The structure of Wntch signalling and the resolution of transition states in development

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Abstract

During development, the emergence of different cell fates and their patterning into tissues and organs requires spatio-temporal coordination that controls the relative number of different cell types. Genetic analysis in different systems have revealed that interactions between Wnt and Notch signalling play pervasive roles in these processes. While many of these interactions can be explained in terms of transcriptional cross-talk between the effectors of these pathways, some of them require a different explanation. Experiments in *Drosophila*, *Xenopus* and mouse have revealed that Notch plays an important role in the modulation of the transcriptional activity of ß-catenin (the main effector of Wnt signalling pathway, independently of its well characterized function as a membrane tethered transcription factor. These studies suggest that rather than two separate pathways, elements of Wnt and Notch signalling configure a single functional module, Wntch, that plays a key role in the resolution of cell fate decisions. Here we review the evidence for Wntch and present a current circuit view of the system, its control and its role in development with a special focus on stem cell populations.

Introduction

A cell can be construed as a computational device where signal transduction and transcription factor networks (STN and TFN) perform calculations about physiology, gene expression, cell division rates or the relative spatial position of a cell. The computational power resides in the molecular fabric of the cell and its effects are cell type specific: for example, a neuron makes calculations about electric inputs and outputs, a cell in the pancreas evaluates whether to secrete insulin on the basis of the glucose concentration in its environment, and stem cells, in the skin or the intestine, perform computations about their transcriptional state and cell cycle timing in relation to the wear and tear of the overall tissue. Computations are therefore central elements in the biology of the cell and this raises questions about the molecular structure of the devices that perform these calculations [1-3]

While these notions are well entrained in physiology and are beginning to be considered in the context of prokaryotic gene regulatory networks [4, 5], the development of an organism is still represented in terms of linear regulatory sequences and loops converging onto cohorts of transcription factors that confer cell identities. And yet, the cell fate decisions that accompany the assembly of a tissue or an organ seem to reflect calculations about the path that the cell follows towards that fate. In these processes cell populations split into defined groups with different and specific gene expression profiles that will interact to give rise to new populations on the way to generate organs. These splits are proportionate i.e. the two populations have precise relative sizes and this underscores the existence of an underlying control over the splitting process. Understanding these processes should be an important aim of modern biology. Here we discuss some aspects of this problem in the context of two signalling pathways, Notch and Wnt.

Signal transduction pathways and calculations in development

The basic process that fuels the generation of tissues and organs during development is the ordered emergence of different kinds of cells. This operation

can be decomposed into two events: a change in gene expression profiles that takes individual cells from an initial to a new state, and the coordination of this event at the level of cell populations that will propagate the new state . In this framework, most accounts of the generation of cell diversity view the emergence of a new pattern of gene expression as a result of deterministic and instructive interactions between the STNs and TFNs: TFNs run transcriptional programmes, while STNs act as parallel information transfer devices that converge onto the regulatory regions of effectors of fates. However, analysis of these processes reveals (i) that they involve interactions between components of different STNs i.e. they are linked so as to integrate information; (ii) that the activity of the TFNs (and probably also of the STNs) is not deterministic but rather probabilistic i.e. within a population of cells a STN or TFN need not be "on" in all cells, but does have a probability to be "on" in each of the cells; this probability can change from cell to cell; (iii) that not all cells that may adopt a fate will adopt that fate i.e. there is some kind of a selective process that determines the size of the pool of cells that will activate the specific TFN that leads to a fate, and, finally (iv) as a consequence of (iii) the pattern of the emerging state cannot be inferred simply by the preceding pattern of gene expression.

The emergence of neural precursors from 'proneural clusters' during insect neurogenesis represent a well understood example of these process[6]. Here, a group of epidermal cells becomes primed to adopt a neural fate, but only one or two cells within this cluster become neural precursors. In *Drosophila*, where this process has been studied in detail, a proneural cluster is defined over a few days through iterative interactions between signalling molecules and region specific transcription factors that lead to the spatially localized expression of the proneural genes *achaete* (*ac*) and *scute* (*sc*). The expression of these genes in the cluster is mosaic, a pattern that remains for several hours and eventually resolves such that only one or two of the cells elevate their levels of *ac* and *sc* expression and become precursors, while the rest lose expression and revert to the epidermal fate[7]. Genetic analysis shows that the resolution of the pattern of *ac* and *sc* expression in the cluster relies on the activity of two STNs, Notch and Wnt: if more Notch signalling, epidermal, and if more Wnt signalling, neural. [8-

12] (Fig. 1). In order to formalize this process, we have proposed that the proneural cluster is an example of a "transition state": an intermediate in a cell fate decision process that represents a developmental checkpoint in which a cell decides whether to adopt a new fate [12-14].

It has been suggested that "rather than being a passive pipe, a signalling pathway undertakes active information processing, the decision to express a gene is the result of a calculation whose complexity is reflected in the structure of the pathways" [3]. Therefore, understanding the nature of the molecular devices that perform this calculation will not only tell us about the nature of the calculation but also about the object of the calculation. The emergence, and particularly the resolution of a proneural cluster, can be construed as an example of a calculation in which a cell decides to adopt or forfeit the neural fate. Here we distinguish between the actual fate of the cells, which is implemented by a network of cell type specific transcription factors that lead to the activation or repression of particular genes, and the implementation of this state i.e. the stabilization of the chromatin structures that will allow the continuous and stable expression of those genes [12, 15]. Cell type specific TFNs and STNs lead to transition states, represented by the unstable establishment of transcriptional complexes. However, the resolution of these states is likely to be cell type independent and respond to a general mechanism that we suggest is tightly linked to interactions between Wnt and Notch signalling.

Wntch: the evidence

The components of the Wnt and Notch signalling pathways were identified in screens for genes involved in developmental processes in *Drosophila* and *C. elegans*. Later on, biochemical studies in mammalian cells, and in particular cancer cells, unravelled the biochemical mechanisms operating in both pathways. As a result, nowadays there is a fairly complete understanding of their backbones and of the flow of information through them.

Wnt signalling came to the fore in the 1980s, nucleated by the observation of a homology between the gene *wingless* from *Drosophila* and the mammary *int-1*

proto-oncogene, which encode ligands for the pathway [16]. Wnt (Wingless+Int-1) signalling uses specific effectors to target different cellular processes e.g transcription and cytoskeletal activity. Transcription is targeted through ß-catenin and it is this branch of Wnt signalling that is the focus of our attention here. Binding of Wnt ligands to LRP5/6 receptors induces the formation of a heterodimer with members of the family of Frizzled receptors and elicits the modulation of the activity of a protein scaffold centered around Axin and APC, whose main function is the GSK3 mediated phosphorylation of ß-catenin that targets it for proteasomal degradation. Inactivation of the Axin/APC based scaffold through the adaptor protein Dishevelled results in a rise of cytosolic levels of ß-catenin and its entry into the nucleus where it modulates transcription through an interaction with members of the Tcf family of DNA binding proteins [17, 18]. Although the mantra of Wnt signalling links directly the levels of cytosolic ß-catenin to its transcriptional function, there is abundant evidence that this association is weak [19-25]. These studies indicate that there are at least two pools of ß-catenin, a cytosolic and a transcriptionally competent, which are represented by different phosphoisoforms and might have different subcellular locations; the transcriptionally competent pool [21] represents less than 1% of the total ß-catenin [26].

Notch, on the other hand, is the name of the founding member of a family of single transmembrane receptors that act as membrane tethered transcription factors [27]. Ligands of the DSL family of transmembrane proteins bind to Notch and trigger a sequence of proteolytic cleavages in the receptor that release its intracellular domain and allow it to enter the nucleus where it mediates transcriptional activation through an interaction with the CSL transcription factor [28-30].

The analysis of developmental events in different organisms has revealed a widespread joint requirement for Wnt and Notch signalling in a range of processes (see e.g [15]). This can be interpreted as resulting from either a convergence of the two signalling pathways and their transcriptional effectors (ß-catenin/Tcf and Nintra/CSL) onto specific promoters of particular genes e.g

[31] or from mutually dependent sequential activations of the two pathways: Wnt/ß-catenin triggers the expression of Notch ligands or Notch/CSL activity leads to expression of Wnt genes (see below). Both modes can be easily framed into conventional models of transcription. However, studies in *Drosophila* and vertebrate embryos and tissues have uncovered a third mode of interaction between the two pathways whereby Notch restricts the activity of ß-catenin [12, 13]. The evidence for this can be summarized as follows:

(i) Loss of function of Notch results in ligand independent activation of Wnt/ß-catenin signalling [32-40]. This regulatory interaction cannot be easily adscribed to transcriptional or epistatic relationships between the components of the two pathways and suggests the existence of a posttranscriptional effect of Notch on Wnt signalling.

(ii) In some systems, gain of function of Notch can downregulate the activity of ß-catenin [32, 35, 41-45].

(iii) The action of Notch on Wnt/ß-catenin signalling is independent of its interaction with CSL and of the release of the intracellular domain of Notch i.e. it does not depend on transcription [35, 43-45].

(iv) Genetic analysis in *Drosophila* has uncovered alleles of Notch encoding proteins that affect specific interactions with Wnt/ß-catenin signalling [42, 46-49].

(v) Structure function analysis reveals functionally different domains, in both the extracellular and intracellular domains of Notch, dedicated either to Wnt or Notch signalling [42, 47, 50-52].

(vi) Notch interacts genetically, and in some instances molecularly, with elements of Wnt signalling: Dishevelled, APC, Axin, Tcf and GSK3 [14, 42, 53-60].

(vii) Notch can associate physically with ß-catenin and both proteins can be found together in endocytic vesicles [31, 35, 43, 44, 61].

The pervasive interactions between Wnt and Notch signalling in mammalian systems are conveniently interpreted in terms of convergent synergistic interactions of their transcriptional effectors. This conclusion is drawn from gain

of function experiment. Given the feedbacks that can be artificially created in these situations and the difficulty to perform sophisticated genetic analysis in systems other than *Drosophila*, these observations cannot rule out other kind of interactions between the two pathways. For example, in mammal Wnt and Notch signalling participate together in the maintenance and differentiation of populations of adult stem cells in the skin [62-64], the intestine [65-67]and the muscles [68]where available tools do not allow a precise dissection of these interactions. The difficulty is highlighted in the case of the intestine where application of gamma secretase inhibitors, suppress the activation of ß-catenin that results from mutations in Apc [65]. This is often interpreted in terms of the sequential requirements for Wnt and Notch signalling in the differentiation of intestinal cells even though loss of CBF function does not suppress the effects of mutations in Apc [69].

Thus, the available evidence suggests that Notch and Wnt signalling are functionally intertwined and are likely to be integrated into a single functional module for which we have suggested the name 'Wntch' [13, 43, 53, 54].

Wntch: the structure

The interactions described above can be used to create a framework reflecting the connectivity of, and information flow through a system configured by elements of both pathways (Fig. 2). While this is useful, it is important to provide a more mechanistic framework for these interactions. As already stated, the transcriptional effectors and their regulatory relationships provide a convenient way to think about these interactions but the role of Notch in the attenuation of Wnt/ß-signalling cannot be easily explained in these terms. An insight into a possible mechanism mediating this interaction is founded upon the observation that the ligand independent traffic of Notch can modulate Wnt/ß-catenin signalling [35, 43, 44].

Experiments in *Drosophila* imaginal discs, and mammalian cells and embryos have shown that Notch mediated suppression of ß-catenin activity can be provided by forms of Notch which cannot interact with DSL ligands, nor engage

in CSL-dependent transcriptional activation [35, 43, 44]. These forms -chimeric receptors in which the extracellular domain has been substituted by that of a heterologous receptor-are endocytosed and trafficked, activities that are essential to their ability to regulate ß-catenin. Surprisingly, Notch specifically targets the transcriptionally competent form of Arm/ß-catenin and has little effect on its cytosolic total levels [35, 43, 44]. This observation is significant because it lends support to the notion that the transcriptional function of ß-catenin is not mediated by the cytosolic pool but rather by a specific phosphoisoform that is likely to be associated with membranes [22] and that this form has specific regulators. This possibility is further supported by reports from *Drosophila* where Axin in addition to acting as a scaffold for GSK3 in the regulation of the total levels of ß-catenin, acts as an anchor for its transcriptionally competent form [23, 70]. In the context of Wntch signalling, genetic interactions have been described between Axin and Notch that converge on Armadillo and Axin, together with APC, can regulate the traffic of Notch [14, 54]. It may be that the anchor function of Axin is related to its interactions with the Notch receptor.

The close relationship between Notch and the transcriptionally competent form of ß-catenin is underpinned by the observation that, in *Drosophila,* the ligand independent traffic of Notch requires Dishevelled (Dsh) [53, 54]. In mammals, there is no evidence for an interaction between Dvl (the mammalian ortholog of Dsh) and Notch, though Numb, an inhibitor of Notch signalling by trafficking membrane bound Notch for lysosome degradation [71] has been implicated in the interactions between Notch and ß-catenin [35, 72]. However, in all instances there is evidence that Deltex, another adaptor protein involved in the traffic of Notch, partcipates in CSL independent Notch signalling and, therefore, could be involved in the regulation of the active form of β-Catenin [42, 46, 73]. These are early days in the analysis of these interactions and more experiments are needed to understand its details but the nature of the proteins involved in the interactions between Notch and Wnt signalling point to the endocytosis and traffic of Notch as central elements in the activity of ß-catenin.

Altogether these observations allow us to propose a model in which Notch is secreted to the apical side of the cell where it interacts with a small pool of ß-catenin –the transcriptionally competent form- located at or near the adherens junctions [53, 54] (Fig. 3). As Notch traffics in a ligand independent manner, it drags the transcriptionally competent form of ß-catenin and promotes its degradation or sequestration. This flow of Notch from the cell surface requires a basal level of Dishevelled and Deltex in *Drosophila* [46, 53], and of Numb, and probably also Deltex, in mammals [35, 74]. Structure-function analysis of the intracellular domain of Notch reveals various sites potentially involved in the interactions with ß-catenin, Dishevelled, Numb and Deltex that will need to be explored further [42, 46, 53, 55].

There is evidence that as Notch affects Wnt/ß-catenin signalling, there is a reciprocal effect of Wnt signalling on Notch. Experiments in *Drosophila* indicate a feedback from the activity of Wnt on the traffic of Notch [53]. Although these effects appear to be mediated by Dishevelled, given the emerging promiscuity of interactions of Wnt ligands with cell surface receptors, it is not possible to rule out interactions between some members of the Wnt family and Notch, as has been described for Wingless in *Drosophila* [75]. The change in Notch traffic mediated by Wnt signalling results in an increase in ß-catenin signalling [53]. Reciprocally, there is evidence that one effect of the activation of Wnt/ß-catenin signalling on Notch/CSL activity is mediated through the activation of the expression of Jagged and Delta [76-79] and that GSK3 inhibits the stability and activity of NICD [80] [81] [56] –thus inhibiting GSK3 activity, Wnt signalling will lead to increased NICD signalling in a ß-catenin and Delta/Serrate/Jagged independent manner-. Intricate links between the two are also supported by the observation that Mastermind, a key element of Notch/CSL activity, is also a coactivator of ß-catenin [82, 83]. All these interactions might have a strong cell type specific component.

Altogether these observations indicate that the elements of Notch and Wnt signalling form a closed, versatile information processing network. An additional level of interaction between the two pathways is derived from the consideration

that there might be a single pool of Notch at the cell surface. In this case it is not difficult to see that the higher the rate of traffic of the receptor, the fewer molecules of Notch will be available for interactions with Delta and Serrate at the cell surface, and that a lower rate of traffic would increase the amount of Notch available for NICD signalling. As Wnt signalling can regulate the traffic of Notch, this would allow a further layer of cross regulation between the two pathways. for interactions with ß-catenin. The possibility of reciprocal effects depending on the amount of Notch at the cell surface are supported by the report that γ-secretase inhibitors enhance the effects of Notch on ß-catenin by increasing the endocytosis and traffic associated activity of Notch [35] i.e. any form of Notch available for traffic will affect Wnt/ß-catenin signalling. It will be interesting to see how general this effect is, as it might provide additional evidence for the difference to separate the two pathways functionally. Interestingly, competitions between the two pathways have been described in mammalian cells [84] and in *Drosophila* [85] [55] and are particularly evident in the patterning of the wing veins of *Drosophila* in different conditions [50] [42]. These competitions could be interpreted as the result of the balanced interactions within the integrated device, Wntch.

The existing set of observations and experiments allow us to outline how Wntch might be organized (for details see Fig. 3) with the possibility of having a self regulated tuning of the inputs that result in a balance of its two outputs: Wnt/ß-catenin and NICD/CBF signalling. Cell type specific regulation of the traffic of Notch, or of the activities of GSK3, Axin and APC will adjust the functioning of the system and determine the impact that the two pathways have on the physiology of the cells. However, there are observations which do not fit well in this framework. A most significant one relates to the effects of soluble forms of the intracellular domain of Notch on the activity of ß-catenin. Experiments in *Drosophila*, *Xenopus* and mouse cells have shown that NICD can downregulate the activity of ß-catenin and that, in some instances, this does not require CSL dependent transcription [35, 41-43, 45]. At the moment there is no clear mechanism to account for this observation.

Wntch, transition states and cell fate decisions

To understand the function of the interactions between Notch and Wnt signalling, whether transcriptional or non-transcriptional, it is important to consider their context. A common denominator of the processes that involve Wnt and Notch signalling is the decision of a cell to adopt or forfeit a state and therefore understanding the details of this process will help understanding the relationship between the two pathways and their interactions (Fig. 4).

A change of state from S1 to S2 can be rationalize as follows: (1) induction of the S2 state in S1 cells; (2) establishment of a transition state, S1/S2; (3) resolution of the transition state and (4) maintenance and/or propagation of the new state, S2 [15] [13]. The first stage is clearly governed by the S1 TFNs, whose components, as all transcription factors, act on cohorts of genes to trigger their activation or repression: thus the S1 TFN will lead to the onset of expression of S2 genes. However, this activity might not be enough to establish a pattern of expression as in many instances gene expression requires combinations of factors and the assembly of chromatin remodelling complexes not all of which are provided by the action of the transcription factors alone. Thus, the action of S1 TFNs leads to unstable transcriptional complexes at the regulatory regions of the S2 genes. Therefore what S1 TFNs achieve is to create an unstable pattern of S2 gene expression. In a second phase, the regulatory regions of the S2 TFNs receive additional inputs from the effectors of signalling pathways to establish a transition state, S1/2, in which genes corresponding to S2 become primed for expression. We surmise that this S1/2 state is very noisy, i.e. transcription might be initiated but is never completed and therefore, the levels are heterogeneous from one cell to another; probably the state of the chromatin is an important element of this transition state. The decision to become S2 or to remain as S1 depends on the resolution of the S1/S2 state at the level of individual cells and we suggest that this is governed by the competition between Wnt and Notch signalling: if Wnt/ß-catenin signalling favours a fate, Notch:CSL signalling will favour the opposite. Wntch ensures that a proportion S1:S2 is as required by the system; it achieves this by setting up the threshold that controls the S1/S2 state, i.e. the probability of the transition from S1 to S2..

The paradigmatic example for this sequence of events is the development of the proneural clusters in *Drosophila* (Fig. 1 and Fig.4B), where an interplay between the different modes of Notch and Wnt signalling (Wnt:ß-catenin and Notch:CSL) provide different fates (epidermal or neural, respectively), while Wntch regulates the stability of the transition state between both fates (see also [12]. This situation can be used as a reference for other binary cell fate decisions and can be translated into the homeostatic maintenance of stem cell populations [13] (Fig.4B,C).

The antagonism between Wnt and Notch signalling also plays a key role in the earliest cell fate decisions in vertebrate embryos: in sea urchins, *Xenopus* and mammals the development of endomesoderm requires Wnt signalling [36, 86-90] while that of neuroectoderm requires Notch [91, 92], a situation recapitulated in embryonic stem cells [93] [94, 95]. These observations demand mechanisms that mediate reciprocal modulations of Wnt and Notch signalling and Wntch provides a framework to think about this. However, while it is possible to see how Wnt signalling can potentiate Notch/CSL activity, for example through the inactivation of GSK3, it is less clear how Wnt signalling could antagonize Notch/CSL signalling. An answer to this question is important to complete the set of interactions that configure Wntch as a generic mechanism that influences cell fate decision. One possibility rests on the observation that, at least in Drosophila, Wnt signalling controls the traffic of Notch and that this may be general (see above).

Although all the interactions described in Fig. 2 are possible and in principle can be found in any cell, this does not mean that all of them will be found in all cells. Variables like the rate of endocytosis and traffic or the activity of GSK3 in specific cells can have cell type specific settings and therefore play a role in the activity of the network. Nonetheless we surmise that Wntch creates a generic information processing device with a central role in cell fate decision making.

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Figure legends

Figure 1. Specification of Sensory Organ Precursors (SOPs) in the proneural clusters of the wing imaginal disc of Drosophila. (A) The third instar wing imaginal disc exhibits a pattern of expression of Achaete and Scute in clusters (green) which outline neural potential. The Dorsocentral cluster (D), that will give rise to the dorsocentral bristles is circled. Other clusters are also highlighted e.g the wing margin (WM) one. (B) In the proneural cluster, shown in the picture with expression of Scute, cells have different levels of expression of proneural genes and the decision to become an SOP is determined by the relative amount of Wingless and Notch signalling (see text for details). The cells that receive more Wingless signal become SOPs. (C) Pattern of the SOPs in the DC cluster (green) with the expression of Wingless in red.

Figure 2. Structure and function of Wntch. **(A)** Summary of the interactions between elements of Wnt and Notch signalling and outline of the network that configures Wntch signalling. For details see text. The transcriptional interactions are labelled in yellow. (1) effects of GSK3 activity that destabilized ß-catenin and NICD. (2) Wnt signalling inhibits GSK3 and thus stimulates ß-catenin and CSL/NICD function. These effects are likely to be cell type specific and depend on basal levels of GSK3 activity. (3) Notch, in a CSL independent manner, inactivates the transcriptional activity of ß-catenin. (4) Wnt signalling inhibits the CSL independent activity of Notch. **(B)** Activity of the network outlined in A in

different conditions. Notice that activation of Wnt signalling can lead to DSL-Notch-CSL signalling.

Figure 3. Mechanism of the interactions between Wnt/ß-catenin and CSL independent Notch signalling. The ligand independent traffic of Notch mediated by Dishevelled and Deltex, promotes the degradation of transcriptionally competent ß-catenin. Changes in the amount of Dishevelled result in a change in the traffic of Notch and an inactivation of this route of ß-catenin degradation. This diagram underlies (3) and (4) in Figure 2. For details see text.

Figure 4. Function of Wntch in the maintenance and resolution of transition states. **(A)** Diagram of a state transition during a process of cell fate assignation. Notice the transition state S1/2 where a cell makes a decision. **(B)** Effects of Wnt and Notch signalling on fate transitions. Notice that by adding a self replicating event on S1, allows us to use the scheme in A to describe a stem cell population. Following the principles laid out in the establishment and resolution of the proneural clusters in Drosophila (Figure 1), it is possible to assign signalling elements to each of the arrows. The literature suggests that (a) is likely to be Wnt signalling as it is likely to be (d), Notch signalling is Notch/CSL signalling and (b) would be Wntch, which will lead to either (a) or (d). Wntch is setting up a threshold and therefore can be seen as the fulcrum of the system. These assignations are inferred from the literature. **(C)** The outlines in (A) and (B) can be used to interpret the changes in cell states associated with the differentiation of a stem cell population as indicated. We suggest that both the stem cell (SC)population and a putative transit amplifying compartment (TA) have as a central element a transition states which is likely to determine the size of the different pools. Cells in the TA always differentiate into one of two cell types and this decision also involves a Wnt/Notch balance.

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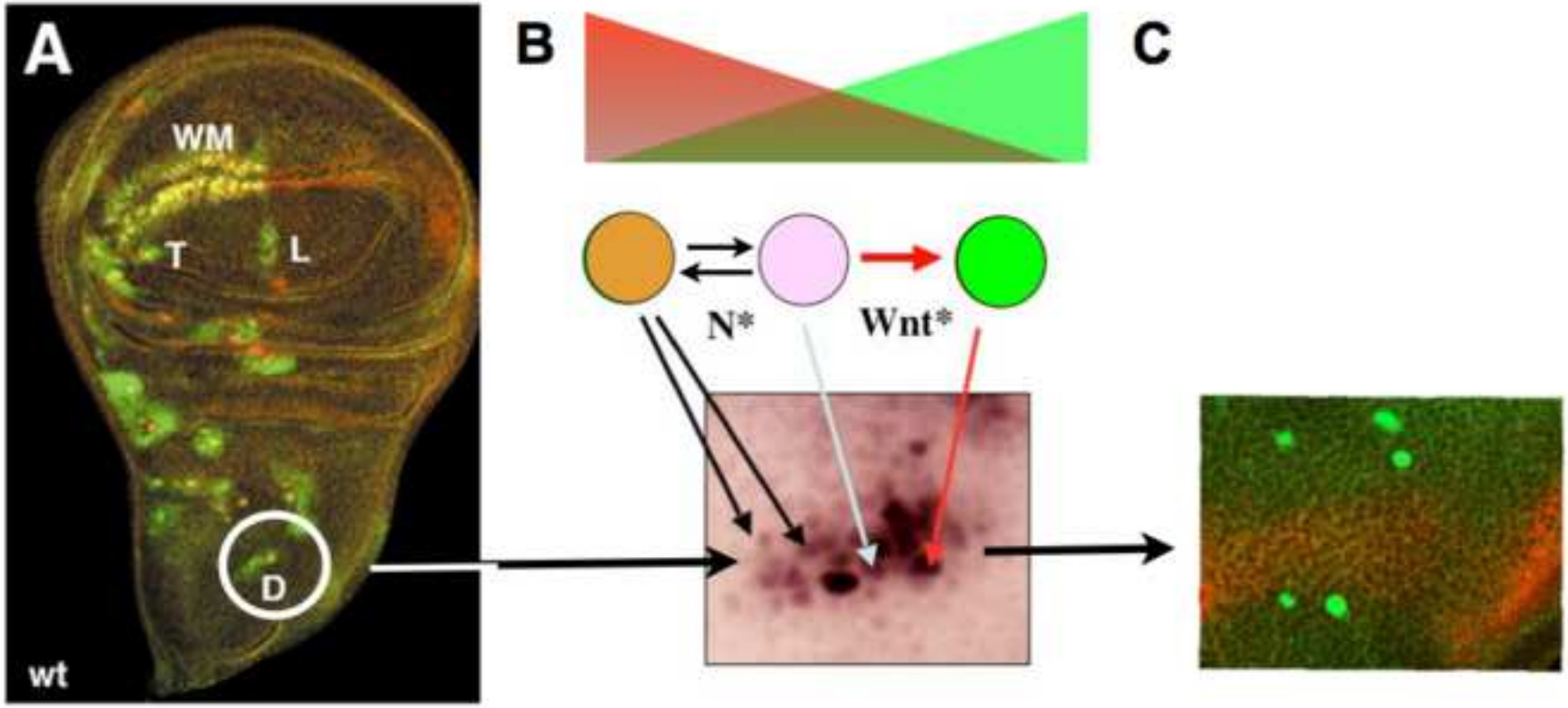

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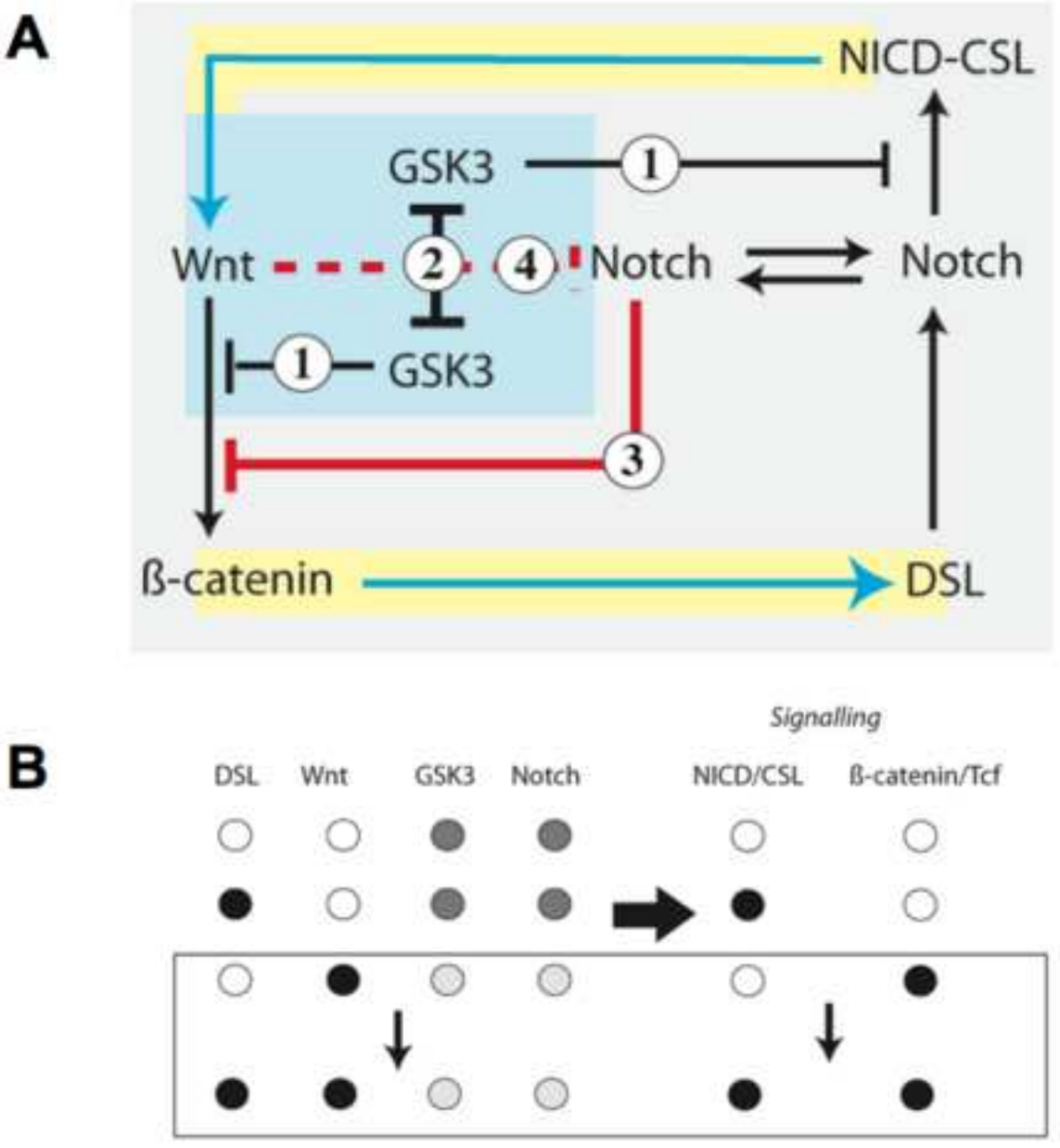

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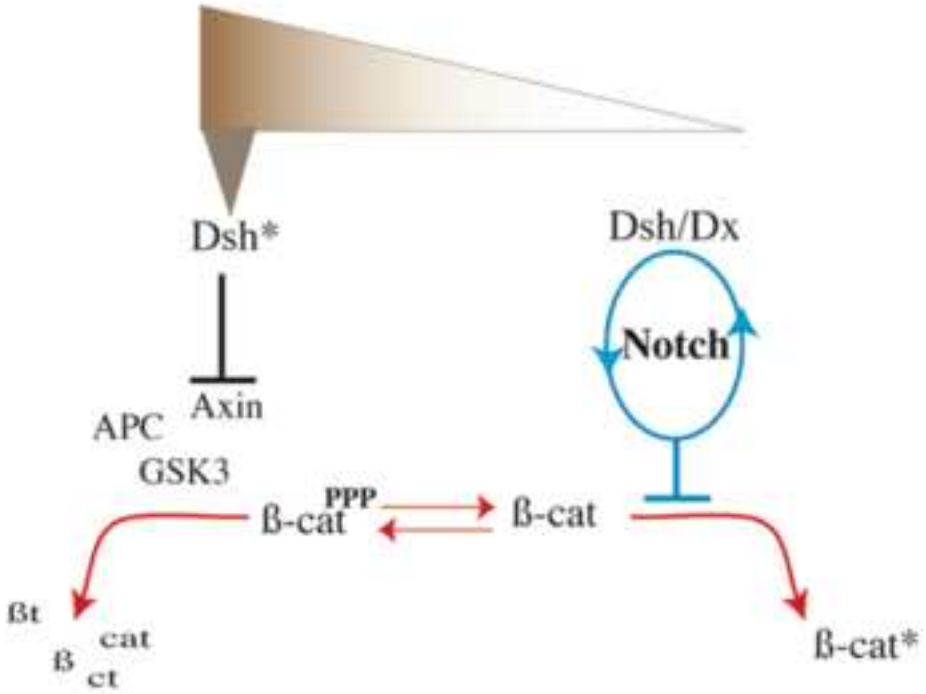

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