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Review

Cell proliferation control by Notch signalling during imaginal discs development in *Drosophila*

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Abstract: The Notch signalling pathway is evolutionary conserved and participates in numerous developmental processes, including the control of cell proliferation. However, Notch signalling can promote or restrain cell division depending on the developmental context, as has been observed in human cancer where Notch can function as a tumor suppressor or an oncogene. Thus, the outcome of Notch signalling can be influenced by the cross-talk between Notch and other signalling pathways. The use of model organisms such as *Drosophila* has been proven to be very valuable to understand the developmental role of the Notch pathway in different tissues and its relationship with other signalling pathways during cell proliferation control. Here we review recent studies in *Drosophila* that shed light in the developmental control of cell proliferation by the Notch pathway in different contexts such as the eye, wing and leg imaginal discs. We also discuss the autonomous and non-autonomous effects of the Notch pathway on cell proliferation and its interactions with different signalling pathways.

Keywords: *Drosophila*; Notch signaling; imaginal discs; cell proliferation

1. Introduction

A wide range of cellular functions including cell proliferation, cell survival and cell fate commitment has been shown to be regulated by the activity of Notch signalling in nearly all multicellular organisms [1-4]. Accordingly with these pleiotropic effects, there is extensive evidence linking the deregulation of Notch pathway with multiple diseases and cancer [4-8]. Despite the

great progress in elucidating the mechanisms and molecular features of this signalling cascade during the last decades, the explanation for the diversity of cellular responses triggered by the pathway is not fully understood.

Since the first allele of *Notch* gene was identified in *Drosophila melanogaster* by T H Morgan in 1917 [9], this genetically powerful model organism has provided solid explanations for much of what we know about Notch-dependent intercellular signalling [6]. In addition, the evolutionary conservation between flies and humans has ensured the functional significance of these findings for obtaining a better understanding of human development and disease. Numerous studies have highlighted the power of using the development of the *Drosophila* imaginal discs as an *in vivo* system model for analyzing how cooperative interactions between the Notch pathway and other signals contribute to regulate cell proliferation in epithelia. Moreover, these structures have been employed to define the molecular mechanisms underlying tumor development and metastasis when Notch signalling is perturbed [10]. As occurs in mammals, *Drosophila* Notch activation can promote tumor growth by causing abnormal cell proliferation, not only autonomously, but also triggering proliferation in the normal surrounding cells. This effect is pleiotropic and context-dependent. Cancer is primarily a disease caused by loss of normal cellular growth regulation and this is often caused by the loss of cell proliferation control. To elucidate how alteration in the activity of Notch leads to the loss of proliferative control is fundamental to define the role that Notch plays in the intricate machinery that regulates the cell cycle and cell proliferation during normal development. In this review we will focus on recent advances and knowledge on the function of Notch signalling in regulating cell proliferation during normal development of the imaginal discs. The mechanisms of normal Notch signalling regulating cell proliferation may provide useful insights to understand how alteration in this pathway can induce tumorogenesis.

2. Description of the Notch signalling pathway

In *Drosophila* the activation of the Notch signalling pathway depends on the interaction between the unique Notch receptor identified in this insect (in mammals there are 4 Notch orthologs, termed NOTCH1–4) with its ligands Delta (Dl) (Delta-like [Dll1], Dll3 and Dll4 in mammals) and Serrate (Ser) (Jagged, Jag 1 and Jag2 in mammals). The Notch receptor is a single-pass transmembrane molecule synthesized as a precursor form that is cleaved by Furin-like convertase (S1 cleavage) to generate a non-covalently linked heterodimer [11]. This heterodimer is composed of two subunits. One of these subunits consists of the major portion of the extracellular domain (NEC) fragment, and the other is the C-terminal region that contains the remainder of the NEC domain, the transmembrane domain and the intracellular domain (NICD) [3,4]. The activation of the receptor initiates a proteolytic process of the receptor and the subsequent release of the Notch intracellular domain (NICD) [4,6,8,12,13]. The cleavage of the receptor is performed by different proteases in a sequential manner, firstly by members of the ADAM metalloprotease Kuzbanian (Kuz)/TACE family, and later by the presenilin (PS)-dependent gamma-secretase complex (S3 cleavage) to release a soluble intercellular Notch fragment (NICD). This domain then translocates to the nucleus to participate in concert with the transcription factor CSL (CBF1-Suppressor of Hairless (Su(H)), and Mastermind (Mam), in the transcriptional activation of Notch target genes [2,3,41].

The Notch (NICD) is subject to a variety of post-translational modifications, including phosphorylation, ubiquitylation, hydroxylation and acetylation. These modifications can modulate

the relative strength of receptor-ligand interaction, as well as the outcome of the signal transduction, and therefore the final cellular response induced by the activation of the pathway [4,6,13,14]. Another process that modulates the activity of Notch signalling is the endocytic trafficking [15-18]. Ligand endocytosis contributes to generate the physical forces necessary to dissociate and activate the receptor, and activated receptors enter endosomes to signal. Moreover, endosomal trafficking is involved in down-regulating Notch receptors that have not been activated. Disruption of the function of genes encoding for endocytic components cause different tumors [15,16,17,19]. According to whether the gene affected is required early or late in the endocytic pathway the tumor phenotype differ. Thus, mutations in genes that act at the level of early endosome fusion, such as *Rab5, the syntaxin, Avalanche (Avl) and Rabenosyn-5*, cause autonomous tumor growth of wholly mutant tissue [20,21], but clones of mutant cells for these genes fail to overgrow. In contrast, loss of later endosomal trafficking components, such as ESCRTI (TSG101) and ESCRTII (Vps25), exhibit a non-autonomous tumor phenotype whereby mutant clones induce proliferation in adjacent wild-type tissue [22-26] reviewed [23,25]. The difference between these two types of endocytic tumors seems to be due to their differential effects on Notch signalling [18,20,22,23,25-28]. Thus, whereas lack of Notch internalization in early endocytic mutants does not appear to promote Notch signalling [18,20,21] the disruption of ESCRTI and ESCRTII results in ectopic Notch activation and the induction of the expression of the secreted JAK/STAT ligand Unpaired (Upd) [18,22,23,25,26,27], which acts to promote growth in adjacent wild-type tissue (see below). The influence of the endocytic trafficking on Notch signalling has been covered in several reviews [15,16,17,19].

3. Notch signalling coordinates the growth of the eye discs

The adult eye, antenna and part of head cuticle of *Drosophila* originate from a common structure known as eye-antennal discs. The discs develop from approximately 70 ectodermic cells that invaginate from the dorsal pouch of the embryo forming a flat epithelial sac-like structure [29,30]. The few cells that constitute the primordium of the eye disc start to proliferate during the larval stages, reaching an approximate number of 97000 cells at end of the pupal stage [31]. During larval development, the eye-antennal disc is divided into different linage units known as compartments [32,33]. The boundaries between different compartments act as a signalling centers necessary for the growth and patterning of the discs [34,35]. One of the borders that play a fundamental role during eye development is the Dorsal-Ventral (D/V) boundary.

In the early stage of eye disc development, before the definition of the D/V border, the function of Notch signalling is necessary to separate the presumptive eye territory from the antenna field and promote proliferation. The elimination of Notch function during this stage truncates completely the development of the eye field and, simultaneously duplicates the presumptive region of the antenna [36,37]. This phenotype can be at least partially rescued by over-expressing the cell cycle regulator *Cyclin-E (CycE)* [37]. Interestingly, this role of Notch signalling participating in the early specification of different territories is not exclusive for the eye field, and a similar mode of action has been reported to take place at early stages of wing development (see below and Figure 1).

Different signalling events occur at the D/V boundary to control and coordinate the growth of the eye discs during larval development. A key proliferative signal is established around a maximal concentration of Notch activity along this boundary, this region is known as ‘organizer’ [35,38]. Although the Notch receptor is expressed in almost all the cells of the eye-antennal disc, its activity

is limited to the D/V boundary due to the function of genes whose expression is restricted either to the dorsal or ventral compartments of the eye disc [38-41]. The sequential and localized expression of genes such as *panier (pnr), wingless (wg*) and *iroquois (iro-C)* determine dorsal fate of the eye primordium [38,42,43]. Concomitantly, the activity of the *iro-C* into the dorsal region represses the expression of *fringe (fng)*, thus restricting its expression to the ventral half of the eye primordium [38,39,40,42] (Figure 2). Opposing *fng*-positive and *fng*-negative cells establish the molecular mechanism that functionally defines the D/V boundary. The *fng* gene encodes for a glycosyltransferase that enzymatically modifies Notch modulating the ability of this receptor to interact with its ligands Dl and Ser [44,45,46]. Fng inhibits the ability of Ser to signal through Notch and at the same time potentiates Notch-Dl interactions, thereby generating the local activation of Notch along the D/V boundary. This localized activation of Notch promotes global growth throughout the eye discs from this early stage of eye development and onwards [38,47]. Any disruption affecting the D/V boundary specification impedes eye formation, while ectopic generation of D/V boundaries hold the potential to create *de novo* organizers that frequently cause eye duplications or enlargements of the eye field [38-41].

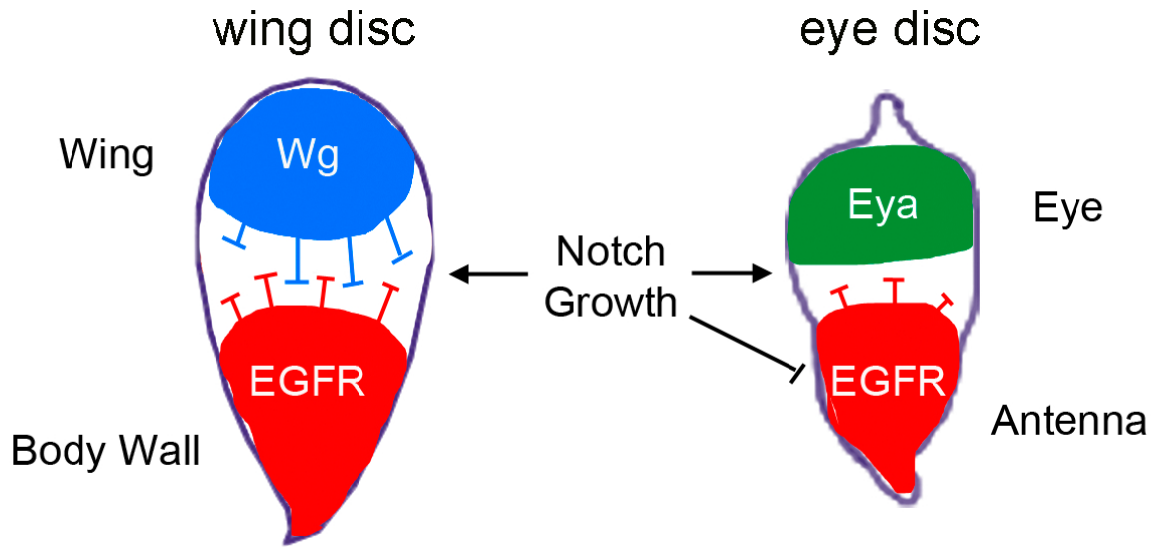

Figure 1. Notch induced growth and territorial specification. Illustration of the subdivision of the wing and eye-antenna imaginal discs into distinct territories. The wing disc is subdivided in wing and body wall primordia by the antagonistic signalling pathways Wg (blue) and EGFR (red). The eye-antenna imaginal disc is subdivided into antenna and eye primordia by the activity of EGFR (red) and Eya (green), respectively. Notch signalling induced cell proliferation promotes the subdivision of the wing and eye-antenna imaginal discs in distinct territories.

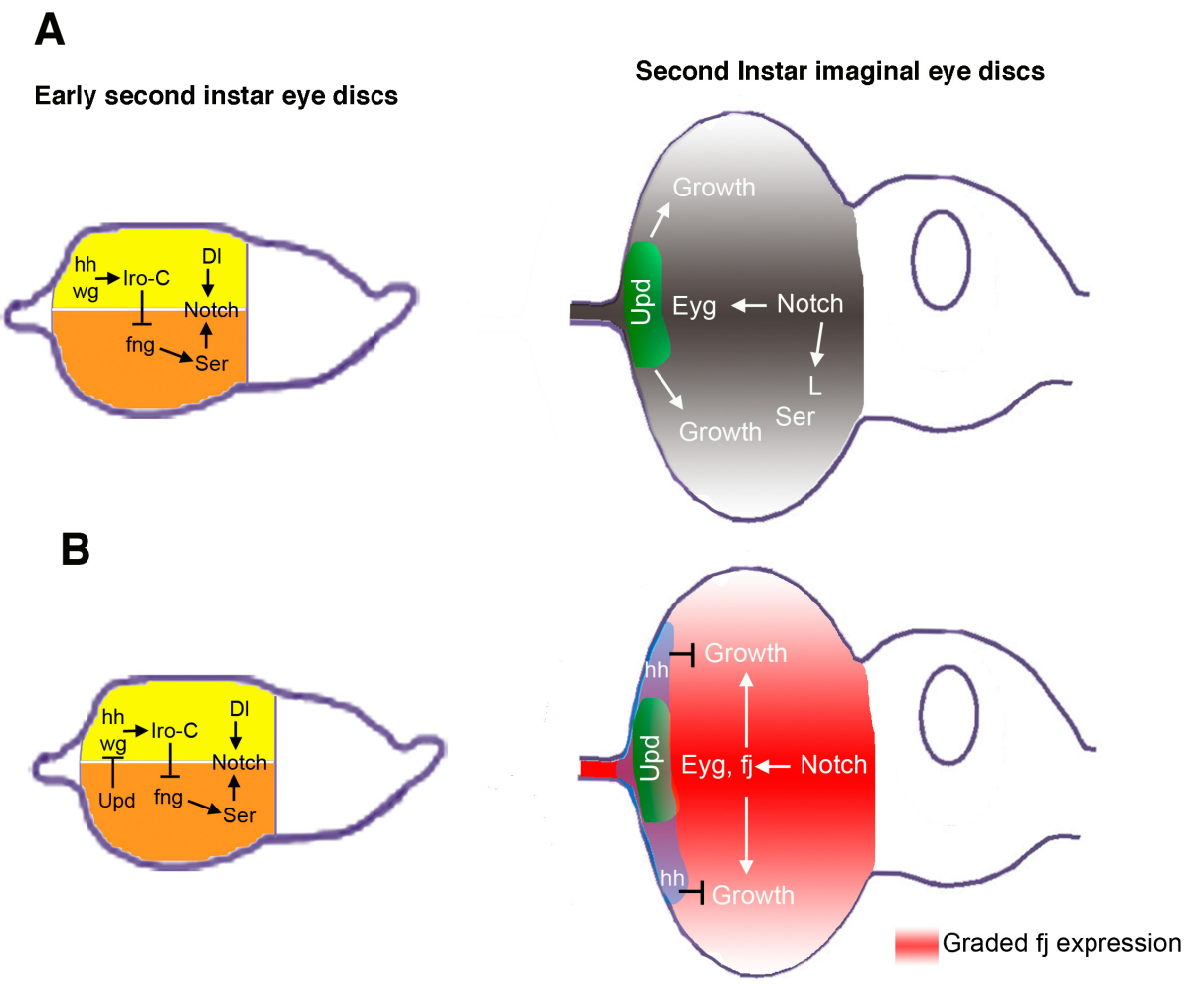

Figure 2. Role of Notch in autonomous and non-autonomous control of cell proliferation in the eye disc. Schematic representation of an early second (left), and third (right) larval instar eye discs. The subdivision of the eye discs in Dorsal (yellow), and Ventral (orange) compartments depends on the restricted expression of different genes that lead to the activation of Notch signalling at the D/V boundary. (A) Model suggesting that the function of the Jak/Stat pathway is required down-stream of the Notch signalling. Accordingly to this model, Upd is activated at the organizer by Notch signalling to promote global eye growth. (B) Alternative model suggesting that Jak/Stat activation is necessary for defining the organizer, and therefore it functions up-stream of Notch signalling.

3. Non-autonomous effects of Notch signalling in control of cell proliferation during eye development

Although Notch activity is restricted to the D/V boundary at the early phases of larval development, the influence of Notch signalling on proliferation spans throughout the eye disc. This implies the existence of a relay signal that expands the growth promoting effect of Notch to the entire eye disc. It has been proposed that the Pax protein encoded by the gene *eye gone (eyg)* is a

major effector of Notch signalling at the D/V boundary [48,49]. In second instar eye discs *eyg* transcription is induced by Notch signalling in a slice within the eye primordium straddling the D/V boundary [48,49]. Over-expressing *eyg* can compensate loss of Notch activity, whereas loss of *eyg* blocked the function of Notch signalling. Accordingly, it has been proposed that *eyg* is required downstream of Notch signalling for eye growth (Figure 2) [48,49]. Since *eyg* encodes for a transcription factor, other protein/s must operate downstream of *eyg* to relay the D/V organizer proliferative signal throughout the eye disc [49]. One of the proteins that might be mediating this function is Unpaired (Upd), the ligand of the JAK/STAT pathway [50]. Considering these data, a model has been proposed that suggests that Notch signalling and *eyg* induce the expression of *upd* at the organizer, then this secreted protein would diffuse throughout the eye disc acting over long distance to promote cell proliferation [48,51,52] (Figure 2A). The fact that in an *eyg* null mutant background the ectopic activation of Notch signalling can still induce proliferation in the eye discs, suggest that Notch could also control proliferation by an *eyg*-independent mechanism. Moreover, as Notch signalling can induce *upd*, but not *eyg*, in the posterior eye discs margin, the activation of *upd* can also be through an *eyg*-independent mechanism [48]. The functional link from Notch signalling to *eyg* and *upd* would explain how the localized activation of Notch could regulate global growth of the eye disc (Figure 2A). However, other authors have questioned this model, as they have found that the growth defects caused by the elimination of Notch signalling cannot be rescued by the activation of the *upd*/JAK/STAT (Figure 2B) [53]. These authors propose that the activation of JAK/STAT pathway by Upd precedes the formation of the organizer. Thus, in early eye development, *upd* would be antagonizing Wg and Hedgehog (Hh) signalling in the ventral domain and therefore repressing *iro-C* in this domain. Consistently with this model, the over-expression of *upd* induces overgrowth associated with *iro-C* inhibition and the appearance of new D/V boundaries [53]. Thus, in an initial phase JAK/STAT pathway would be necessary to define the position of the organizer, and therefore it would be functioning upstream of Notch signalling. Once the D/V boundary is defined Notch signalling would be locally activated along this border to establish the organizer, and therefore to induce global eye growth [53]. Interestingly, these authors have reported that one of the factors that is, at least partially, executing this global growth response induced by Notch signalling is the Golgi transmembrane type II glycoprotein Four-jointed (Fj) (Figure 2B)[54]. The ectopic expression of *fj* partially rescues the proliferative defects caused by reduced Notch activity [53]. The expression of *fj* is activated by Notch and JAK/STAT pathway at the D/V boundary in a graded manner (Figure 2B). This expression is complementary to the graded expression of the tumor suppressor gene *dachsous (ds)*. This gene encodes for an atypical cadherin that binds to, and activates the protocadherin Fat, that in turn initiates the Hippo pathway signal. The function of this pathway restrains disc growth by inhibiting cell proliferation [55,56]. Fj kinase activity phosphorylates the cadherin domains of Fat and Ds during their transit through the Golgi, decreasing the affinity of Ds for Ft, and therefore reducing the activity of Hippo pathway [56]. Thus, Fj might be functioning as a regulatory hub, integrating Notch and Hippo pathway during the growth of the eye discs [53].

During eye disc development Hh and Notch signalling display an antagonistic interaction that plays an important role in the definition of the final size of the eye. In early second larval stage *hh* is expressed in marginal cells of the eye disc, and its product is secreted in an opposite gradient to that of the growth-promoting factors produced in the organizer [47,57] (Figure 2B). The function of this signalling pathway is crucial for retinal patterning and also to regulate eye growth [47]. In a

recent genetic screening, Da Ros and co-workers [57] have found that the over-expression of the conserved microRNA *mir-7* enhances the overgrowth eye phenotype caused by the ectopic expression of *Dl*. These authors proposed that the cooperative action between *mir-7* and Notch pathway reduces the activity of Hh signalling pathway by down-regulating two functionally redundant Hh receptors, Interference hedgehog (Ihog) and Brother of Ihog (Boi) [58-61]. *ihog* is directly silenced by *miR-7*, whereas *boi* is repressed by Notch signalling during eye growth. In this context Hh signalling would be necessary to reduce cell proliferation (Figure 2B). These results contrast with the function that it has been proposed for Hh signalling in promoting cell proliferation during eye discs development [62]. However, recent reports suggest that when Hh pathway is activated in clones of cells, the mutant cells are eliminated by apoptosis, whereas the surrounding wild-type cells over-proliferate [63]. Interestingly, mutations in members of the Hh pathway have been associated with human cancers [64]. This suggests that in addition to the well-known oncogenic role of Hh signalling, this pathway might also have a tumor-suppressor function. Accordingly, the down-regulation of components of Hh signalling enhances the phenotype caused by the ectopic activation of Notch, resulting in severe disc overgrowth. Moreover, the tumor-like overgrowths induced by the ectopic expression of *Dl* and *mir-7* are suppressed when Hh signal is increased [57]. These results reveal a cooperative antagonistic interaction between these two pathways in the control of cell proliferation. Considering that these signalling pathways, and their regulation, are highly conserved between flies and humans, it is likely that this mechanism might play a role in the development of some human cancers.

All these models coincide with the idea that different signalling pathways mediate the Notch-induced global eye growth (Figure 2). These signals promote cell proliferation throughout the eye discs in response to the local activation of Notch at the D/V boundary. Thus, the mechanisms underlying Notch-induced tumorigenesis might be, at least partially, explained by the interaction between Notch signalling and these signalling pathways (Hh, JAK/STAT and the Hippo pathway).

4. Cell autonomous effect of Notch regulating cell proliferation during eye disc development

4.1. Second mitotic wave

During eye disc development the onset of retinal differentiation is associated with the formation of an indentation in the most posterior region of the eye disc, known as morphogenetic furrow (MF). Since progressively anterior cells undergo this constriction, the furrow takes on a wave-like appearance moving from posterior region of the eye discs to the anterior [65,66,67]. The furrow sweeps across the disc, leaving in its wake the developing clusters of cells that will become the individual units of the compound eye, known as ommatidia. Moreover, furrow initiation promotes cell cycle synchronization, as cells anterior to the furrow proliferate randomly, but as soon as they enter the MF they arrest in G1 [68,69,70]. After the passage of the furrow, most of the cells that are not part of the cluster of cells that have initiated the process of differentiation undergo a single round of cell division, known as the second mitotic wave (SMW). After this, most cells will not divide again [66,71]. This additional round of cell division supplies unspecified precursor cells to each ommatidium [66]. Because the cells only undergo one round of division at this stage, and because of the progressive mode of development, all stages of the cell cycle can be observed easily in a single eye disc (Figure 3).

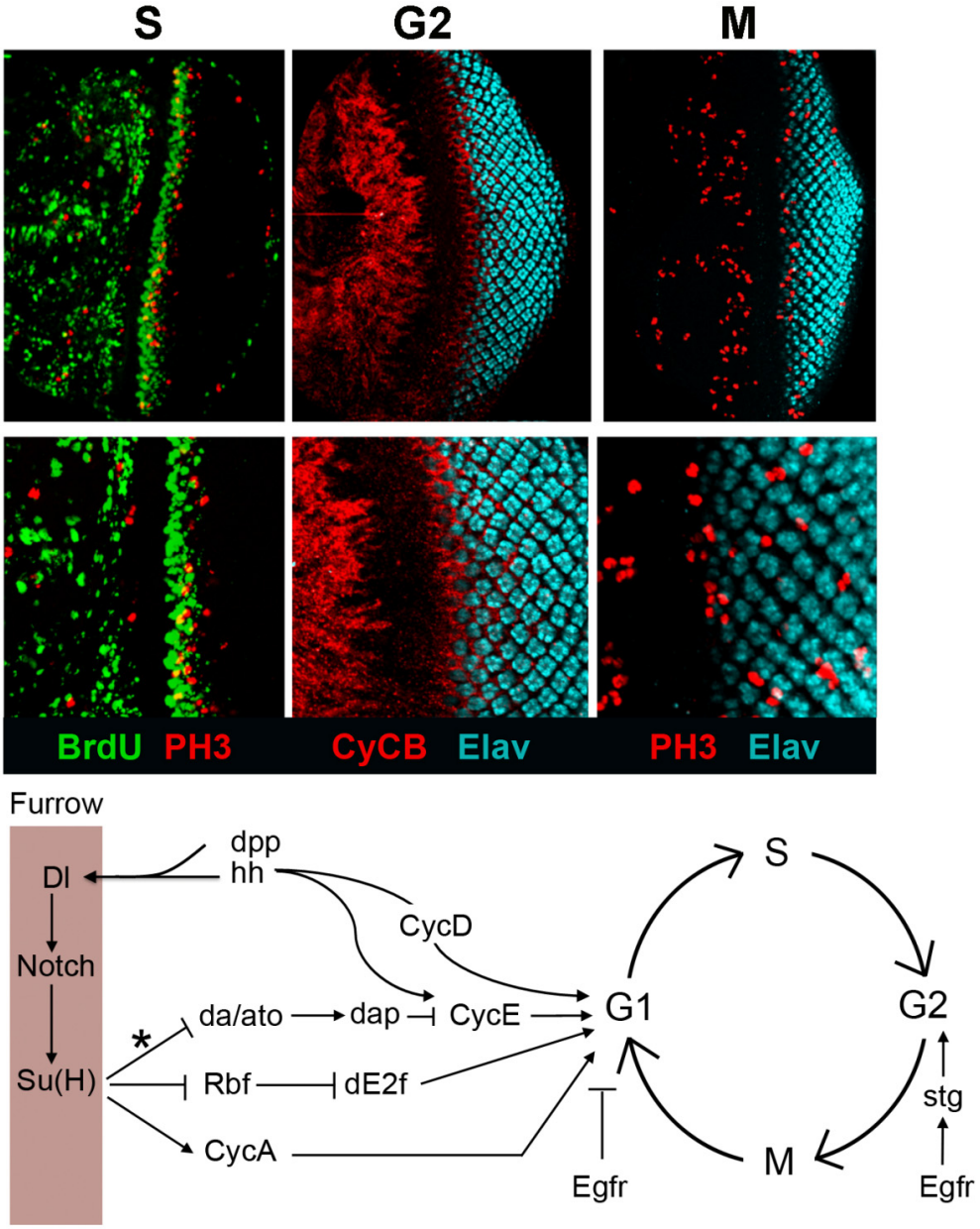

Figure 3. Second Mitotic wave regulation. Third instar eye imaginal discs, showing the different phases of the cell cycle. BrdU incorporation (green) marks S phase, CycB staining (red) indicates G2 phase and staining with antibodies against PH3 (red) marks mitosis. Photoreceptors are staining with Elav (blue). In the morphogenetic furrow cells are arrested in G1, and are therefore devoid of mitotic cells expressing PH3; posterior to the furrow, the SMW is marked by a narrow band of mitotic cells. Model of SMW regulation by different signals: Delta/Notch signalling, controls the onset of S-phase in the SMW by regulating the activity of dE2F, and the expression of CycA. Su(H) (asterisk) independently of Notch blocks the activity of Dap for promoting CycE function. The later G2/M checkpoint in the SMW is overcome by EGFR signalling that triggers the expression of Stg. Model modified from [72].

Different studies report that there is a specific Notch requirement for cells to enter S-phase in the SMW [52,72,73,74]. These studies have shown that clones of cells containing loss of function alleles of *Notch, Dl* or *Su(H)* do not enter S-phase and consequently fail to incorporate Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) or express *Cyclin-B* (*CycB*) in the region posterior to the MF [72,73]. Consistently, the ectopic activation of Notch signalling or the over-expression of an activated form of *Su(H)* in cells posterior to the MF expands the zone of BrdU incorporation in the SMW, and increases the number of mitotic cells posterior to the MF [52,72,73]. This cell-autonomous effect of Notch is independent of the function of this signalling pathway inducing the proliferative signals associated with the organizer [52]. The function of Notch signalling promoting S phase entry in the SMW depends on at least two processes (Figure 3). Firstly, the de-repression of the inhibition of dE2F1 by Retinoblastoma factor (Rbf). Secondly, the activation of the expression of *Cyclin-A* (*CycA*) by Notch signalling [72]. However, the co-over-expression of *CycA* and *dE2F1* is not sufficient to rescue S-phase in *Notch* mutant cells, implying the existence of, at least, one other target gene by which Notch triggers the onset of S-phase [72]. An obvious candidate for being a Notch effector in this transition is *CycE*, which is a primary trigger of S-phase [75]. However, it has been suggested that Notch signalling might be controlling this transition independently of this cyclin, since high levels of CycE are observed in *Notch* mutant cells [72,73]. CycE functions through regulating the activity of its partner Cdk2. Thus, the level of expression of *CycE* does not always correlate with the activity of the complex formed by CycE/Cdk2 kinase, since the function or expression of Cdk2 can be modified independently of CycE. In fact *Cdk2* mutant cells accumulate high levels of CycE but fail to enter S phase in the SMW [74]. In addition, CycE/Cdk2 kinase activity can be inhibited by the p21/p27 family of Cdk inhibitor, Dacapo (Dap). The over-expression of *dap* blocks the S phase transition, though mutant cells accumulate high levels of CycE protein [76,77]. It has been reported that *Su(H)* mutant cells are arrested in G1 in the SMW, in part because *dap* is up-regulated. Thus, G1/S progression in *Su(H)* mutant cells can be induced by expressing *CycE* together with *Cdk2* or eliminating *dap* in *Su(H)* mutant cells [74]. The up-regulation of *dap* in *Su(H)* mutant cells is dependent upon the basic helix-loop-helix (bHLH) proneural protein Daughterless (Da) [74]. Surprisingly, in clones of *Notch* mutant cells the levels of Dap are reduced [72,73], suggesting that Dap accumulation is not contributing to the G1 arrest in these mutant cells. These observations suggest that the mechanisms involved in the retention of *Notch* and *Su(H)* mutant cells in G1 in the SMW might be different (Figure 3)[74]. This possible mechanism should be further analyzed, since it might be a novel regulatory process, through which Su(H) could regulate cell proliferation.

The movement of the MF depends on the secreted proteins Hh and Decapentaplegic (Dpp). Hh is secreted from differentiating cells behind the MF and induces the expression of *Dpp*, which then also participates in furrow propagation [78-82]. Posterior-to-anterior spread of both proteins (Hh and Dpp) is also required to arrest cells in G1 ahead of the MF, and to activate the expression of *Dl* in an uniform band of cells [62,68,72,73,82]. As cells emerge from the MF, Dl activates to the Notch receptor to promote G1/S progression in all cells, although only uncommitted cells enter S-phase in the SMW, since Epidermal growth factor receptor (EGFR) activity blocks the cell cycle progression in G1 in the cells that are part of the precluster [72,73,83].

4.2. Hh and Notch signalling in the control of cell proliferation in the SMW

Hh signalling plays a fundamental role in initiating and coordinating the onset of S-phase in the SMW. This signalling pathway activates several branches to promote G1/S transition during this coordinated division. On one hand, it promotes transcription of *CycE* and *CycD* [84], principal regulators of the S-phase onset, and on the other hand, together with Dpp, leads to the expression of *Dl* in the furrow (Figure 3) [72,73]. In addition to this function, Hh signalling is also required to regulate the expression of the basic helix-loop-helix (bHLH) protein Atonal (Ato) [80,85,86]. This transcription factor is essential for the differentiation of the photoreceptors [87]. Interestingly, Bossuyt and co-workers [88] have identified an anti-oncogenic function for *ato* during eye development. These authors report that the loss of *ato* strongly enhances the formation and progression of tumors generated when Notch is activated in ‘‘sensitized’’ flies, whereas, ectopic expression of *ato* strongly inhibits tumor formation in these flies [88]. This suggests that tumor formation might require loss of the capacity of the cells to induce cell fate commitment and differentiation.

ato is first expressed in a band of cells in front of the MF in response to Hh signalling. In the MF, this stripe of cells is refined into clusters of cells expressing high levels of *ato* (intermediate groups). Finally, from these groups the expression of *ato* is restricted to one single cell in each cluster, which will become the R8 photoreceptor [47]. The function of Notch is necessary to restrict the expression of *ato* to only one cell in each group. The Hh pathway is required to repress *ato* expression between the nascent proneural clusters [86]. Thus, both signalling pathways coordinately define the final pattern of expression of *ato* [47]. Interestingly, this repressive function is mediated by the bHLH protein Da (Figure 4). This factor is dynamically regulated in the furrow by several mechanisms, including Hh, and Notch signalling pathways. In the region posterior to the MF, Notch signalling up-regulates the expression of *da* in some cells to repress the expression of *ato,* to define the R8 pattern (Figure 4) [89]. Interestingly, the regulation of the expression of the cyclin

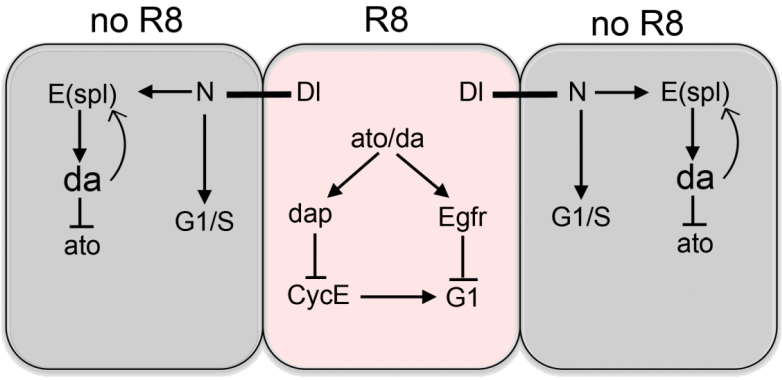

Figure 4. Schematic representation of the genetic interactions between *Notch*/*atonal*/*da* in the R8 equivalence group. The function of Notch signalling regulating cell proliferation in the equivalence group is tightly linked with its function controlling the differentiation of the photoreceptors. In the cells of the equivalence group that do not differentiate as R8s, Notch signalling is involved in the down-regulation of Atonal at the same time as it promotes G1/S progression.

inhibitor *dap* in the SMW depends on Da [74,90]. All these data suggest a tight link between the role of Notch signalling regulating proliferation in the SMW and its function controlling differentiation.

5. Autonomous requirements of Notch signalling in the regulation of cell proliferation in the ventral region of the eye discs

The cell autonomous effect of Notch signalling regulating cell proliferation seems to be restricted to cells posterior to the MF, as cell proliferation ahead of the furrow is not detectably impaired by loss of *Notch*. The fact that clones of *Notch* and *Dl* mutant cells can be generated implies that they are not required for the earlier, unpatterned proliferation in the region anterior to the MF [52,72]. However, different reports have shown that the growth of the ventral region of the eye discs depends on the function of ventral genes like *Lobe* (*L*) and its downstream target *Ser*. The depletion of the function of these genes in the whole eye disc result in selective growth defects in the ventral half of the eye [38,91]. In addition, other reports suggest that ventral *Notch* clones show disrupted *upd* transcription. These results argue that there is Notch activation not only at the D/V-midline but also in ventral tissue, and that Notch activity in ventral tissue is necessary for *upd* expression [52]. Surprisingly, *Ser* loss-of-function clones do not exhibit any phenotypes in the eye [41,92,93]. It has been suggested that Ser might function as a diffusible factor, or there may be other functionally redundant *Ser*-like genes. The function of Ser and Notch in this context is not clear, further analysis is necessary to clarify the role of these factors in the control of cell proliferation in the ventral region of the eye disc.

6. Notch pathway activation in the wing disc

The dynamic pattern of Notch pathway activation, as observed by the transcriptional response of its targets like *E(spl)mβ* or the synthetic Notch Responding Element (NRE) [94,95,96] reflects the different Notch requirements during wing development. As previously described for the eye disc, as development progresses the wing discs is subdivided into different compartments. In early second larval stage the discs is subdivided into two compartments, Dorsal and Ventral. This division depends on the function of the gene *apterous (ap)*, which is expressed specifically in dorsal cells [97-101]. Some aspects of the definition of the D/V border are conserved in the developing eye and the wing, although the precise activating mechanisms differ. One important difference between both discs is that in the eye discs, compared to the wing disc, the D/V axis is inverted. Thus, the restricted expression of *ap* in the dorsal region activates the dorsal-specific expression of *Ser* and *fng*. As in the eye discs, Fng inhibits Notch activation by Ser, but in this case in the dorsal compartment, restricting Ser to signal to ventral cells [102-107]. Contrary to the negative function of Fng in Notch-Ser interaction, Fng binds to Notch and promote Notch-Dl interaction, therefore restricting Notch activation at the D/V border [44,106-110]. As previously described in the eye discs, the D/V boundary in the wing discs also functions as an organizing "center" influencing global growth and patterning of the wing [97,101]. The growth response induced by Notch at the D/V boundary of the wing disc is, at least partially, mediated through the expression of target genes such as *wg*, *Cut* and *vestigial* (*vg*) [104,108,109,111-114]. Wg promotes wing development at least in part, by feeding an auto-regulatory loop of *vg*, which is required for wing specification and growth [111,115]. Therefore in the absence of Notch activity at the D/V boundary, the wing does not grow due to a failure of wing

cell fate specification [96,109,111,112,114,116,117,118]. In the wing disc, it has been proposed that the effects of Notch signalling on cell proliferation is not just a consequence of the activation of these target genes [96,117,119,120,121] (see below). In accordance to this more general role of Notch controlling wing growth, mutant clones for *Notch* do not proliferate normally in the wing, even when they are far away from the D/V border, suggesting that Notch is required for the proliferation of the whole wing primordia [122].

The effects of Notch in control of the cell cycle and proliferation are context-dependent as it occurs in the eye discs. For example at the D/V boundary Notch has a negative impact on cell proliferation defining the so-called Zone of Non-Proliferation Cells (ZNCs), while in other regions Notch can influence growth autonomously and non-autonomously (see below). Therefore Notch pathway can have a very different outcome on cell proliferation depending on the time and spatial location of its activation, and its synergy with other signalling pathways.

7. Early Notch induced cell proliferation promotes territorial subdivision of wing disc

An important developmental decision that occurs during wing discs development, apart from the A/P and D/V compartmentalization, is the specification of the wing pouch and the body wall fates [123,124,125]. One of the earliest roles of Notch during wing disc development is to separate the mutually antagonistic effects of two signalling pathways, EGFR and Wg that are required for body wall and wing specification, respectively [96,123,125]. In the early wing disc primordium, Vein the ligand of the EGFR pathway, is expressed in dorsal body wall cells, and restricts the expression of Wg to the distal domain of the disc, delimiting the region of the disc that is specified as wing cell fate (Figure 1) [126,125,127]. Growth induced by Notch activity pull apart the mutually repressive effects of the EGFR and Wg pathways leading to wing cell fate specification [96]. Interestingly, this effect of Notch in early wing disc growth, and wing development can be rescue by the expression of the cell cycle regulator *CycE* (G1/S) or the universal G2/M regulator *string* (*stg*, the *cdc25* homologue) in conditions of reduced *Notch* function [96]. Therefore Notch seems to control wing disc growth modulating the expression of these cell cycle regulators. This early role of Notch precedes the function of this pathway inducing the D/V organizer centre. It is remarkable the similarities that exist between this process and that observed during the definition of eye primordium (see above) (Figure 1). This suggests that likely the mechanisms involved in the regulation of both processes are conserved between both discs.

8. Autonomous control of cell proliferation by Notch and the zone of non-proliferation cells

Proliferation of the wing disc proceeds randomly at a rapid rate that slowly decays at the end of third instar stage. Just before pupal formation, the cells of the discs are arrested in the G2 phase of the cell cycle. However, a band of cells at the D/V boundary are arrested in G1 around 30h before the rest of the cells of the discs stop proliferate. This effect is as a consequence of a regulatory program involved in sensory organ formation, and defines the ZNCs [128,129]. The pattern of cell proliferation at these stages correlates with the pattern of activity of the mutually exclusive Notch and Wg signalling pathways. The activation of Notch at the D/V boundary activates *wg* that signals to non-boundary cells, while boundary cells are refractory to Wg-signalling through *Cut* repression [130,131]. At the D/V boundary Notch activity blocks cells in G1 by repressing the G1/S regulators *bantam*

microRNA and *dmyc*, both factors are necessary to activate the dE2F1 transcription factor. In non-boundary cells, which are actively proliferating, the response to Notch is reduced by the activity of Wg alleviating the block in G1 (Figure 5) [131,130]. Interestingly, cells that are unable to respond to Notch and Wg signalling are still able to proliferate [131]. It has been suggested that in anterior cells, Wg induces a G2 arrest in Dorsal and Ventral cells close to the D/V boundary by inducing the expression of the bHLH proneural transcription factors *achaete* (*ac*) and *scute* (*sc*) leading to the repression of *stg* [129]. However, in at recent report [132] it has been proposed that the capacity of Sc to repress *stg* expression in the ZNC could be mediated by Da. Da functions as a transcriptional repressor of *stg*, thereby regulating G2/M transition. The over-expression of *sc* in the wing discs induces high levels of Da that in turn block the expression of *stg* [132]. These data suggest that, as occurs during the SMW, the function of Notch signalling regulating cell proliferation in the wing margin is tightly linked with its function controlling the differentiation of the sensory organs that appear in this region. The bHLH family of transcription factors plays an important role mediating the function of Notch signalling in the regulation of these processes [133]. This family of proteins coordinate cell cycle control, cell lineage commitment and cell differentiation [134].

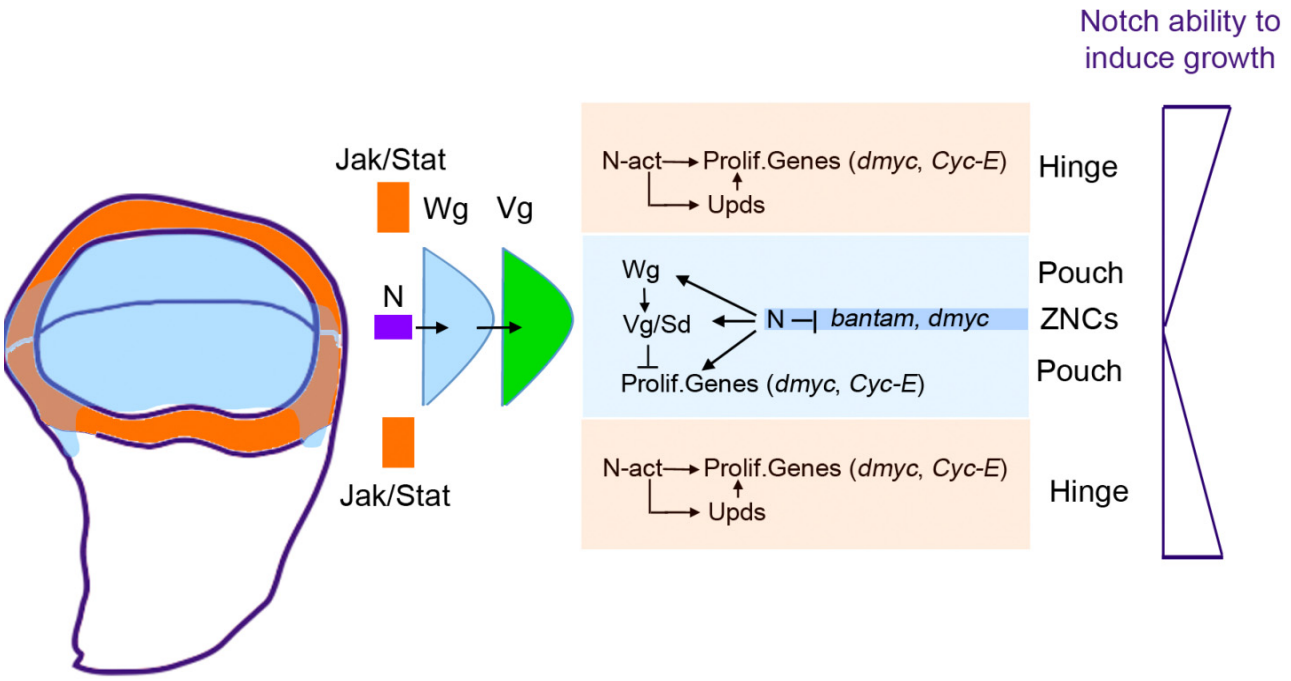

Figure 5. Role of Notch in autonomous and non-autonomous control of cell proliferation in the wing imaginal disc. Schematic representation of a wing imaginal disc with the activity of Notch pathway in purple at the D/V boundary, Wg (blue) and Vg (green) in the pouch and Jak/Stat activation pathway in orange. At the zone of non-proliferation cells, Notch blocks cell proliferation autonomously though the repression of the *bantam* microRNA and *dmyc*. At the pouch, Notch's ability to induce the expression of *dmyc* and *CycE* is repressed by Vg in a feed-forward repression mechanisms. In the proximal pouch and the hinge, Notch cooperates with the Jak/Stat pathway to promote cell proliferation.

Interestingly, it has been reported that most of Notch functions during wing disc development seem to be mediated by this family of transcription factors. Thus, the bHLH proteins encoding by *E(spl)-C* and *deadpan (dpn*) are required at the wing margin for *wg* and *Cut* expression while repress *dmyc* and therefore G1/S transtition. Moreover, these factors also mediate the function of Notch signalling regulating cell proliferation in the rest of the wing pouch, and in the hinge region [135,136].

9. Notch autonomous and non-autonomous control of cell proliferation

The analysis of the effects caused by the ectopic activation of Notch during wing disc proliferation reflects the context-dependent nature of Notch signalling. Thus, clones of cells expressing, either the intracellular domain of *Notch (NICD)*, that function as a constitutively activated form of the receptor, or the ligand *Dl,* stimulate cell proliferation [119,121,120]. In the distal region of the wing pouch, these clones induce non-autonomous over-growth, as result of the induction of ectopic D/V boundaries. However the size of these clones is smaller than control clones. This later effect is likely caused by the function of Notch in the specification of the ZNC [119,120,121,129,131,135]. Clones located in the proximal region of the wing and in the wing hinge, induce large over-growths, both autonomously and non-autonomously. The proliferative effects of these clones are not caused by the generation of ectopic D/V boundaries [119,121].

The non-autonomous effects on cell proliferation produced by Notch signalling from the D/V boundary, or when this pathway is ectopically expressed in the hinge, likely depend on a secreted relay factor/s. The obvious candidates to be mediating these non-autonomous effects are the Notch target genes *wg* and *vg,* which are expressed at the D/V boundary [102,104,105,108,109,137]. However, different results suggest that only part of the proliferative effects induced by Notch can be attributed to *wg* expression. Thus, *wg* is not ectopically expressed in all proximal clones, and in the absence of Wg function, Notch activation can induce proliferation in some proximal clones and in the hinge [121]. In addition, the ectopic expression of *wg* is not sufficient to reproduce the effects caused when Notch signalling is activated [117,119,121,138]. Wg's role in controlling cell proliferation is controversial and has been extensively discussed elsewhere [117,119,121,138,139,140].

All the complex effects on wing disc growth mediated by Notch are indicative of the context dependence that this pathway has regulating cell proliferation. To solve this problem Djiane and co-workers [136] have used a genome-wide approach to characterize the repertoire of genes directly activated by Notch that could be mediating Notch's effects on tissue growth. Among the genes identified in this analysis they have found: cell proliferation regulators (*CycE* and *stg*), cell signalling molecules (*wg* and *upd*), and wing growth regulators (*sd* and *vg*). RNAi knockdown of most of the genes identified in this study suppressed to some extent the growth defects caused by Notch pathway activation. The authors defined 4 different classes of target genes. The first was a largely cell-autonomous response genes (e.g. *wg*). The second group comprised genes that respond to Notch activation both autonomously and non-autonomously (e.g. *sd* and *fj*). Interestingly *fj* has been also identified as a Notch target gene during eye development (see above). The third group includes genes that present a complex response to Notch signalling activation. Thus, these genes are not autonomously expressed in all the cells where Notch is active in the wing pouch, whereas are both autonomously and non-autonomously up-regulated in the hinge and pleural regions (e.g. *CycE*). Finally, group four is constituted by genes that are up-regulated in broad domains at the hinge and pleural regions tips, but are not responsive to Notch in the wing pouch (e.g. *dmyc*). These data

implies the existence of mechanisms to limit the capacity of Notch signalling to up-regulate the expression of the target genes of group 3 and 4 in central wing pouch. One of the factors that is mediating this effect is the TAE transcriptional factor Scalloped (Sd) [141]. This gene is activated by Notch, and it has been classified in group 2 [136]. Sd forms a complex with the nuclear protein Vg [141,142]. This complex is required for wing specification and growth [118]. Sd is also one of the binding partners of Yorkie, the transcriptional effector of the Hippo pathway that regulates tissue growth and cell survival (reviewed [143]). Therefore, changes in the balance between the amounts of Sd/Vg/Yki proteins might influence the target genes activated, and the proliferative response of wing cells. In this way, Notch pathway activation or down-regulation has a profound impact in the expression of *expanded* (*ex*), one of the best-characterized Yki targets [144,145]. Notch activation represses *ex* expression just in the wing pouch while Notch down-regulation de-represses it in the D/V margin where it is not expressed [144]. According to this feed-forward repression model, Notch dependent activation of *vg*, induces the formation of the Sd/Vg repressive complex in the pouch preventing the activation of Yorkie targets such as *ex* and the cell cycle regulators *dmyc* and *CycE* or the apoptosis regulator *diap1* (Figure 5) [136].

Similarly, the proteins of the E(spl)-C, well known targets of Notch, are also involved in a negative feed-forward onto other Notch targets like *dmyc,* restricting the capacity of Notch signalling to activate gene expression to particular regions of wing pouch [136]. However, other Notch targets like *ex* or *diap1* are not regulated by the E(spl)-C, suggesting that different targets depend on different negative feed-forward mechanisms [136,144].

The ectopic activation of Notch in the hinge and pleural regions promotes non-autonomous cell proliferation and activation of *dmyc*, *CycE* or *diap1* [119,120,121,131,135]. In this region the activation of Notch induces high levels of Jak/Stat pathway activity [136,146]. A feed-forward mechanism between the Notch and Jak/Stat pathways has been proposed to coordinate the non-autonomous growth at the periphery of the pouch. This process depends on the secreted Jak/Stat ligands Upd1, Upd2 and Upd3 that would function as a secreted relay factors. Interestingly, these Jak/Stat ligands are also regulated by Notch. Therefore, a feed-forward mechanism between these two pathways has been proposed to explain the non-autonomous growth and expression of *CycE*, *dmyc* and *diap1* (Figure 5) [136]. However, a functional link between these pathways is still missing.

10. Notch growth regulation in the leg disc

Notch signaling establishes boundaries that separate each leg segment, as it does in the D/V boundary of the wing and in the eye discs. In the leg disc, the Notch pathway is activated in 9 concentric rings that prefigure the leg joints along the Proximo-Distal (PD) axis (Figure 6) [95,147,148]. The expression of the Notch ligands, *Dl* and *Ser*, is also segmentally controlled by the combination of the leg "gap" genes and PD tarsal genes, in proximal cells to the end of each segment [147,149]. The Notch pathway is activated in a row of cells adjacent and distal to *Dl* and *Ser* expression domains at the presumptive joints. Apart for being completely required for the formation of all joints, Notch also controls the growth of the leg in a non-autonomous manner. Mutant cells for components of the Notch pathway that span two segments are usually associated with joint and growth defects [95,147,148]. Interestingly, the leg size reduction involved mutant and non-mutant tissue. Moreover, clones that locally activate the pathway induced leg overgrowths composed of mutant and

wild type tissue, suggesting that as in the wing and eye discs, Notch control leg growth in an autonomous and non-autonomous manner [95,147,148]. It has been proposed that Notch pathway activation at the joint induces an unidentified signal that acts non-autonomously to promote cell survival and growth [150]. However it is still unknown if in the leg disc, Notch controls cell proliferation, cell shape changes or oriented cell division that could be the cause of the growth defects observed in *Notch* mutant legs.

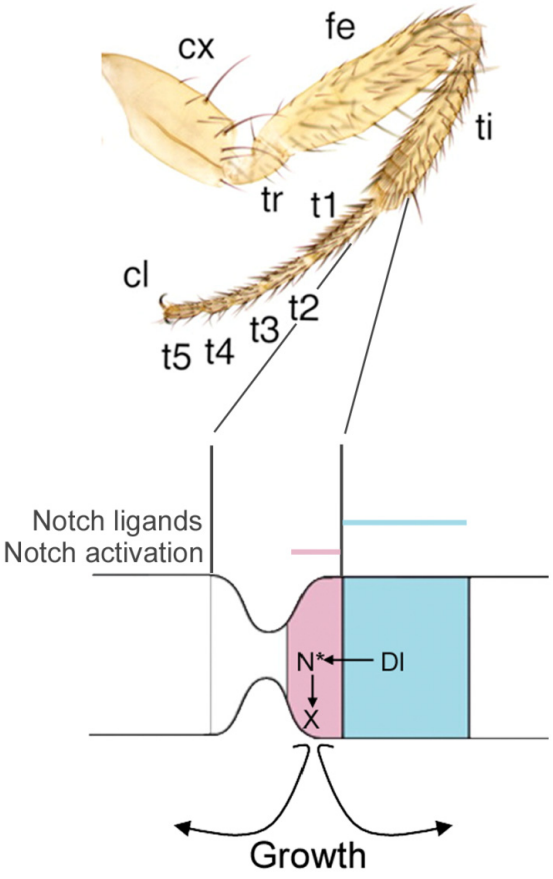

Figure 6. Leg growth control by Notch: *Drosophila* leg with the 10 segments separated by the joints. The cartoon represents a joint where the proximal expression of the Notch ligand (Dl) activate the Notch pathway (N*) in the joint and controls the growth of the adjacent segments.

11. Notch and its relationship with epigenetic modifiers

The combination of different *ex vivo* and *in vivo* RNAi screenings [151], as well as genome-wide [136] approaches have identified distinct epigenetic factors that are functionally related to Notch signalling. Interestingly the alteration of the function of some of these factors synergize with the gain of Notch signalling to foment hyperplastic and neoplastic growth. One example is the BTB/POZ containing transcriptional repressors *longitudinals lacking* (*lola*) and *pipsqueak* (*psq*). It has been shown that these proteins cooperate with Notch in the formation of tumors and invasion after *Dl* over-expression in the wing and eye imaginal disc [152]. Both *lola* and

psq function as epigenetic silencers that together with co-expression with *Dl* shut down the expression of *Rbf*, a tumor suppressor gene that negatively regulates *dE2F1*, promoting metastatic tumors [152]. The importance of epigenetic regulation in the control of the function of Notch signalling, and its relation with tumor formation has been recently reviewed [10].

12. Effects of loss of apico-basal polarity in Notch signaling

In *Drosophila*, Scribble (Scrib), Discs large (Dlg) and Lethal giant larvae (Lgl), cooperatively establish, and maintain apico-basal cell polarity. Loss-of-function mutations in these genes result in neoplastic overgrowth, along with a loss of apico-basal cell polarity and differentiation. Though *scrib* mutant clones shown ectopic expression of *CycE,* and excessive cell proliferation, they do not become overgrown because they are eliminated by Jun N-terminal kinase (JNK)-dependent apoptosis [153]. However, the ectopic activation of Notch in these clones induces unrestrained tissue overgrowth [153-158]. The mechanistic details about how Notch promotes this effect is unknown, however it has been proposed that the activation of Notch in *scrib* mutant cells blocks differentiation and apoptosis, and also promotes JNK-mediated tumor overgrowth and invasion. aPKC signalling is also required to promote tumor overgrowth when Notch is ectopically expressed in *scrib* mutant clones, through either increased cell proliferation or cell survival [158].

It is not clear what the relationship is between cell polarity and tissue growth. Different publications have suggested that at least the apico-basal polarity determinants Lgl/aPKC and Crb can establish apico-basal cell polarity, and control tissue growth, independently [159-162]. The effects on proliferation and survival of these factors are via the Salvador/Warts/Hippo (SWH) tumor suppressor pathway [160,161,162]. Therefore, Notch signalling might be cooperating with the Hippo pathway to promote tumor growth in mutant cells rather than loss of apico-basal cell polarity. In addition, it has been recently published that Lgl regulates endocytosis to restrict vesicle acidification and prevent ectopic ligand-dependent Notch signaling [28]. Thus, the disruption of apico-basal cell polarity can affect different processes, and compromise the activity of multiple signalling pathways, that eventually can cooperate with Notch signalling to promote tumor formation.

13. Notch and tumor formation

Although it is well defined that Notch 1 is an oncogene in human T lymphoblastic leukemias/lymphomas (T-ALL), there is little evidence to support a causative role for Notch in the initiation of tumorigenesis in human solid cancers [5-8]. Considering the clear relationship between proliferative events and Notch activation, it has been proposed that the hyper-proliferative states induced by Notch, frequently in synergy with other signals, can eventually lead to bona fide oncogenic events, even though Notch activation does not lead to cancer *per se* [5]. Therefore, the identification of the mechanisms by which Notch signalling control cell proliferation during normal development can help us to better understand how alterations in the activity of Notch signalling may lead to oncogenic events in different pathological conditions. The analysis of the function of Notch signalling during the development of *Drosophila* has been fundamental for the identification of such mechanisms. Interestingly the development of some tumors in humans seems to be initiated by mechanisms similar to those by which Notch signalling induces normal growth. For example, the function of Notch regulating the activity of different cell cycle regulators and cyclins, seems to be

involved in the initiation of some cancers. Thus, increased levels of the Notch ligand JAG1, which commonly occur in breast cancers, promotes cell cycle progression by inducing *CycD1* through Notch signalling [163]. Moreover, in mice homozygous-null for *Ccnd3* (*CycD*), the ectopic expression of *Notch* failed to induce T-ALL, suggesting a fundamental role for D type cyclins in Notch-mediated transformation [8,164]. Notch signalling also drives cell cycle entry by transcriptionally activating *myc*, that is a direct target of Notch and contributes to cell cycle progression in T-ALL [8,165,166], as well as in Notch-induced mouse mammary tumors [167]. Moreover, Notch can promote cell cycle progression repressing the transcription of CDKIs *p27* and *p57* through HES1 in different cell types [8]. In T-ALL, Notch directs the transcription of the E3 ubiquitin ligase *S phase kinase-associated protein 2* (*SKP2*), which leads to decreased p27 protein levels and increased cell proliferation [168].

Potential involvement of Notch signalling in other human cancers is frequently associated with the cooperative action between Notch and other signalling pathways (Wg, Jak/Stat). As we have described in this review, these pathways are functionally related to Notch signalling in the control of cell proliferation during normal development [6,8].

14. Conclusions

There are many precedents to demonstrate the utility of the development of the imaginal discs of Drosophila as a model to reveal the mechanistic details of how Notch signalling controls cell proliferation. The definition of these mechanisms is fundamental to establish how perturbations in the activity of Notch signalling can lead to an excess of cell proliferation, the primary caused of cancer. The studies in Drosophila have shown that Notch can regulate the activity and/or function of different factors that control the cell cycle. Interestingly the function of some of these cell cycle regulators have been found that to be altered in human cancer associated with disruption of Notch signalling. In addition the function of Notch in synergy with other signalling pathways can regulate different factors involved in the control of cell proliferation.

An important aspect of the function of Notch regulating cell proliferation is its context-dependent nature. Different studies in Drosophila have revealed that this effect is at least partially caused by different feed-forward mechanism between Notch signalling and other genes and signalling pathways. Therefore the final outcome of the activity of Notch signalling in the regulation of cell proliferation depends on the interaction between this pathway and different genes, and signals.

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Conflict of Interest

The authors declare no conflict of interest for this work.

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