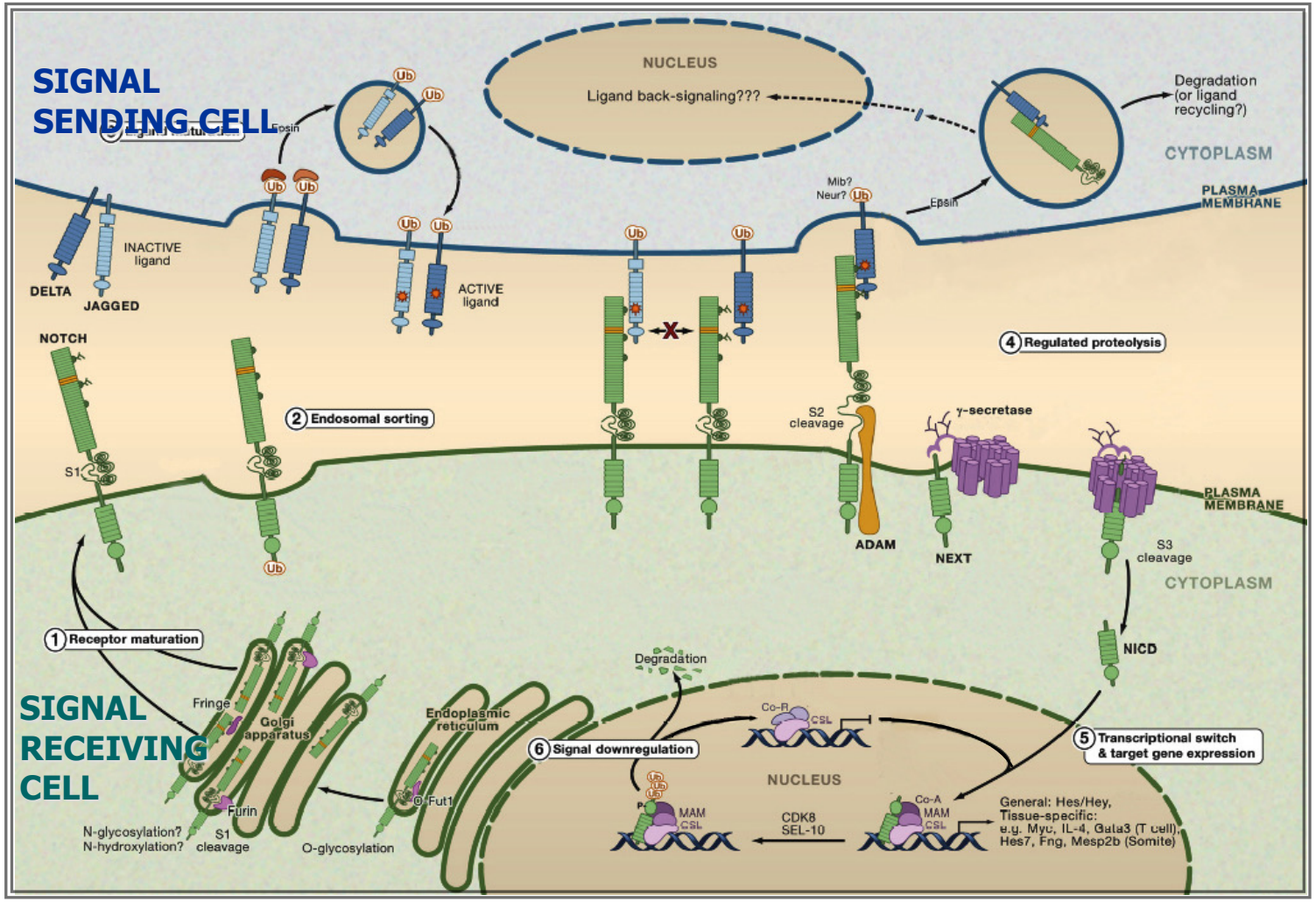
Notch Signalling Pathway

A brief history

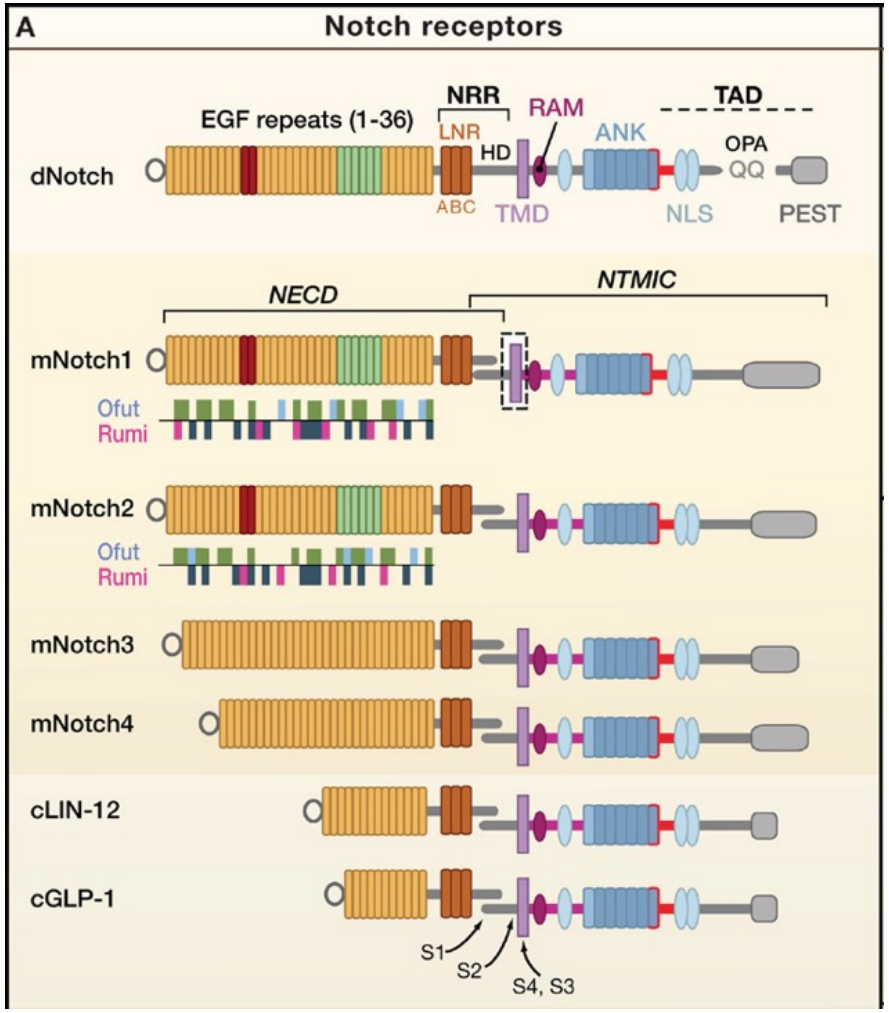
- In 1917, Thomas Hunt Morgan described a strain of *Drosophila* with notches at the end of their wing blades, which result from haploinsufficiency
- Notch gene was cloned in the mid-1980s

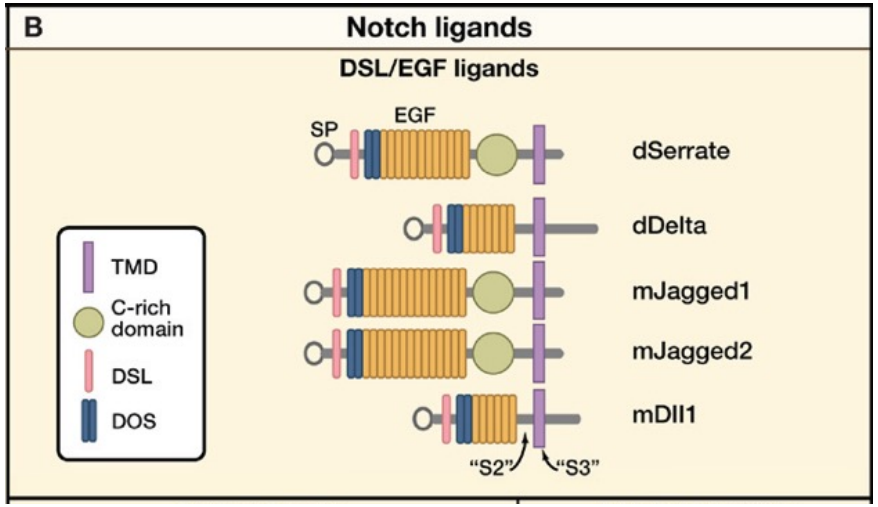
The Notch signalling pathway



UNIQUE FEATURES

- each Notch molecule is irreversibly activated by proteolysis
- signals only once without amplification by secondary messenger cascades

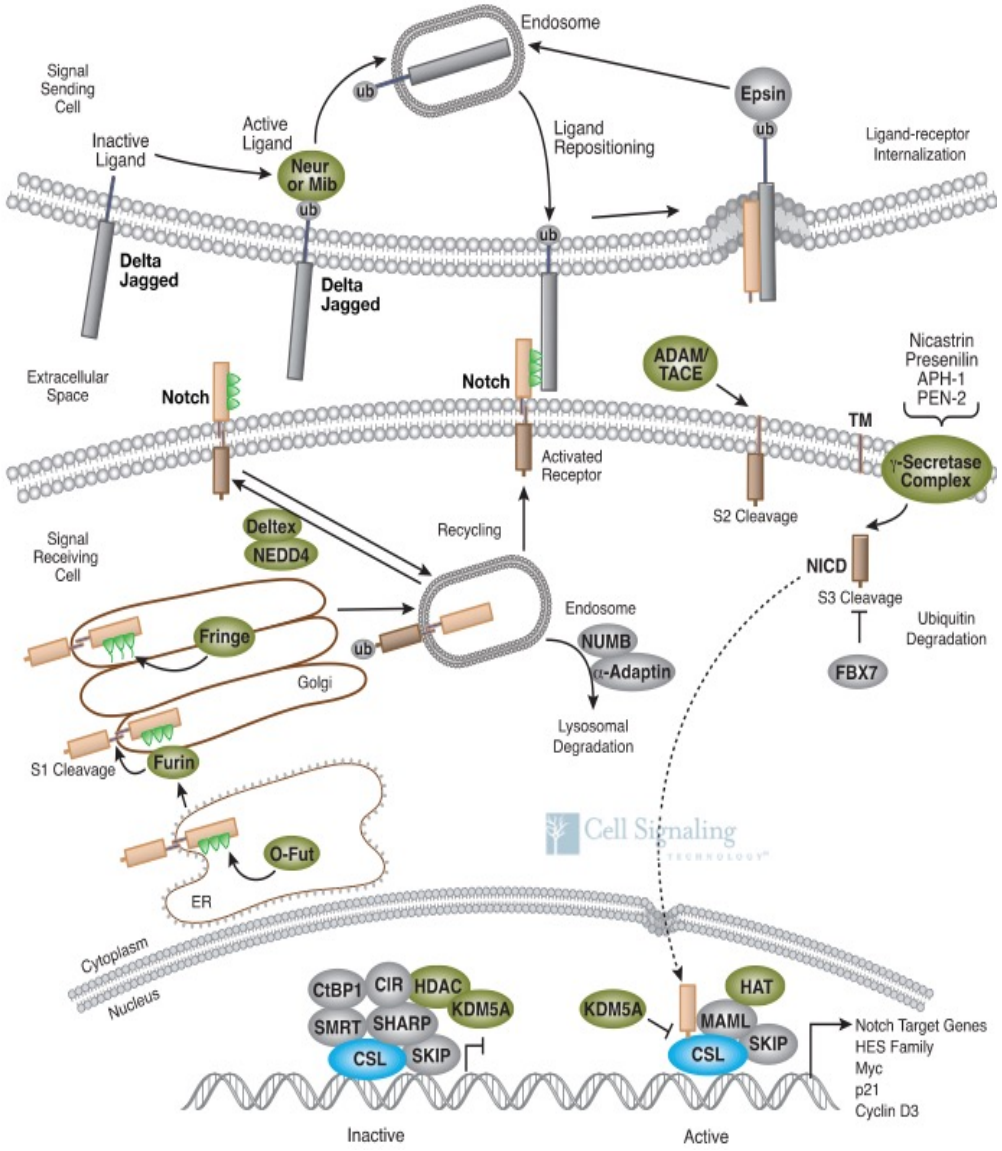




(A) Notch receptors are transmembrane proteins that contain multiple EGF-like repeats, involved in ligand interactions, fucosylation and glucosylation. They also contains a transmembrane domain (TMD), a RAM (RBPjκ association module) domain, nuclear localization sequences (NLSs), seven ankyrin repeats (ANK) domain, and a transactivation domain (TAD) that habors a PEST domain.

(B) Known and putative ligands of Notch receptors can be divided into several groups on the basis of their domain composition.

The Notch Signaling Pathway Is Mediated by Regulated Proteolysis



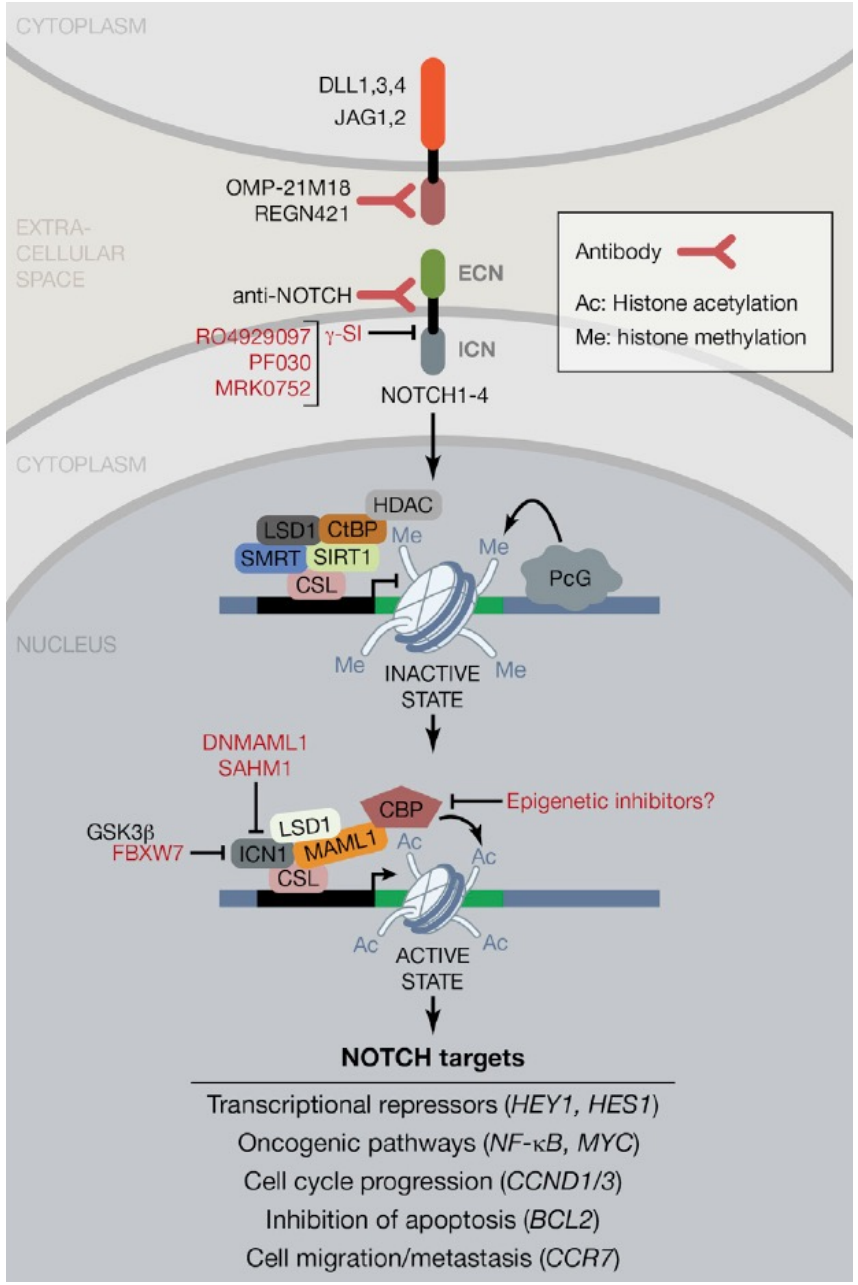
The mature Notch receptor is produced after glycosylation and proteolytic cleavage by PC5/furin at (S1) site and is targeted to the cell surface as a heterodimer.

Notch is activated by binding to the ligand presented by a neighboring cell. Ligand endocytosis generates the mechanical force to promote a conformational change in the bound Notch receptor.

This conformational change exposes site S2 in Notch for cleavage by ADAM metalloproteases. Juxtamembrane Notch cleavage at site 2 generates the NEXT fragment, which is cleaved by the γ-secretase complex to release the Notch intracellular domain (NICD) and Nβ peptide. NICD enters the nucleus where it associates with the DNA-binding protein CSL (CBF1/RBPjk in vertebrates. The coactivator Mastermind (MAM) recognizes the NICD/CSL interface, and this triprotein complex recruits additional coactivators to activate transcription.

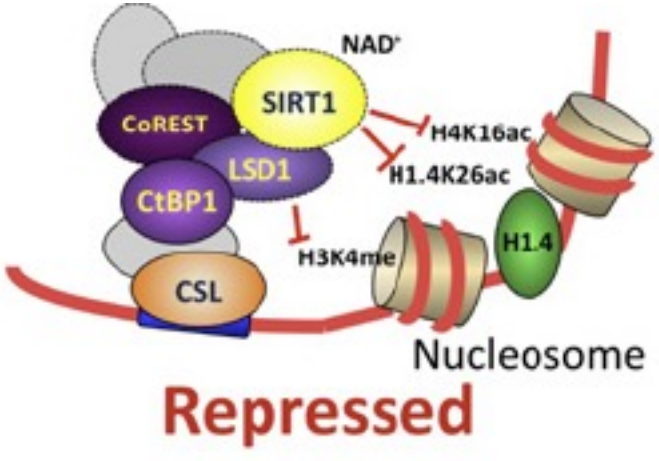
In the absence of NICD,CSL may associate with ubiquitous corepressors (Co-R) and HDACs to repress transcription of the target genes.

Overview of the Notch Signaling Pathway



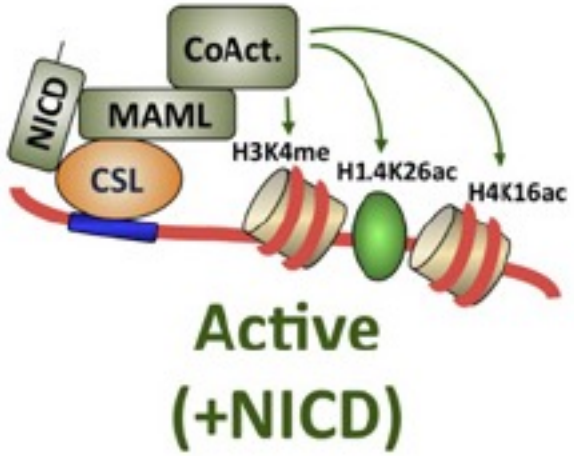
HDAC, histone deacetylase; ICN1, intracellular part of NOTCH1; LSD1, lysine-specific demethylase 1; SMRT, Silencing-Mediator for Retinoid/Thyroid hormone receptors; GSK3b, glycogen synthase kinase 3 beta; DNMAML1, dominant-negative MAML1

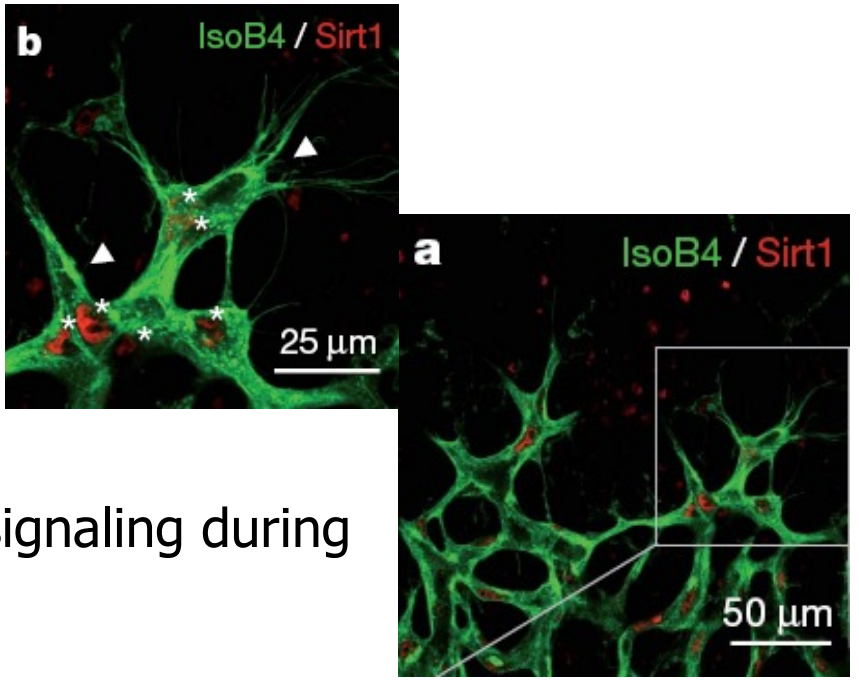
SirT'N repression for Notch



SIRT1 deacetylase acts in concert with the LSD1 demethylase to repress Notch-induced transcription

Mulligan P. et al., 2011





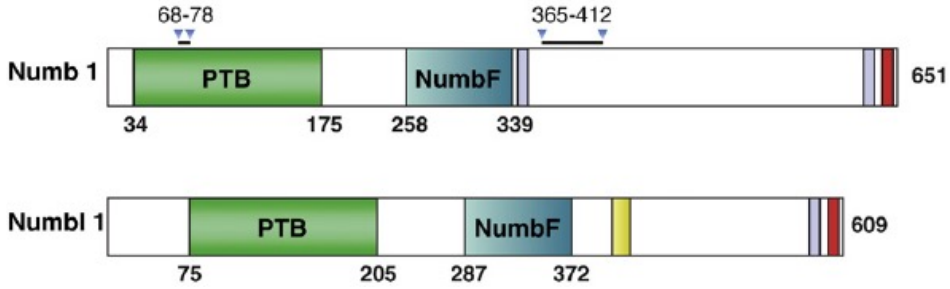
SIRT1 inhibits endothelial cell Notch signaling during angiogenesis in zebrafish and mice.

Guarani V. et al., 2012

Modulators of Notch signalling

- Fringe: regulate notch
- Numb: notch inhibitor
- Neuralized (E3 ubiquitin ligases): regulate ligand Delta.
- Mib (mindbomb): (E3 ubiquitin ligase): function on Delta

Modulators of Notch signalling: Numb



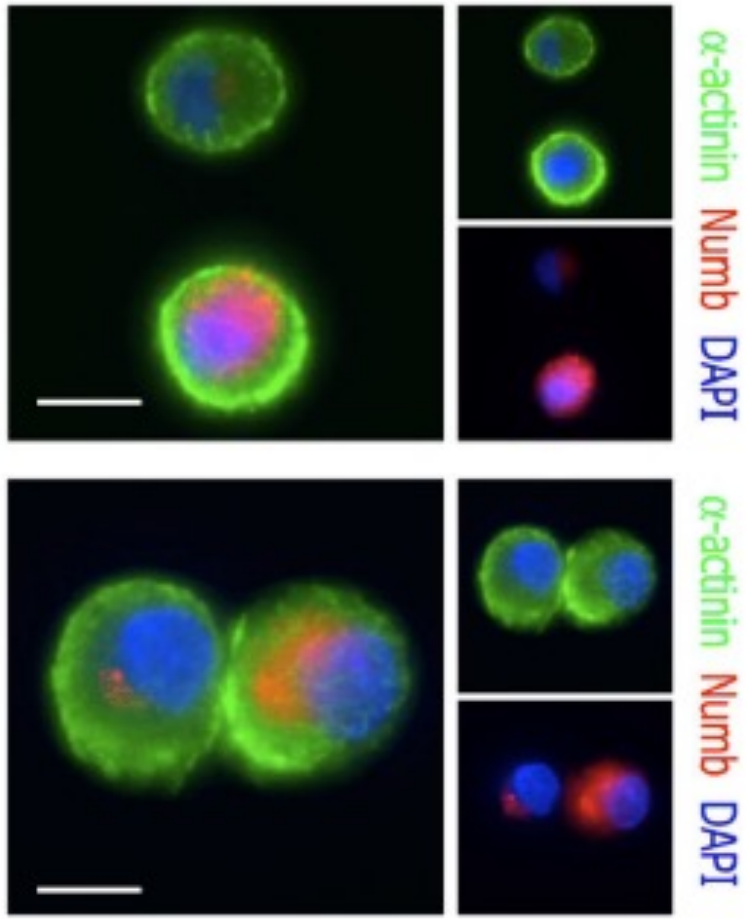
Pece et al., 2011

- Numb domains:
  - 1. PTB domain; N-terminal phosphotyrosine binding domain
  - 2. proline-rich C-terminal region.

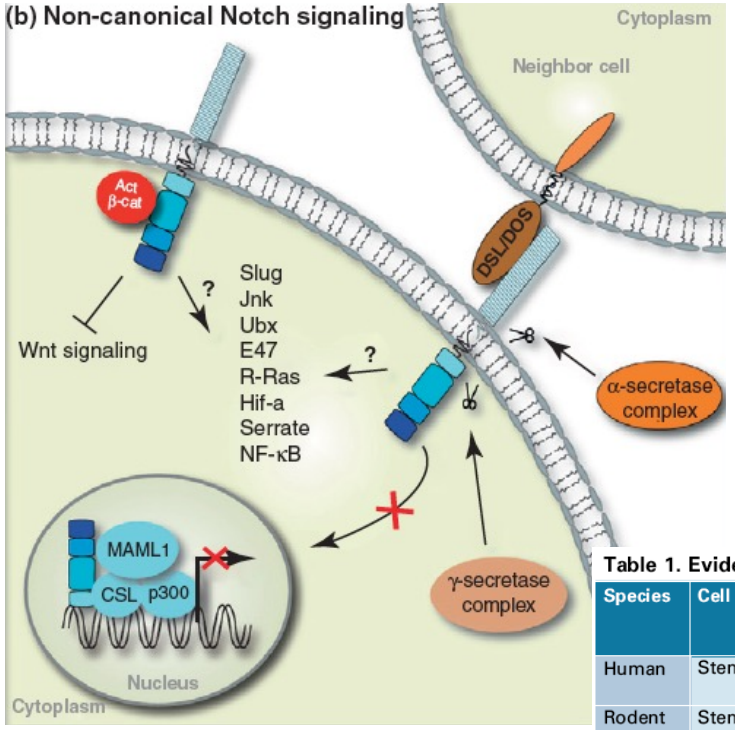
- Numb binds directly to NICD. The Cterminal half of the PTB domain and the N-terminus of Numb are required to inhibit Notch. Numb also has two motifs associated with endocytic proteins.

- mammalian Numb (mNumb) localizes to clathrin coated pits and early endosomes, might target endocytosed NICD for proteosomal destruction.

- Numb acts either upstream of S3 cleavage site of Notch or inhibit the endocytosis of membrane-bound activated Notch.



Non-canonical Notch Signaling Pathway

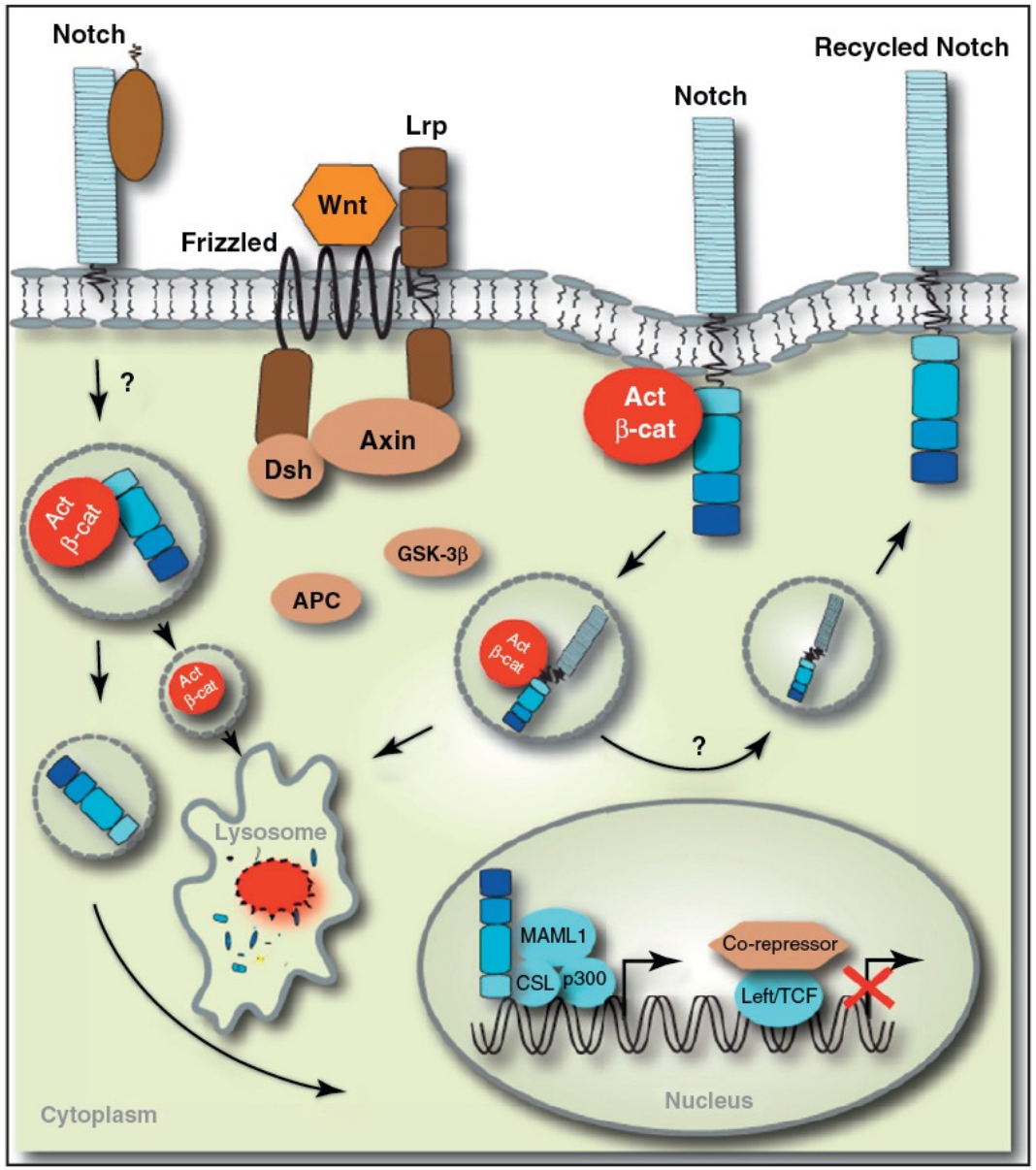


Non-canonical Notch signaling is CSL-independent and can be either ligand-dependent or independent. In most cases the mediators of non-canonical Notch signaling are unknown.

The most well-studied and conserved effect of non-canonical Notch function is regulation of **Wnt/β-catenin signaling**: Notch binds and titrate levels of the active β-catenin. Therefore, active β-catenin activity is a readout for non-canonical Notch signals.

Table 1. Evidence of CSL/ligand-independent Notch signaling

Species	Cell type	System	Independence	Function	Interacting molecule/ signaling (direct or indirect)	Refs
Human	Stem cells (hESCs), Cancer	*in vitro*	Ligand, CSL	Negative regulation of Wnt signaling	Active β-catenin/ Wnt signaling	[5]
Rodent	Stem cells (mESCs, NSCs, MSCs), Progenitors (CPCs)	*in vivo, in vitro*	Ligand, CSL	Negative regulation of Wnt signaling	Active β-catenin/ Wnt signaling	[5]
	T cells	*In vitro*	CSL	Notch-1 stimulates NF-κB	NF-κB pathway	[28]
	Primary embryonic cells	*in vitro*	PS, Ligand	HES1 activation and MCK inhibition	HES1 and MCK	[6]
	Skin progenitors	*in vivo*	CSL	Leukocytosis, longevity	nd	[7]
	Muscle stem cells (C2C12)	*in vitro*	CSL	Inhibition of muscle cell differentiation	nd	[8–10]
	Fibroblasts (3T3)	*in vitro*	CSL	Inhibition of E47	E47	[11]
	CHO cell line	*in vitro*	CSL	b1 integrin activation	R-Ras	[12]
Avian	Neural crest (stem cells)	*in vivo*	CSL	Slug expression	Slug	[13,14]
Frog	Embryo	*in vivo*	CSL	Negative regulation of Wnt signaling	β-catenin/ Wnt signaling	[15]
Fly	Wing primordium	*in vivo*	Ligand, CSL	Negative regulation of Wnt signaling	Active β-catenin/ Wnt signaling	[16,17,27]
	Muscle progenitors	*in vivo*	Ligand, CSL	Muscle precursor selection	Wnt signaling	[18,19]
	Neural progenitors	*in vivo*	Ligand, CSL	Neuronal Cell (MP2) selection	nd	[20]
	Blood cells	*in vivo*	Ligand	Hemocyte survival	Hif-a	[21]
	Wing primordium	*in vivo, in vitro*	CSL	Inhibition of ligand function	Serrate	[22]
	Embryo	*in vivo*	CSL	Dorsal epidermis patterning (closure)	JNK pathway	[23]
	Visceral mesoderm progenitors	*in vivo*	CSL	Inhibition of Wnt signaling	Ubx	[24]
	Neural precursors	*in vivo*	CSL	Repression of neural fate	Wnt signaling	[25,26]



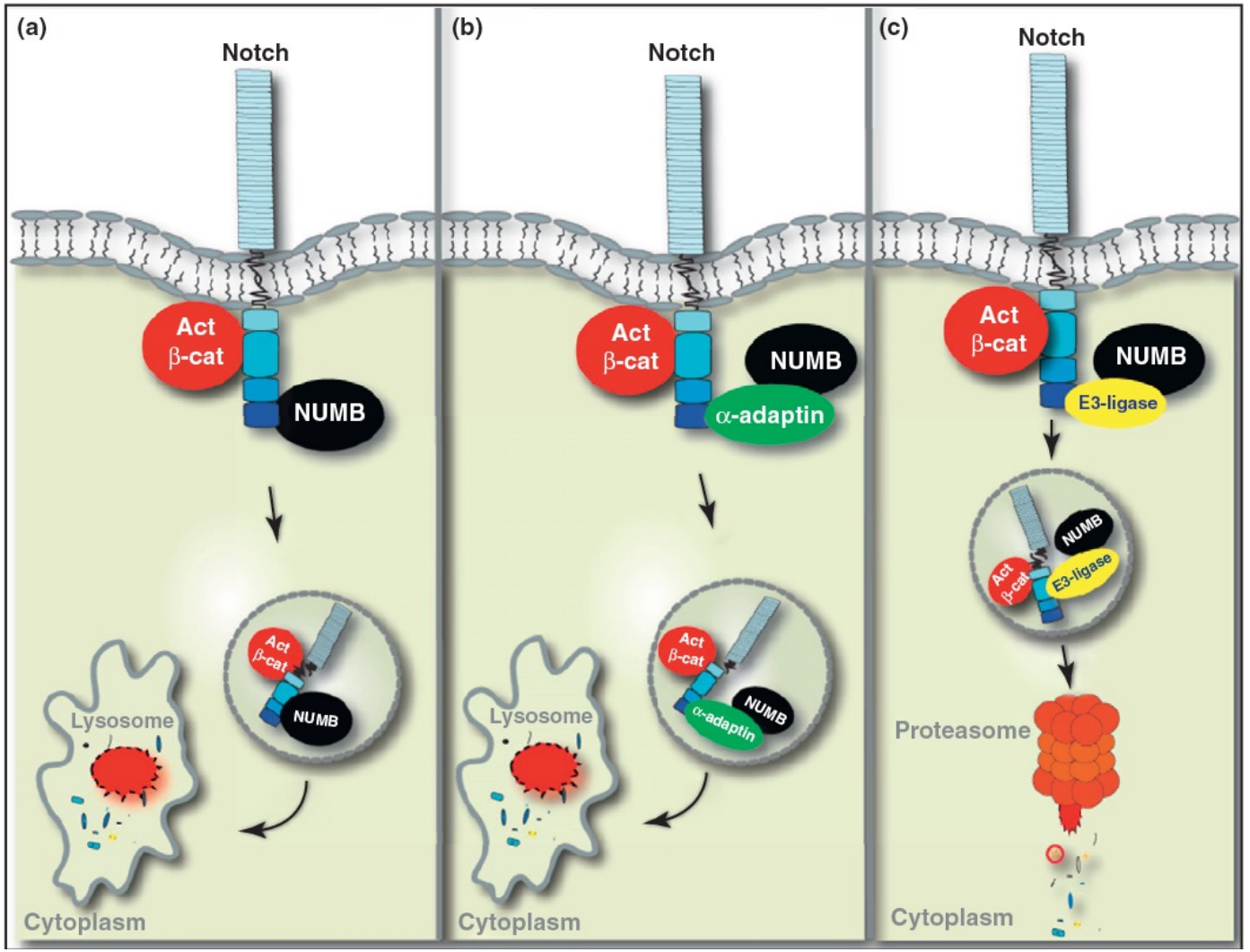
Post-translational regulation of β-catenin by Notch.

In the presence of Wnts, membrane-bound Notch forms a complex with active β-catenin and degrades active β-catenin through an endo-lysosomal pathway.

The degradation is independent of GSK3β-dependent destruction complex.

Whether Notch is recycled back to the membrane is unclear.

Numb regulation of Notch and β-catenin

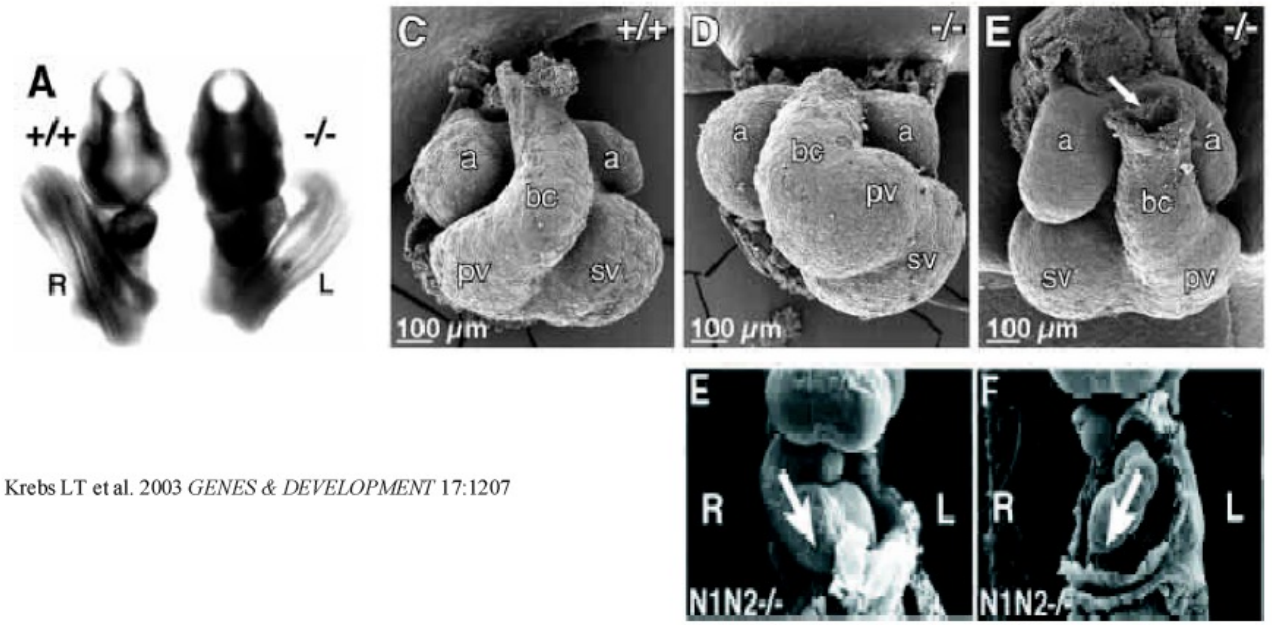


Numb could bind to Notch either directly (a) or dependently from α-adaptin (b), with targeting of the Numb-Notch complex for lysosomal degradation. In both cases it might be possible that activated β-catenin could be targeted for lysosomal destruction. Downregulation of Notch may occur through proteasome-mediated degradation (c).

Notch signaling has effects in many different organs

- Notch signalling can maintain stem cells or precursor populations in an undifferentiated state
- Notch signalling influences binary cell-fate decisions via lateral or inductive signalling
- A third property of Notch is its ability to influence differentiation and cell-cycle progression

Notch and left-right asymmetry

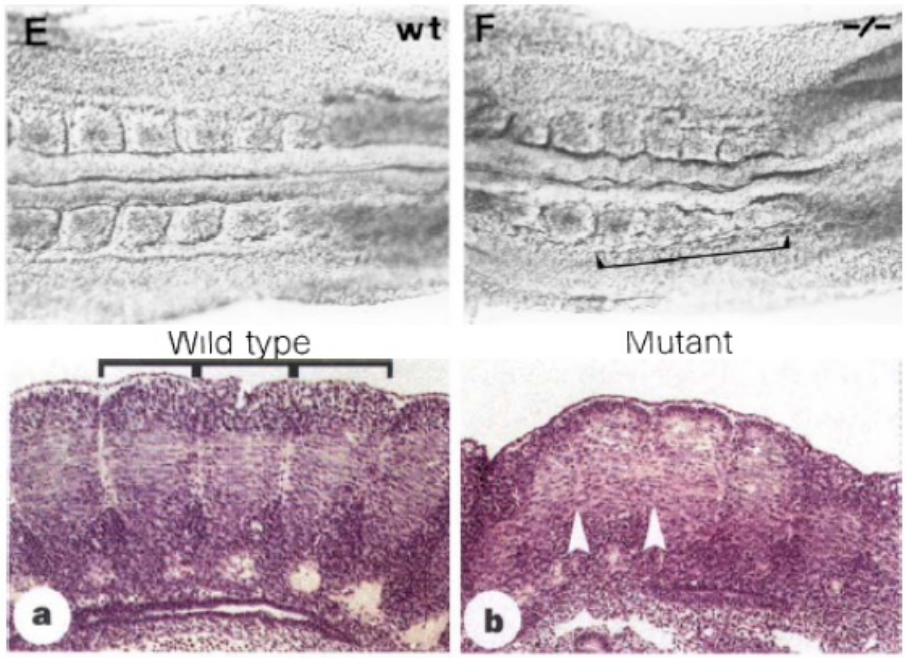


Krebs LT et al. 2003 *GENES & DEVELOPMENT* 17:1207

Embryos mutant for the Notch ligand Dll1 or doubly mutant for the Notch1 and Notch2 receptors exhibit multiple defects in left–right asymmetry.

The Notch signaling pathway plays a primary role in the establishment of left–right asymmetry in mice by directly regulating expression of the *Nodal* gene.

Notch and somitogenesis



Notch1-/-

Conlon RA et al. 1995
Development 121: 1533–45

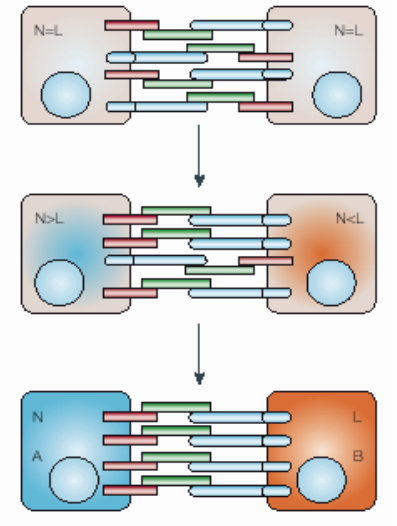
Dll1-/-

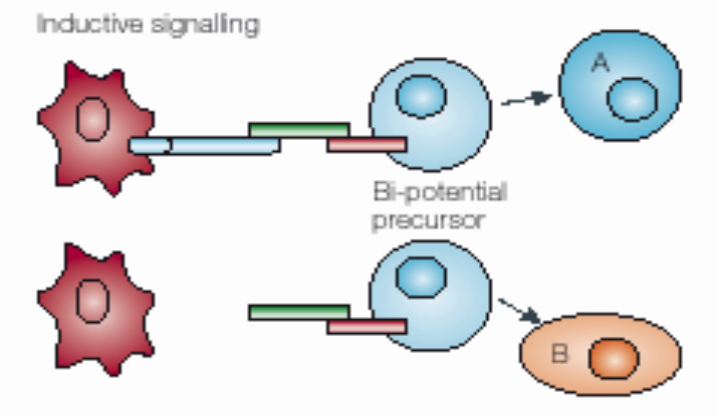
Hrabe de Angelis M et al. 1997
Nature 386:717–21

The positioning of segmental boundaries is chaotic, resulting in a large variation in somite size.

Notch signaling and cell-fate decisions

Notch signaling can have different effects depending on the timing and the tissue context. It is acting on cell fate decisions either through lateral signaling or through inductive signaling .

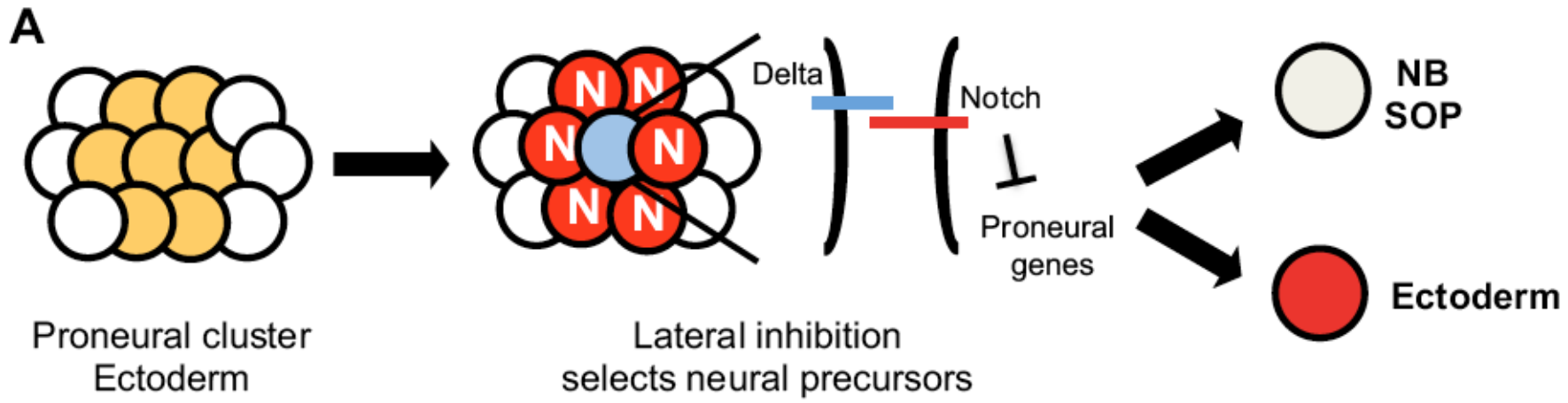




In lateral signaling, equipotent cells initially express both Notch receptors and their ligands, but the concentrations of these proteins start to differ between neighboring cells.
Differences in receptor and/or ligand concentrations in cells are amplified over time, leading to cells that exclusively express either the receptors or their ligands, thus guiding the specification of the cell fate and differentiation.

In inductive signaling, two distinct cells express exclusively either the receptor or the ligand. The fate of the bi-potential precursor cell is decided by the occurrence of this interaction. The cell expressing the receptor, and therefore the recipient of the Notch signal, is induced to differentiate into a particular cell lineage.

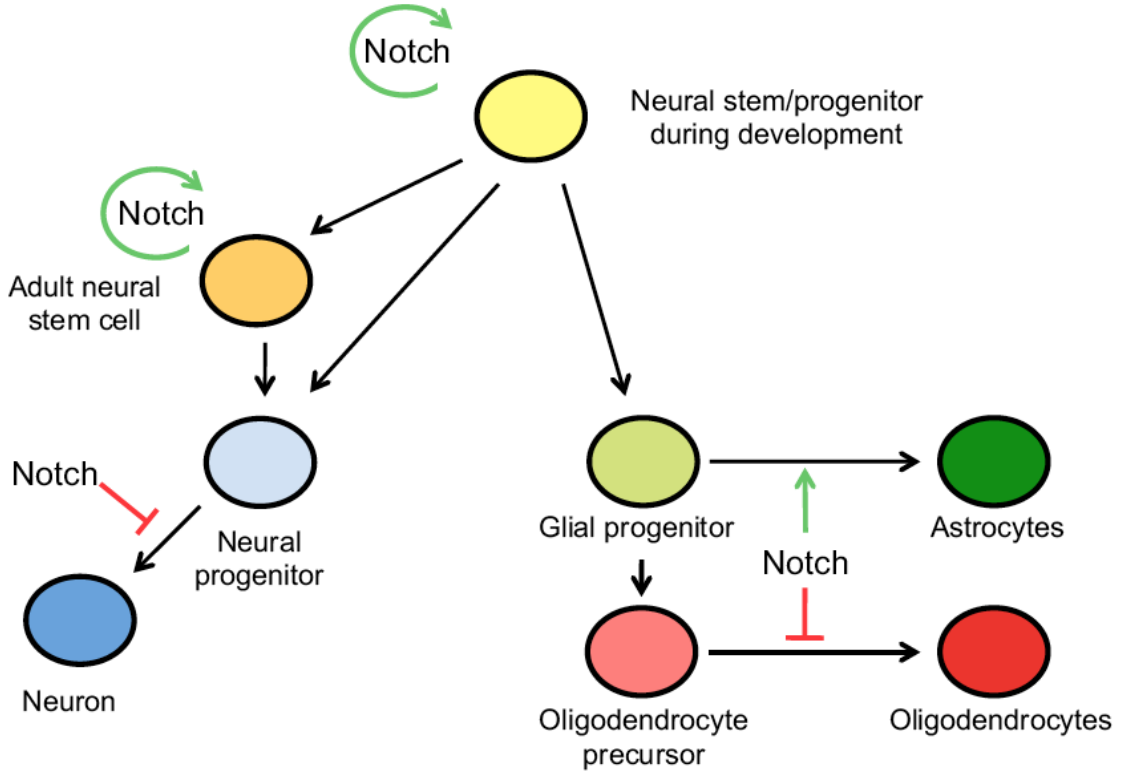
Notch function in the developing nervous system of Drosophila.



Lateral inhibition within the developing nervous system of Drosophila.

The role of Notch signaling during selection of neuroblasts (NBs) in the CNS or sensory organ precursor (SOP) cells in the peripheral nervous system from a proneural cluster of equipotent cells is shown (yellow). These equipotent cells express the same amount of receptors and ligands. As a result of a stochastic event, individual cells within the cluster start expressing higher levels of Notch ligands (blue). More ligands on the cell surface (blue) can now engage more receptors in neighbouring cells (red) and thereby elicit a stronger Notch signal compared with the ligand-expressing cell (blue). Notch signal receiving cells (red) are inhibited from developing into either a NB or SOP cell, and therefore differentiate into ectoderm (red). By contrast, Delta-like-expressing cells (blue, Notch signal initiating cells) will adopt either a NB or SOP cell fate. At the molecular level, Notch signaling results in the repression of proneural genes. Proneural genes will only be activated in neuroectodermal cells that have low or no Notch signaling and as a result become NBs or SOP cells.

Notch function in the developing nervous system of vertebrates.



Notch signaling regulates self-renewal (curved green arrows) of developing and adult neural stem cells, preserving the neural stem cell pool. Notch also promotes gliogenesis (straight green arrow), whereas oligodendrocyte and terminal differentiation of neurons are inhibited (red capped bars).

Development 140, 689-704 (2013) doi:10.1242/dev.080614

Stem cells living with a Notch

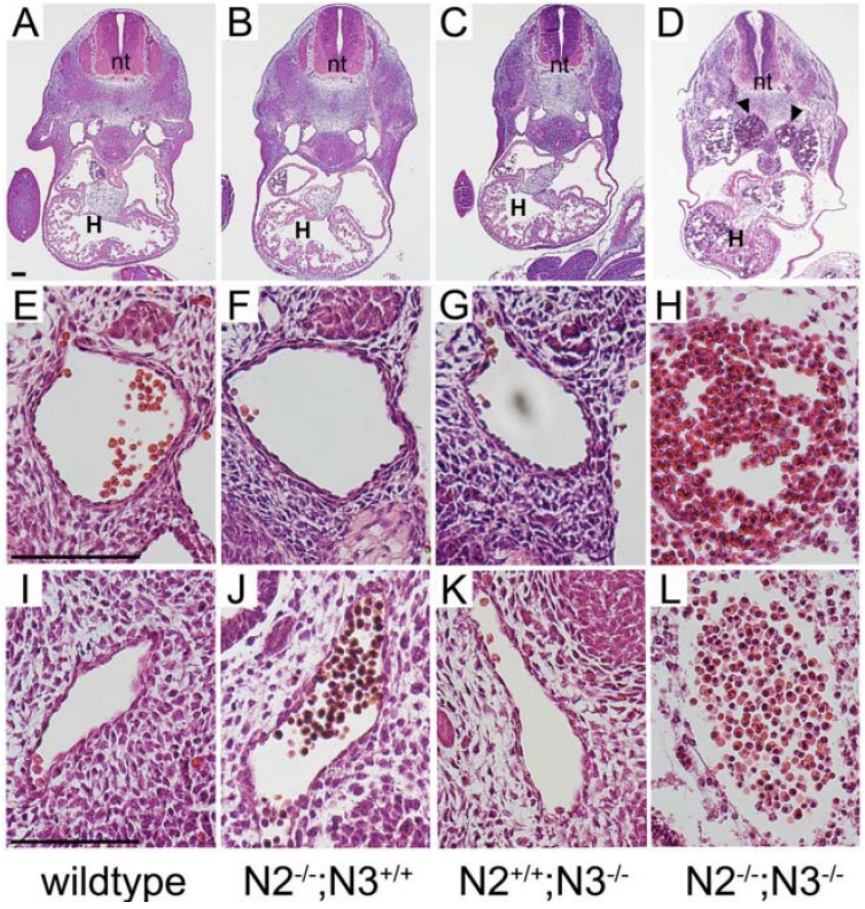
Ute Koch, Rajwinder Lehal and Freddy Radtke*

May 2012 | Volume 7 | Issue 5 | e37365

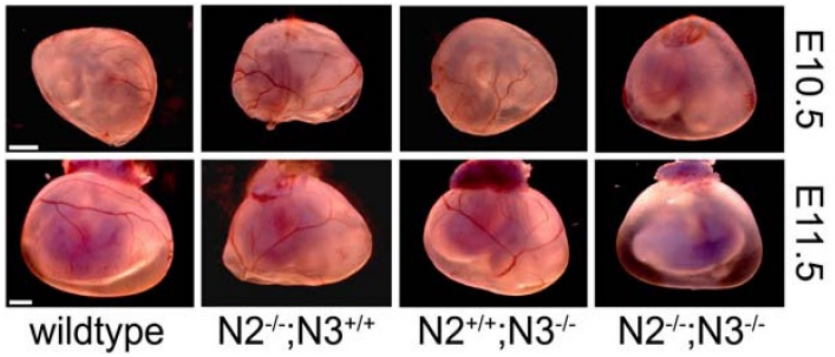
Notch2 and Notch3 Function Together to Regulate Vascular Smooth Muscle Development

Qingqing Wang[1], Ning Zhao[1,2], Simone Kennard[3], Brenda Lilly[1,2]*

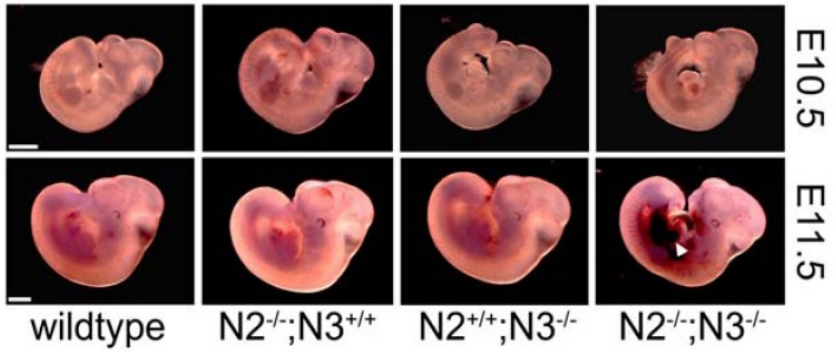
Embryos lacking both Notch2 and Notch3 have disrupted blood vessels



Yolk sac



Embryos



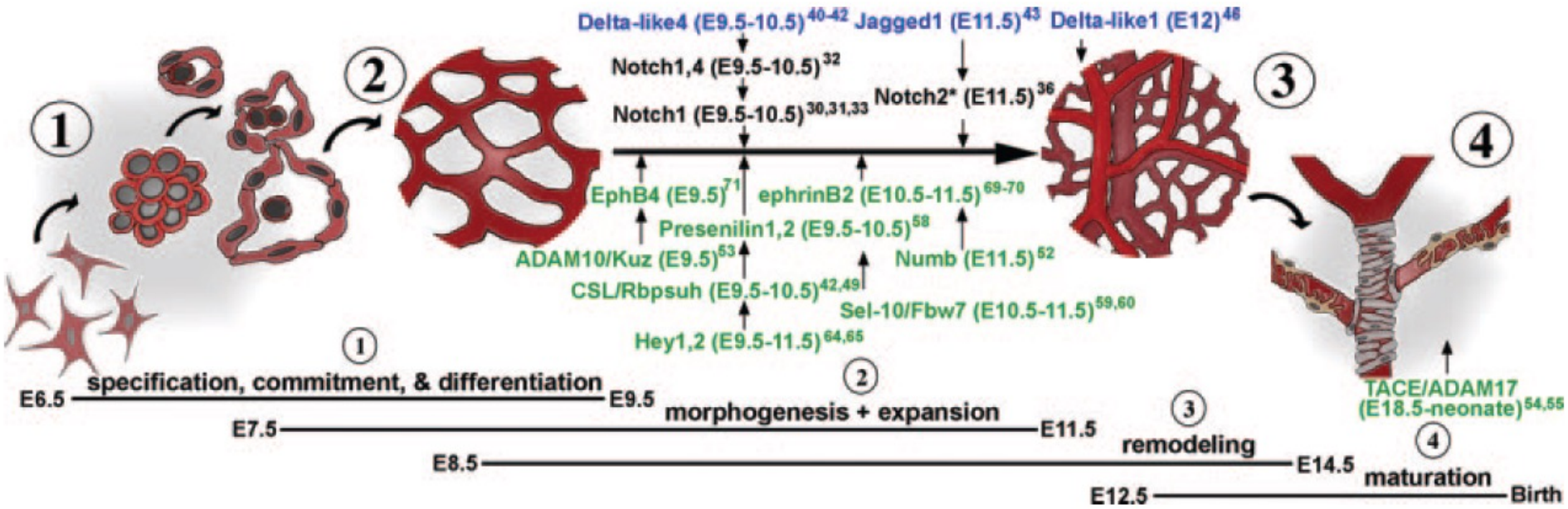
At E10.5, Notch2-/-;Notch3-/-; (N2-/-;N3-/-) embryos exhibit a decrease in yolk sac blood vessels, while the embryo is relatively normal in appearance.

At E11.5, Notch2-/-;Notch3-/- mice show severe vascular defects in both yolk sac and embryo. Yolk sac blood vessels are not visible and extensive hemorrhaging is seen in the embryo (arrowhead).

Notch Signaling in Blood Vessels
Who Is Talking to Whom About What?

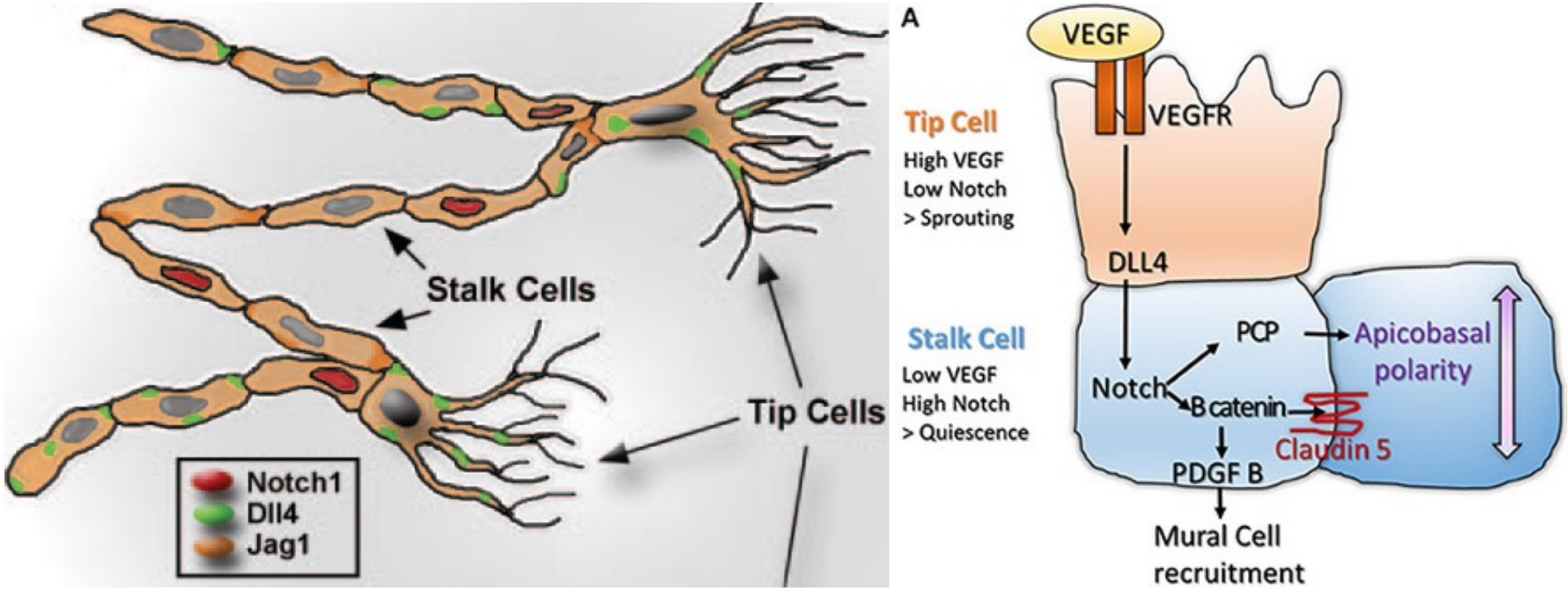
Circulation Research June 8, 2007

Jennifer J. Hofmann, M. Luisa Iruela-Arispe



The vascular system develops from mesenchymal progenitor cells that differentiate into hemangioblasts (1) and subsequently form the primitive vascular plexus. (2) Later, this uniform network remodels into a hierarchical vascular system. (3) It is at this stage that the functional consequences of Notch signaling are most notable.
Inactivation of several Notch receptors, ligands, and genes associated with Notch signaling result in embryonic lethality at the developmental stages indicated in parenthesis.

Active Notch signaling during angiogenesis



Relative distribution of active Notch1 (red), Delta-like4 (Dll4) (green), and Jagged1 (Jag1) (orange) in tip and stalk cells of angiogenic sprouts.

The pattern of active Notch is scattered in stalk cells.

In contrast, Dll4 expression solely marks the tip cells at the leading edge of the vascular front.

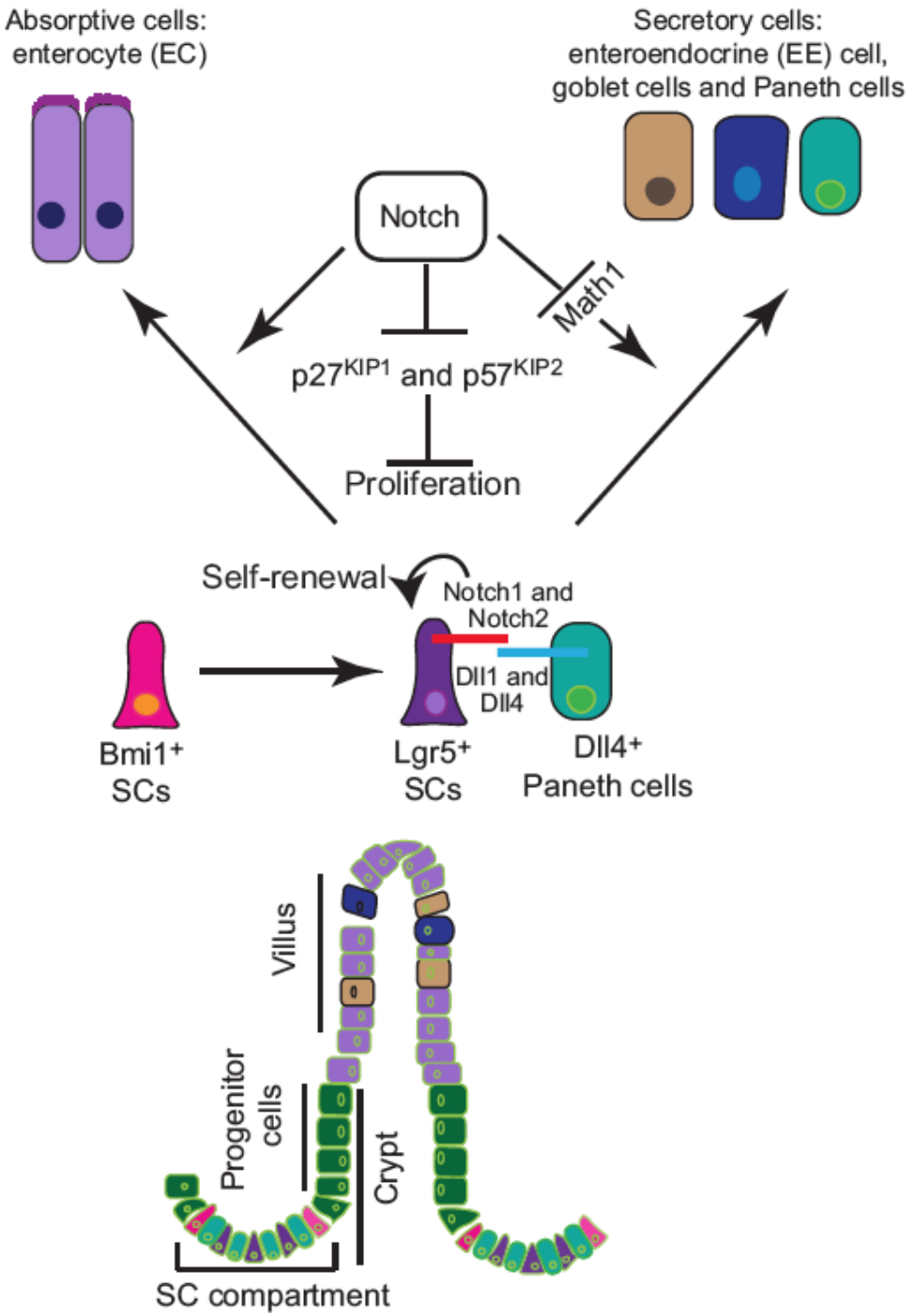
Notch in murine intestine

Long-lived Bmi1^{+} SCs give rise to mitotically active Lgr5^{+} SCs.

In the crypt compartment, Notch expressing Lgr5^{+} SCs are sandwiched between Delta-like 4^{+} Paneth cells.

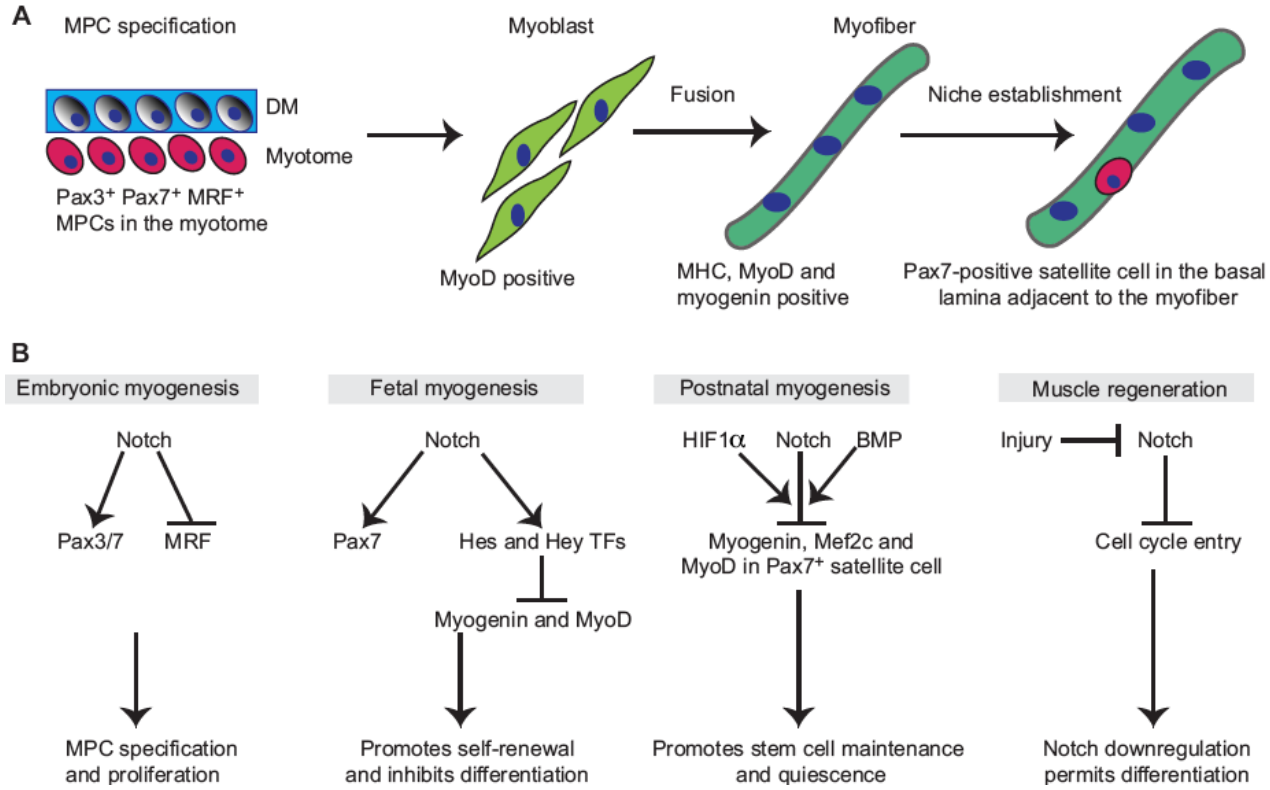
Activation of Notch signaling in Lgr5^{+} cells maintains SC self-renewal and proliferation by negative regulation of the cyclin-dependent kinase inhibitors p27 and p57, and the transcription factor Math1, which is necessary for differentiation of the secretory cell lineages.

Notch thus biases cell fate choice towards the absorptive lineage.



Notch in muscle development and stem cell maintenance

Notch signaling contributes to muscle development by regulating the muscle stem/progenitor cells during embryonic, fetal and postnatal myogenesis.



- During embryonic myogenesis, Notch signaling determines the number of muscle stem/progenitor cells and inhibits lineage commitment by repression of muscle regulatory factors (MRFs).
- During fetal and postnatal myogenesis, Notch-driven transcriptional activation of Pax7 ensures self-renewal of MPCs and satellite cells. Notch signaling also inhibits their premature terminal differentiation into skeletal muscle via transcriptional repression of myogenic genes.
- In case of muscle regeneration, Notch signaling is downregulated, accompanied by an upregulation of Wnt signaling. This allows the satellite cells to enter the cell cycle; activation of myogenic genes such as MyoD, myogenin and MHC further drive their terminal differentiation into multinucleated muscle fibers.

Notch signaling drives hematopoietic stem cell specification during development.

Notch signaling drives cell-autonomous hematopoietic stem cell (HSC) specification in the dorsal aorta (DA).
During vertebrate embryonic development, aortic endothelial cells of the DA express Jagged1, Delta-like 1 and Delta-like 4, whereas cells destined to emerge as HSCs express the Notch1 receptor.
Interaction between Jag1 and Notch1 is essential for HSC specification.
Notch signaling is dispensable for HSC maintenance in the bone marrow.

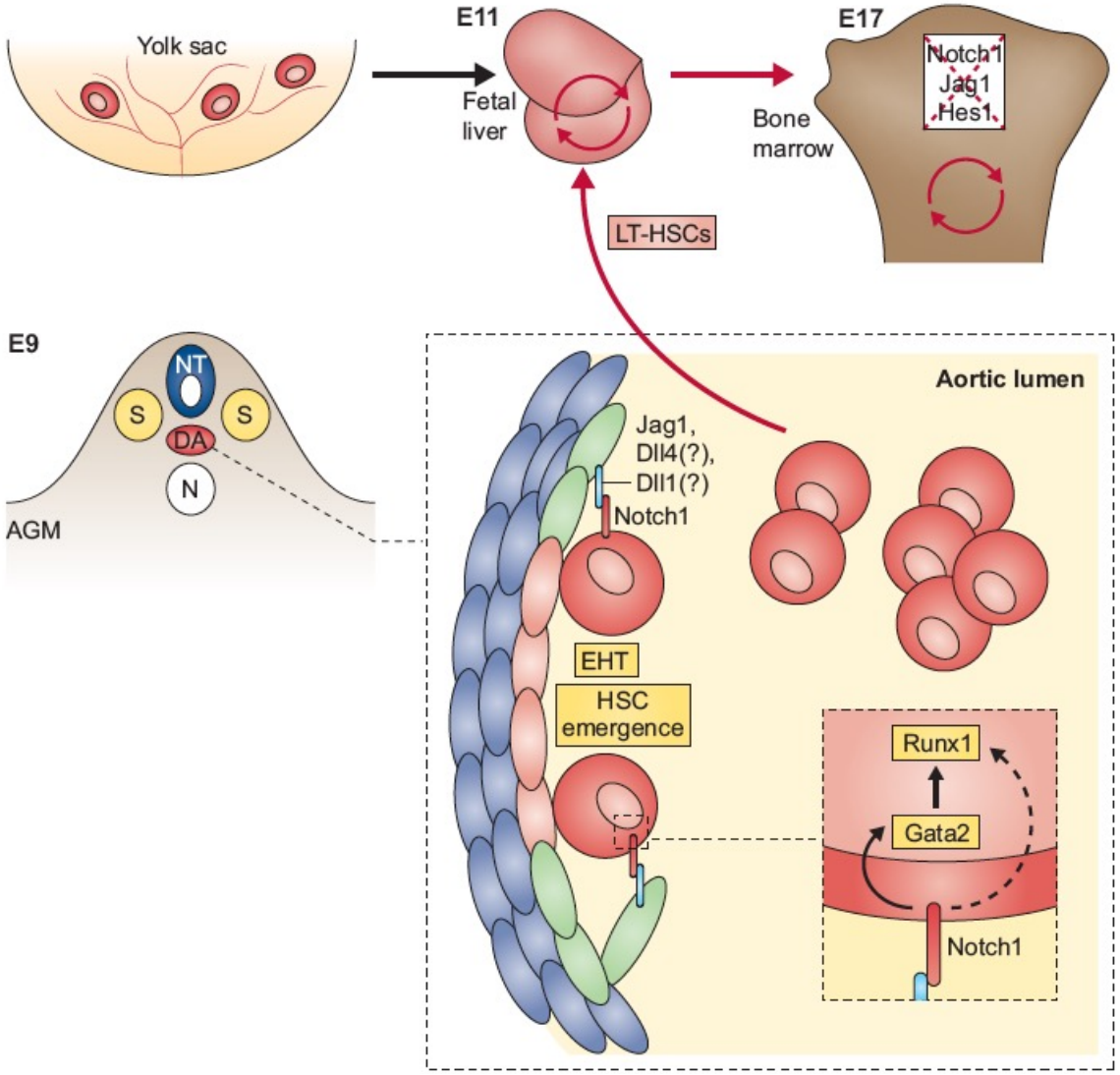


Table 1. Relationship between Notch signaling and congenital heart disease.

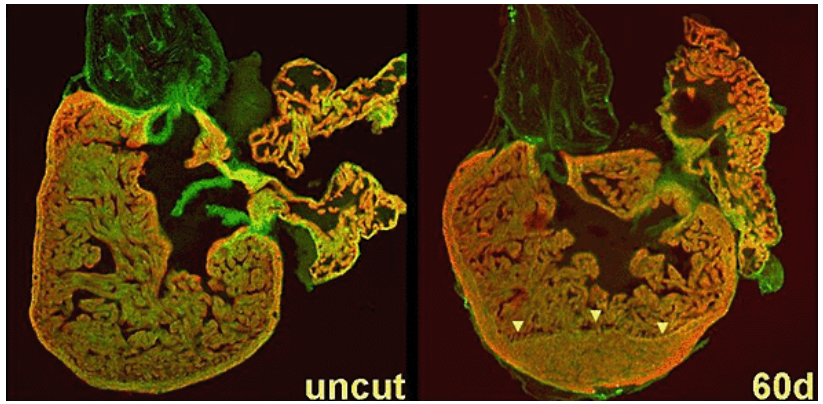
Congenital heart disease	Gene mutation
Aortic valve degenerative disease	RBP-Jκ
Left ventricular outflow tract defects	Notch 1
Bicuspid aortic valve disease	Notch 1-4, Jagged 1, Hes 1, Hey 1, Hey 2
Aortic valve calcification	Notch 1, Hey 1, Hey 2
Pulmonic stenosis	Jagged 1
Tetralogy of Fallot	Jagged 1
Mitral valve disease	HRT 2
Tricuspid valve disease	HRT 2
Ventricular septal defect	HRT 2
Atrial septal defect	HRT 2
Pericardial distension	Notch 1, RBP-Jκ
Alagille syndrome	Notch 2, Jagged 1, HRT 2, Hey 2

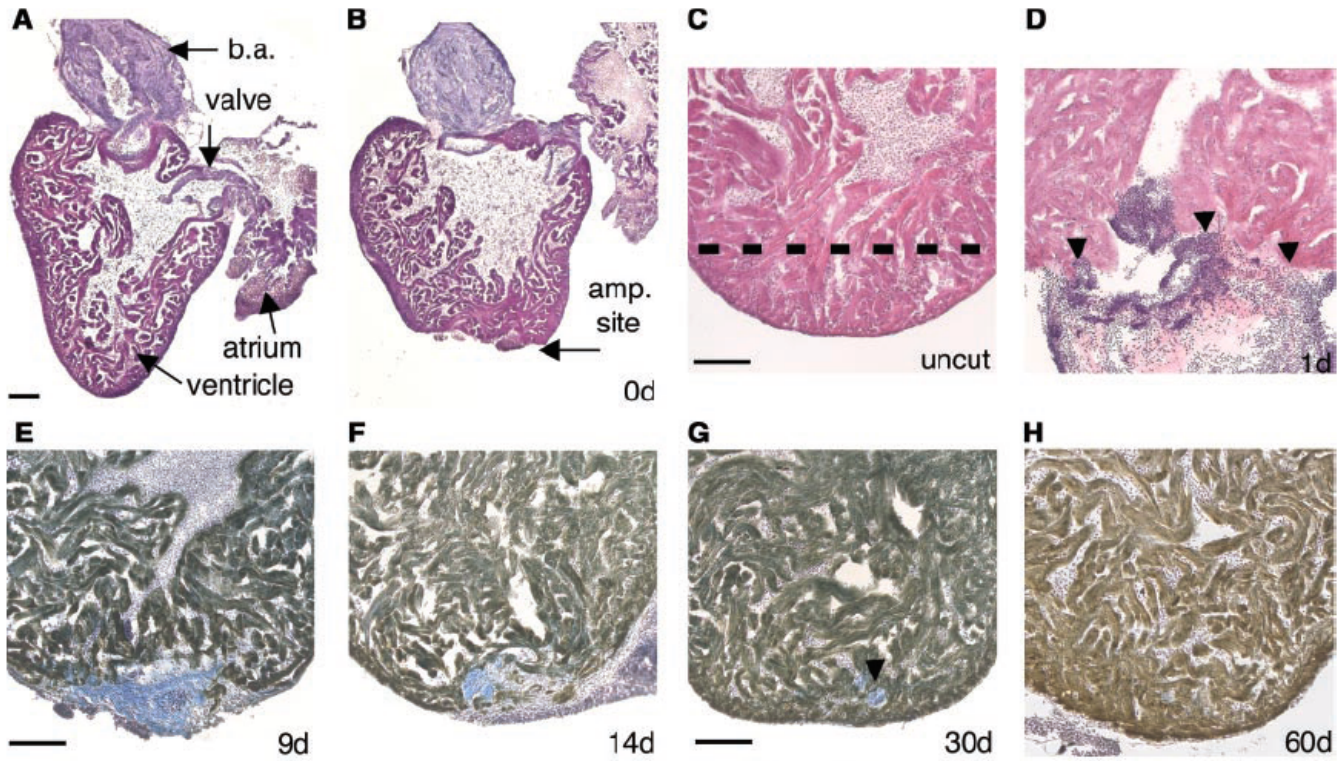
RBP-Jκ: recombination signal binding protein for immunoglobulin Jκ region; Hes: hairy and enhancer of split: Hey: hairy/enhancer of split-related with YRPW motif; HRT: hairy-related transcription.

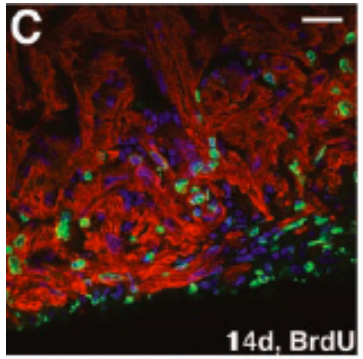
Heart Regeneration in Zebrafish

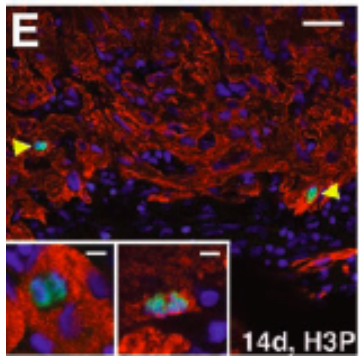
Kenneth D. Poss,* Lindsay G. Wilson, Mark T. Keating*

Cardiac injury in mammals and amphibians typically leads to scarring, with minimal regeneration of heart muscle. Here, we demonstrate histologically that zebrafish fully regenerate hearts within 2 months of 20% ventricular resection. Regeneration occurs through robust proliferation of cardiomyocytes localized at the leading epicardial edge of the new myocardium. The hearts of zebrafish with mutations in the Mps1 mitotic checkpoint kinase, a critical cell cycle regulator, failed to regenerate and formed scars. Thus, injury-induced cardiomyocyte proliferation in zebrafish can overcome scar formation, allowing cardiac muscle regeneration. These findings indicate that zebrafish will be useful for genetically dissecting the molecular mechanisms of cardiac regeneration.









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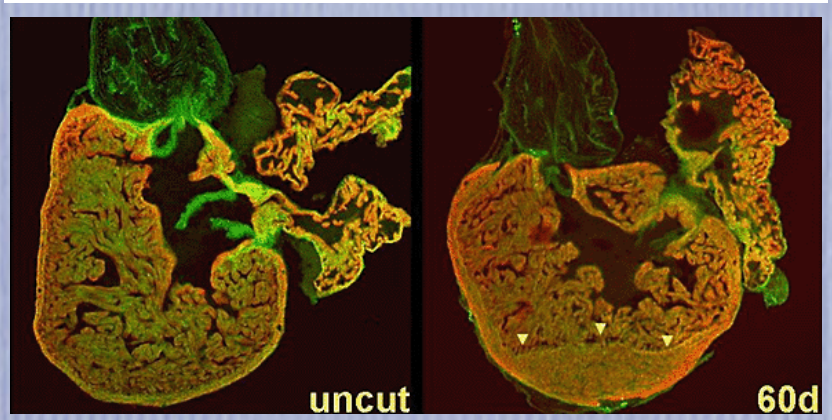
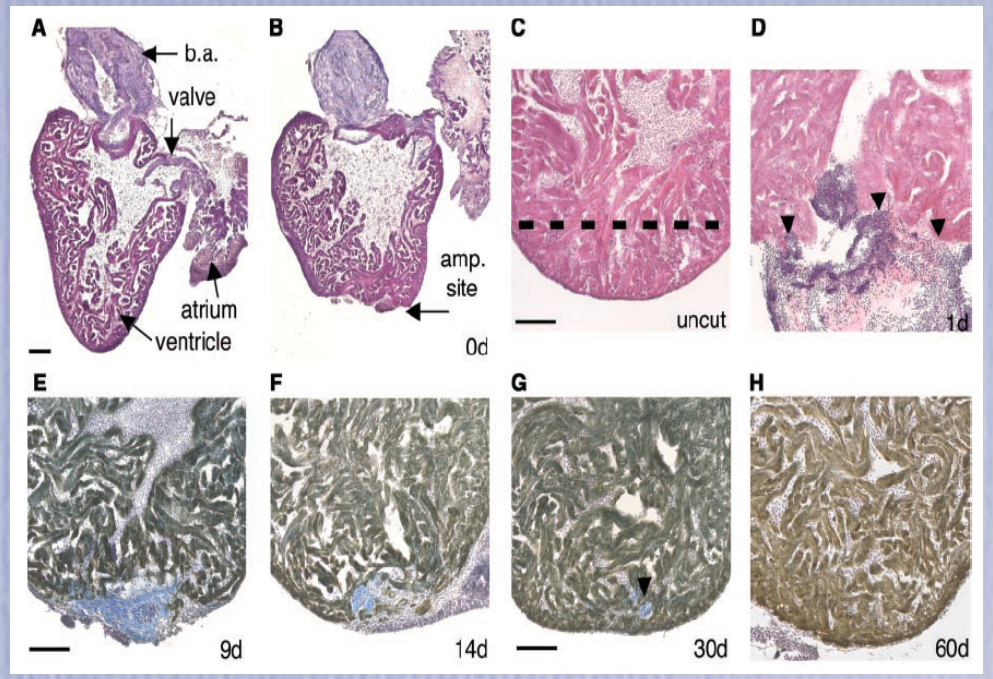
Kenneth D. Poss,* Lindsay G. Wilson, Mark T. Keating*

Cardiac injury in mammals and amphibians typically leads to scarring, with minimal regeneration of heart muscle. Here, we demonstrate histologically that zebrafish fully regenerate hearts within 2 months of 20% ventricular resection. Regeneration occurs through robust proliferation of cardiomyocytes localized at the leading epicardial edge of the new myocardium. The hearts of zebrafish with mutations in the Mps1 mitotic checkpoint kinase, a critical cell cycle regulator, failed to regenerate and formed scars. Thus, injury-induced cardiomyocyte proliferation in zebrafish can overcome scar formation, allowing cardiac muscle regeneration. These findings indicate that zebrafish will be useful for genetically dissecting the molecular mechanisms of cardiac regeneration.



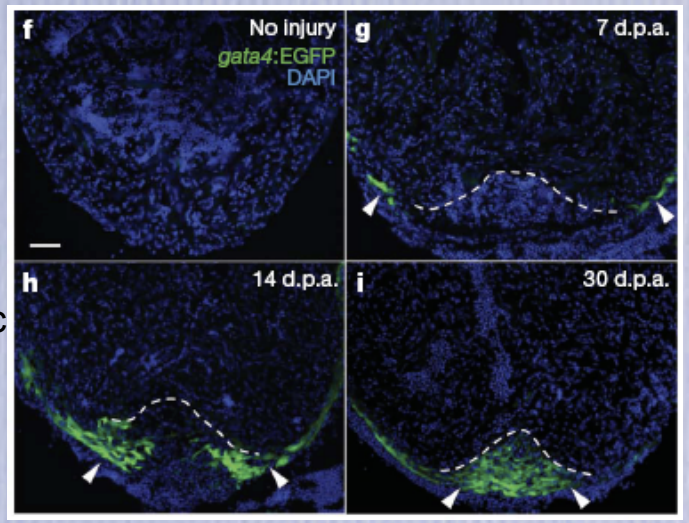


SCIENCE VOL 298 13 DECEMBER 2002

Primary contribution to zebrafish heart regeneration by *gata4*$^{+}$ cardiomyocytes

Kazu Kikuchi[1,2], Jennifer E. Holdway[1,2], Andreas A. Werdich[4], Ryan M. Anderson[5], Yi Fang[1,2], Gregory F. Egnaczyk[1,2,3], Todd Evans[6], Calum A. MacRae[4], Didier Y. R. Stainier[5] & Kenneth D. Poss[1,2]

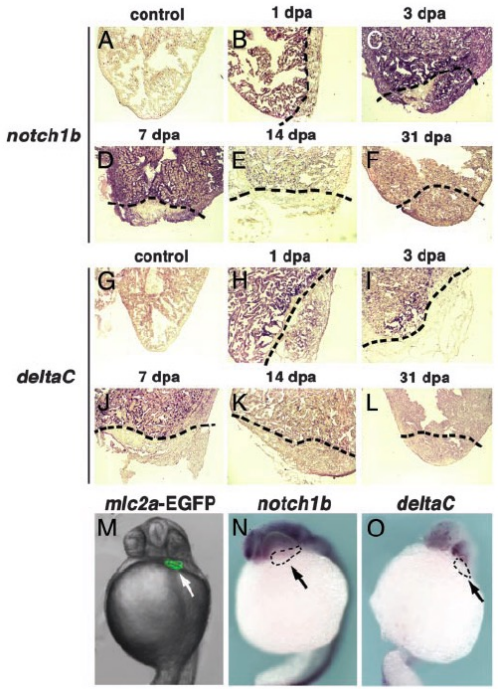
Cardiomyocytes are the source of the regenerating tissues and expressed a marker of regeneration called *gata4*—a transcription fac involved in normal development of the heart



NATURE|Vol 464|25 March 2010

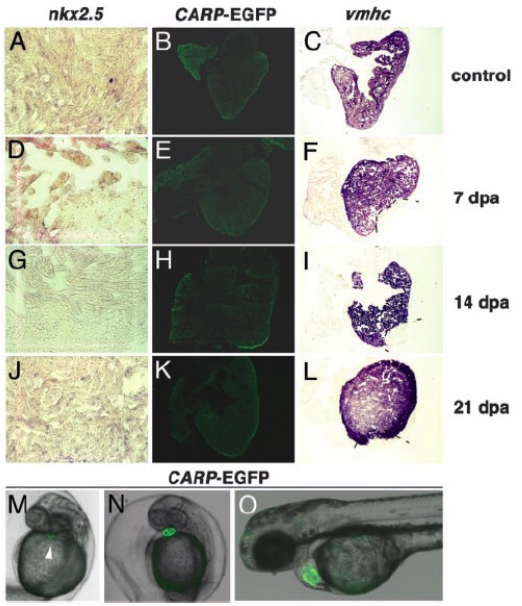
Activation of Notch signaling pathway precedes heart regeneration in zebrafish

Ángel Raya*†, Christopher M. Koth*†, Dirk Büscher*†, Yasuhiko Kawakami*†, Tohru Itoh*†, R. Marina Raya*, Gabriel Sternik*, Huai-Jen Tsai‡, Concepción Rodríguez-Esteban*, and Juan Carlos Izpisúa-Belmonte*§

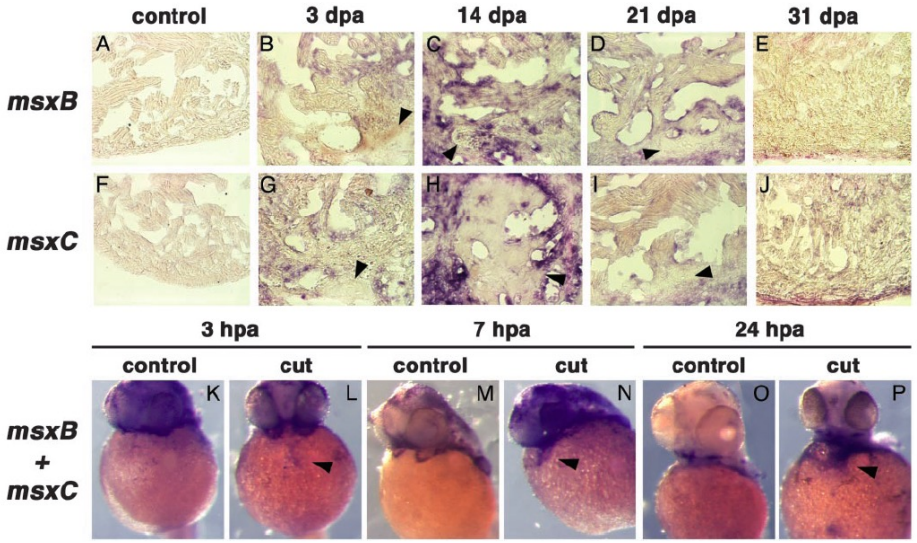


notch1b and ***deltaC*** are up-regulated during heart regeneration but not in the developing heart

Heart regeneration in zebrafish is accompanied by up-regulation of components of the Notch pathway, followed by members of the Msx family. These genes are not expressed during zebrafish heart development, indicating that **regeneration** involves the execution of a specific genetic program, rather than redeployment of a developmental program.



Markers of early cardiac development are not up-regulated during heart regeneration



msxC and msxB are expressed in the regenerating heart but not in 24- to 48-hpf embryos. However, both genes are expressed after removal of 50% of the developing heart

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Many different hypothesis for the BrdU labeling results:

- First, differentiated, contracting CMs in existing myofibers could be stimulated to enter the cell cycle, divide, and reform the apex.
- Second, regeneration could proceed through the recruitment of undifferentiated progenitor cells that form new, proliferative CMs.
- A third conceivable mechanism for the origin of regenerative muscle is a chimera of these two mechanisms called “dedifferentiation”, in which existing muscle would downregulate contractile genes toward creation of undifferentiated or poorly differentiated cells.

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LETTERS

Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation

Chris Jopling[1], Eduard Sleep[1,2]†, Marina Raya[1]†, Mercè Martí[1], Angel Raya[1,2,3]† & Juan Carlos Izpisúa Belmonte[1,2,4]

Although mammalian hearts show almost no ability to regenerate, there is a growing initiative to determine whether existing cardiomyocytes or progenitor cells can be coaxed into eliciting a regenerative response. In contrast to mammals, several non-mammalian vertebrate species are able to regenerate their hearts[1–3], including the zebrafish[4,5], which can fully regenerate its heart after amputation of up to 20% of the ventricle. To address directly the source of newly formed cardiomyocytes during zebrafish heart regeneration, we first established a genetic strategy to trace the lineage of cardiomyocytes in the adult fish, on the basis of the Cre/*lox* system widely used in the mouse[6]. Here we use this system to show that regenerated heart muscle cells are derived from the proliferation of differentiated cardiomyocytes. Furthermore, we show that proliferating cardiomyocytes undergo limited dedifferentiation characterized by the disassembly of their sarcomeric structure, detachment from one another and the expression of regulators of cell-cycle progression. Specifically, we show that the gene product of *polo-like kinase 1* (*plk1*) is an essential component of cardiomyocyte proliferation during heart regeneration. Our data provide the first direct evidence for the source of proliferating cardiomyocytes during zebrafish heart regeneration and indicate that stem or progenitor cells are not significantly involved in this process.

Regenerated cardiomyocytes are derived from differentiated, pre-existing cardiomyocytes

has been regenerated by cardiomyocytes. The exact source of these new cardiomyocytes is not yet known definitively. To address this question we developed and successfully implemented the 4-hydroxy-tamoxifen (4-OHT)-inducible Cre/*lox* approach in zebrafish to label regenerating cardiomyocytes genetically (for a detailed description of the lines generated and/or methodologies, see Methods and Supplementary Figs 1–9).

genetically labelled 48 h after fertilization. About 20% of the ventricle was removed, and cardiac regeneration was subsequently assessed at 7, 14 and 30 days after amputation. At 7 days after amputation, the remaining cardiac tissue was uniformly positive for green fluorescent protein (GFP) (Fig. 1a, b), with much of the missing tissue now replaced by a fibrin/collagen clot ($n = 5$ hearts) (Fig. 1c). At 14 days

($n = 7$ hearts; Fig. 1f). These results suggest that the regenerated cardiomyocytes arise from differentiated GFP-positive cardiomyocytes. These findings were substantiated at 30 days after amputation, when regeneration is nearly complete; all of the cardiomyocytes within the

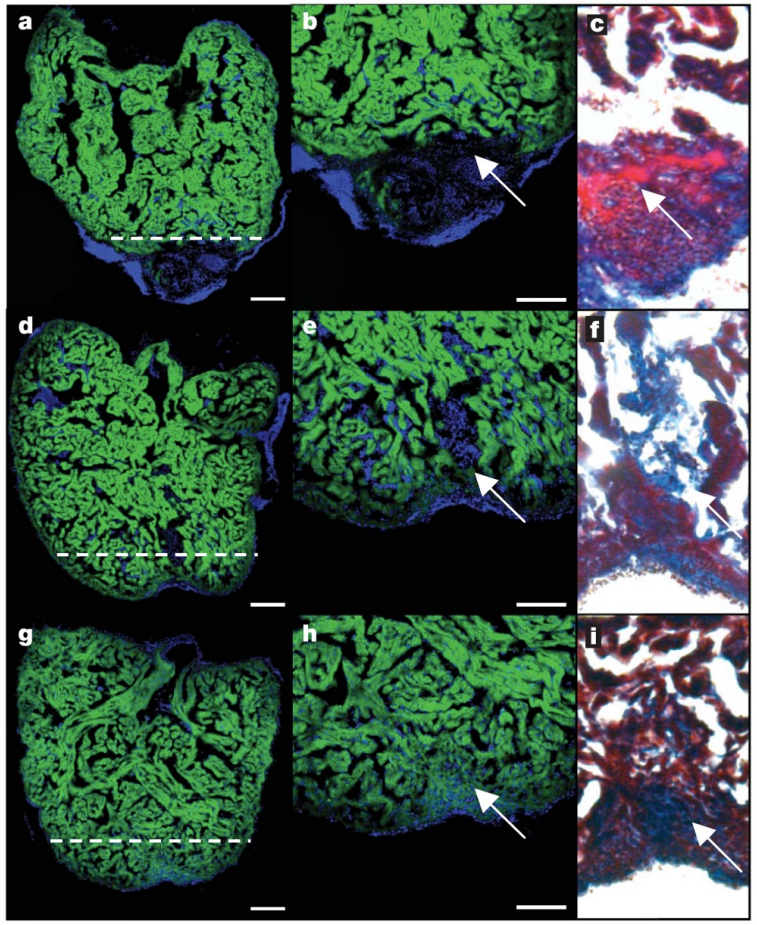
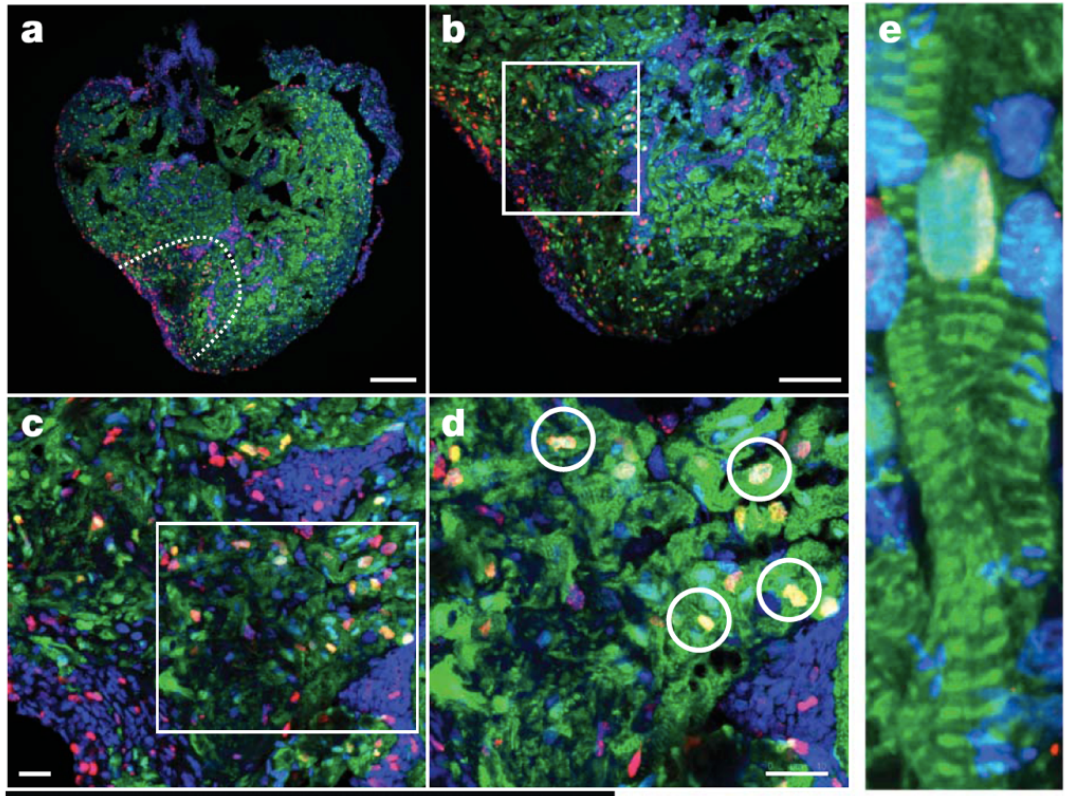
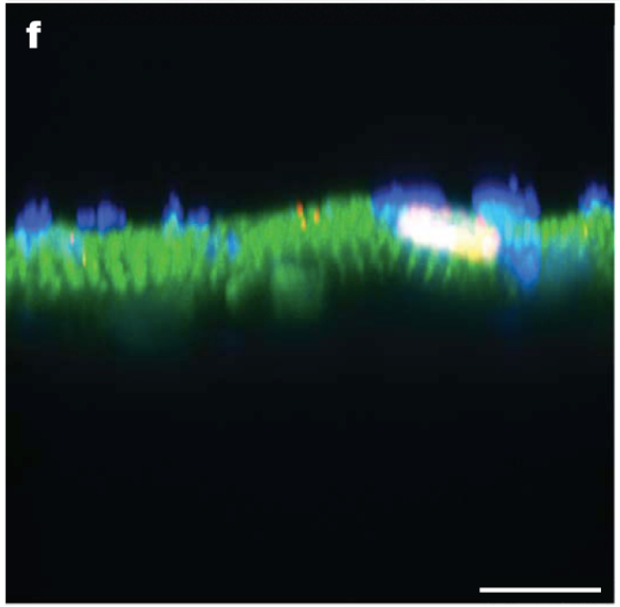


Figure 1 | Regenerated cardiomyocytes are derived from differentiated cardiomyocytes. Cardiomyocytes in transgenic zebrafish (tg-cmlc2a-Cre-Ert2: tg-cmlc2a-LnL–GFP) were genetically labelled at 48 h after fertilization by inducing Cre activity with tamoxifen. These embryos were then grown to adulthood (3 months or sexually mature), at which point the heart was amputated and allowed to regenerate for 7 (**a**–**c**), 14 (**d**–**f**) or 30 (**g**–**i**) days. The dashed white line represents the plane of amputation. At 7 days after amputation (**a**; enlargement in **b**) relatively little regeneration has occurred. Trichromic staining indicates that a fibrin clot has formed adjacent to the wound (**c**). By 14 days after amputation, GFP-positive cardiomyocytes have regenerated a substantial amount of new cardiac tissue (**d**; enlargement in **e**) and the fibrin clot was decreased in size (**f**). At 30 days after amputation, heart regeneration is virtually complete (**g**; enlargement in **h**) and all of the regenerated tissue is composed of GFP-positive cardiomyocytes. The clot has been replaced by a small scar (**h**). Scale bars, 100 μm (**a**, **d**, **g**) and 75 μm (**b**, **e**, **h**). Panels **c**, **f** and **i** are ×2 magnifications of the areas indicated with a white arrow in **b**, **e** and **h**.

Differentiating cardiomyocytes re-enter the cell cycle

We next sought to determine whether GFP-positive cardiomyocytes had re-entered the cell cycle. Adult GFP-positive transgenic zebrafish were treated with bromodeoxyuridine (BrdU) for 7 days after amputation (Fig. 2a–f). Subsequently, at 14 days after amputation, we found a significant increase in the number of BrdU-positive/GFP-positive cardiomyocytes in regenerating hearts compared with non-amputated controls (Fig. 2g). From this we conclude that differentiated GFP-positive cardiomyocytes had re-entered the cell cycle and engaged in DNA replication. We also analysed the position of BrdU-labelled GFP-positive cardiomyocytes within the regenerating heart (Fig. 2h and inset). Whereas most BrdU-positive/GFP-positive labelled cardiomyocytes were concentrated around the wound, a proportion could also be found in regions far from the site of amputation. This suggests that the response to the injury affects the heart in a global manner.





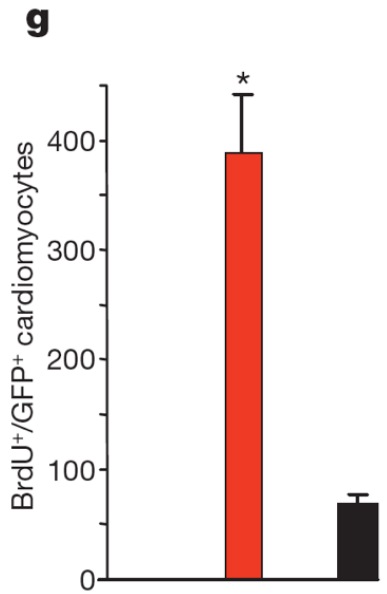


Figure 2 | Differentiated cardiomyocytes re-enter the cell cycle. (**a**–**f**) Transgenic zebrafish (tg-cmlc2a-Cre-Ert2: tg-cmlc2a-LnL–GFP) genetically labelled at 48 h after fertilization and grown to adulthood underwent cardiac amputation and were then treated with BrdU for 7 days after amputation. Hearts were isolated and processed at 14 days after amputation. Green, GFP-positive cardiomyocytes; red, BrdU-positive cells; blue, 4,6-diamidino-2-phenylindole stain for DNA; yellow, BrdU-positive/GFP-positive cardiomyocytes (white rings in **d**). **a**, Section of the entire heart, with a dashed white line representing the regenerating area. **b**, Enlargement of the regenerating area. **c**, **d**, Enlargements of the boxed areas in **b** and **c**, respectively. **e**, An *XY* reconstruction of an individual BrdU-positive/GFP-positive cardiomyocyte within a regenerating heart 14 days after amputation. **f**, An *XZ* reconstruction of the BrdU-positive/GFP-positive cardiomyocyte shown in **e**. **g**, The average number of BrdU-positive/GFP-positive cardiomyocytes per section (means and s.e.m.). Asterisk, $P < 0.01$ (*t*-test). Amputated (red bar), $n = 17$ sections from seven different animals; control (black bar), $n = 9$ sections from three different animals.

Regenerating cardiomyocyte partially disassemble the contractile apparatus but not revert to an embryonic stage

lineage they regress[7,8]. An increase in the expression of the cardiac-progenitor-associated genes *nkx2.5* and *hand2* during zebrafish heart regeneration has been reported[9]. However, our own *in situ* hybridization analyses failed to detect any significant upregulation of either transcript (data not shown), confirming previous results from our laboratory[5]. Furthermore, genome-wide transcriptome data[10,11] also failed to detect significant changes in the expression of either transcript during zebrafish heart regeneration. These results argue against an extensive dedifferentiation of cardiomyocytes as a prerequisite for their proliferation in the context of heart regeneration.

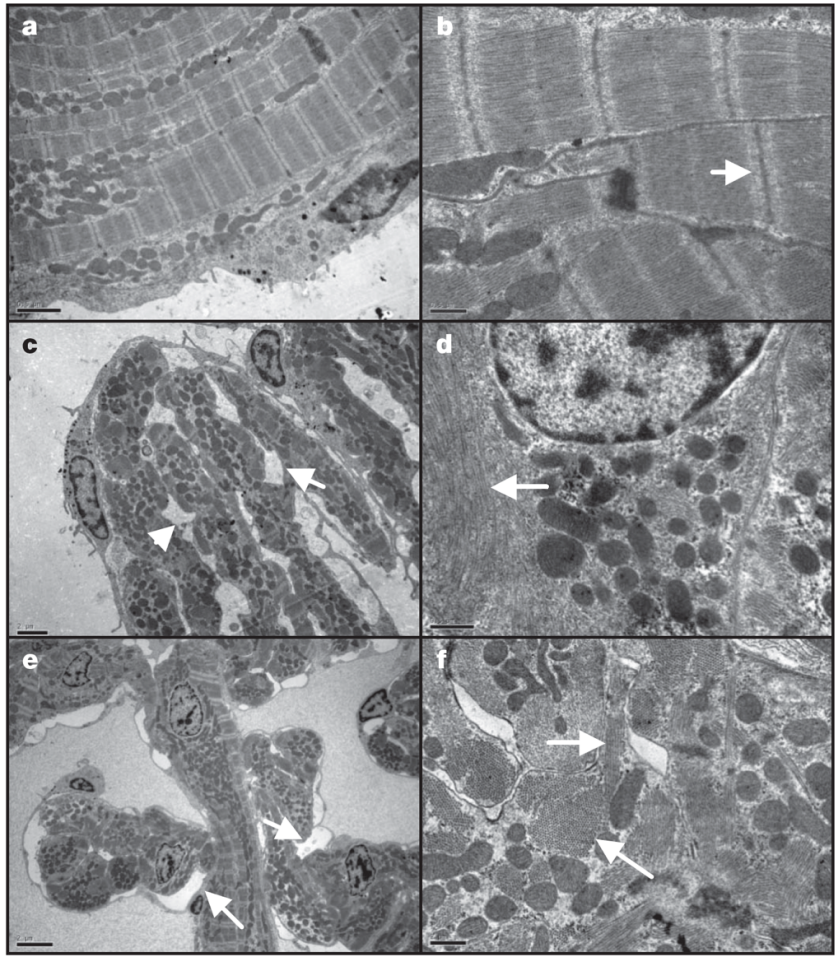


Figure 3 | Cardiomyocytes dedifferentiate, resulting in the disassembly of sarcomeric structure and detachment. Electron microscopy of sections of a control heart (**a**, **b**) and a regenerating heart at 5 days (**c**, **d**) and 7 days (**e**, **f**) after amputation. Cardiomyocytes in unamputated control samples show a tightly organized sarcomeric structure (**a**); at higher magnification (**b**) the Z-lines are clearly visible (arrow). At 5 days after amputation many of the cardiomyocytes have a disorganized sarcomeric structure (**c**) along with the appearance of intercellular spaces (arrows). Closer examination reveals a loss of Z-lines (**d**, arrow). At 7 days after amputation there is a similar loss of structure and appearance of intercellular spaces (**e**, arrows). At higher magnification (**f**) myosin fibres are visible (arrows); however, both longitudinal (upper arrow) and transverse (lower arrow) fibres are present within the same cardiomyocyte, indicating disorganized sarcomeric structure. Scale bars, 0.5 μm (**a**, **b**, **d**) and 2 μm (**c**, **e**, **f**).