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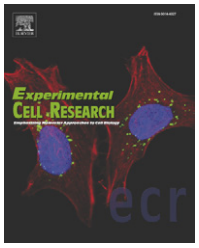

Review Article

Notch signaling in intestinal homeostasis across species: the cases of Drosophila, Zebrafish and the mouse

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ABSTRACT

Notch signaling has been recently shown to have a fundamental role in stem cell maintenance and control of proper homeostasis in the intestine of different species. Here, we briefly review the current literature on Notch signals in the intestine of Drosophila, Zebrafish and the mouse, and try to highlight conserved and divergent Notch functions across species. Notch signals show a remarkably conserved role in skewing cell fate choices in intestinal lineages throughout evolution. Genetic analysis demonstrates that loss of Notch signaling invariably leads to increased numbers of secretory cells and loss of enterocytes, while gain of Notch function will completely block secretory cell differentiation. Finally, we discuss the potential contribution of Notch signaling to the initiation of colorectal cancer by controlling the maintenance of the undifferentiated state of intestinal neoplastic cells and speculate on the therapeutic consequences of affecting cancer stem cells.

Contents

Introduction

Notch signaling plays a fundamental and evolutionarily conserved role in metazoan development. Extensive molecular and genetic analyses, based primarily on invertebrate studies, established that the general developmental role of Notch signals is to link cell fate choices of one cell to those of a cellular neighbor. Notch signals are highly pleiotropic, dictating cellular fates in a way that depends on

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cellular context. Thus, Notch activity can influence differentiation, proliferation or apoptotic events in a wide range of tissues depending on the presence of other cellular elements and the ability of Notch signals to integrate with other signaling pathways [1,2]. The fundamental nature of Notch signals in metazoan development is reflected by the fact that aberrant Notch signaling invariably leads to mutant phenotypes in every system examined and in humans it underlies a growing spectrum of pathologies [3–7], including cancer [8–13].

In this short review, we focus on the role of Notch signals in the maintenance of proper homeostasis in the intestine of different species: Drosophila, Zebrafish and the mouse.

Notch signals in the fly

The adult intestine of *Drosophila melanogaster* has been recently exploited as a simple model system to study intestinal biology (see accompanying review in this issue for more details). Though lacking crypt-villus structures, the fly intestinal epithelial cells use a brush border luminal surface to perform absorptive functions. Like its mammalian counterpart, the fly intestine hosts a large number of multipotent stem cells, which self-renew and continuously replenish the epithelium. Stem cells have been identified in various regions of the fly digestive tract, but those of the posterior midgut are, to date, the best characterized and will be further discussed here as "intestinal stem cells". In contrast to vertebrates, though, fly intestinal stem cells appear to produce only two types of terminally differentiated cells, enteroendocrine cells and enterocytes [14,15]. The lumen of the fly intestine is lined by a chitinous peritrophic membrane, acting as a barrier that may perform some of the physiological activities of goblet and paneth cells. Enteroendocrine cells are also heterogeneous in their expression of peptide hormones and therefore may have some degree of specialization [14,15]. The niche-like function of paneth cells may be filled by the enterocytes and surrounding muscles cell, both of which secrete mitogens important to control stem cell proliferation ([16–19]; see also review in this issue). Unlike the mouse intestine, the Drosophila midgut does not contain a transit-amplifying population of progenitors, but rather the stem cells give rise to intermediate progenitor cells, enteroblasts, that will terminally differentiate into one of the two differentiated cell types, the secretory or absorptive cells without further divisions.

Compared to its mammalian counterpart, the Drosophila midgut represents a greatly simplified model system in terms of cell types and lineages with which to investigate how Notch signaling mediates cell fate decisions and cell fate specification. However, morphological and histological markers of specific cell types are less well defined in the fly and the identification of different cells relies on a combination of epitope (including the Notch ligand Delta) and enhancer trap markers, lineage tracing techniques, and mitotic cell state. Enteroendocrine cells are post-mitotic secretory cells; they express the transcription factor Prospero, and often produce peptide hormones such as tachykinin and allatostatin. The enterocytes express the transcription factor Pdm1 and undergo endoreplication of their DNA (a common feature of many post-mitotic fly cells); consequently, these cells become very large and present a characteristic big nucleus, they have an actin-rich apical brush border that contributes to the majority of the luminal surface.

The Notch signaling pathway controls cell fate decisions of the intestinal stem cell and its daughter cells. Both the Notch ligand Delta and the Notch receptor are expressed in the stem cells and are present in both daughter cells immediately after stem cell division. Delta expression is then lost in the differentiating daughter cell, while Notch expression is maintained in the EB and young differentiating enterocytes, but not in mature enterocytes or enteroendocrine cells [15,20]. Delta sends a signal from one daughter cell to activate the Notch transcriptional response in its adjacent sister cell, thereby promoting its commitment to differentiation, while the signal sending cell does not activate Notch and retains stem cell identity [14,15,20]. The exact mechanisms allowing activation of Notch in one cell and preventing activation in the other cell are not fully understood. Transcriptional repression of Notch target genes is mediated by the Hairless/Suppressor of Hairless repression complex and by chromatin modifications via the histone ubiquitin protease Scrawny, which helps maintaining stem cells by preventing Notch target gene activation. Loss of either *Hairless* or *Scrawny* leads to a loss of Delta positive stem cells [21,22]. Whether other factors impact on the Notch switch is unclear, though the negative regulator Numb does not influence this decision [21].

Genetic analysis of loss and gain of Notch signaling demonstrates the important role of this pathway in cell fate specification in the intestine. The clonal loss of Notch in adult stem cells results in the aberrant production of cell types. While wild-type stem cells self-renew, keeping their number relatively constant, and produce new terminally differentiated enterocytes and enteroendocrine cells, Notch mutant mitotic clones contain many more stem cell-like cells (Delta+, mitotic cells), many more enteroendocrine cells (Prospero+, allatostatin+), and lack enterocytes (large polyploid, Pdm1+ cells) [14,15] (Fig. 1). The reverse is also true: expression of a constitutively active form of Notch in single stem cells drives their terminal differentiation into large, polyploid enterocytes [14] (Fig. 1). It should be noted, however, that distinction of the cell types in mutant contexts relies on the expression of relatively few known markers, leaving the possibility open that these cells may differ from their wild-type counterparts.

In the fly intestine, the only identified Notch target genes to date are the Hes orthologs, the Enhancer of split-Complex [E(spl)-C] [18,21]. Loss of the *E(spl)-C* leads to an increase in the number of Delta + stem-cell-like cells and to a loss of enteroendocrine cells, without affecting the enterocytes [21]. Interestingly, the number of Prospero + enteroendocrine cells can be restored if Notch signaling is impaired in *E(spl)-C* mutants, suggesting that the *E(spl)-C* genes are not directly required for enteroendocrine fate and that other Notch targets may inhibit this fate. In addition, the presence of enterocytes in the *E(spl)-C* mutants indicates that yet unidentified target genes are required for enterocyte specification. It has also been suggested that the level of Notch signaling may have an impact on cell fate specification [20]; it will therefore be important to identify Notch target genes in the fly intestine and to understand how they may differentially respond to Notch levels.

The E(spl) family of proteins has been shown in many contexts to inhibit the activity of basic helix-loop-helix (bHLH) transcription factors. Of note, during lateral inhibition in the fly, the bHLH proteins are part of a regulatory loop acting both upstream and downstream of Notch signaling: they can directly promote Delta expression but they are repressed downstream of Notch signaling by E(spl) proteins [23,24]. In the fly intestine, loss of the bHLH

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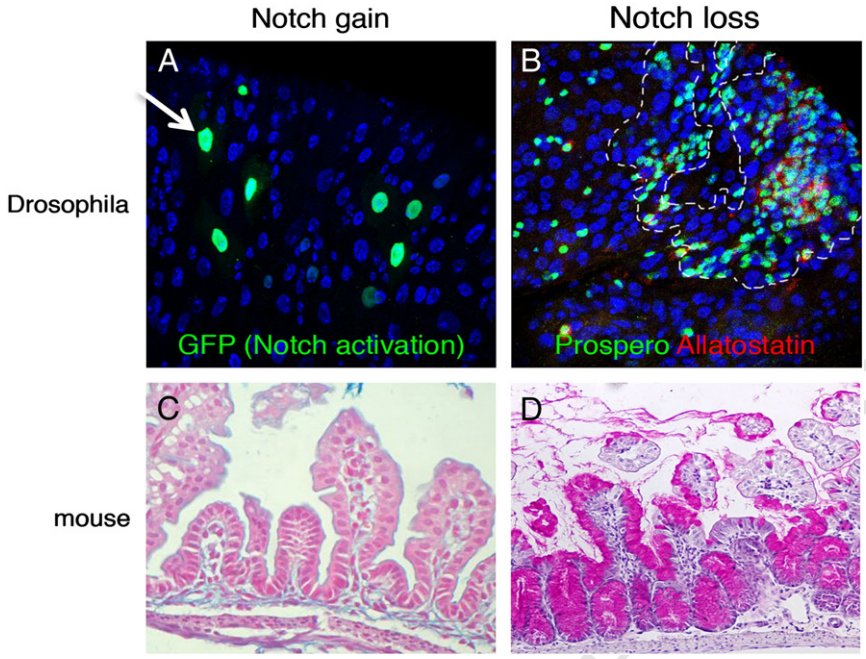

Fig. 1 – Conserved role of Notch signaling in controlling cell fate specification in the intestine. Over expression of activated Notch (green in A) in Drosophila stem cells drives their differentiation into enterocytes (large, polyploid cells, an example of which is indicated by a white arrow). The loss of Notch signaling (illustrated in B in *Delta* mutant tissue outlined in white) causes excess enteroendocrine cells (green Prospero+, a subset of which are also Allatostatin+ in red) and a loss of enterocytes (A.B., Carolina Perdigoto and François Schweisguth, unpublished data). Similarly, ectopic expression of active Notch in the intestinal epithelium (C) completely blocks secretory cell differentiation as shown by the absence of PAS + Goblet cells. On the contrary, loss of Notch signaling achieved by deletion of RBPJ (D) leads to a dramatic increase of PAS + secretory cells (labeled in purple in D).

protein *Daughterless* in stem cells results in their terminal differentiation as enterocytes suggesting that *Daughterless* is required to maintain intestinal stem cell identity. Additional bHLH factors are important for enteroendocrine differentiation as cells lacking the *achaete-scute-complex* fail to produce enteroendocrine cells. Conversely, the overexpression of *scute* is sufficient to produce Prospero + enteroendocrine cells, suggesting that this family of bHLH genes is specifically important for enteroendocrine fate acquisition [21]. The role of bHLH factors for both stem cell maintenance (*Daughterless*) and secretory cell differentiation (*achaete-scute-complex*) may be similar to the mouse where the bHLH homologue Ascl2 (achaete-scute-like 2) is required for intestinal stem cell identity [25], while the bHLH genes Math1 and Ngn3 play specific roles in secretory lineage specification [26–28] (see below). The shared features of Notch signaling in the fly and mouse intestine suggest that the use of the fly as a genetic model system to understand Notch pathway modulation will be fruitful (Fig. 2).

Notch signals in the Zebrafish intestine

As in the fly and mouse systems, Notch signals appear to be required in the developing Zebrafish intestine. Loss of Notch signaling in *mindbomb* homozygous fish leads to increased numbers of secretory cells and a loss of enterocytes, indicating a strikingly conserved role in cell fate specification throughout evolution [29]. Conversely, *bronbones* mutants, which exhibit increased expression of Notch target genes, show defects in goblet cell specification and an increase in proliferative cells; the identity of the proliferating cells is unclear though, due to lack of appropriate markers [30]. The Hes1 homologs *her6* and *her9* appear to be expressed in response to Notch signaling in the fish intestine [30]. The further development and use of Zebrafish as a model system to study intestinal biology will undoubtedly provide valuable insights.

Notch signals in the mouse intestine

The first demonstration of a direct role for Notch signals in controlling the segregation of each mature intestinal lineage from undifferentiated progenitor cells, as well as in the maintenance of the proliferating intestinal cell pool, came from the analysis of transgenic mice carrying either a loss of function [31] or a gain of function [32] Notch pathway mutation. When Notch signaling is inhibited by deletion of the core transcriptional effector of Notch signaling RBPJκ [33], all crypt cells cease to proliferate and differentiate into secretory cells (Fig. 1) [31,34]. Reciprocally, expression of a constitutively active form of the Notch receptor in the intestinal epithelium dramatically impairs cell differentiation and increases the proportion of dividing cells, that extend outside of the crypt proliferative compartment, and are found all along the vertical axis of the villi [32]. These studies revealed two essential functions of Notch in gut homestasis: on one hand, Notch is required to promote stem cell self-renewal in the intestinal crypts,

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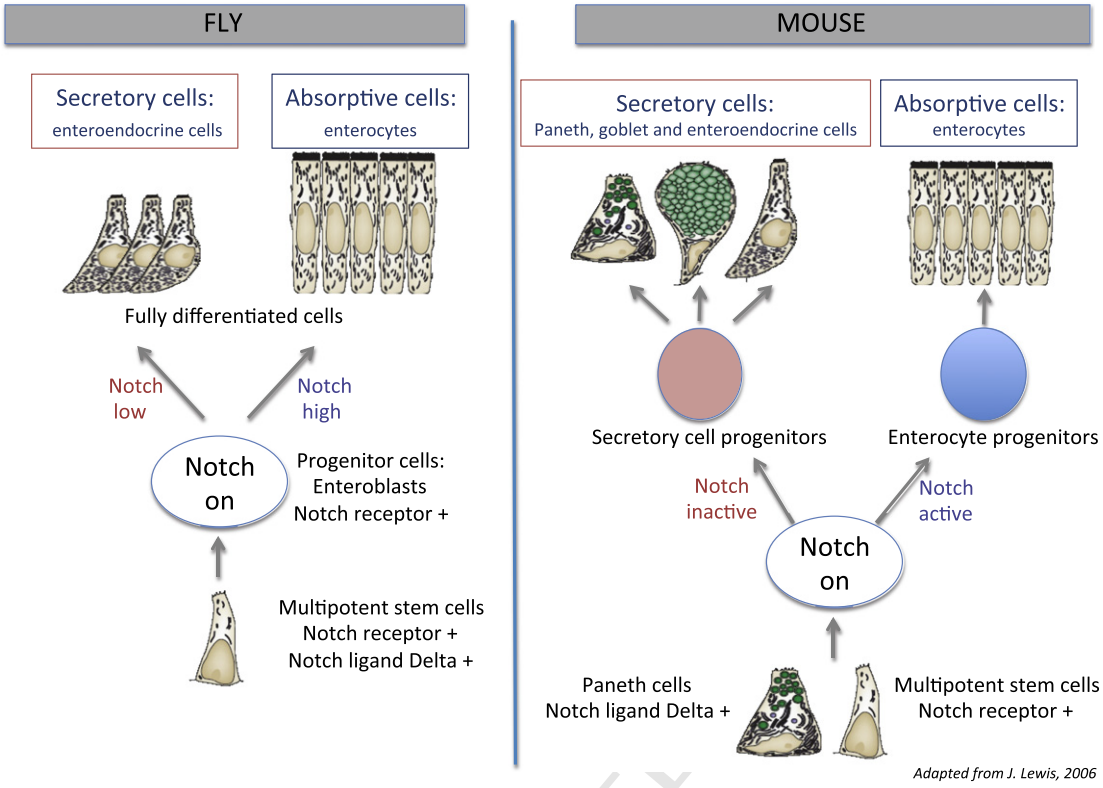

Fig. 2 – Schematic view of intestinal homeostasis in the fly and the mouse. In the fly, multipotent stem cells express both the Notch receptor and the ligand Delta. Notch is active in the enteroblast progenitor. During the differentiation process, low Notch activity gives rise to the secretory lineage (enteroendocrine cells) while high Notch activity leads to the absorptive lineage. In the mouse, the Notch receptor is probably active in stem cells and progenitors, while Paneth cells appear to express the ligand Delta. During cell fate acquisition, Notch signaling blocks the secretory lineage, while it is permissive for enterocyte differentiation.

and on the other Notch signals are dictating the differentiation fate of cells at commitment by a lateral inhibition mechanism [35].

The role of Notch in promoting intestinal proliferation requires Wnt signals, since only cells with intact Wnt signaling are "competent" to respond to the Notch-dependent mitogenic stimulus. On the contrary, Notch controls cell differentiation independently of Wnt signals [36] and through the transcriptional activation of Hes1 that in turns represses the secretory cell determinant Math1. The bHLH transcription factor Math1 is absolutely required to specify intestinal secretory cells [28] and indeed mediates the function of Notch on cell fate specification in the crypt. Genetic evidence that Math1 functions downstream of Notch signaling in allocating intestinal epithelial lineages comes from the study of conditional compound mutant mice that lack intestinal expression of both RBPJκ and Math1. The combined genetic loss of Math1 and RBPJκ completely rescues the intestinal phenotypes associated with loss of RBPJκ and establishes that Math1 is epistatic to Notch signaling in secretory cell specification [34]. Importantly, Math1 activation also leads to increased expression of the Notch ligand Delta-like 1, possibly mediating a negative regulatory loop blocking Notch receptor expression and activation in the same cell, thus allowing the Math1-expressing cell to acquire a secretory fate [37].

Interestingly, γ-secretase inhibitors of Notch activation can both reduce proliferation and induce secretory cells differentiation; however, the simultaneous targeting of Notch1 and Notch2 with an siRNA approach is sufficient to induce markers of goblet cell differentiation, but fails to alter proliferation in colon cancer cells [38]. These results may be related to our findings that cells respond to a Notch-dependent mitogenic stimulus only when the Wnt cascade is intact, while the effect of Notch on cell differentiation is independent of Wnt [36]. Thus, a lack of effect on proliferation by siRNA reduction of Notch1 and Notch2, but modulation of differentiation, could be due to altered Wnt signaling in colon cancer cells. Similar results were reported by Rodilla et al. [39] who showed that expression of the active Notch in cells depleted for Tcf4 are able to regulate differentiation but not proliferation.

Two of the four mammalian Notch receptor paralogues have been found to be involved in mediating a gatekeeper function in the undifferentiated cells of the crypt: Notch1 and Notch2. Through inducible and tissue-specific loss-of-function approaches, Notch1 and Notch2 have been found to have redundant functions in the intestine [40]. On the other hand, Wu et al. recently reported that the specific inhibition of Notch1, but not Notch2, by antagonistic antibodies is sufficient to reveal a partial Notch block phenotype in the intestinal crypt, marked by a decrease of proliferative cells and an increase of secretory goblet cells [41]. The existing evidence therefore suggests an overlapping, but not completely redundant function of the two Notch receptors in the intestinal epithelium.

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Activation of Notch signaling in intestinal stem cells has recently been described using a Notch1 activation-dependent knock-in mouse line, NIP1::CreERT2 [42]. In this report, stem cell labeling has been indirectly assessed, by tracing the progeny of cells in which Notch activation had occurred. Importantly, the authors show that tissue-specific deletion of the two Notch ligands Delta-like 1 and Delta-like 4, as well as of the major Notch effector RBPJκ, leads to loss of stem cell markers (Lgr5, Olfm4, Ascl2), strongly suggesting an essential function for Notch signaling in stem cell maintenance. Further genetic evidence for Notch activation in crypt stem cells comes from lineace tracing data showing that the Notch target Hes1 labels multipotent intestinal stem cells [43].

Notwithstanding previous reports suggesting that intestinal stem cells are completely autonomous and self-organizing units [44], the existence of a cellular niche for crypt stem cells has been recently demonstrated [45]. Paneth cells, representing a specialized type of secretory cells residing at the bottom of crypts in close contact with stem cells, have been indeed shown to be essential for stem cell survival, since *in vivo* genetic ablation of this cell type results in stem cell loss. Interestingly, Paneth cells express several ligands for major signaling pathways, such as EGF, TGF-a, Wnt3 and the Notch ligand Delta-like 4. The above mentioned disappearance of stem cells upon genetic deletion of Delta-like 1 and Delta-like 4, therefore, could be explained by the loss of niche support due to impairment of Paneth cell specification and function in this mutant background.

While the identity of the Notch-expressing cells in the intestine is still unclear due to the lack of reliable antibodies, the expression pattern of Hes1 in the crypts tightly correlates with the expression of the RNA-binding protein Musashi-1 (Msi-1) [46], a marker of neural and intestinal stem cells. In the embryonic central nervous system, Msi-1 appears to be able of activating Notch signaling through repression of translation of the mRNA of the Notch inhibitor m-Numb [47,48]. However, it is currently unknown if Msi-1 can activate the Notch pathway in the intestinal crypts. Importantly, in the mouse intestine, both Msi-1 and Hes1 are expressed in cells just above the Paneth cells, as well as in the crypt base columnar cells intercalated between Paneth cells at the base of the crypt [46]. Both these types of cells have been shown to represent intestinal stem cells responsible for the renewal of the gut epithelium that occurs throughout adulthood [49].

There exists a striking similarity between the fly, the fish and the mouse in the conserved role of Notch signaling in dictating intestinal cell fate specification. One apparent difference, however, is the impact that Notch signals have on the self-renewing properties of intestinal stem cells in Drosophila and in the mouse crypt. While the loss of Notch signaling in Drosophila expands the number of stem cell-like cells, it depletes the stem cell compartment in the mouse. Conversely, Notch activation in the mouse intestine increases the numbers of proliferating stem cells and progenitors, while preventing their terminal differentiation; Notch activation in the fly intestinal stem cells forces their differentiation as enterocytes. At a closer examination, however, what Notch signaling does in both systems is to promote the fate of early progenitors (a transit-amplifying compartment in the mouse crypt and the enteroblasts in flies). The major difference is that the enteroblasts do not divide, while mouse progenitors do, and therefore the nature of the Notch transcriptional program likely differs in control of cell cycle regulation.

Notch signals in intestinal tumors

In spite of the paucity of current data directly implicating Notch signaling in colorectal cancer, several lines of evidence support the hypothesis that Notch signals have a positive impact on intestinal tumorigenesis.

A connection between Notch and intestinal tumors arises from studies showing that Hes-1, a signature of Notch signal activation whose expression is normally restricted to the crypt proliferative compartment, is also uniformly observed in adenomas formed in the intestine of mice carrying Apc mutations that lead to constitutive activation of the Wnt pathway [31,36]. Indeed there is a strong symmetry between crypts and intestinal neoplasia and these observations imply that Notch signaling is active not only in crypt progenitors, but also in some tumor cells. In addition, Notch inhibition has been reported to significantly reduce tumor size and incidence in adenomas that spontaneously occur in Multiple intestinal neoplasia (Min) mice [31,39,50], suggesting that the maintenance of the proliferative potential of intestinal adenoma cells depends on Notch activity.

These observations strongly suggest an important role for Notch signaling in regulating stem cell self-renewal during normal homeostasis and imply the value of exploring if Notch signals can contribute to the maintenance of cancer stem cells in this organ.

In other tissues, such as the hematopoietic system, it has been clearly established that the primary events in tumorigenesis are linked to stem cell transformation [51,52]. The existence of cancer stem cells in human colorectal tumors has recently been shown [53,54] and they represent a rare population of undifferentiated cells, responsible for tumor formation and for sustaining tumor growth. This subset of cells, referred to as cancer initiating stem cells (CISC), share many properties with physiological stem cells, but it is still unclear whether they originate from normal stem cells or by dedifferentiation of somatic tumor cells to stem-like cells.

The analogy between physiological stem cells and CISC is reflected in the intestine by the strong expression of both Msi-1 and Hes-1 in intestinal tumors of Apc mutant mice as well as in human colorectal tumors [36,55,56]. Indeed, siRNA-mediated knockdown of Msi-1 in colon adenocarcinoma xenografts induces tumor growth arrest. Inhibition of Msi-1 in these xenografts results in decreased cancer cell proliferation, increased apoptosis and down-regulation of activated Notch-1 [55]. In addition, both pharmacological and siRNA-mediated Notch inhibition in human colon cancer initiating cells has shown the critical requirement for Notch signals in maintaining self-renewal and preventing secretory lineage differentiation genes [57]. Taken together, these observations suggest that elevated Notch signaling may contribute to the initiation of colorectal cancer, by controlling the maintenance of the undifferentiated state of intestinal neoplastic cells.

While more than 50% of T cell Acute Lymphoblastic Leukemia (T-ALL) patients exhibit activating Notch receptor mutations [58], no one has yet reported genetic mutations in Notch receptors, ligands or in the effector RBPJ in colorectal cancer. Our findings in mouse and human tumor specimens show aberrant Notch activation almost exclusively in early stages of benign intestinal adenomas, suggesting that Notch may not play a role during carcinoma progression [36]. We therefore propose that aberrant Notch activation in a specific subset of cells can provide a

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developmental context that is favorable for the accumulation of oncogenic mutations that will trigger intestinal tumorigenesis.

Conclusive remarks

The analysis of Notch function in the same organ of different species has proven extremely valuable as it allows exploiting the specific advantages of each model system to highlight both conserved and divergent functions across species barriers. The results obtained to date comparing Drosophila, Zebrafish and mouse suggest that the essential role of Notch signaling in the control of intestinal homeostasis reflects a fundamental mechanism.

The genetic events that underlie the initiation and progression of colorectal tumors provide a remarkable example of the multi-step process at the origin of human cancers formalized in classic papers from the group of B. Vogelstein [59,60].

Accordingly, the sequential nature of colorectal tumor development is directly linked to loss or gain of function mutations occurring in well-known tumor suppressor genes or oncogenes. Yet so far no mutations of the Notch gene itself, or of the other major components of the Notch pathway, have been reported. Future studies should aim at identifying the molecular mechanisms triggering Notch activation in the gut. One may anticipate that various acute or chronic physio-pathological conditions may drive Notch activation (as in inflammatory bowel or infectious diseases).

The role of Notch signaling in colon cancer implies a distinct mode of action from that of conventional oncogenes. Specifically, its high context specificity and the frequently contradictory action in different tissues suggest that it does not behave as an oncogene *per se*. It is more likely that it exerts its oncogenic potential by facilitating the expansion or depletion of certain cell subpopulations in a given tissue with different susceptibility to tumorigenesis. A transient increase of cell proliferation in the crypt would indeed amplify a cell population that could be a dangerous target for mutations (*i.e.* stem cells or early progenitors). In summary, despite the fundamental role that Notch signaling plays in maintaining a proliferating cell phenotype in the normal intestinal epithelium, and despite its role in tumorigenesis, it is likely that effective therapeutic targeting will require multiple strategies based on the mechanisms underlying Notch deregulation in different subsets of colorectal tumors.

A generality that can be drawn regarding the action of Notch signals in development is that modulation of the Notch pathway in cells that are not terminally differentiated provides them with the plasticity to change developmental fate. Based on this rationale, it has been suggested that modulation of Notch signals in tumor cells may force cell fate changes that, depending on the context, may have therapeutic value, as they will possibly force tumor cells into a less malignant state. Future studies are expected to clarify these issues and elucidate the precise mode of Notch action in tumorigenesis.

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