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**NOTCH SIGNALING AND DISEASES: AN EVOLUTIONARY JOURNEY
FROM A SIMPLE BEGINNING TO COMPLEX OUTCOMES**

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Abstract

Notch signaling pathway regulates a wide variety of cellular processes during development and it also plays a crucial role in human diseases. This important link is firmly established in cancer, since a rare T-ALL-associated genetic lesion has been initially reported to result in deletion of Notch1 ectodomain and constitutive activation of its intracellular region. Interestingly, the cellular response to Notch signaling can be extremely variable depending on the cell type and activation context. Notch signaling triggers signals implicated in promoting carcinogenesis and autoimmune diseases, whereas it can also sustain responses that are critical to suppress carcinogenesis and to negatively regulate immune response. However, Notch signaling induces all these effects via an apparently simple signal transduction pathway, diversified into a complex network along evolution from *Drosophila* to mammals. Indeed, an explanation of this paradox comes from a number of evidences accumulated during the last few years, which dissected the intrinsic canonical and non-canonical components of the Notch pathway as well as several modulatory extrinsic signaling events. The identification of these signals has shed light onto the mechanisms whereby Notch and other pathways collaborate to induce a particular cellular phenotype. In this article, we review the role of Notch signaling in cells as diverse as T lymphocytes and epithelial cells of the epidermis, with the main focus on understanding the mechanisms of Notch versatility.

Notch signaling: not only a canonical pathway.

The Notch signaling pathway appears as a paradigm of simplicity. Indeed, the core signaling relies on relatively few components to convey the signal from the cell surface to the transcriptional machinery. This is exemplified by the *Drosophila* pathway in which a single Notch receptor signals through the transcriptional regulator Suppressor of Hairless (Su(H)). In mammals, the pathway diversified into four Notch receptors (Notch 1-4), and all of them are regulated through a common processing mechanism. After its synthesis in the ER, the Notch receptor is transported through the secretory pathway to the trans-Golgi network, where it is constitutively cleaved by a furin-like convertase [1, 2]. Following this proteolytic processing event, referred as S1 cleavage, the Notch receptor proceeds to the cell surface, where, as a trans-membrane receptor, it can interact with ligands presented on neighboring cells. Notch ligands are also trans-membrane proteins comprising two different sub-families, Delta and Serrate/Jagged, each including several members [3]. Upon ligand–receptor interaction between cells in physical contact, Notch receptors become susceptible to cleavage by ADAM-type metalloproteases at site S2, creating a short-lived intermediate, Notch-Transmembrane (NTM). Two ADAMs have been implicated in the S2 cleavage of Notch. In *Drosophila*, ADAM10 ortholog Kuzbanian is the main protease mediating Notch processing [4-7] (for Review see [8]). In mouse cells *in vitro*, ADAM17, and not ADAM10, appears to be a protease responsible for Notch cleavage [9, 10]. ADAM17-deficient mice do not show, however, a Notch-deficient-like phenotype [11]. In contrast, ADAM10 deficiency leads to embryonic lethality at E9.5 and multiple malformations [12] resembling those observed in Notch1 knockout mice, in mice homozygous for a γ -secretase processing-deficient allele of Notch1, or in presenilin1/presenilin2 double-knockout mice [13-15]. Thus, as proposed by Hartmann et al. [12], different ADAMs may contribute to the S2 cleavage in a tissue-specific manner, with ADAM10 playing the major role in this process *in vivo*. NTM is recognized and rapidly cleaved at site S3 by a multiprotein protease complex known as γ -secretase, which releases the active intracellular form of Notch, (NICD) [16, 17]. Once cleaved, the Notch's intracellular domain (NICD) is released and translocates to the nucleus, where it can bind the DNA-binding protein CSL (also referred to as RBP-Jk and CBF-1 in mammals and Suppressor of Hairless (Su(H) in *Drosophila*). In the absence of NICD, CSL protein acts as a transcriptional repressor by binding ubiquitous co-repressor proteins, such as silencing mediator of retinoic and thyroid hormone receptors (SMRT) or Mint (also called SHARP and Spen in human) [18]. The NICD generated upon ligand binding compete with the repressor proteins and form a NICD/CSL complex converting CSL from a repressor into an activator of transcription. The NICD/CSL module is recognized by Mastermind (MAM)/Lag-3 protein, and this complex is important to assemble an active transcription complex on target promoters by recruiting positive regulator of transcription such as the ARC-L/MED mediator complex [19], the histone ubiquitin Ligase, Bre-1 [20] and histone acetyltransferases (HAT). This signaling module is referred commonly as the canonical effector of Notch signaling.

Components of the two families of basic helix-loop-helix transcription factors, Hes and Herp (the latter also known as Hey, Hesr, HRT, CHF and gridlock) have been identified as immediate transcriptional targets [21].

In recent years, a detailed picture of the canonical Notch pathway in flies and

vertebrates has emerged [22]. Central to this pathway is, as mentioned above, the effector CSL. However, there is accumulating evidence for aspects of Notch signaling that occur in a CSL-independent manner. Although key genetic data in favor of a non-canonical Notch signaling have been presented, the experimental evidence for CSL-independent signaling is less direct, with few biochemical elements identified up to date. Interestingly, PI3K was identified as one intracellular mediator of Notch-dependent anti-apoptotic activity [23]. Moreover, activation of AKT pathway downstream of Notch signaling has been shown in differentiation and neoplastic transformation of several tissues [24, 25]. However, data from myogenic differentiation argue that Notch receptors that have not undergone S1 cleavage, and hence no other cleavages, still block myogenesis, and that Notch ICD constructs, lacking the CSL-interacting domain, exert effects in blocking myogenesis [26-29]. Moreover, certain *Drosophila* Notch alleles, the *mcd* alleles, appear to operate in a Deltex-dependent but Su(H)-independent manner [30]. Furthermore, a recent report provides genetic and biochemical evidence that Notch directs axon growth and guidance in *Drosophila* via a non-canonical, signaling pathway, characterized by association with the adaptor protein Disabled, and Trio, an accessory factor of the Abl tyrosine kinase [31]. Interestingly, Disabled and Trio were found associated with full-length Notch prior to cleavage, suggesting the possibility that γ -secretase cleavage terminates the Notch/Abl signal by separating the receptor-bound complex from membrane-tethered components of the pathway such as Abl kinase and Rho GTPases [31]. Moreover, deletion from Notch of the binding site for Disabled impairs its action in axon patterning without disturbing cell fate control [31]. However, additional experiments will be necessary to assess the various possible models. In light of this possible molecular duality for the Notch signaling, it is important to understand in detail how Notch exerts its action. It will be of great interest to learn whether the context-sensitive and flexible outputs of Notch signaling in various cellular contexts reflect a balanced contribution from both canonical and non-canonical Notch signaling pathways. In addition, we could hypothesize that different Notch receptors, possibly based on structural (i.e. presence or absence of TAD domain) or functional differences (i.e. binding affinity with MAM; physical interaction with several components of the NF- κ B signaling pathway [32-35]), may have differential ability in triggering canonical and non-canonical signaling pathways, thus resulting in intersection and/or reciprocal control with each other. This could justify the spatial and temporal differential expression of several Notches during differentiation, as well as the apparently opposite roles of Notch signaling in different cell compartments and or functions.

Notch and autoimmunity

The development of autoimmune diseases rises from the breakdown of unresponsiveness to self antigens. `Central tolerance` processes, via clonal deletion of autoreactive T cells in the thymus, are largely effective in avoiding autoimmunity, but incomplete. Thus, mechanisms of `peripheral tolerance` are essential to prevent harmful immune responses: the active suppression of self-reactive T lymphocytes by a highly specialized subpopulation of CD4⁺ T cells, known as CD4⁺CD25⁺FoxP3⁺ regulatory T cells (T_{reg}) [36], as well as the regulation of T effector functions, are both directly implicated in the control of autoimmunity.

The role of Notch signaling in regulating thymocyte cell-fate decisions is well established [37] and accumulating evidence indicates that it is also involved in the differentiation of peripheral CD4⁺ T cell subsets, including TH1, TH2 and T_{reg} [38]. Thus, an emerging and still controversial role in autoimmunity has been recently suggested for Notch receptors.

An important issue concerns a putative role of Notch signaling activation in driving the generation and/or induction of T cells with regulatory functions. Initially, it was shown that Notch signaling can impose a regulatory phenotype on murine peripheral CD4⁺ T cells, by means of their exposure to antigen presenting cells (APC), overexpressing the human Jagged1 Notch ligand [39]. These `induced` regulatory T cells can inhibit primary and secondary immune responses and transfer antigen-specific tolerance to recipient mice. Similar results were obtained with human T cells, which were co-cultured with EBV-positive human B cell lines, engineered to over-express human Jagged1 and used as APCs [40]. This treatment induces antigen-specific, IL-10-producing regulatory T cells (in both CD4⁺ and CD8⁺ subsets), that specifically inhibit the proliferative response and the cytotoxic-T-cell activity to EBV proteins. These cells block the responses of fresh T lymphocytes that have themselves never been exposed to Notch ligand, and importantly, spare responses to third-party stimulatory cells. The same researcher group indeed, demonstrated that these cells may be useful in the context of allogeneic hematopoietic stem cell transplantation, to avoid graft-versus-host disease (GVHD), while preserving activity against tumoral or infective antigens [41].

Since then, different approaches revealed that Notch signalling can be also involved in influencing the `naturally occurring` subset of T_{reg}. Interestingly, expression of Notch transcriptional target, Deltex, in naturally arising CD4⁺CD25⁺ regulatory T cells isolated from peripheral blood of adult volunteers was significantly higher when compared with their CD4⁺CD25⁻ counterparts, suggesting that Notch signaling is active in these cells. Further, upon stimulation, the transcription of Notch pathway components (such as, Notch4, Delta1 and Hes1) was highly up-regulated only in CD4⁺CD25⁺ subset. These cells can thus exert suppressive function by a cell-to-cell contact mechanism that may involve Notch signaling between neighboring T cells.

Moreover, stimulation of naive CD4⁺ T cells with Delta1 involving interactions with Notch3 promotes the differentiation toward the Th1 pathway [42], whereas Jagged1-mediated Notch1 activation is critical in driving Th2 differentiation [43]. Thus, the expression of multiple Notch receptors and ligands in a range of different T cell types indicates that Notch signaling pathway supports many of the processes involved in the maintenance and function of different mature T cell populations. However, the precise role of Notch signaling and the specific contribution of the different Notch-receptors and ligands is still controversial.

Recently, Notch3 receptor up-regulation was reported on naturally occurring CD4⁺CD25⁺ regulatory T cells from spleens of *wild-type* mice, when compared to CD4⁺CD25⁻ population [44]. Consistently, the analysis of *lck*-Notch3-IC transgenic mice revealed a significant increase in the generation and function of CD4⁺CD25⁺ regulatory T cells. Interestingly, these mice failed to develop streptozotocyn-induced autoimmune diabetes, and the adoptive transfer of *lck*-Notch3-IC transgenic CD4⁺ cells to immunocompetent *wild-type* recipients prevents the progression of the disease [44]

The putative role of Notch3 in T_{reg} biology has been also suggested by demonstrating that the exposure of T_{reg} to Jagged2-expressing hematopoietic progenitor cells (HPC), selectively activates Notch3 signaling and directly drives the expansion of functional host-derived T_{reg} that halt autoimmune diabetes onset in NOD mice, the spontaneous model of type 1 diabetes [45]. Intriguingly, the same group reported an association between up-regulated expression of Notch3 protein and enhanced FoxP3 expression in $CD4^+CD25^+$ regulatory T cells, following the treatment with Jagged2-overexpressing hematopoietic progenitors.

However, despite the above findings, it should be noted that other groups reported that the modulation of Notch signaling does not always impact on thymic generation of naturally occurring T_{reg} or induction of regulatory T cells from naïve $CD4^+$ T subset. In particular, it has been recently reported that selective inhibition of Notch3 in myelin-reactive T cells attenuates experimental autoimmune encephalomyelitis (EAE) [46].

Furthermore, to date there is no report about any quantitative or functional deficiency of regulatory T cell subset in Notch *knock-out* mouse models, perhaps because of the redundancy between different receptors and ligands of Notch family or the potential involvement of non-canonical transduction pathways of Notch signaling in regulatory T cell development.

Notably, the Notch signaling has also been linked to autoimmunity because of its proposed and controversial function in driving activation and differentiation towards TH1 fate of peripheral $CD4^+$ T cells (reviewed in [38]). Indeed, a dysregulated TH1 response may perpetuate destructive inflammation and contribute to the pathogenesis of different human autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis [47, 48]. This issue has been extensively studied by several groups in the pathogenesis of EAE, a mouse model of multiple sclerosis. Firstly, B. Osborne's group demonstrated that Notch activation facilitates TH1 development by directly promoting the transcription of T-bet, an essential factor for TH1 differentiation [49]. They showed that blocking Notch signaling by γ -secretase inhibitors (GSI) treatment *in vitro* or by expression of Notch1 anti-sense construct *in vivo*, significantly inhibits the expression of T-bet and the production of Interferon γ (IFN- γ) in TH1-polarized $CD4^+$ T cells. Furthermore, *in vivo* administration of GSI significantly attenuates the progression of TH1-mediated disease in the EAE mouse model. Then, another group confirmed the critical role of Notch signaling in the control of peripheral T cell functions, but demonstrated that Jagged1 and Delta1 differentially regulate the outcome of autoimmunity in EAE model [50]. While Jagged1 signaling activation protected from EAE and increased the frequency of IL-10-producing cells, the Delta1 activation worsened the EAE disease and increased the frequency of IFN- γ -producing cells.

From another point of view, a loss of function mutation in the *Itch* ubiquitin ligase, previously shown to be involved in Notch1 degradation [51], leads homozygous *Itch*^{-/-} (*itchy*) mice to develop a fatal, late-onset autoimmune-like disease [52]. Moreover, homozygous *itch*^{-/-} mice, carrying an activated Notch1 transgene, developed a more severe autoimmune disease, supporting the assertion that Notch1 and *Itch* mutations genetically interact [53]. Interestingly, the activation of canonical Notch signaling does not correlate with the severity of the observed autoimmune disease, suggesting that this phenotype may arise from a non-canonical, CSL-independent mechanism.

Overall, the evidence collected so far, clearly shows that the Notch signaling interferes with the generation and function of different T cell subsets. However, the mechanisms underlying these effects remain largely elusive and challenging. To this regard, the heterogeneity of cellular outcomes is likely to arise from the complex interactions between canonical and non-canonical Notch signaling, possibly sustained by different ligand/receptor pairing and intersecting extrinsic pathways.

Notch and lymphomagenesis: the cases of T and B cell malignancies.

The oncogenic potential of Notch signaling was initially identified by showing that a chromosomal alteration in a subset of T cell lymphoblastic leukemias/lymphomas containing a t(7;9)(q34;q34.3) chromosomal translocation [54], resulted in the truncation of Notch1 transcript and the generation of a constitutively active Notch1-ICD. In subsequent studies, it appeared that <1% of all human T cell leukemias or lymphomas contain this translocation [55]. However, aberrant Notch signaling was subsequently found in several human T cell leukemias and lymphomas that lacked genomic rearrangements [56, 57], implying that up-regulated Notch signaling might have a common role in human leukemogenesis.

Accordingly, constitutively active intracellular domains of Notch1 and 3 have been shown to cause T cell Acute Lymphoblastic Leukemia (T-ALL) in animals, and activating *Notch1* mutations are found in human and murine T-ALL (reviewed in [58]). Furthermore, high levels of Notch3 were consistently expressed in samples of human T cell acute leukemias, and dramatically reduced at clinical remission [59]. Interestingly, in T-ALL, Notch3 is associated with the expression of its target gene, HES1, and of the gene encoding pT α , important in pre-TCR signaling [59].

The molecular link between Notch and pre-TCR signaling pathways assumes particular relevance taking into account that combined expression of Notch3 and pT α has been reported in virtually 100% of T-ALL patients examined [59], and that bone marrow transplantation (BMT) with Notch1 overexpressing hematopoietic stem cells (HSCs) from mice lacking a functional pre-TCR, failed to give rise to any T cell transformation [60]. However, more recent evidence suggests that Notch1 promotes transformation through both pre-TCR-dependent and independent pathways (reviewed in [58]). In particular, it was shown that Notch1-dependent T-ALL is not essentially dependent on pre-TCR signalling, but that pre-TCR signalling may facilitate the early onset of the disease [61]. Indeed, the transient mimicking of pre-TCR signals (by injection of anti-CD3 ϵ antibodies), accelerates leukemia development in BMT experiments with Notch1 overexpressing Rag2^{-/-} hematopoietic stem cells (HSCs). Intriguingly, Notch1 activation causes transformation of Rag2^{-/-} HSCs without any induction of pre-TCR signaling, though with delayed kinetics. Furthermore, in this system Notch1 constitutive activation significantly up-regulates expression of Notch3, previously shown to induce T-ALL in experimental models [62]. Strikingly, Notch3 was reported as a Notch1 target also in human T-ALL cell lines [63, 64]. These data argue that high levels of Notch activity, possibly achieved through Notch1-dependent overexpression of Notch3, could overcome the β -selection checkpoint. In line with this idea, the absolute and pre-TCR-independent requirement of Notch signaling to drive differentiation of DN cells to DP stage, was

recently demonstrated both *in vitro* and *in vivo* [65, 66], confirming previous genetically-based observations [59]. Moreover, von Boehmer's group recently proposed that thymic precursors expressing various TCR complexes (preTCR, $\alpha\beta$ TCR or $\gamma\delta$ TCR), may all induce differentiation of $\alpha\beta$ CD4⁺CD8⁺ T cells, by synergizing with Notch signaling of different intensity [67, 68]. Interestingly, pre-TCR interacts more efficiently than the other two receptors [67], thus requiring weaker Notch signaling to drive precursors through the β -selection phase. Furthermore, a crucial role for pre-TCR signaling has been demonstrated in mediating the Notch3-dependent activation of a number of oncogenic transcription factors, involved in the initiation and/or the progression of T cell leukemia in transgenic mice (i.e. NF- κ B, Tal1, Ikaros) [35, 69, 70]. However, pre-TCR signaling seems not to be a prerequisite for NF- κ B activation triggered by Notch receptors. Indeed, while Notch1 appears to be able to activate NF- κ B independently on the presence of pre-TCR [71], the presence of pT α discriminates between canonical and alternative NF- κ B pathway activation, regulating either cell differentiation or proliferation/leukemogenesis, in Notch3-IC transgenic mice [35].

Although the observations reported above do not definitely establish a role for Notch1 signaling in promoting Notch3 expression, there is the possibility that a combination of activated Notch1 and Notch3 may be important in the development of T cell leukemia.

In contrast to T-ALL, *Notch1* mutations are not found in B-ALL and activated Notch signaling is a potent inducer of growth arrest and apoptosis in B-cell malignancies [72]. Indeed, it has been shown that expression of constitutively-active, truncated forms of the four mammalian Notch receptors inhibited growth and induced apoptosis in both murine and human B-cell lines, but not T-cell lines [72]. Similar results were obtained in human precursor B-cell acute lymphoblastic leukemia cell lines when Notch activation was achieved by co-culture with fibroblasts expressing the Notch ligands Jagged1 or Jagged2 [72]. Notably, all four truncated Notch receptors, as well as the Jagged-mediated Notch activation, induce Hes1 transcription and retroviral expression of Hes1 recapitulates the Notch effects, suggesting that Hes1 is an important mediator of Notch-induced growth arrest and apoptosis in B-cells [72].

It is however of note that over-expression of Notch2 is observed in malignant cells from patients with B cell chronic lymphocytic leukemia (B-CLL), and is associated with increased B cell survival [73]. Moreover, it has been shown that oncogenic viral proteins utilize the Notch signaling pathway to induce B cell immortalization [74].

Altogether, it is tempting to speculate that corrupt Notch activation plays opposite roles in different B cell malignancies, acute or chronic, possibly depending on the B cell differentiation stage, at which the main leukemogenic hit takes place.

In summary, activation of Notch signaling has different outcomes underlying T versus B cell malignancies, being always oncogenic in T cell. In contrast, Notch appears to mediate growth arrest and apoptosis in human precursor B-cell acute lymphoblastic leukemia lines while it increases survival in B-CLL.

Notch signaling and skin: sustaining cell differentiation to stop tumor growth

Notch activation has been mainly associated to its multiple effects in sustaining oncogenesis, including the control of tumor cell proliferation, migration, cell cycle progression, and inhibition of apoptosis. Indeed, Notch signaling is constitutively

activated in several types of cancer cells and it is generally regarded as an anti-apoptotic and pro-oncogenic signal. The most studied and well-established function of Notch signaling in promoting oncogenesis is the ability (i) to induce growth promoting genes such as cyclin D1 and c-myc and (ii) to trigger anti-apoptotic pathways such as PI3-Kinase/AKT. Moreover, several data indicate that in tissues in which Notch exhibits growth-promoting functions, it acts as a critical mediator of self-renewal and stem cells maintenance. In contrast, in normal epidermis, Notch1, Notch2 and Notch3 are all expressed suprabasally, indicating that their physiological role is mostly associated with keratinocyte differentiation. For example, blockade of Notch1 predisposes murine skin to basal cell carcinoma-like tumors. This observation could be explained by the fact that in normal keratinocytes, Notch activation induces cell cycle arrest [75]. Many hypotheses have been put forward to explain this dual activity. The emerging conclusion is that the final outcome of Notch activation depends on the cell type, the stimulus, and the context of activation.

Hence, although initially it appeared counter-intuitive that the same signaling cascade mediates different outcomes, consideration gathered from other cellular systems indicates that the archetypal model in which Notch signaling activation can serve in stem cell maintenance and inhibition of cellular differentiation, might be re-evaluated.

Notch signaling can indeed play different roles based on cellular context as well as on differential involvement of different Notch receptors. Notch1 receptor activation can inhibit the growth of hepatocellular carcinoma, small-cell lung cancer, and prostate cancer cells [76-80]. Although this growth inhibitory role of Notch has mainly been suggested on the basis of constitutively active Notch1-IC overexpression studies, data generated by inactivation of endogenous Notch signaling further support the notion that Notch has tumor suppressive functions in prostate cancer cells [80]. Furthermore, Notch1 deficiency in the liver results in the spontaneous proliferation of Notch1 *-/-* hepatocytes. Although Notch1 deficiency in the liver did not result in the development of liver carcinomas, this observation together with the ability of *Notch1 overexpression* to inhibit hepatocellular carcinoma cells proliferation support the hypothesis that Notch1 signaling may be part of a tumor suppressor program in the liver [82].

Conversely, Notch3 overexpression has been reported to be responsible for increased in vitro tumor cell growth in human lung cancer and Notch3 constitutive activation was reported to inhibit terminal differentiation in lungs of transgenic mice [83]. Moreover, increased expression of Notch3 has been observed in spontaneous human pancreas tumors [84] and in T-ALL [59]. Overall, the above observations support the notion that in addition to maintaining precursor cells, Notch signaling also participates in the induction of terminal differentiation and growth arrest.

Notch in keratinocyte differentiation

In mouse skin the Notch pathway is essential for the normal growth control of the epidermis and in the maintenance of the normal border between basal and upper differentiating layers[85]. Notch1 deletion in the epidermis results in increased cell proliferation, thus reflecting the growth inhibitory effects exerted by activated Notch1 in primary keratinocytes in culture [85]. This is consistent with the finding that p21WAF1/Cip1 expression can be induced by activated Notch1 expression [85]. Consistently with the model that the Notch pathway plays an important role in regulating

epidermal homeostasis, Notch1 deficiency in the epidermis results in a pleiotropic phenotype, with hair loss, hyperproliferation, deregulated expression of multiple differentiation markers and spontaneous basal cell carcinomas [85-87]. Thus, consideration gathered from human and mouse epidermis indicates a simple model in which Notch signaling activation regulates epidermis homeostasis by first promoting stem cell- transit amplifying cells transition and thereafter inducing a differentiation-associated growth arrest.

However, even this apparently simple model can exhibit complicated dynamics, generated not only by cross-talk between Notch and other signaling molecules but also by an additional complexity, intrinsic to the Notch signaling. There is growing evidence that signals transmitted through Delta or Jagged ligands can differentially affect the target cell [88]. Analyses using keratinocytes from human and mouse skin indicate that multiple Notch receptors and ligands are expressed in keratinocytes. In human epidermis, it has been proposed that high expression of Dll1 by clusters of stem cells protects them from undergoing terminal differentiation, while stimulating neighboring cells to become transit amplifying cells [89, 90] and other observations indicate that Dll1 expression also has cell-autonomous effects [91, 92]. Additionally, different ligands mediate different effects, not only *in vivo* [93], but also in culture, where loss of Jagged1 or Dll1 has different effects on the expression of integrins and spinous layer markers. Such differences are likely to reflect not only the different cell populations that express the ligands, but also the ability of the same cell population to respond differently to Dll1 and Jagged1 [94].

In vitro studies have suggested that NICD1 overexpression promotes keratinocyte differentiation, including some spinous layer genes such as K1, through a mechanism which appeared to be independent of RBP-Jk, thus suggestive of a non-canonical Notch signaling pathway [75]. However, more recent studies *in vivo* have indicated that loss of canonical Notch signaling by conditional loss of RBP-Jk, rather than promoting proliferation, resulted in a marked thinning of embryonic epidermis and by an marked decrease in proliferation within the RBP-Jk deficient epidermis [91]. According to these data, *in vivo*, the spinous fate induced by Notch is dependent on the canonical RBP-J-dependent-pathway. Thus, it has been suggested that it could be formerly possible that Notch1 functions as a tumor suppressor through a non-canonical pathway or as an alternative possibility perturbations in canonical Notch signaling compromise epidermal barrier function, which in turn leads to hyperproliferation as an indirect secondary reaction [92]. However, blockade of Notch1 but not loss of RBP-Jk predisposes murine skin to basal cell carcinoma-like tumors. This observation simply indicates that removing one member of the Notch family, namely Notch1, has not equal effects that the removal of RBP-J. Thus, a complex possibility is that loss of either RBP-Jk or Notch1 differently affects both balance and strength of Notch canonical and non-canonical pathway during keratinocyte differentiation. In this model, keratinocytes can normally differentiate in the presence of a strong signaling through the RBP-Jk, whereas the synergism between the non-canonical and canonical signaling restricts the growth potential of differentiating keratinocyte. In the absence of RBP-Jk, the balance between canonical and non-canonical Notch signaling is shifted towards the non-canonical one, which might be important to actively repress expression of differentiation markers. Notably, among the Notch family members, Notch1 elicits the strongest transcriptional activation on promoters whose activation is dependent on RBP-Jk-dependent canonical Notch signaling [95]. Similarly,

we could hypothesize that Notch1, elicits the strongest activation of non-canonical signaling in keratinocytes. Thus, loss of Notch-1 activity does not influence significantly the balance between canonical and non-canonical Notch signaling, but instead strongly decreases the strength of both canonical and non-canonical signaling required to restrict the growth potential of differentiating keratinocyte.

A complex picture of keratinocyte differentiation emerges from these considerations, including the involvement of non-canonical Notch signal strength. Further studies are required to fully elucidate the mechanism underlying this process.

Notch in skin cancer.

Tissue-specific conditional ablation of Notch1 under the control of the keratin-5 promoter in the basal layer of murine epidermis results in early hyperplasia followed by the spontaneous development of basal cell carcinoma (BCC)-like tumors [87]. In this context of Notch1 deficiency, the Wnt pathway was derepressed and beta-catenin-mediated signaling was re-activated, resulting in tumorigenesis.

Previous studies also pointed to a tumor-suppressive role for Notch signaling in mouse skin. In a presenilin-deficient mouse model, loss of presenilin, a component of gamma-secretase complex, results in epidermal hyperplasia and the development of keratinocyte-derived skin cancers [96]. In presenilin deficient mice, beta-catenin signaling was also upregulated and the downstream target cyclin D1 was activated. Thus, the interruption of Notch1 signaling in murine skin via two independent approaches, direct targeting of the gene or inhibition of cleavage of the encoded protein, produces similar consequences and highlights the importance of Notch-mediated regulation of beta-catenin signaling in murine keratinocytes. In a recent report it has been shown that the pan-inhibition of canonical Notch signaling by dominant negative (DN)MAML1 results in the spontaneous formation of cutaneous squamous cell carcinoma (SCC) [97]. Moreover, DN MAML1 expressing mice have accumulation of nuclear beta-catenin and up-regulation of cyclin D1 gene expression [97]. These observations suggest that Notch signaling may play a critical role in the pathogenesis of both BCC and SCC, originating from epidermal cells that exhibit profoundly different clinical manifestations. Consequently, understanding how selective inhibition of Notch1 in the skin gives rise to BCC while pan-inhibition of canonical Notch signaling in the skin gives rise to de novo development of SCC could provide important information in order to identify the mechanisms that govern the behavior of Notch signaling in both skin differentiation and cancer. The cytostatic and differentiative effects of Notch signaling are a barrier to tumor emergence and progression. Thus, the simplest hypothesis could be that loss of canonical Notch signaling represents a mechanism for evasion of Notch growth inhibition potential. Alternatively, since the non-canonical Notch signaling remain intact, as a consequence of the inhibition of canonical Notch signaling, only a specific subset of genes may be activated and/or repressed (i.e. genes involved in anti-apoptotic pathways, such as PI3K [23]). Thus, the balance between non-canonical and canonical Notch signaling determines the correct unfolding of the keratinocyte differentiation process.

Notch and cervical cancer: a dual role of the same Notch or two distinct signaling pathways?

HPV infection plays a critical role in cervical cancer, through the expression of the viral proteins E6/7 which are able to inactivate p53 and Rb-dependent tumor suppressor pathways. Several reports are consistent with the view that Notch signaling plays a role in development of cervical cancer [98-100]. However, it was shown that Notch1 expression is downregulated in late stage HPV-induced tumors, and that Notch signals counteract the HPV-induced neoplastic transformation by promoting E6 and E7 downmodulation [101]. Immunohistochemical data have indicated that Notch1 expression is elevated in squamous metaplasia of the columnar epithelium and in early HPV-induced lesions (CINI-III), as well as in differentiated superficial carcinomas of the cervix [98]. Conversely, Notch1 expression is substantially reduced in invasive cervical cancers [101]. This controversy could arise two conclusion: firstly, the dual pattern of Notch1 expression reveals a dual function of Notch signaling, depending on the cell biology context: namely, early stage versus later stage of cervical carcinogenesis. Secondly, the functional interaction between Notch and other signaling molecules determines a qualitatively different Notch signaling, sensed and interpreted differentially by the cells and resulting in a different outcome. Interestingly, it has been shown that E6 binds human MAML1, the transcriptional co-activator of Notch receptors, perhaps preventing MAML1 from amplifying Notch signaling and activating target genes [76, 102]. Interestingly, the promoter activity of the HES-1 gene, which is classically used to measure endogenous Notch1/RBP-Jk activity, is increased in HPV-negative cervical cancer cell lines, when compared to HPV-positive cells (Talora et al unpublished observation). Thus, suggesting that HPV-infection actively promotes downmodulation of Notch signaling. Consistently with this observation, it has been recently reported that the Notch1 gene is a target of p53 and can be down-regulated by E6 through p53 degradation in normal human epithelial cells [103]. Therefore, hypo-activity of Notch1 signaling may serve to counteract Notch1 mediated-repression of E6 and E7 expression [101]. Paradoxically, a synergic role of Notch1 with E6/E7 in the protection of HaCat epidermal cells, against apoptotic stimuli has also been reported [75]. However, full ablation of Notch activity by DN-RBP-Jk somewhat boosts proliferation of cervical cancer cells, but this takes place at the expenses of an increased susceptibility to pro-apoptotic stimuli [104], likely reflecting a dual role of Notch1 in the biology of cervical cancer cells. A model that may integrate these findings is as follows. HPV cancer cells can translate a low dose of Notch1 signals into an anti-apoptotic response. The cell cycle machinery of cervical cancer cells, on the other hand, would filter this level of Notch1 activity as noise and therefore implement a growth arrest program only at a higher level on Notch1 activity. In the normal epithelium of the cervix only Notch1 and Notch2 are expressed [101]. Interestingly, overexpression of Notch1, Notch2 and Notch3 resulted in a marked decrease of cervical cancer cells proliferation (Talora et al., unpublished observation). In contrast only Notch1 but not Notch2 results down-modulated in cervical cancer cells and primary tumors [101, 103]. Notably, among the Notch family members, Notch1 elicited the strongest transcriptional activation on promoters whose activation is dependent on canonical Notch signaling [95]. Hence, we could hypothesize that enforced overexpression of either Notch1, 2 or 3 elicits a Notch signaling, whose strength is able to overcome the active HPV-mediate repression of canonical Notch signaling. However, at physiological levels of expression, only Notch1, with its strongest activation of canonical Notch signaling, has the ability to counteract HPV activity. Thus, an attractive

hypothesis could be that HPV adopts a selective strategy for the evasion of the antiproliferative function of the canonical Notch signaling generated by Notch1, as this capacity enables the corrupt use of the non-canonical Notch pathway in tumor progression.

Conclusion and perspectives

Over the last several years, a great deal of progress has been made in our understanding of the signal transduction pathways that mediate the various cellular responses to Notch signaling. The realization that Notch's family members can execute different functions and may be distinctly regulated has further increased the complexity of Notch signaling. For instance, Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron [105]. Moreover, Notch2 cannot compensate for the loss of Notch1 function during T cell maturation [106]. Finally, Notch3 but not Notch1 seems to be able to sustain Ikaros alternative splicing in the experimental model of T-ALL [70]. Thus, these results establish distinct and non-redundant roles for Notch receptors. In addition, Delta and Jagged can evoke different responses. In T cells, for example, it has been suggested that these different ligands preferentially activate different Notch receptors [43, 88]. However, despite the conceptual appeal of differential Notch usage by Jagged or Delta, an alternative possibility is a differential signaling through the same Notch receptor paired with different ligands. Indeed, in *Drosophila* only a single Notch gene is expressed, thus the response to different ligands may involve signaling differences [107-109].

Certainly, different Notch family members seem to have distinct preferences for transcriptional regulatory elements [95], which could lead to distinct effects on proliferation and differentiation. Differences may therefore lie in other pathways activated by Notch, perhaps not involving RBP-Jk. However, the mechanisms regulating non-canonical pathways also require additional elucidation. Of primary interest is the relationship between intracellular versus membrane-bound forms of the Notch receptors, which could lead to distinct effects of Notch signaling. As an example, recent data indicate that Notch3 is involved in vascular injury and has an important role in the cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) pathology but its exact function is unknown, as yet. Investigation of CADASIL mutant Notch3 shows that the majority of mutations do not change CBF1/RBP-Jkappa-mediated canonical Notch pathway activation, so that the pathological consequences of NOTCH3 mutations in CADASIL patients can not be simply explained by loss- or gain-of-function in the classic Notch signalling pathway. This suggests that a novel Notch3-mediated signalling pathway may be present in Vascular Smooth Muscle Cells [110].

In summary, the current model of Notch pathway leads to a number of challenging questions: the answers are likely to reveal the mechanisms underlying critical cellular responses, and hopefully will lead to the development of more effective therapeutics for a number of human diseases.

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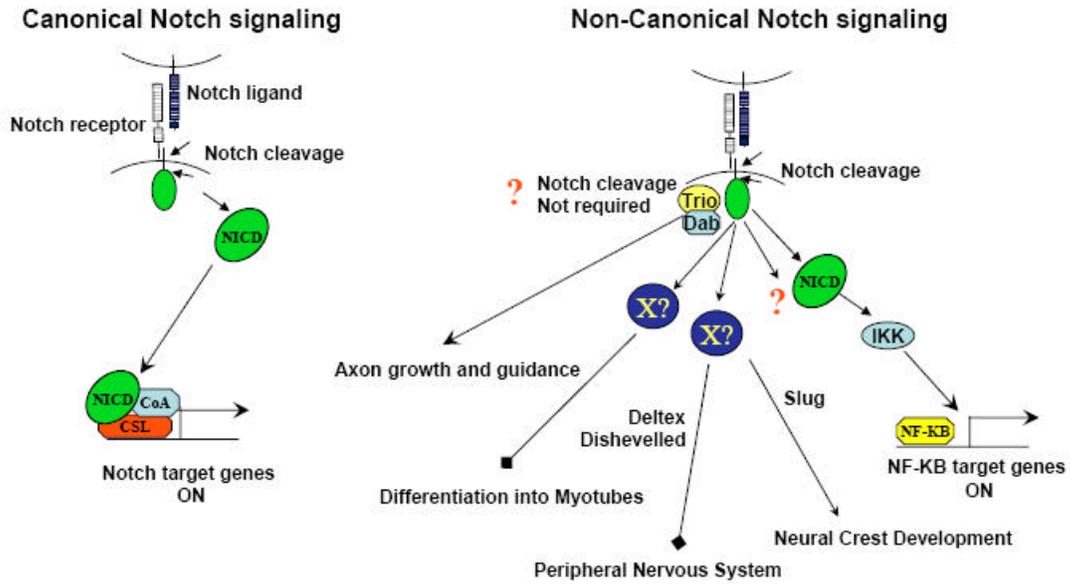
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Figure Legends

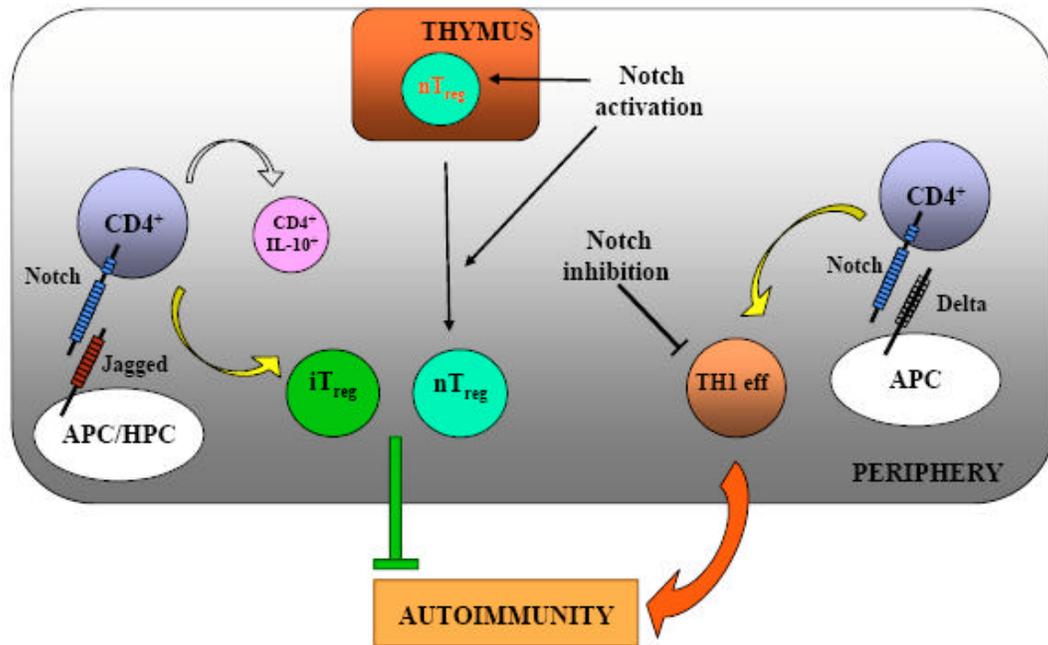
Figure 1. Canonical and non-canonical Notch signaling. A diagram of Notch canonical signaling pathways is compared to the non-canonical Notch pathway. Note that the canonical Notch pathway has been simplified to include only the main components. The interaction of ligands with Notch leads to the release of the Notch intracellular domain. This fragment of Notch enters the cell nucleus where it interacts with CSL to modulate transcription (Canonical Notch pathway). However, Notch-ligand interaction can also or alternatively activate a non-canonical signalling pathway. Currently it is not clear whether this is an intrinsic property of the membrane bound Notch receptor or whether Notch activated by its ligand, requires additional unknown factors in order to transduce non-canonical signaling. Signaling through this pathway has been shown to block differentiation in Myotubes [28, 29]. Peripheral Nervous system development [30, 111], Neural Crest development [112] and to promotes Axon growth ad guidance [31], moreover, through a direct interaction with IKKalpha Notch3 has been shown to be able to promote NF-KB activation in T-cell [35].

Figure 2. Notch and autoimmunity. Schematic representation of the controversial role of Notch signaling in regulating autoimmunity. Several reports suggest that activation of Notch signaling results in generation of both thymic-derived naturally occurring (nT_{reg}) and peripherally-induced (iT_{reg}) regulatory T cells, as well as IL-10-producing (CD4⁺IL-10⁺) regulatory cells. However, other data report that inhibition of Notch signaling leads to the blockade of generation of TH1 lymphocytes, responsible of development of autoimmunity, thus suggesting a protection against autoimmunity.

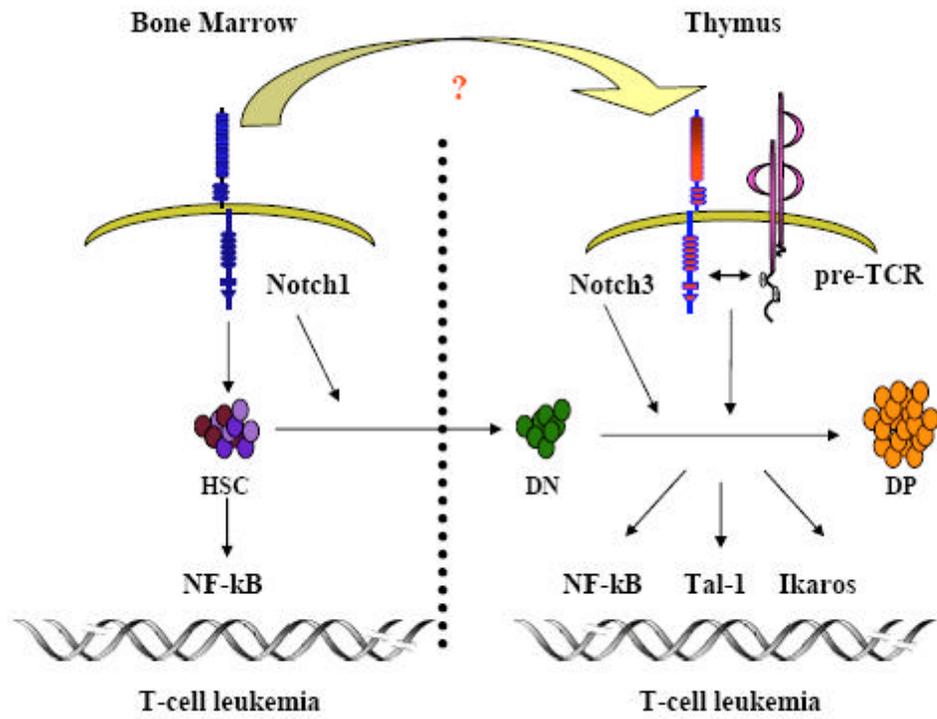
Figure 3. Notch signaling and T cell leukemogenesis. The cartoon illustrates schematically the differences between Notch1 and Notch3 in sustaining T cell leukemogenesis. Notch1 would be able to sustain neoplastic transformation of bone marrow-derived HSC towards T cell leukemia, independently on pre-TCR. In contrast, Notch3, through relationships with pre-TCR in thymocytes, would activate a number of oncogenic pathways, involved in the development of T cell leukemia. The possibility that Notch1 may directly activate Notch3 and in this way, through the interaction with pre-TCR, sustain the maintenance and progression of T cell leukemia, remains to be elucidated and is indicated by the question mark.



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