

# "Notch-Off": a perspective on the termination of Notch signalling

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**ABSTRACT** During Notch mediated lateral inhibition, interacting cells establish or amplify differences in Notch signalling, which are then translated into distinct cell fate decisions according to the developmental context. In recent years, several mechanisms that increase the signalling capacity of interacting cells have been uncovered (reviewed in Bray, 2006, Le Borgne, 2006, Schweisguth, 2004). However, mechanisms specifically targeted to downregulate receptor activity are also at work during lateral inhibition, contributing decisively to generate definitive differences between interacting cells. In this review, we discuss some of these mechanisms and their relevance for the overall architecture of the Notch pathway. We further highlight the importance of properly terminating Notch activity during cell fate decisions mediated by this pathway.

**KEY WORDS:** *Notch signaling, neurogenesis, transcriptional regulation, robustness, hes gene*

Cell-cell communication mediated by the Notch receptor is essential for the correct generation and patterning of most animal tissues. The initial characterization of *Notch* mutants in *Drosophila*, by Donald Poulson (Poulson, 1937, Poulson, 1945), led to the finding that absence of *Notch* activity in the embryo causes hyperplasia of the neural tissue at the expense of epidermis, a phenotype that was named "neurogenic" and later shown to be characteristic of several other *Drosophila* mutants (Lehmann *et al.*, 1983, Poulson, 1937, Poulson, 1945). This led to a genetic definition of the *Drosophila* Notch pathway as a cascade of interacting neurogenic genes that function to control the formation of the fly nervous system (Vässin *et al.*, 1985). However, Poulson also noted that *Notch* mutants have several other defects in embryonic and adult tissues, indicating that the pathway is involved not only in the development of the nervous system but also in most cell fate decisions that occur in the developing animal. Today, it is known that this pleiotropic function of the Notch pathway applies not only to *Drosophila* but to all metazoa, where it regulates a myriad of cell fate decisions, affecting almost all animal tissues (reviewed in Artavanis-Tsakonas *et al.*, 1999).

Notch-mediated cell communication seems to operate in two main ways: lateral inhibition and lateral induction. In the first case, Notch signalling mediates binary cell fate decisions and generates fine-grained patterns of cellular differentiation, while in the second case the Notch pathway is used to coordinate interactions between groups of cells and usually results in the establishment

of boundaries separating different cellular fields. Although these two distinct operations rely on the existence of a few dedicated components, there is a number of core players that participate in both processes. These include the transmembrane Notch receptor and the interacting transmembrane ligands, both characterized by the presence of several EGF-like repeats in their extracellular domains. Notch is a large type-I transmembrane receptor that accumulates at the plasma membrane as a heterodimer, composed of the extracellular domain (NECD) and a membrane bound intracellular domain. These two polypeptides are formed in the trans-golgi as the result of proteolytic activity by a Furin protease that constitutively cleaves Notch molecules at the S1 site (Logeat *et al.*, 1998) (Fig. 1). The Notch receptor heterodimer is then formed through a non-covalent Ca<sup>2+</sup> dependent ligation and inserted at the plasma membrane. In *Drosophila*, however, it seems that the Notch transmembrane receptor is not a heterodimer as in vertebrates, being instead composed of just one single polypeptide (Kidd and Lieber, 2002).

The best characterized Notch ligands belong to the DSL family (Delta-Serrate-Lag2) and are also type-I transmembrane pro-

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*Abbreviations used in this paper:* CSL, CBF-1, *Drosophila* Suppressor of Hairless and *C. elegans* Lag-1 transcription factor; DSL, delta-serrate-lag2; LGD, lethal giant disc; MAM, mastermind co-activator, NECD, notch extracellular domain; NICD, notch intracellular domain; RIP, regulated intramembrane proteolysis.

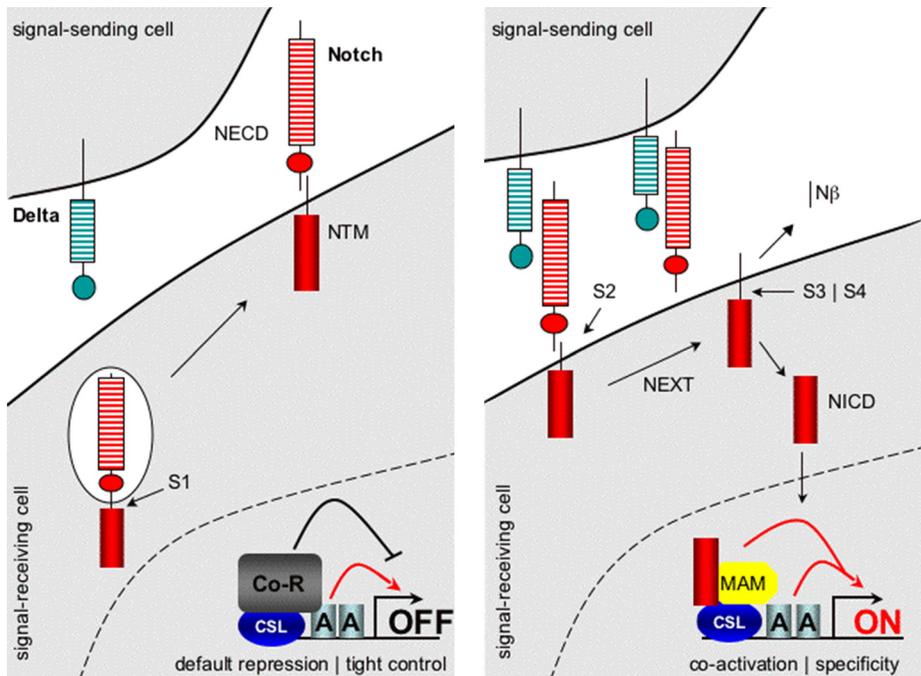
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**Fig. 1. A partial view of Notch in action, mediating communication between two adjacent cells.** The mechanisms that lead to Notch activity in the receiving cell are depicted, including the cleavage of Notch at the cell membrane and the assembly of a tripartite nuclear complex with the transcription factors CSL (CBF-1, *Drosophila* Suppressor of Hairless and *C. elegans* Lag-1) and MAM (mastermind co-activator).

teins, although with much smaller and less conserved intracellular domains than Notch. Upon ligand-receptor interaction, the Notch receptor undergoes successive proteolytic cleavages that lead to the release of its intracellular domain (NICD) (reviewed in Schweisguth, 2004), which translocates to the nucleus (Fig. 1). Here, NICD binds to the CSL transcription factor (an acronym for the mammalian CBF-1, *Drosophila* Suppressor of Hairless and *C. elegans* Lag-1) and to the Mastermind co-activator (MAM), forming a ternary complex (Fig. 1). This complex then recruits other factors, like the histone acetylase p300, the co-regulator SKIP (Ski-interacting protein) and the CDK8-Mediator complex, to activate transcription of target genes (reviewed in Barrick and Kopan, 2006).

In the absence of nuclear NICD, a repressor complex is instead assembled around the DNA-bound CSL protein, resulting in transcriptional repression of Notch target genes. Following ligand-triggered Notch activation and nuclear translocation of NICD, the interaction with CSL triggers a structural conformation that leads to the displacement of CSL-bound co-repressors and recruitment of MAM and other co-factors, forming a different transcriptional complex with activator properties. Thus, the Notch target genes which were previously repressed now become transcriptionally active.

There are many putative binding sites for the CSL transcription factor throughout the genome (Rebeiz *et al.*, 2002), but the best characterized Notch targets are genes of the *hes* (*hairly* and *Enhancer of split*) and *hrt* (*hairly-related*) families, encoding bHLH (basic Helix-Loop-Helix) transcriptional repressors (reviewed in Davis and Turner, 2001), which function as effectors to implement the cell fate decisions mediated by Notch signalling.

Although this core pathway is evolutionary conserved in the metazoan phyla, the number of paralogues for each element differs in various animal models. For instance, whereas *Drosophila* has only one *Notch* gene, mice have four different genes encoding Notch receptors (see Table 1). Even between vertebrates, the variation on the number of genes encoding core components of the Notch pathway is noteworthy, as for instance the different number of *Delta* and *Notch* genes in birds and mammals. Despite this variability in molecular components, the Notch pathway has essential and conserved roles in several developmental processes that are common to all vertebrates, like neurogenesis and somitogenesis (reviewed in Louvi and Artavanis-Tsakonas, 2006, Pourquie, 2003).

The Notch mediated lateral inhibition (LI) mechanism regulates interactions between cells with equivalent potential, ensuring that they acquire one of two alternative fates. In this process, Notch signalling has no instructive role on the decision, functioning mainly to guarantee that the interacting cells follow alternative fates and, simultaneously, that both fates are in the end adopted. This involves a competitive

process whereby interacting cells compete not only by sending as much signal as possible, but also by preventing signal reception. So, any mechanism that leads the competing cells to produce more signal and/or to receive less signal might contribute to generate the normal outcome of LI: a "winner" cell with little receptor activity and strong signalling capacity, versus the "loser" cells that can only receive signal and activate the Notch cascade, being unable to signal back.

What is unique about the Notch pathway is its capacity to generate this stable outcome in so many different cell fate decisions. This "robustness" (Meir *et al.*, 2002) arises from the unique architecture of the underlying genetic network, where an inter-cellular feedback loop plays a central role in connecting signal reception in one cell with the capacity to produce and send signal to neighbouring cells. In other words, when cells exchange signals during LI, the more signal a cell receives, the less signal it is able to produce, so that any small difference in Notch activity between interacting cells is amplified and leads invariably to a distinct outcome in each of them: one cell becomes a signalling cell whereas the others become net receivers (reviewed in Greenwald and Rubin, 1992, Lewis, 1996). The starting point in these cellular interactions may be random, in case of stochastic decisions where any cell can become a signalling cell, or may be biased, in which case the inter-cellular feedback loop serves to reinforce an initial bias on signal directionality.

In recent years, various mechanisms have been uncovered that modulate the activity of the Notch pathway, from transcription to the intra-cellular trafficking of receptor and ligands, with an emphasis on the mechanisms that promote signal generation and receptor activation in interacting cells (reviewed in Bray, 2006,

Schweisguth, 2004). However, how positive and negative mechanisms are integrated to regulate the temporal, spatial and directional aspects of LI-mediated cell interactions is still not completely understood. Here, we focus our attention on the mechanisms that negatively regulate Notch activity, discussing their importance for the functional architecture of the Notch pathway.

### Notch signalling: from the cell surface to the nucleus

During Notch signalling, NICD release to the nucleus involves a two-step regulated intramembrane proteolysis (RIP) of the receptor, triggered by ligand binding. The Notch receptor can thus be viewed as a membrane bound transcription co-factor that integrates signalling events at the membrane and transduces directly to the nucleus, without any second messengers. Since RIP cleavage removes the receptor from the cell's membrane, each Notch molecule can transduce signal only once, implying that the regulation of the number and availability of Notch receptors at the cell surface must be critical to modulate signal strength. In addition, as the pathway does not rely on second messengers, no signal amplification is possible and signalling capacity must thus be highly dependent on the nuclear concentration of NICD, providing another nodal point for regulating the intensity and duration of Notch signalling.

### Regulation of Notch availability at the cell surface

In recent years, it has become clear that endocytosis and intracellular trafficking of Notch receptors play a major role in signal modulation, controlling not only the availability but also the "quality" of receptors and ligands. For example, the enzyme O-fucosyl transferase (O-Fut) initiates a glycosylation cascade that is needed to generate a functional Notch receptor (Okajima and Irvine, 2002, Sasamura *et al.*, 2003, Shi and Stanley, 2003), contributing also to promote the folding and exit of receptors from the endoplasmic reticulum (Okajima *et al.*, 2005). In addition, O-Fut may control the removal of Notch molecules from the plasma membrane and their entry into the degradation compartment, thereby preventing an excessive accumulation of free receptors at the cell membrane (Sasamura *et al.*, 2007).

Other mechanisms are known that regulate the presence of Notch receptors at the cell surface, involving the activity of various HECT domain E3 ubiquitin-ligases, like those belonging to the Nedd4 family (*Drosophila* Nedd4 and Suppressor of Deltex, and mammalian Itch). These E3-ligases target Notch molecules to late endosomes and subsequent degradation by the lysosome (reviewed in Le Borgne, 2006). In contrast, another E3 ubiquitin-ligase, Deltex, is a positive regulator of Notch activity in *Drosophila* (Hori *et al.*, 2004, Matsuno *et al.*, 1995), although in mammalian cells it may also act to antagonize Notch (Izon *et al.*, 2002). Altogether, the outcome of these ubiquitin modifications of the Notch receptor controls its availability at the cell surface and, thereby, the strength and duration of the signal.

There are still other mechanisms, not mediated by ubiquitylation, that also control endosomal sorting of the receptor and promote its targeting to lysosomal degradation. These involve the activity of the ESCRT complex (Moberg *et al.*, 2005,

Thompson *et al.*, 2005, Vaccari and Bilder, 2005) and of a newly identified protein - Lethal Giant Discs (LGD), that play important roles in restricting Notch signalling (Childress *et al.*, 2006, Jaekel and Klein, 2006, Klein, 2003).

Numb is a membrane associated protein that also antagonizes Notch signalling in the *Drosophila* nervous system, by downregulating the activity of a positive regulator of Notch, Sanpodo. Numb is an endocytic regulator and triggers the alpha-adaptin mediated endocytosis of Sanpodo, targeting it to late endosomes, thereby inhibiting its positive interaction with Notch at the membrane (Hutterer and Knoblich, 2005). In vertebrates, however, the role of Numb in regulating Notch activity is still controversial. Although some studies point to Numb as a negative regulator that targets vertebrate Notch receptors for endocytosis and subsequent degradation (McGill and McGlade, 2003), the analysis of mutant mice without Numb activity during neurogenesis is difficult to reconcile with this view of Numb as antagonist of Notch signalling (Kuo *et al.*, 2006, Li *et al.*, 2003, Petersen *et al.*, 2002, Zhong *et al.*, 2000).

Another mechanism to prevent activation of the Notch pathway might involve a postulated dominant-activity of Delta ligands, which could bind Notch receptors in *cis* (within the same cell) and prevent their interaction with ligands from neighbouring cells (in *trans*). These *cis*-interactions are supposed to be non-activating and result in down-regulation of Notch signalling in cells with high levels of Delta ligands. This mechanism has been suggested from the analysis of mosaic mutants in *Drosophila* (Heitzler and Simpson, 1993), but it has been difficult to gather biochemical data to support it. Still, it makes sense that during LI the winning cells use their excess of ligand not only to signal neighbouring cells but also to avoid Notch activation, by reducing the availability of the receptors at their cell membrane.

In other situations, removal of Notch receptors from the membrane is a pre-requisite to increase the signalling capacity of the cell, avoiding the occurrence of *cis*-interactions between ligands and receptors that might sequester ligand activity. This has been described during vulva formation in *C.elegans*, where Lin-3/EGF-Ras-MAPK signalling is known to induce Lin-12/Notch removal from the membrane of the "winner" cell (P6.p) (Shaye and Greenwald, 2002). Actually, the signalling capacity of P6.p is severely decreased in mutants that fail to remove Lin-12/Notch from the membrane, and no LI occurs to sort out vulva

TABLE 1

#### COMPONENTS OF THE NOTCH SIGNALLING PATHWAY ARE EVOLUTIONARILY CONSERVED

	<i>Drosophila</i>	<i>C. elegans</i>	Chick	Mammals
Notch Receptor	<i>Notch</i>	<i>lin-12</i> <i>glp-1</i>	<i>Notch1</i> <i>Notch2</i>	<i>Notch1</i> <i>Notch2</i> <i>Notch3</i> <i>Notch4</i>
LIGAND	<i>Delta</i> <i>Serrate</i>	<i>lag-2</i> <i>apx-1</i> <i>arg-2</i> <i>f16b12.2</i>	<i>Delta1</i> <i>Delta4</i> <i>Jagged1</i> <i>Jagged2</i>	<i>Dll-1</i> <i>Dll-3</i> <i>Dll-4</i> <i>Jagged1</i> <i>Jagged2</i>
CSL	<i>Su(H)</i>	<i>Lag-1</i>	<i>CBF1/RBPJK</i>	<i>CBF1/RBPJK</i>
MAM	<i>Mam</i>	<i>Lag-3</i>	<i>Mam1</i> <i>Mam2</i> <i>Mam3</i>	<i>Mam1</i> <i>Mam2</i> <i>Mam3</i>

fates (Shaye and Greenwald, 2005). Since this phenotype is dependent on the presence of the Lin-12/Notch extracellular domain, it has been suggested that an excess of receptor at the surface of mutant cells might sequester DSL ligands in *cis* and prevent their activity, thus blocking lateral inhibition of neighbouring cells (Shaye and Greenwald, 2005).

### Notch in the nucleus: stability and degradation

Following Notch activation and NICD translocation to the nucleus, a tripartite complex is assembled around the DNA-bound CSL transcription factor, leading to the expression of Notch target genes. The MAM co-factor is essential to organize this activator complex, recruiting other proteins that contribute to the transcriptional up-regulation of target genes. However, MAM also recruits the complex of cyclin C and cyclin-dependent kinase 8 (CycC:CDK8), which directly phosphorylates NICD and targets it to proteasome degradation, after ubiquitylation by the Fbw7/Sel10 ubiquitin ligase (Fryer *et al.*, 2002, Fryer *et al.*, 2004). Thus, MAM acts like a “kamikaze” co-factor that when recruited to the transcription complex brings also the means to swiftly terminate its activity, allowing a tight control of the level and timing of Notch signalling.

The C-terminal PEST domain of NICD is directly involved in these phosphorylation events, with three Serine residues known to be phosphorylated by the CycC:CDK8 kinase (Fryer *et al.*, 2004). However, another region of the PEST domain has been shown to be involved in NICD turnover, containing a string of four Ser residues (WSSSSP) that were not identified as CycC:CDK8 targets. Deletion of this small region in the Notch molecule leads to NICD hypophosphorylation, decreased turnover and stabilization of the CSL/NICD/MAM complex, with a consequent increase in Notch signalling (Chiang *et al.*, 2006). These deletions result in strong “gain-of-function” phenotypes and can be detected in T-cell acute lymphoblastic leukemia (T-ALL), a tumor derived from T-cell progenitors which correlates with uncontrolled Notch activity (Chiang *et al.*, 2006, Weng *et al.*, 2004).

NICD can be also phosphorylated by another kinase – GSK-3 $\beta$  (Espinosa *et al.*, 2003, Foltz *et al.*, 2002), although this does not seem to happen when NICD is present in the DNA-bound complex with CSL and MAM (Fryer *et al.*, 2004). Also, the outcome of this phosphorylation is not yet clear, as it can lead to higher or lower NICD activity depending on the cellular context (Espinosa *et al.*, 2003, Foltz *et al.*, 2002).

### Target genes and termination of Notch signalling

Although NICD is a short-lived transcription co-factor, it is possible that its target genes encode stable transcription factors that generate a sustained activity of the pathway. This seems unlikely, as the main Notch effectors, belonging to the HES family of bHLH transcriptional repressors, are also subjected to a tight temporal regulation of their activity (reviewed in Kageyama *et al.*, 2007b). Actually, not only HES proteins are short-lived (Hirata *et al.*, 2004) but they are also able to repress transcription of their own genes, thereby ensuring that their activity is limited in time (Cooper *et al.*, 2000, Fior and Henrique, 2005, Gajewski *et al.*, 2003, Hirata *et al.*, 2004). Furthermore,

some HES proteins have been reported to be phosphorylated, leading to a decrease in their capacity to bind DNA and repress transcription (Strom *et al.*, 1997), revealing that post-translational mechanisms at the level of Notch effectors might also contribute to restrict the duration of Notch signalling.

### The importance of a clean termination

The described mechanisms to down-regulate Notch activity are important components of the LI intercellular feedback loop that amplifies differences between interacting cells. Their relative contribution may vary in different developmental contexts but, together with the mechanisms that positively regulate Notch signalling, they end up imposing a bias on the directionality of signal transduction, an essential feature of LI-mediated cell-cell interactions.

Actually, to achieve signal directionality in a competitive mechanism like LI, it is important not only to generate an excess of signalling activity delivered by “winner” cells but also to maximize reception of the signal in “loser” cells. Thus, it might seem paradoxical that LI is able to operate efficiently at limiting concentrations of Notch receptors at the cell surface and of NICD in the nucleus, as discussed above. However, this paradox is misguided and we would argue instead that this is a key feature of the LI mechanism, required to achieve an effective amplification of small fluctuations in Notch activity and establish clear differences between cells with equivalent potential. This applies, for instance, to the rapid turnover of NICD, which is needed to prevent its accumulation in the nucleus to levels that would otherwise buffer the system against small fluctuations and block the emergence of “winners” and “losers”.

Another important consequence of the rapid NICD turnover in the nucleus is that it prevents NICD from being reused for target gene transcription, thereby producing bursts of Notch activity, instead of a sustained and prolonged activation. In addition, the negative auto-regulation of the target/effector *hes* genes, together with the short-lived nature of *hes* mRNAs and HES proteins, contribute also to this very transient character of the Notch response.

In summary, the Notch pathway’s architecture is designed to produce an effective but highly transient response in interacting cells, with various mechanisms that negatively regulate Notch signalling contributing to this end. A recurrent theme in these mechanisms is that the players which drive the activity of the pathway are also instrumental in subsequent steps to restrict such activity. This is true, for instance, for the O-Fut protein, which is essential to generate an active Notch receptor but triggers also other modifications that restrict the receptor’s capacity to interact with specific DSL ligands. Another striking example is the MAM co-factor, which is absolutely required to assemble a transcriptionally active complex at Notch target genes but recruits also the CycC:CDK8 kinase to trigger NICD proteasomal degradation.

This particular architecture of the Notch pathway allows a precise control of the amount of signalling experienced by interacting cells and is fundamental for the functioning of the intercellular feedback loop that amplifies signalling differences during LI. In addition, the capacity to effectively terminate Notch signalling by modifying or eliminating some of its components is important for the events that follow LI. Cells where Notch activity reached a

peak can quickly terminate it, reset their potential and enter a new round of LI, involving the same or a different set of alternative fates. This enables LI to regulate various histogenic processes where a pool of progenitors is maintained during an extended period of time, while giving rise to cells with specific and diverse fates. A good example of this happens during *Drosophila* and vertebrate neurogenesis, where neural progenitors (neuroblasts in *Drosophila* and neuroepithelial progenitors in vertebrates) can participate in several rounds of Notch-mediated decisions, resetting their levels of Notch activity after each round. If these cells are simultaneously able to “retune” their potential, by intrinsic or extrinsic mechanisms, the capacity to generate consecutive rounds of Notch-mediated LI can produce an additional outcome: the acquisition of different neural fates along the period of neurogenesis, i.e., neuronal diversity.

Another developmental process where progenitors go through several rounds of Notch activity occurs during vertebrate somitogenesis. Here, cells in the presomitic mesoderm (PSM) experience synchronized oscillations in Notch activity, a finding first revealed by the cyclic expression of *hes* genes in these cells (Palmeirim *et al.*, 1997). More recently, using fluorescent reporters for Notch activity, these cycles have been visualized *in vivo* by time-lapse imaging of the developing presomitic mesoderm (Masamizu *et al.*, 2006). Analysis of the network’s architecture underlying these cycles of Notch signalling confirm the key role played by the mechanisms that terminate Notch activity (reviewed in Kageyama *et al.*, 2007a, Pourquie, 2003), with a major contribution arising from the mechanism that controls the half-life of *hes* mRNAs and proteins (Hirata *et al.*, 2004, Lewis, 2003).

## Final remarks

In recent years, several mechanisms that positively regulate Notch signalling have been described, involving processes like transcriptional up-regulation of DSL ligands, their selective recycling to the cell membrane, and post-translational modifications of ligands and receptors to increase their mutual affinity (reviewed in Bray, 2006, Le Borgne, 2006, Schweisguth, 2004). However, in a competitive mechanism like LI, the final outcome is dictated by the balance of positive and negative mechanisms, regulated globally in the ensemble of interacting cells through a unique intercellular feedback loop. Here, we discussed some of the mechanisms which are known to negatively regulate Notch signalling, at different steps of the pathway. We propose that these mechanisms are crucial to regulate timing and duration of Notch activity during LI, underlying the generation of signalling directionality during cell interactions. We further propose that switching-off Notch activity after signalling has occurred is a critical aspect of the Notch pathway architecture that allows it to regulate progenitor maintenance and cell type diversification in several histogenic processes.

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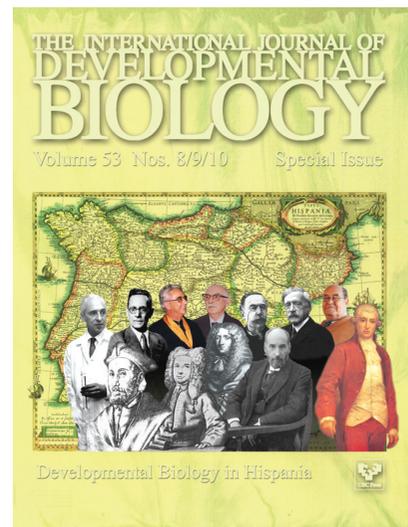
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