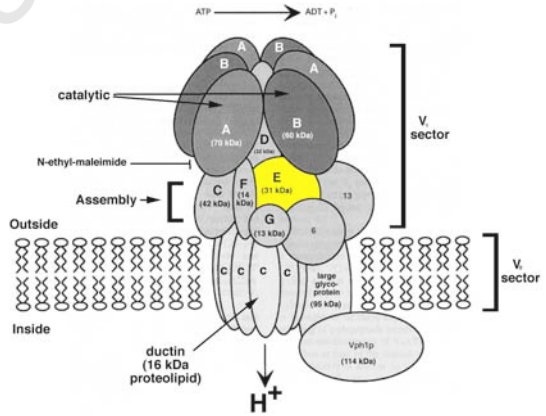
Molecular loss-of-function experiments:

Like the inhibitor studies (which allowed a dissection of timing), misexpression of dominant-negative constructs randomizes LR

V-ATPase

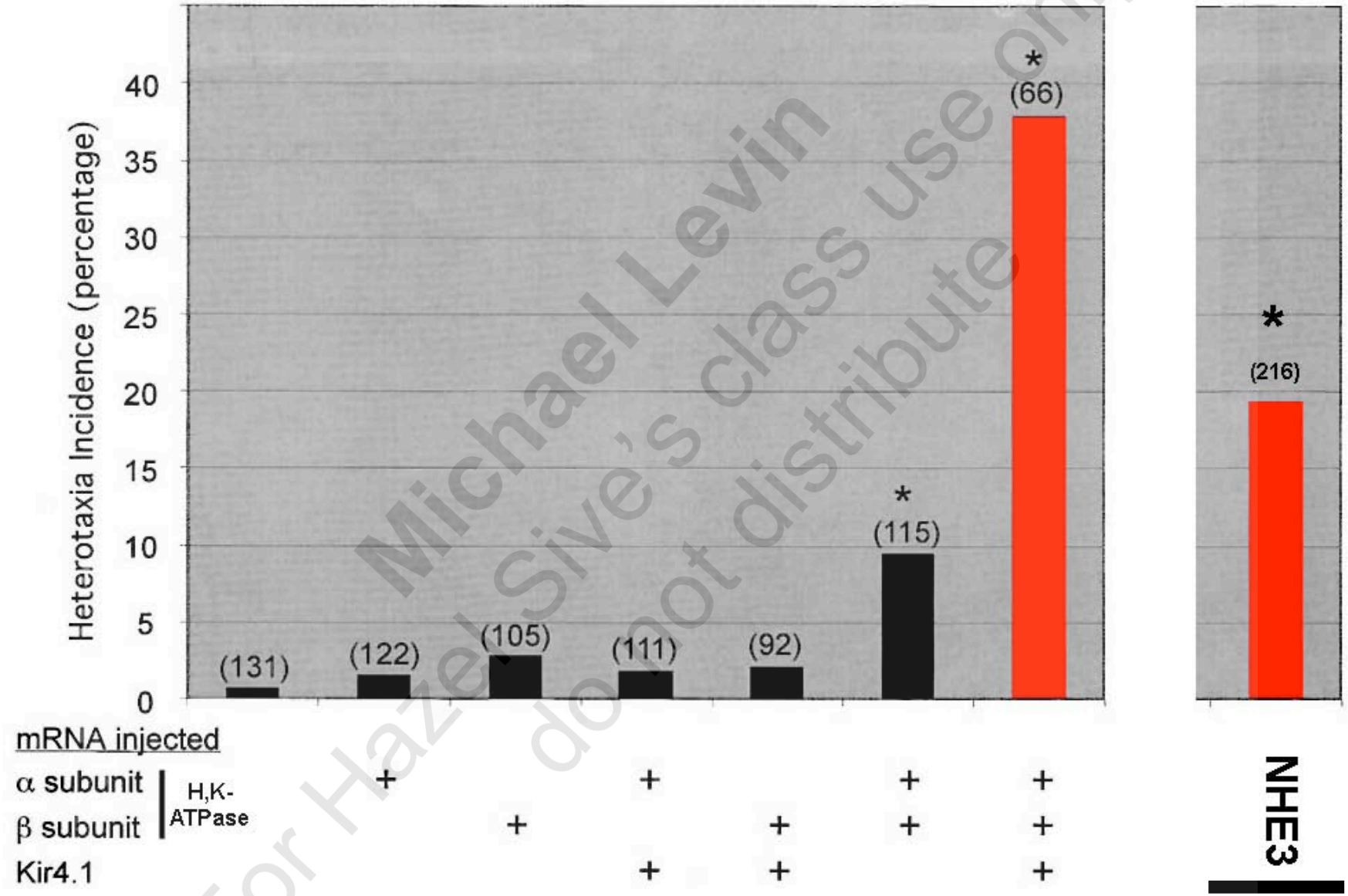






Pore mutants,
ER-retention signals

Gain-of-function experiments using electrogenic genes to induce ectopic ion flux

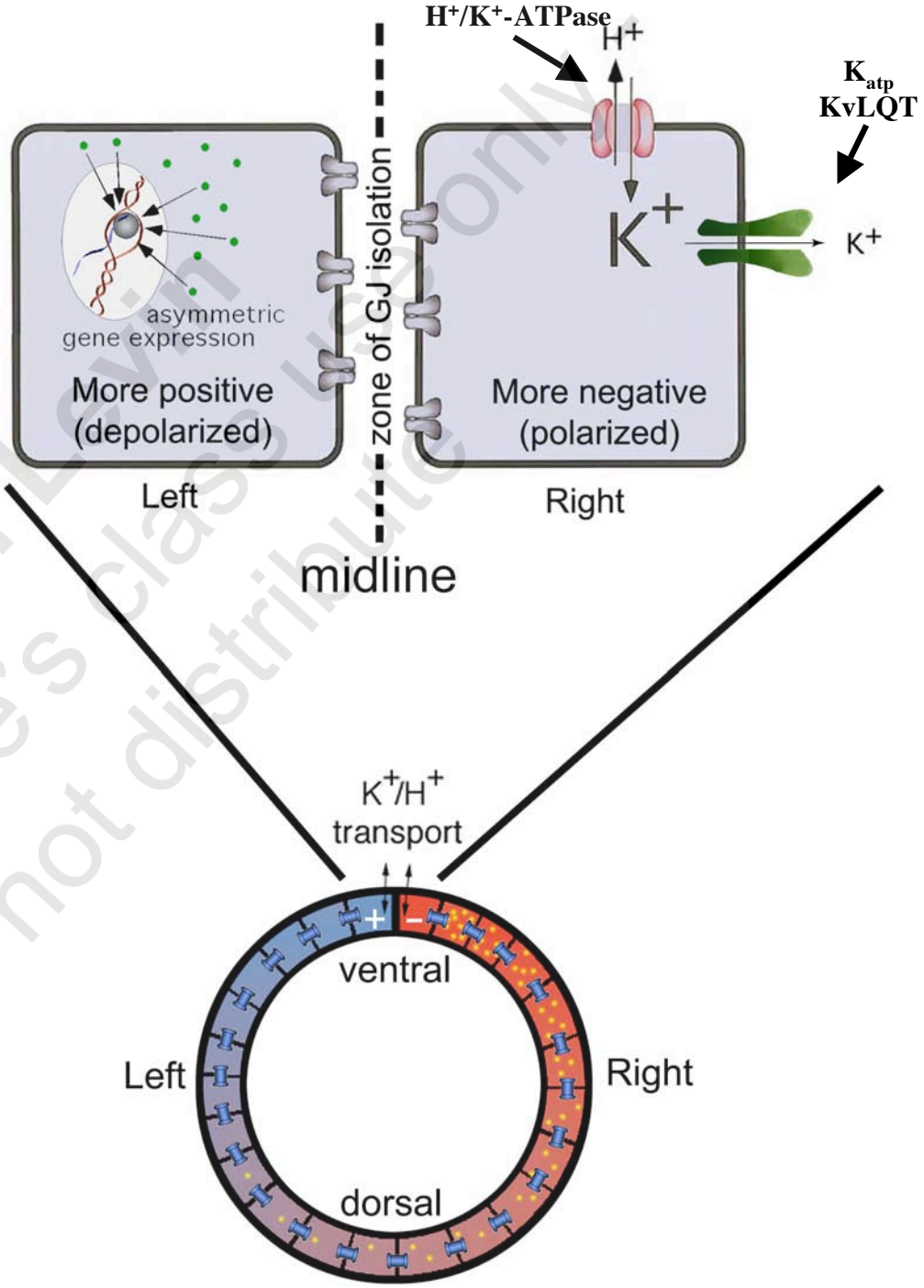


Why 2 targets together? Individual contributions sum to net flux.

Summary:

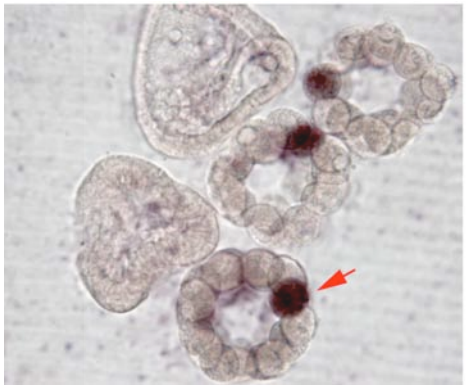
- Pharmacological inhibitor screen
- W.T. construct gain-of-function
- Dominant-negative loss-of-function
- *In vivo* characterization of early asymmetric voltage/ion flux
-Characterization of asymmetric endogenous early localization of ion channel and pump protein/mRNA

Taken together, the data suggest a consistent model of ion flux mechanisms upstream of the asymmetric gene cascade



Expression and function of the ion flux system is conserved to primitive chordates and even invertebrates

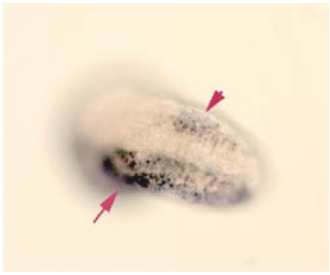
Sea Urchin H^+/K^+-ATPase
(Atsuo Nishino)



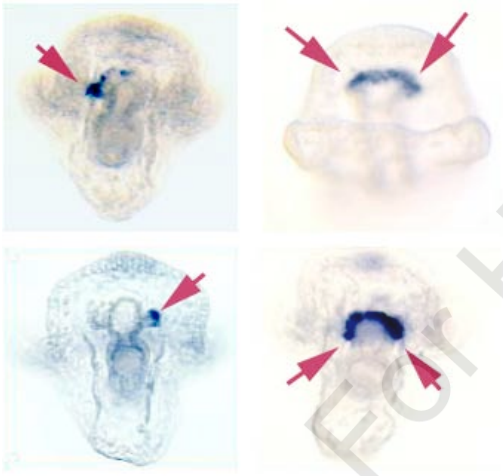
Ciona H^+/K^+-ATPase
(Seb Shimeld)

Ctrl

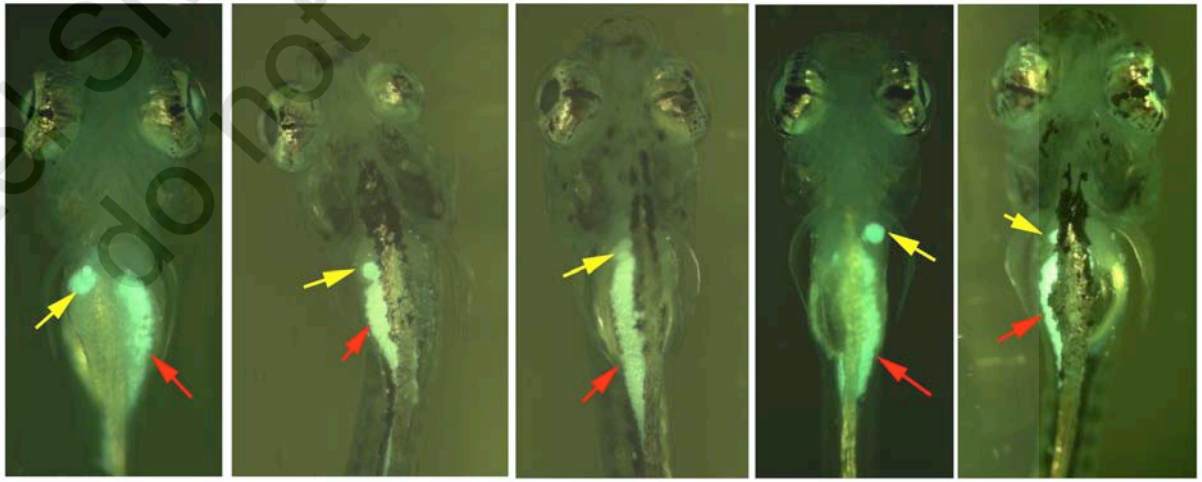
K^+ channel
inhibited

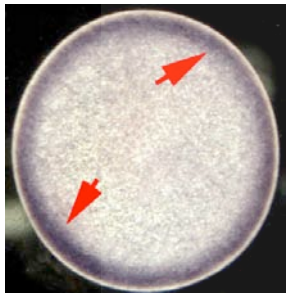


Ctrl **H^+/K^+ inhibited**

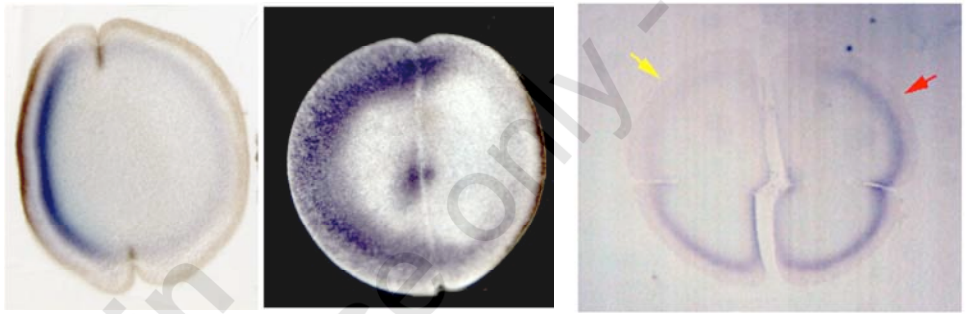


Zebrafish (Pam Yelick)

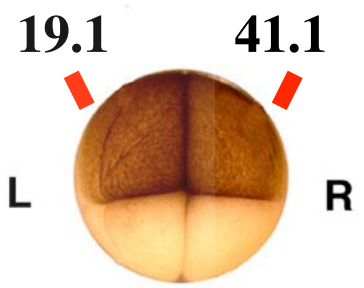




Unfertilized egg

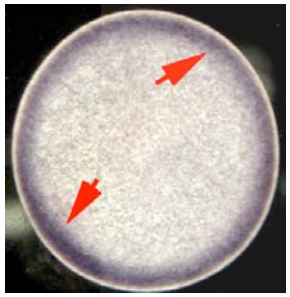


Ventral

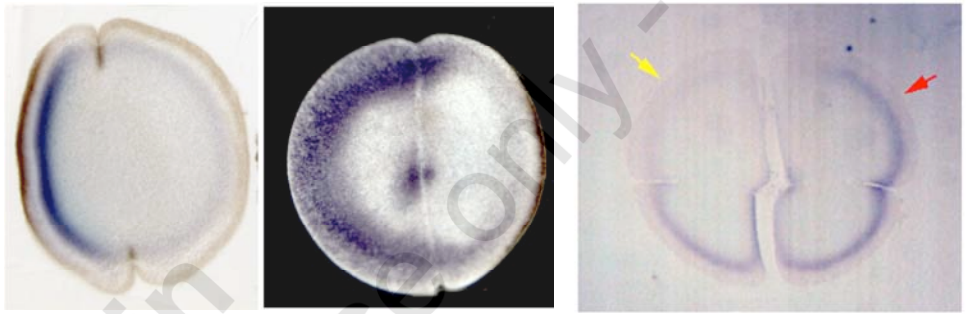


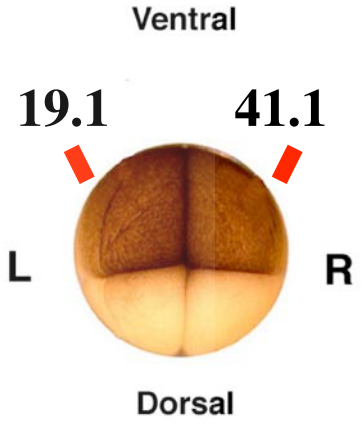
Dorsal

Is LR asymmetry now solved?



Unfertilized egg

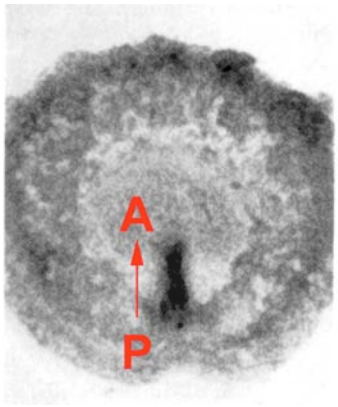




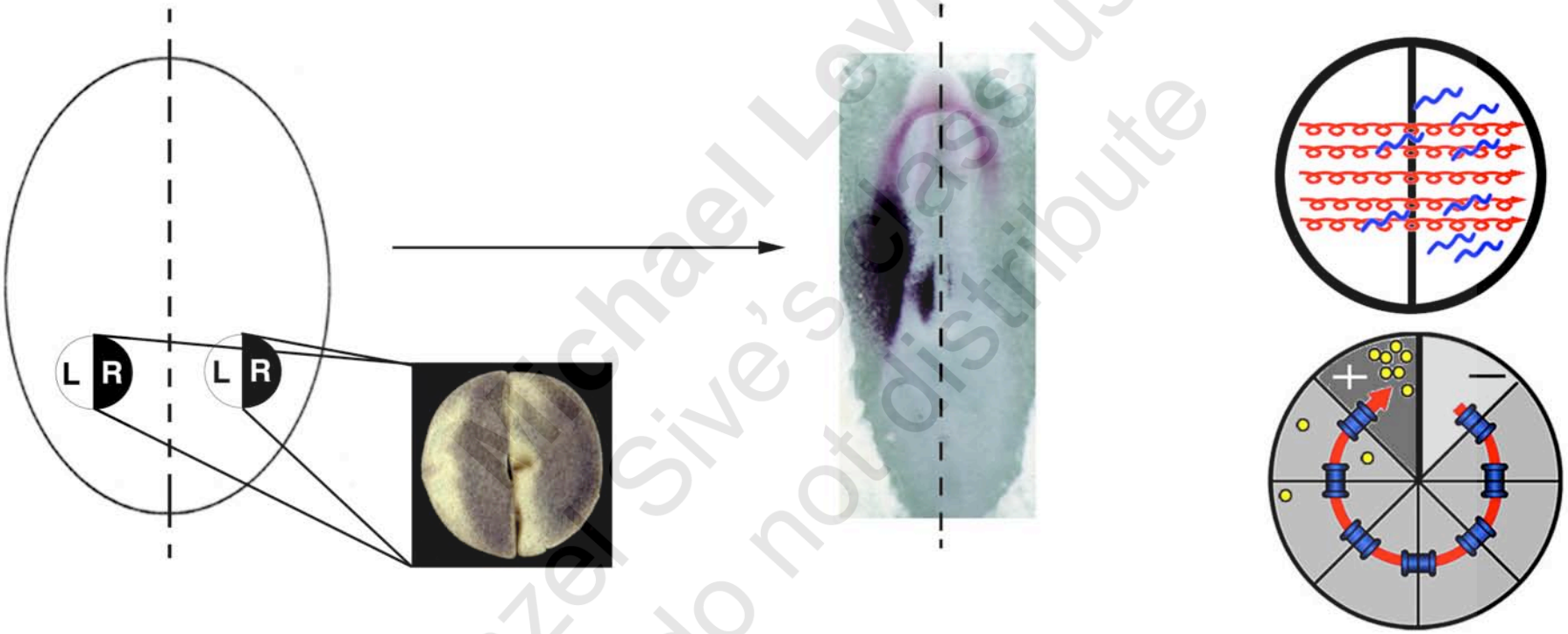
Is LR asymmetry now solved? No:

1. Because asymmetric localization of ion transporter mRNA and protein itself requires explanation, this mechanism is not "Step 1" of LR asymmetry. But, since our data constrains Step 1 to the first 2 hours of development, it is likely that ion flux is very close to the initial chirality breaking event. Thus we have the process boxed in, and have a handle on how to proceed upstream. My guess: a cytoskeletal motor leverages large-scale LR asymmetry by directing the asymmetric localization of ion transport proteins with respect to an oriented cytoskeletal structure.

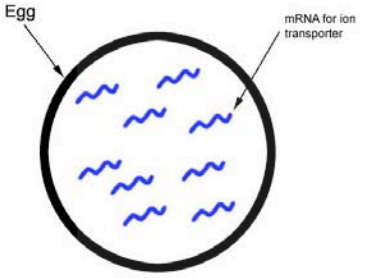
2. This basic model has to be modified in the case of the chick, and will first require some understanding of how the AP axis is controlled in the chick blastoderm (it is not known how cells know in which direction to grow the primitive streak).



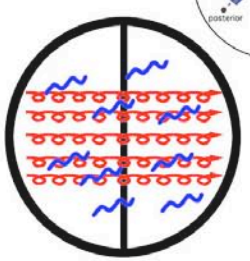
The ion pump mRNA + GJC mechanism allows asymmetries generated at the level of the single cell to be imposed on large multi-cellular fields in the embryo



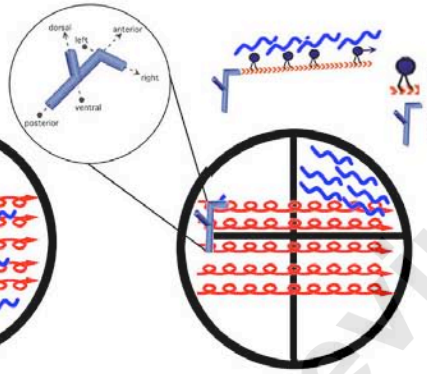
A grand-unified-theory of LR pattern formation



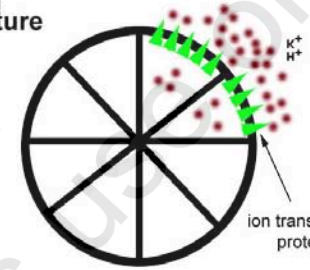
mRNAs for ion transporters are randomly distributed



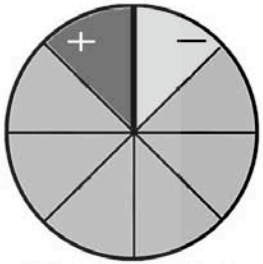
Fertilization and 1st cleavage allow LR computation to be made by a chiral component (cytoskeleton?)



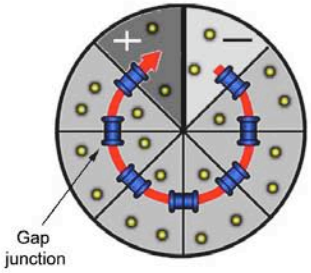
This allows mRNAs to become localized with a LR asymmetry (cytoplasmic motor?)



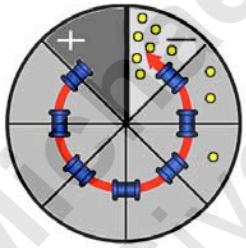
This leads to asymmetric localization of ion channels and pumps



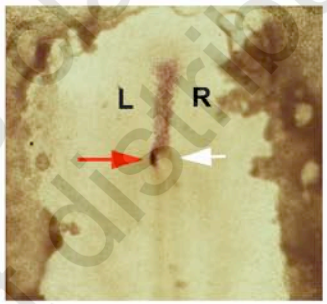
Differential ion exchange with the outside world leads to a LR voltage gradient



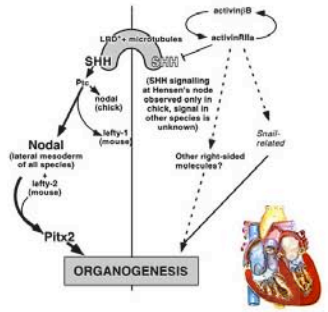
DV-patterning pathways (Wnts, etc.) set up a large-scale GJC region surrounding a zone of isolation. Small molecule determinants are randomly distributed.



The existing voltage difference electrophoreses charged small molecule determinants in a preferred direction, subject to gap junctional selectivity.

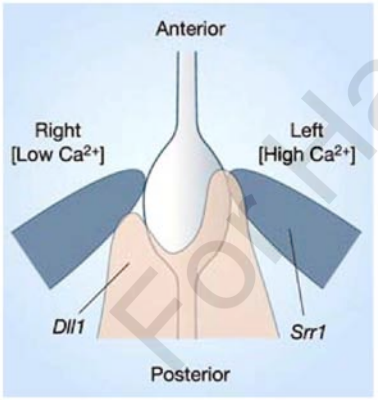


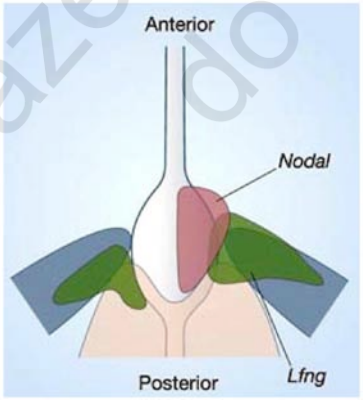
The LR gradient of small molecules results in the asymmetric induction of markers (such as Shh) in the midline



Early asymmetric markers initiate cascade of asymmetric signaling molecules which in turn guide the chiral morphogenesis of the viscera

Or, direct role of ion flux integrating at the node and feeding into Notch cascade (Juan-Carlos Izpisua Belmonte)



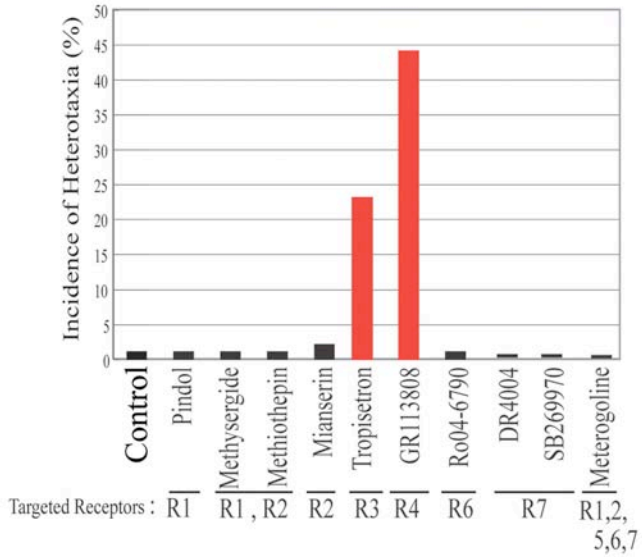


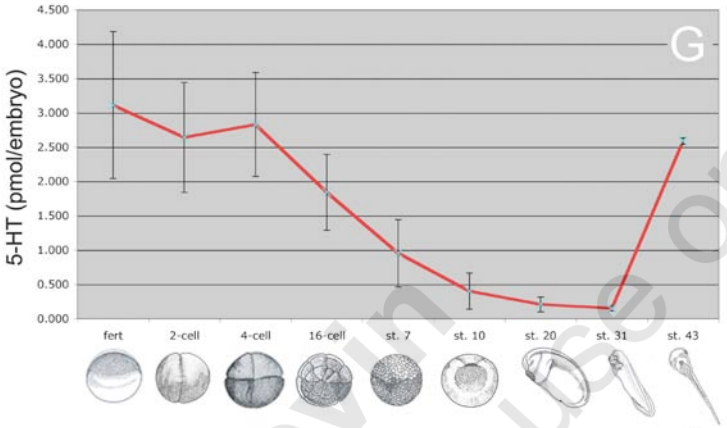
(Ventral view of embryo) Nodal expression on the left of the 'node' (oval) depends on the Notch pathway, which is in turn activated by Dll1 and Srr1. (A), At stage 5 Dll1 expression extends further towards the head (the anterior) on the left than on the right. This is the earliest indication that Notch activity is higher on the left (as Dll1 is a target of Notch activity). (B), During stage 6, the Dll1 and Srr1 expression domains are symmetrical. But, as the fifth pulse of expression of the Lfng gene sweeps up the embryo, it moves further to the anterior on the left. Nodal expression is then induced around the boundary between Dll1 and Srr1 expression. This occurs only on the left, where the Ca2+ concentration is high; this might enhance the affinity of Notch for its ligands. Note that the node 'regresses' posteriorly between stages 5 and 6.

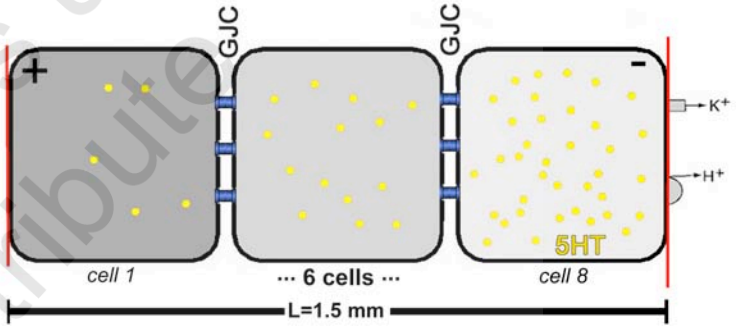
(after Monk, Nature, 2004)

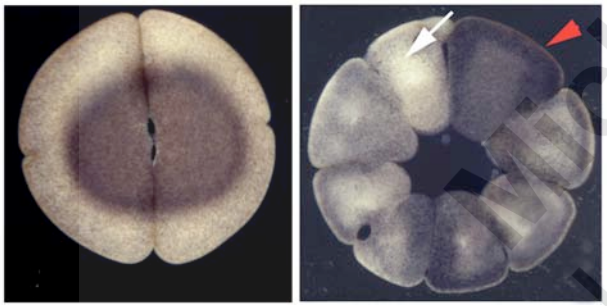
Serotonin and asymmetry

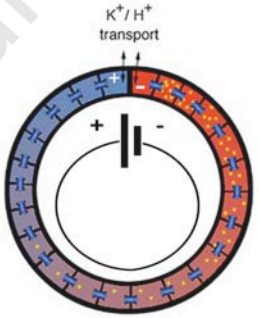
Current Biology, 2005, 15: 794-803
Developmental Neuroscience, 2005, 27:349-363

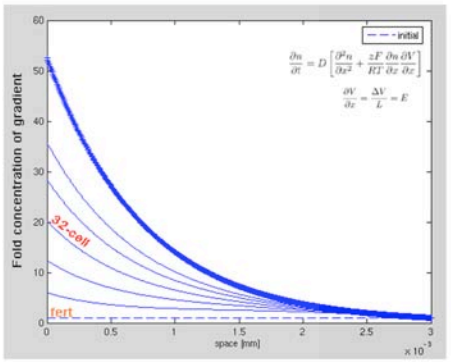




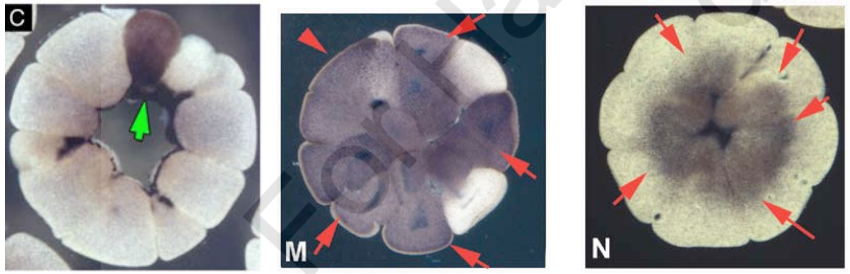


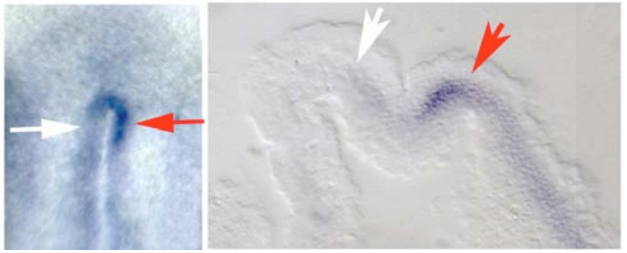






w.t. embryos: GJC inhibited: H,K-ATPase inhibited:





Problem 1: LR regulation at later stages

Early mechanisms:

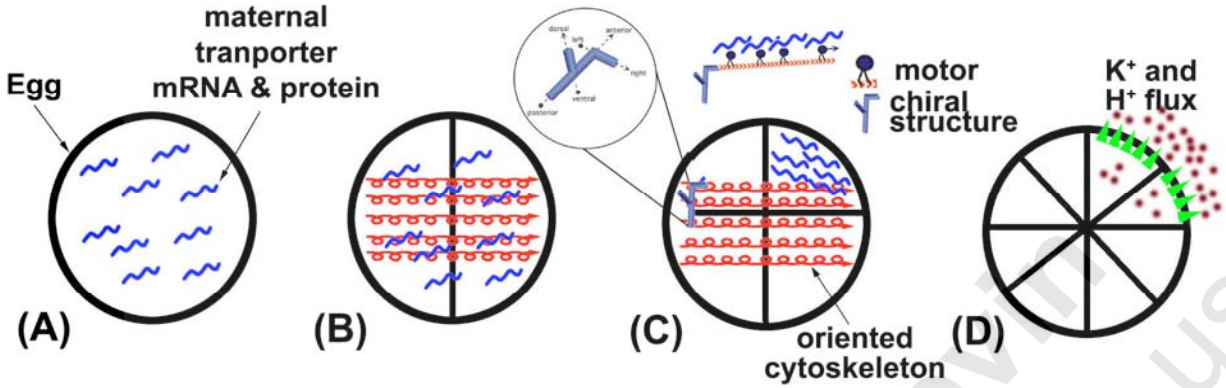
- syndecans
- Vg-1
- ion flux
- GJC

Yet,

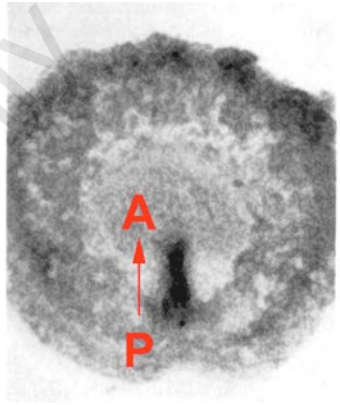
Nascone and Mercola, 1997, "Organizer Induction Determines Left–Right Asymmetry in *Xenopus,* Developmental Biology, **189,** 68–78 (1997)

show that normal asymmetry is present in an ectopic axis induced after MBT - too many cells to rely on large cleavage planes to segregate pumps.

What's the basis of this regulative ability? Different (parallel) mechanisms, or same mechanisms functioning later? How to test?

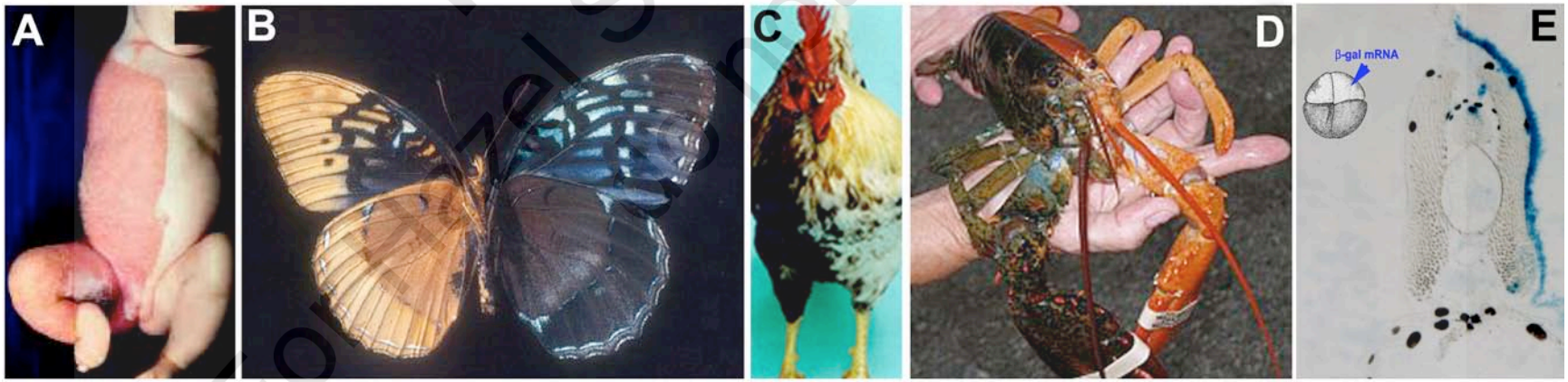


vs.



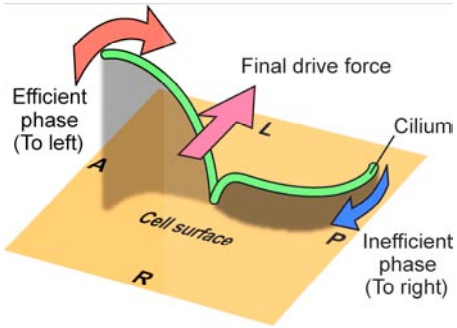
A big problem; but, is it possible that midline is actually set very early?!?

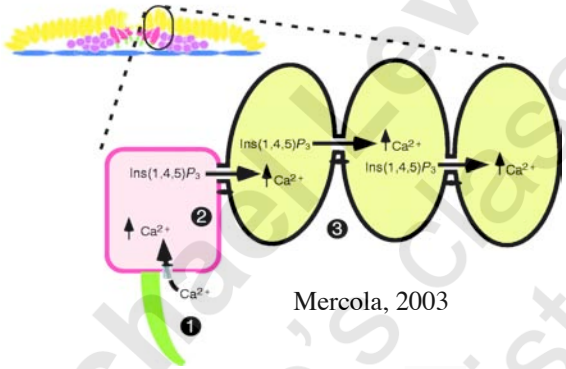
Bilateral gynandromorphs suggest midline is inked to 1st cleavages



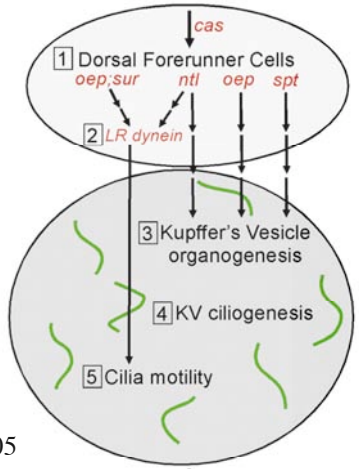
Problem #2: cilia - a case study in controversy

1) Kartagener's patients had immotile cilia and heterotaxia
2) Several mouse laterality mutants mapped to ciliary or motor protein proteins (*lrd*, KIF3B, etc.)
3) Experiments in cultured mice showed that exogenous flow across node could randomize asymmetric marker sidedness

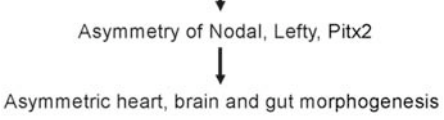




Mercola, 2003



Bisgrove and Yost, 2005



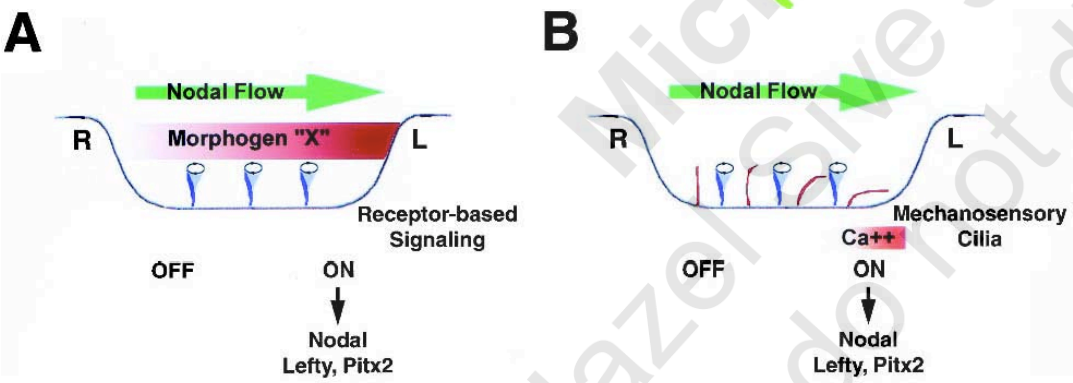
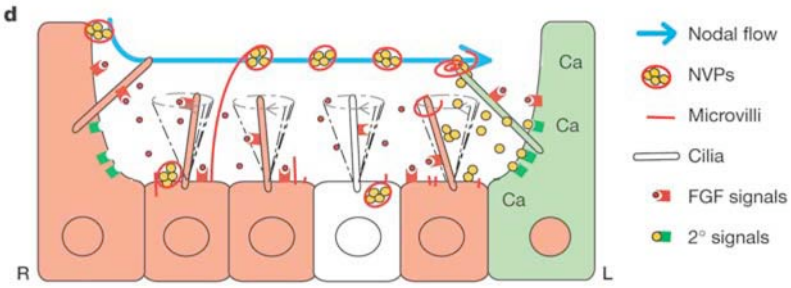


Figure 1. Alternative models of the proposed signaling events functioning downstream of nodal flow. (*A*) According to the morphogen flow hypothesis (Nonaka et al. 1998; Okada et al. 1999), a critical left determinant, morphogen "X" (red), is released symmetrically into the node region and subsequently becomes concentrated to the left side of the node in response to nodal flow (green arrow) generated by the clockwise vortical rotation of motile node cilia (blue). Morphogen "X" subsequently interacts with its receptor on the left side of the node to initiate expression of left-sided genes such *Nodal*, *Lefty*, and *Pitx2*. (*B*) According to the two-cilia model described here, nodal flow (green arrow) generated by the activity of motile node cilia (blue) produces differential fluid pressure at the two sides of the node, leading to asymmetric stimulation of mechanosensory cilia (red) distributed symmetrically in the node region. In response to stimulation, these mechanosensory cilia initiate a calcium-mediated signal transduction event that leads to the asymmetric induction of *Nodal* and other left-sided genes. Both panels illustrate transverse sections through the node region (ventral to the *top*). L, left; R, right.

GENES & DEVELOPMENT 17:1–6 © 2003

A two-cilia model for vertebrate left-right axis specification

Clifford J. Tabin and Kyle J. Vogan



Tanaka et al., 2005

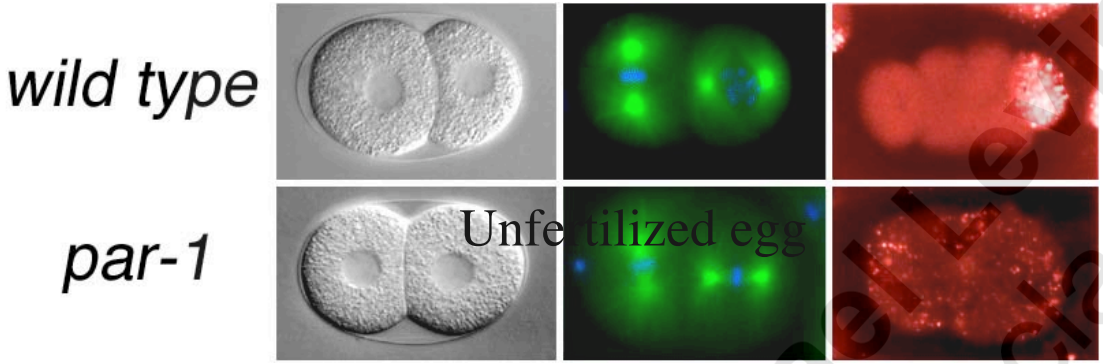
What is the role of ciliary motion in LR asymmetry?

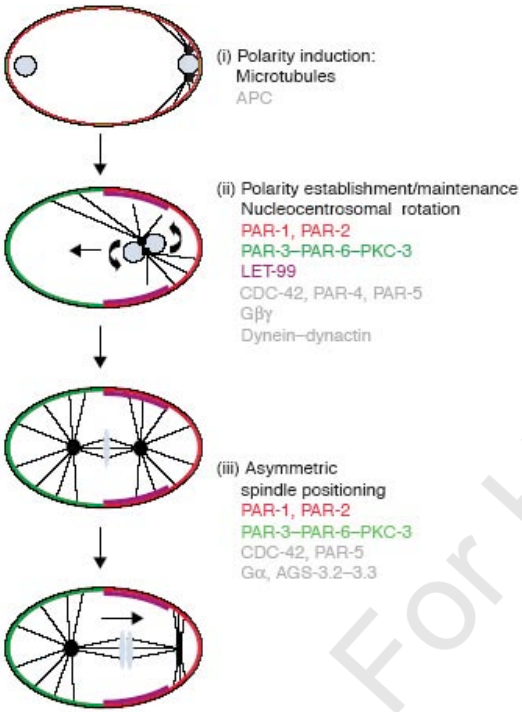
Is ciliary motion causal or an epiphenomenon?
Is ciliary motion the origin of asymmetry or a later step?
Is this mechanism unique to rodents or more general?

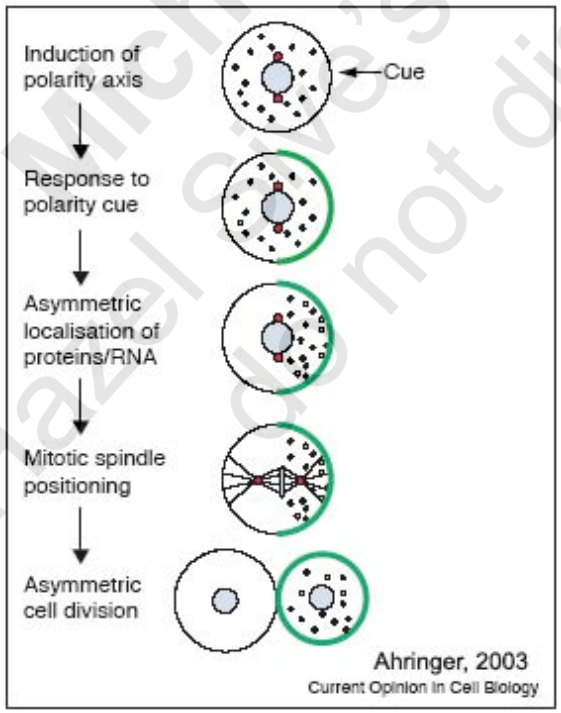
1) GJC and ion flux model has been studied in chick, *Xenopus,* zebrafish, *Ciona*, urchin; functional cilia (as distinct from knockouts of motors and cilia element genes) data are confined to the mouse. However, there is some overlap: PCKD Ca^{++} channel mouse knockout which has a LR phenotype, and asymmetric Ca^{++} flux exists at mouse node.
2) Timing data suggest that GJC/ion flux operates at stages long prior to the appearance of cilia, suggesting that cilia cannot be "Step 1" in chick or frog embryos.
3) The cilia model predicts that node cells generate LR information intrinsically. Data show that LR signals move along large-scale paths; several labs have shown that in the chick, the node is instructed by lateral tissue with respect to LR polarity.
4) Ultrastructure data argue against ciliary motion in the chick node. Conversely, the rodent embryo has an atypical morphology. Rabbit embryos (flat blastodiscs) are a better model (more similar to human embryos and most mammals).
5) Control culture of rodent embryo destabilizes asymmetry (Fujinaga's work in the 1990's). Despite elegant recent attempts, no one has shown a causal effect by manipulating ciliary function directly including a "no flow" control condition.
6) Mouse LR phenotypes which have been interpreted to support the ciliary model result from knockouts or mutations which affect motor protein function and other targets which may have cytoplasmic ion transport roles. A definitive test would require a mechanical block of ciliary motion in the absence of motor effects, or a K.O. acting after node formation.

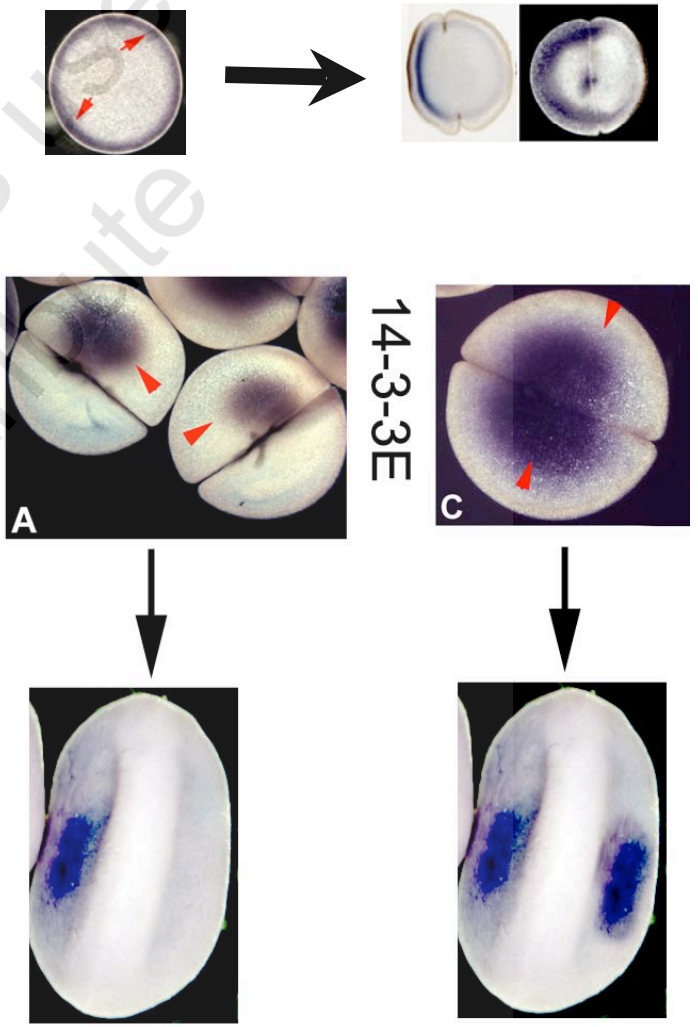
A common point between vertebrate LR asymmetry and basic mechanisms of cell polarity in *C. elegans*/*Drosophila*

(from Kemphues lab)



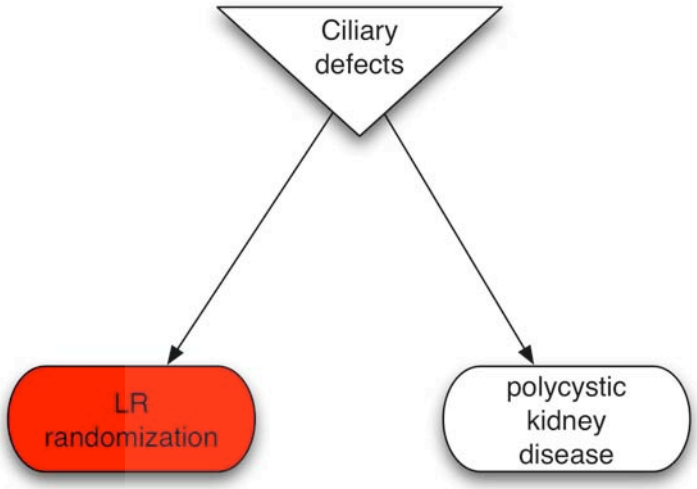




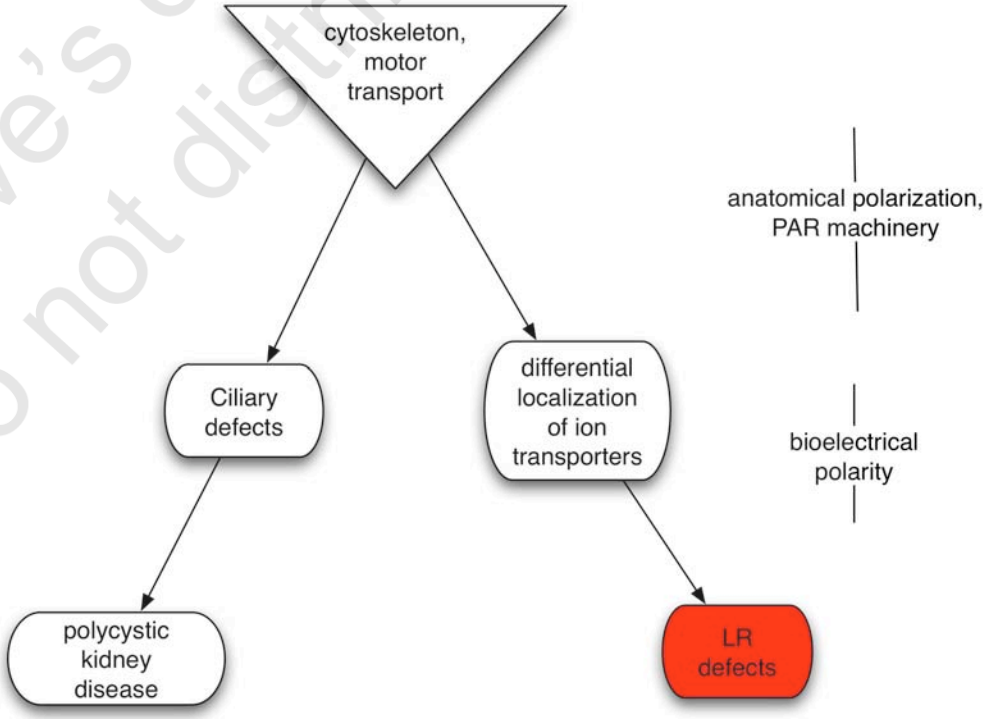


Development, 2003, 130: 4847-4858

Why do LR defects and kidney problems occur together?



nodal flow, Calcium signaling



anatomical polarization, PAR machinery

bioelectrical polarity

Kidney cells, like many epithelia, are highly polarized with respect to their apical-basal axis, using cytoskeleton and motor proteins to localize ion transporters (and other proteins) to specific parts of the cell.

Problem #3:

How conserved are the LR mechanisms upstream of *Nodal* expression?

Are there any evolutionary trends?

If so, what needs to be done?

Embryonic time: →

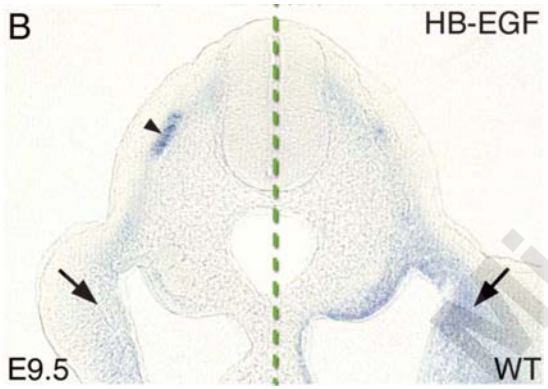
Complexity ↓

Evolutionary Relationships

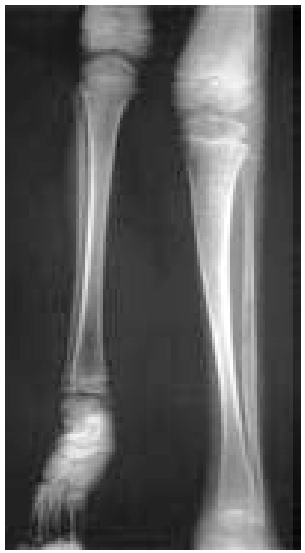
Model system	Cytoskeleton/ Motor protein	Ion flux	GJC	5HT	Cilia
Ciliates	√		X		
Arabidopsis	√	√	X		X
Lymnaea	√				X
C. elegans	√				X
Drosophila	√				X
Sea urchin larvae		√			
Ciona		√			
Zebrafish		√			√
Xenopus	√	√	√	√	
Chick		√	√	√	X
Human					√
Rabbit			√		
Mouse					√

Problem #4: cryptic asymmetries

1) Hemihypertrophy syndromes - which tissues have LR information and for how long? LR asymmetry matters far longer than just for embryogenesis.
2) Unilateral presentation of genetic syndromes, and one-sided drug effects, affecting morphologically-symmetrical paired organs
3) Asymmetric gene expression in symmetrical organs



(Golding lab)



How might this issue be addressed?

Now what?

1) What is the 1st step of asymmetry?
   - mouse: are cilia causal, are they 1st step?
   - other model systems: what determines the asymmetry of ion flux?

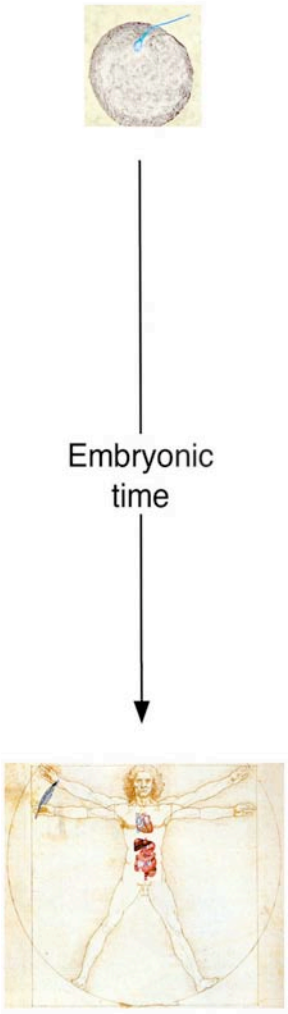
2) What mechanisms link early steps to asymmetric gene expression?
   - what is the very first asymmetric gene?
   - what is the role of gap junctions, syndecans, etc. in this process?
   - what is the small GJC morphogen? Serotonin?

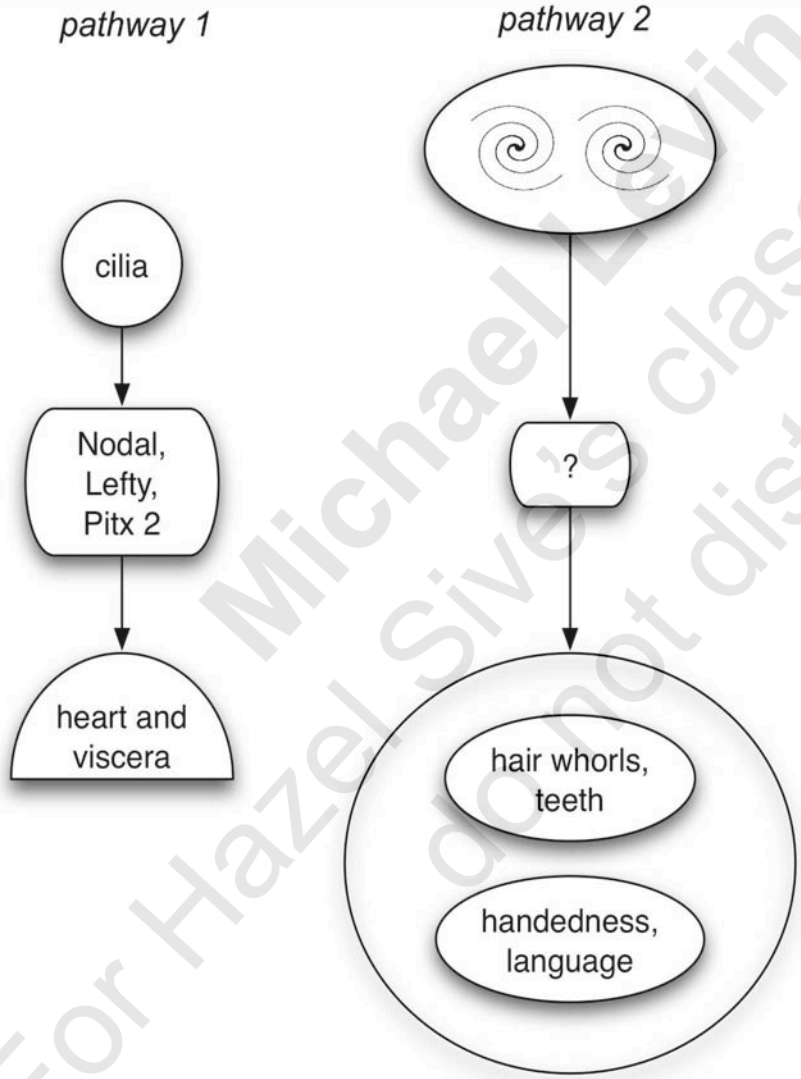
3) How conserved are the mechanisms?
   - do cilia play a role in any organism other than mice/fish?
   - do GJC/ion flux/serotonin function in mammals?
   - how do different early mechanisms all converge on left-sided *Nodal* cassette?

4) What is the nature of LR identity in symmetrical tissues?

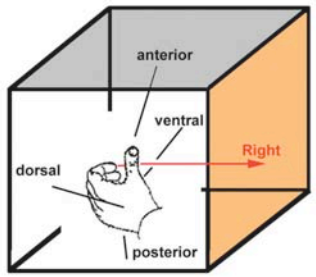
5) What is "randomization", and what exactly is "integration" at the node?

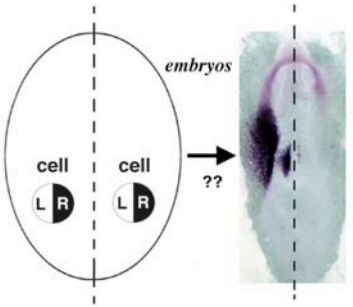
6) What pathway determines the chirality of hair whorls, brain laterality, and other non-visceral asymmetries in human embryos?





Developmental Task	Likely Mechanism	Molecular Candidate
differentiate L from R by orienting 3rd axis with respect to AP & DV	cytoskeleton, cleavage planes, chiral molecules	inv, polaris, dextral snail factor, cilia
transduce chiral information into LR position, transmit to multicellular fields	protein localization, small molecule flow, ion flux, ciliary motion	connexins, ion transporters, syndecans, adhesion molecules, 5HT
pass LR identity to numerous domains within embryo	cascade of asymmetric gene expression	asymmetric secreted proteins in the TGF-β pathway, Shh, etc.
asymmetric organogenesis	differential proliferation, adhesion, migration	Pitx-2, dhand, ehand





Consider our lab for rotations, Ph.D., and post-doc work!

We have projects in:

1) Ion transporter roles in asymmetry
2) Biophysical mechanisms controlling vertebrate regeneration
3) Neurotransmitter roles in pre-neural morphogenesis
4) Non-neural mechanisms of memory and learning
5) Gap junction-mediated signals in stem cell regulation

We use model systems including:

- Chick
- Frog (*Xenopus*)
- Planaria (flatworm)
- Axolotl
- Zebrafish

We use approaches including:

- Molecular, cell, developmental biology
- Physiology, pharmacology, biophysics
- Computer/mathematical modeling

http://www.drmichaellevin.org
mlevin@forsyth.org

The Levin Lab: working to understand biophysical mechanisms of morphogenesis

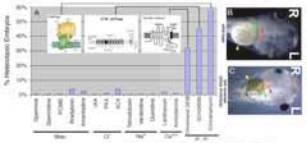
We use a combination of molecular genetics, pharmacology, cell biology, and physiology to understand and learn to control important large-scale morphogenetic modules which function in embryonic development, cancer, and regeneration. We specifically focus on the patterning roles and information content of endogenous ion flows and voltage/pH gradients. Model systems used in our lab: Xenopus, chick, planaria, axolotl, zebrafish. Please see **http://www.drmichaellevin.org/** for more information on our lab and reprints.

Ion flows, serotonin, and the establishment of left-right asymmetry

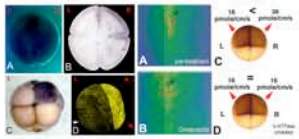
Many body-plans superimpose a consistently-biased chirality upon a basically bilaterally-symmetrical bodyplan. The origin, significance, and mechanisms of this left-right asymmetry raise fascinating problems in developmental and evolutionary biology. It is also of considerable biomedical relevance because of a number of laterality-based birth defects.

While a cascade of asymmetrically-expressed genes has been found, early (upstream) mechanisms are poorly-understood and highly controversial. Our earlier work showed that signals mediated by gap junctions were crucial at very early stages in two vertebrate species.

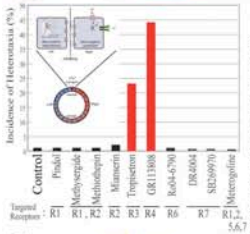
Recently, we pursued 2 directions: what provides the motive force for directing small-molecule signals through long-range gap junctional paths, and the molecular nature of the signal.



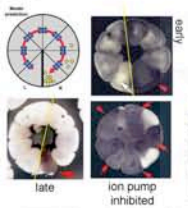
A pharmacological screen allowed us to rapidly implicate four ion transporters. We then used specific dominant-negatives to validate the involvement of these targets. Inhibition of two H^+ pumps and two K^+ channels specifically results in randomization of early asymmetric markers and of the heart and viscera. These targets have now been shown to be involved in LR asymmetry in other species, including sea urchin, zebrafish, and chick.



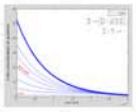
By characterizing the localization of these ion transporters, we discovered a novel, early, motor protein- and cytoskeleton- dependent asymmetry in protein localization. Also, by characterizing membrane voltage using *in vivo* voltage-sensitive dyes, and extracellular ion flux using ion-selective vibrating probes (with Ken Robinson at Purdue), we found consistently-biased asymmetries in membrane voltage and ion efflux which are dependant upon the activity of the implicated transporters.



The data suggested a model whereby voltage gradients across a long-range circumferential path of cells coupled by gap junctions provide the motive force for small charged morphogens to accumulate on one side of the midline. We used a pharmacological approach to ask whether serotonin (5HT) might be a candidate target. Serotonin signaling via receptor types R3 and R4 was implicated. Molecular gain- and loss-of-function experiments using dominant negative and constitutively active serotonin pathway molecules confirmed the involvement of maternal serotonin signaling in LR asymmetry long before neuronal cells appear. Moreover, 5HT is endogenously present in a circumferential LR gradient and accumulates on one side of the midline in a GJC- and ion pump-dependent process.

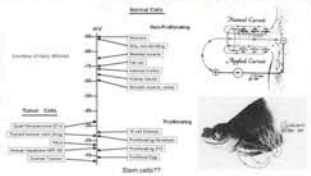


Our data implicate a novel intracellular serotonin receptor; we are pursuing array approaches to identify immediate downstream targets of serotonergic signaling in very early (pre-neural) cells.



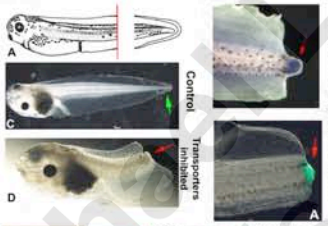
We are also (with James Weaver @ MIT) pursuing mathematical modeling approaches to synthesize this physiological and molecular data into a predictive mechanistic model of control of large-scale axial patterning by early flows of small molecules controled by bioelectric gradients.

Ion flows and the control of regeneration

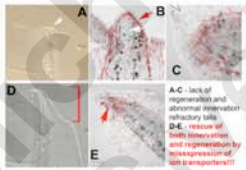


Intriguing older data suggest that membrane voltage potential in non-excitable cells is an important parameter controling cell proliferation, migration, and differentiation. Moreover, strong currents driven out of regenerating systems have been suggested to underlie the ability of some animals to regenerate whole limbs. Indeed, some promising data shows that regeneration can be induced in normally non-regenerating species by imposition of appropriate currents.

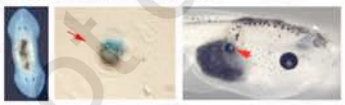
We have brought this paradigm to two molecularly-tractable model systems, to understand how endogenous bioelectric events control pattern and the growth profile of different tissues.



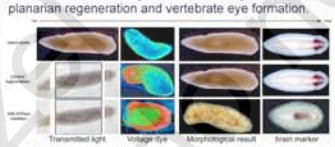
The Xenopus embryo regenerates its tail - a complex appendage including muscle and spinal cord. Our pharmacological screen implicated an H^+ pump, a Na^+ channel, and a K^+ channel as specifically required for regeneration but not for primary tail growth, wound healing, or normal patterning of the rest of the embryo. All three of the implicated targets are specifically expressed in the blastema within 12 hours of amputation. We also characterized the specific depolarization which is dependent on the activity of these three transporters and required for regeneration.



The abrogation of regeneration by inhibition of the function of these 3 ion transporters does not occur by apoptosis, but rather through an inhibition of appearance of proliferating cells in the blastema. Our data suggest a detailed epistatic pathway of biophysical events and protein function and reveal long-range influence to/from remote signaling sites upon regenerative ability. We are working to apply these data to gain-of-function approaches to induce regeneration in species which normally do not regenerate.



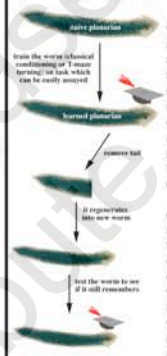
By specific misexpression of ion transporters designed to alter bioelectrical properties of cells in predicable ways, we have shown that such gain-of-function approaches can be used to alter the large-scale patterning of planarian regeneration and vertebrate eye formation.



We have also identified novel gap junction-mediated signals for the control of adult stem cell properties. We are now pursuing mathematical and computer modeling approaches to understand how bioelectrical properties of cells instruct their neighbors to assemble complex structures (initiation of high-level morphogenetic programs); this work is intended to eventually allow fine control over tissue growth in biomedical contexts to repair injury and combat senescence.

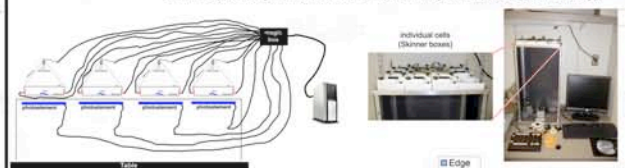
Memory and morphogenesis: what does half a worm know?

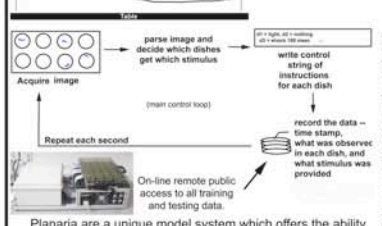
Planaria offer an unprecedented opporunity to study regeneration and memory in the same molecularly-tractable model system; we are investigating where and how memory may be stored and the mechanisms by which memories may be transferred to the regenerating brain from other tissues.

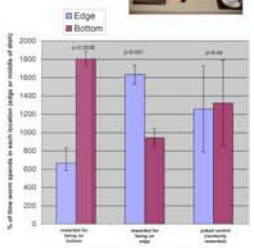


Older work suggested that worms which arise from the tail end of a trained worm do in fact remember tasks upon which they were trained before amputation. This is a striking finding and suggests that (1) memories may not be localized to the brain, and (2) this information can be imprinted on the regenerating brain (enabling it to control the new worm correctly during recall testing). The ability to train the worms and assay memories after regeneration and other genetic and pharmacological manipulations would enable addressing questions such as: (1) does memory require neuronal activity or gap-junctional communication? (2) does memory require any active process at all or is it encoded exclusively in structural changes (does cryogenic freezing abolish memories)? (3) which tissues can store memories, and does this extend to non-neuronal cells?

However, the older work degenerated into work on implausible models of molecular coding of arbitrary memories in RNA (cannibalism studies), and more importantly, were subject to a number of problems stemming from the fact that the tedious training and testing had to be done by hand. We are developing this system into a powerful assay to study the biophysical properties of memory by establishing an automated, computer-controlled, parallel device which enables worms to be trained on an instrumental learning task and tested, all without human intervention and with full on-line data recording. This device will allow large numbers of worms to be trained and the data to be publically available, to demonstrate robust learning (necessary for future experiments) and unequivocally demonstrate the most striking claim (extra-CNS memory).

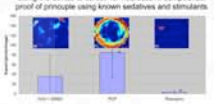






Planaria are a unique model system which offers the ability to investigate memory and behavior in a completely viable organism with bipolar brains or no brains. Our ability to induce predictable and radical large-scale changes in the CNS architecture of the worms, together with the automated device, will allow us to investigate plasticity of neural control mechanisms.

Moreover, our device is a prototype for a large-scale system for screening small molecule or RNAi libraries for novel compounds with neuroactive effects (e.g., improve memory, counteract drug addiction or neurotoxins, enhance sensory modalities, etc.); planaria offer complex behavior and CNS structure (unlike yeast/cell culture screens).



Our work was supported by NIH, NSF, American Heart Association, American Cancer Society, March of Dimes.