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An Overview of Notch Signaling in Adult Tissue Renewal and Maintenance

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Abstract

The Notch pathway is a critical mediator of short-range cell-cell communication that is reiteratively used to regulate a diverse array of cellular processes during embryonic development and the renewal and maintenance of adult tissues. Most Notch-dependent processes utilize a core signaling mechanism that is dependent on regulated intramembrane proteolysis: Upon ligand binding, Notch receptors undergo ectodomain shedding by ADAM metalloproteases, followed by γ -secretase-mediated intramembrane proteolysis. This releases the Notch intracellular domain, which translocates to the nucleus to activate transcription. In this review, we highlight the roles of Notch signaling particularly in self-renewing tissues in adults and several human diseases and raise some key considerations when targeting ADAMs and γ -secretase as disease-modifying strategies for Alzheimer's Disease.

Keywords

ADAM; CADASIL; γ -secretase; multiple sclerosis; Notch; T-ALL; stem cell; tissue renewal

Overview of the Notch signaling pathway

The Notch pathway is one of several conserved signaling pathways that regulate cell proliferation, cell fate decisions and induction of differentiation during embryonic and postnatal development [1-4]. The Notch receptors (Notch1-4 in mammals) are large Type I transmembrane proteins (Fig 1a). The extracellular domain is composed of 29-36 EGF repeats, three conserved cysteine-rich Lin12-Notch repeats (LNR) and the heterodimerization domain (HD). Together, the LNR and heterodimerization domains constitute the Negative regulatory region (NRR), which functions to keep the receptor “off” in the absence of ligand [5-9]. The single transmembrane domain (TMD) is then followed by a large intracellular region, which contains the RAM (RBPjk association module) domain, nuclear localization signals (NLS), seven ankyrin repeats (ANK) and the PEST (Proline/ GLutamic acid/Serine/Threonine-rich) domain.

Most receptors undergo processing at cleavage site1 (S1) by furin-like proteases in the Golgi and are therefore targeted to the cell surface as an intramolecular heterodimer held together

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by non-covalent interactions between the N- and C-terminal HD regions (Fig 1b). Recent studies using structure-guided deletions of the S1 sites in human Notch1 and Notch2 demonstrated that S1 cleavage is not absolutely required for receptor activation but it can have differential effects on cell surface trafficking [10], suggesting that this could be a mechanism for regulating availability at the cell surface (and thereby, activation potential) of different paralogs.

The Notch receptor is activated by binding to a ligand presented by a neighboring cell (Fig 1b). The major Notch ligands are also Type I transmembrane proteins that are characterized by an N-terminal DSL (Delta/Serrate/Jagged/Lag-2) domain and can be further classified based on the presence or absence of a cysteine-rich domain (Serrate/Jagged ligands have this domain, Delta ligands do not) (Fig 1a) [9, 11]. The mechanistic details of how ligand binding promotes Notch receptor proteolysis and activation are emerging (Fig 1b) [7, 9, 12]. Ligand endocytosis is thought to be required for ligand maturation [13-15] and also, after binding to the receptor, to generate sufficient force to produce a conformational change in the NRR [5, 8, 16]. The unfolding of the NRR (perhaps following heterodimer dissociation at S1 [17]) will expose site 2 (S2) to proteolysis by ADAM10/Kuz, the major metalloprotease involved in S2 cleavage of ligand-activated Notch receptors [18, 19]. This is consistent with the ADAM10/Kuz/sup-17 loss-of-function phenotypes (but not those of ADAM17/TACE) phenocopying the defects associated with Notch deficiency [20-22]. However, other metalloproteases may also play a role in tissues where ADAM10 is not expressed and in disease states. Elevated metalloprotease levels can lead to ligand-independent activation of Notch receptors [23, 24]. Interestingly, Notch receptors that are activated in a ligand-independent manner (specifically, by calcium chelation [25], or by T-ALL-associated and other activating mutations that destabilize the NRR [26, 27]) are no longer mainly dependent on ADAM10 for ectodomain shedding. ADAM17/TACE (and perhaps other metalloproteases) appears to play a more prominent role under these conditions [18, 19]. The molecular mechanisms underlying this selectivity are still unclear. This could reflect differences in structures between the various ADAMs and/or differences in the conformational changes of the receptor under different modes of receptor activation. Differential spatial distribution of the various ADAMs within the cell (relative to γ -secretase, for instance) may also be critical.

S2 cleavage is a major regulatory step in the Notch activation process. Notch ectodomain shedding creates the membrane-anchored intermediate fragment NEXT (Notch extracellular truncation), which is a direct substrate for the γ -secretase enzyme (Fig 1b). γ -Secretase is a multi-component intramembrane-cleaving protease composed of the catalytic component Presenilin (PS), along with Nicastrin, Pen2 and Aph1 [28, 29]. The γ -secretase complex initially cleaves the Notch TMD at the cytoplasmic end (site S3), which releases the Notch intracellular domain (NICD). Progressive cleavages towards the middle of the TMD eventually release the N β peptides (from site S4) (Fig 1c) [30]. The precise cellular location where γ -secretase cleaves Notch remains a subject of intense investigation [3, 9, 15]. Early genetic studies demonstrated the requirement of endocytosis in both the signal-sending and signal-receiving cells [16, 31]. As mentioned earlier, endocytosis of ligand during the activation process could be providing the force to unfold the NRR for exposure of S2 to ADAM. In the signal-receiving cell, endocytosis could also be contributing to this process

[9, 32]. Alternatively or in addition, endocytosis might be required for cleavage to occur (at least in some contexts) [33]. γ -Secretase is active in many cellular membranes and studies have shown that γ -secretase cleavage of Notch can occur at the cell surface or in an endosomal compartment (Fig 1b). Moreover, the cellular location where cleavage occurs can influence the precise position of S3 cleavage and as a consequence, modulate the stability of released NICD molecules [34]. This too can potentially be a point of pathway regulation. After NICD is released by γ -secretase, it travels to the nucleus where it associates with the DNA-binding protein CSL (CBF-1/RBPjk, Su(H), Lag-1). Together, they recruit the coactivator Mastermind, and further assemble a transcriptional complex that activates downstream targets (Fig 1b) [2, 7, 9, 35, 36].

Interestingly, Notch ligands also undergo sequential proteolytic processing by ADAMs and γ -secretase (Fig 1b) [11, 37]. Ligand proteolysis can be constitutive and likely depends on the availability of ADAMs. Indeed, activating ADAMs with p-aminophenylmercuric acetate increases ligand cleavage [38]. Notch receptor binding can also enhance ligand cleavage [38, 39]. ADAM-mediated ectodomain shedding of ligands is thought to be important for their downregulation, which helps promote and maintain unidirectional signaling [38, 40-42] or alleviate *cis*-inhibition [41, 43]. Ligand ectodomain shedding by ADAM appears to be dispensable during T-cell development [44] but is critical during cortical neurogenesis [45] and for maintaining the balance between self-renewal and differentiation in muscle satellite cells [46]. In contrast, the functional significance of the γ -secretase cleavage of ligands is less clear. γ -Secretase cleavage might just be facilitating disposal of the membrane-tethered carboxy-terminal fragments that are left after ADAM-mediated shedding [47, 48]. Alternatively, ligand proteolysis could be releasing functional ICD fragments (analogous to NICD) that could regulate transcription and be involved in ligand back signaling [39, 49]. Because most of the studies regarding the functions of released ligand ICD fragments have relied on overexpressed or engineered protein fragments, additional studies will have to address whether such a bidirectional mode of signaling from endogenously expressed and cleaved ligands plays a role in specific contexts *in vivo*. Notably, γ -secretase-deficient ligand-expressing cells appear to signal well for most Notch-mediated decisions, and display no obvious phenotypes [50, 51].

The core or canonical pathway is conserved for all Notch receptors and is utilized in most Notch-dependent processes. This direct membrane-to-nucleus signaling mechanism occurs without any signal amplification by secondary messengers, but is regulated by post-translational modifications (e.g., glycosylation, phosphorylation, ubiquitination) and endocytic trafficking of both ligands and receptors [2, 3, 11, 14, 15, 52-57]. In a context-dependent manner, these regulatory mechanisms modulate the strength and timing of Notch signals, and ultimately their biological consequences.

The essential function of Notch signaling in development is evident in the early embryonic lethality associated with loss of Notch signaling. Global knockouts of Notch1 or 2 in mice are embryonic lethal mainly due to the early defects in neurons, somites, and vascular integrity both in the embryo and placenta [58-61]. In addition, mice homozygous for the Notch1 V1744G allele, which produce highly unstable NICD molecules, phenocopy the Notch1 null mice [34, 62]. Ablation of RBPjk abolishes all canonical Notch signaling and

RBPjk global knockout mice exhibit similar or more severe phenotypes than those of Notch1 null embryos [63]. Knockouts of the other key components and modifiers of the Notch signaling pathway also support the view that Notch signaling is dispensable for gastrulation but essential for post-implantation mammalian development, starting at E9 [64]. It is important to note in this context that ablation of γ -secretase components, especially PS1, is almost identical to Notch-deficient mice [65, 66]. PS2 is dispensable for embryogenesis, but the double knockout of PS1 and PS2 is more severe and nearly identical to Notch signaling knockout mice [67]. Nicastrin- and Aph1a-null mice also exhibit embryonic lethal phenotypes, although Aph1b and c are dispensable for embryogenesis [68-72]. These studies strongly suggest that γ -secretase inhibition can phenocopy the loss of Notch signaling, and confirms the central role of γ -secretase as a mediator of the Notch signaling mechanism.

A growing number of studies have begun to investigate the roles of Notch signaling in adult mammals, thanks to the recent advances in inducible Cre-loxP targeting technology, which makes it possible to study both temporal and spatial regulation and functions of Notch signaling *in vivo*. This review will primarily focus on the biological role of Notch signaling in adult mammals in an attempt to delineate the possible complications that could be caused by γ -secretase inhibition and/or modulation in Alzheimer's disease (AD) therapy. In addition, we wish to identify other possible clinical applications wherein Notch modulation by γ -secretase inhibitors (GSIs) might be useful.

Notch Signaling in Adult Tissue Renewal and Maintenance

It has been demonstrated that Notch signaling is critical in tissue renewal and maintenance in various organs, including but not limited to, the intestine, skin, blood, liver, kidney, nervous system, bone and muscle. Thus, one can easily imagine that acute or chronic disruption of the pathway can lead to various complications in multiple organs. However, important questions such as how much reduction of Notch signaling can be tolerated in each tissue remain largely unanswered. In addition, Notch signaling functions in a highly context-dependent manner; biological consequences of pathway activation can vary depending on multiple factors, such as cell type, timing, and mode of signaling. Therefore, understanding the role of Notch signaling in each organ is critical for identifying a therapeutic window for GSIs. In the following sections, we highlight some of the key roles of Notch signaling in actively self-renewing tissues, such as the intestine, hematopoietic system, and skin. In addition, we discuss the potential roles of Notch signaling in cell renewal systems within the brain and muscle, which go through slower turnover.

Notch signaling in adult intestine

The epithelium of the intestine is one of the most rapidly and dynamically renewed tissues in the adult. It renews every four to five days, and failure to maintain this homeostasis can lead to death. Mild disruptions can lead to malnutrition, infection, and cancer. Cell renewal of the epithelium relies heavily on its proliferating LGR5+ intestinal stem cells (ISCs), which reside at the bottom of the crypt, and quiescent +4 position, Bmi+ ISCs [73]. LGR5+ cells first divide symmetrically to stochastically generate transit amplifying (TA) cells [74, 75], which upon leaving the TA compartment at the crypt-villus junction, subsequently give rise

to all four different terminally differentiated cells: absorptive enterocyte, secretory goblet cells, enteroendocrine, and Paneth cells (Fig 2a) [76-79]. Notch signaling has been shown to regulate cell renewal and binary fate decisions in the adult intestine in concert with Wnt signaling, which functions as the master switch that promotes cell proliferation and suppresses differentiation (Fig 2a) [77-83]. Ablation of Notch signaling in the intestine using conditional Notch1/Notch2 double knockout, or conditional RBPjk knockout mice, or the GSI dibenzazepine, was shown to specifically increase the number of secretory goblet cells at the expense of absorptive enterocyte cells [84, 85]. This suggests that Notch signaling regulates binary cell fate decision between these two cell lineages. In a reciprocal experiment, forced expression of NICD in the intestine led to reduction of secretory cells and increased cell proliferation [86]. These results suggest that there are two roles of Notch signaling in the adult intestine: it promotes proliferation either in the ISC or TA cell compartment, and it regulates binary fate decisions between absorptive and secretory cells. The first role was shown to be Wnt signaling-dependent while the second is independent of Wnt signaling [87]. In zebrafish, lateral inhibition through Delta-mediated Notch signaling has been implicated to play a role in these binary cell fate decisions [88]. Notch1 and Notch2 have been shown to function redundantly in the gut [84, 89].

Notch activation in the intestine was also shown to promote tumorigenesis in a sensitized background (Apc^{min/+}) that has a propensity for developing intestinal adenomas [87]. Treatment with GSIs promoted goblet cell differentiation and reduced proliferation in adenomas [85], suggesting that GSIs may also be useful in the treatment of neoplastic diseases in the gut [90]. However, severe gut toxicity has already been demonstrated in multiple independent rodent toxicological studies with GSIs [85, 91-93], suggesting that dosage or regional control of γ -secretase inhibition is critical. Recent *in vivo* studies using a combination of the GSI compound E, and the glucocorticoid dexamethasone, have successfully improved the anti-leukemic effects in T-ALL while reducing intestinal toxicity, showing that dosage control is feasible [94].

Notch signaling in adult hematopoiesis

The hematopoietic system undergoes rapid and robust cell renewal throughout life, and its dysregulation can lead to various hematologic diseases such as anemia, leukemia, and immune disorders. During embryonic development, hematopoietic stem cells (HSCs) primitively arise from the yolk sac and aorta-gonad mesonephros, which is followed by its generation in the fetal liver and bone marrow. In adult animals, HSCs primarily reside in the bone marrow, and they can give rise to all hematopoietic cell lineages, including myeloid cells (monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes, dendritic cells) and lymphoid cells (T cells, B cells, natural killer (NK) cells) [95, 96].

Notch signaling has been implicated in multiple cell fate decisions during hematopoiesis (Fig 2b) [80, 97-99]. Examples include cell differentiation decisions toward marginal zone B/follicular B cell [100, 101], CD8+ cytotoxic T cell [102, 103], Th1/Th2 cell [104], megakaryocyte [105], mast cell [106], NK cell [107, 108], and dendritic cell [109, 110] fates, but the best-studied role of Notch signaling is in T cell lineage commitment owing to

the discovery of its causative role in human T cell acute lymphoblastic leukemia (T-ALL), which will be further discussed later. A number of loss- and gain-of-function studies of Notch1 in bone marrow progenitors have demonstrated that Notch signaling instructs T cell fate at the expense of B cell fate [111-114]. Importantly, early versions of GSIs inhibited T cell development in mouse fetal thymus organ cultures [115, 116]. In follow up studies with human-mouse fetal thymus organ cultures, the GSI DAPT was shown to dose-dependently affect different hematopoietic cell lineage development: T cells required a higher dose of Notch signaling, NK cells were generated with a low dosage of Notch signaling whereas B-cells were generated in the absence of Notch signaling [117]. T cell lineage commitment was shown to be mediated through Dll4 expressed in thymic epithelial cells, implicating a thymic niche for T cell development [118, 119]. Proto-oncogene leukemia/lymphoma related factor (LRF) was identified as a negative regulator of Notch signaling in bone marrow progenitors, possibly providing a mechanism for suppressing the level of Notch signaling in order to prevent ectopic differentiation into T cell lineages [120]. It has been further shown that Notch signaling inhibits additional cell fate decisions including myeloid cells and dendritic cells to ensure T cell lineage commitment [121, 122].

Notch signaling has also been suggested to regulate HSC renewal (Fig 2b), but this is still highly controversial. Notch1 was shown to be required for generating HSCs during embryonic development [123-125]. In addition, osteoblast-specific expression of parathyroid hormone related protein receptor increased Jagged1 expression, which resulted in HSC expansion, implicating that Jagged1-mediated Notch signaling creates a HSC niche in the bone [126]. Overexpression of either NICD or the Notch downstream target Hes1 resulted in increased self-renewal of HSCs [127, 128]. In contrast, loss-of-function studies using a dominant negative form of the coactivator protein MAML or a conditional RBPjk knockout in adult HSCs did not show any phenotypes, suggesting that Notch signaling is dispensable for maintaining HSC homeostasis in the adult *in vivo* [129]. In summary, these studies suggest that γ -secretase inhibition is likely to be tolerated in self-renewal of adult HSCs, but will impair T cell development and suppress or alter many hematopoietic lineages in the adult.

Notch signaling in skin barrier defects

The skin is another organ that goes through robust self-renewal throughout life, and dysfunction in the skin barrier can result in dehydration, infection, or cancer. As in the gut, adult stem cells can be found in two places in the skin, a proliferative population in the epidermis and the hair bulb, and a quiescent population in the bulge region of the hair follicles [73]. The epidermis consists of four layers: basal (innermost), spinous, granular, and cornified layers, which express different cellular markers. The adult skin epidermal stem cells reside in the basal layer, and as they detach and migrate outward, they commit to terminal differentiation [130, 131].

Notch signaling regulates differentiation and proliferation of adult stem cells in the epidermis (Fig 2c) [80, 132-134]. Conditional gain- (NICD overexpression) and loss-of-function (Notch1^{-/-} or RBPjk^{-/-}) of Notch signaling in the epidermis during development and in the adult suggests that canonical Notch signaling contributes to spinous cell

differentiation *in vivo* [135-138]. However, while embryonic ablation of RBPj κ in the epidermis resulted in hypoplasia [135], its postnatal deletion resulted in hyperplasia and increased susceptibility to tumorigenesis [137, 139], suggesting that different mechanisms underlie the proliferative effects of the Notch signaling in the adult stem cells. This led to a surprising proposal that Notch1 functions in the adult skin as a “tumor suppressor”, in contrast to its well known oncogenic functions in many other tissues [139, 140]. However, this view was challenged by a recent study using a multistage chemical skin carcinogenesis paradigm on a chimeric mouse model of Notch deletion in the skin. Rather than Notch functioning as a “tumor suppressor” in the skin, loss of Notch1 was shown to act non-cell autonomously by creating a microenvironment that promotes carcinogenesis [137].

Moderate reduction of Notch signaling in the skin can dose-dependently increase the susceptibility to tumorigenesis. While conditional knockout of either Notch2 or 3 alone in the postnatal epidermis exhibited no phenotype, stepwise deletion of Notch paralogs dose-dependently accelerated skin carcinogenesis, suggesting that Notch1, 2 and 3 were not redundant but additive [137]. Similarly, moderate reduction of γ -secretase activity (either PS1^{+/-}, PS1^{+/-};PS2^{-/-}, Nicastrin^{+/-}, or Aph1a^{+/-}) increased the risk of developing squamous cell carcinoma [141, 142]. Another study has shown that skin barrier defects caused by loss of Notch signaling can induce systemic B-lymphoproliferative disorder in newborn mice through a dose-dependent secretion of thymic stromal lymphopoietin (TSLP), which can eventually lead to atopic dermatitis and asthma in adult animals [143, 144]. While TSLP can be useful as a biomarker for Notch-mediated skin barrier defects, these results raise the concern that even a moderate reduction of Notch signaling can increase susceptibility to carcinogenesis in the skin and simultaneously add risks of atopic dermatitis and asthma. Indeed, PS1^{+/-};PS2^{-/-} mice with reduced dosages of γ -secretase *in vivo* developed autoimmune phenotypes [142, 145], most likely in synergy with skin barrier defects. Recently, haploinsufficiency of the γ -secretase component genes PS1, Nicastrin and Pen2 have been associated with familial Acne Inversa, highlighting the sensitivity of the skin to reduction in γ -secretase activity [146].

Notch signaling in adult neurogenesis and synaptic plasticity

Unlike the intestine, blood, or skin where massive cell renewal is continuous throughout life, the vast majority of neurogenesis takes place during embryonic development. Adult neurogenesis occurs in two restricted regions of the brain - in the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricle. Although the functional role of adult neurogenesis is not fully understood, it has been implicated in hippocampus-dependent learning and memory, and neurological disorders such as epilepsy and AD. Moreover, adult neurogenesis can be enhanced upon injury, exercise, or an enriched environment [147, 148].

Notch signaling has classically been shown to play a critical role in the maintenance of neural progenitor identity and the inhibition of neuronal differentiation during early development [149]. The current view holds that while Notch signaling may inhibit the neural fate in the early developmental stage, it can also instruct or permissively allow fate choices between different subtypes of neural cells in the later stage, serving as a binary

switch of cell fate determination [150]. Emerging evidence also suggests multiple roles for Notch signaling in adult neurogenesis (Fig 2d) [80, 151]. During adult neurogenesis, neural stem cells (NSCs), which specifically express cellular markers such as glial fibrillary acidic protein (GFAP), glutamate-aspartate transporter (GLAST), and Nestin, divide slowly and asymmetrically to produce NSCs and TA cells. TA cells proliferate rapidly, exit from cell cycle and produce neural progenitor cells (NPCs), which migrate from SGZ and SVZ to the granule cell layer or olfactory bulb, respectively, where they mature into neurons. In the SGZ, a study using GFAP-CreERT Notch1 knockout mice and reciprocal experiments employing NICD transgenic mice suggested that Notch signaling promotes proliferation and inhibits cell cycle exit from TA cells to NPCs [152]. Notably, two recent studies using GLAST-CreERT and Nestin-CreERT inducible RBPjk knockout mice, which completely ablate canonical Notch signaling in the NSCs within the SGZ or SVZ, suggest that Notch signaling instead inhibits entry from NSC pools into TA status and is critical in long-term maintenance of adult NSCs [153, 154]. The discrepancy from the previous study may be due to residual signaling by other Notch receptors in the Notch1 knockout mice. Although forebrain ependymal cells are not formally considered as stem cells, they were shown to give rise to neuroblasts and astrocytes upon recovery from stroke, and Notch signaling plays an active role in maintaining their quiescence [155].

Notch signaling has also been shown to be involved in neurite remodeling and synaptic plasticity in the adult brain [156]. Several studies have indicated that Notch signaling modulates neurite outgrowth *in vitro* as well as *in vivo*, although the underlying mechanism still remains unknown (Fig 2d) [152, 157, 158]. A study using hippocampal slices from Notch antisense transgenic mice has suggested that Notch is involved in long-term potentiation [159]. Roles for Notch signaling were also implicated in learning and memory. In *Drosophila*, Notch signaling was shown to play a role in long-term memory [160, 161]. In mice, postnatal forebrain-specific deletion of PS1, both PS1 and PS2, or Nicastrin resulted in impairment in spatial memory and/or age-related neurodegeneration [162-164], although direct evidence of whether these phenotypes can be attributed to the lack of Notch signaling has not yet been provided. Notch1^{+/-} and RBPjk^{+/-} mice were both shown to exhibit spatial memory deficits [165]; however, hippocampus-specific and postnatal deletion of Notch signaling will have to be examined to exclude the possibility that it may be secondary to developmental effects caused by the reduction of Notch signaling.

Many questions still remain, such as how much reduction of Notch signaling can be tolerated, is this threshold different in various individuals (a quantitative trait) or how different Notch paralogs contribute to adult neurogenesis, neuritogenesis, or memory and learning. Lastly, the role of adult neurogenesis in AD has been recently discussed in several reviews; however, how Notch signaling may be involved in its pathogenesis is still largely unknown [156, 166-169].

Notch signaling in adult skeletal muscle

Satellite cells are stem cells of mature skeletal muscle that are responsible for its regenerative potential, and dysfunction in this regenerative capacity can lead to sarcopenia (loss of muscle mass). Although the majority of satellite cells are mitotically quiescent,

muscles can go through robust regeneration throughout life, upon homeostatic demand, hypertrophy, or need for repair [170].

Loss of Delta1 ligands during muscle development leads to the premature differentiation of muscle progenitors and a concomitant loss of satellite cells, causing severe muscle hypotrophy in newborns [171]. Notch signaling also plays a key role in regulating adult muscle regeneration (Fig 2e) [80, 170, 172-174]. In a myofiber explant culture system that mimics muscle injury, Notch1 was rapidly activated in satellite cells and promoted cell proliferation of myogenic precursor cells that express the premyoblast marker, Pax3 [175]. It was also shown that the Notch antagonist, Numb, was asymmetrically localized in dividing cells, and that attenuation of Notch signaling was required for fate determination toward myoblasts. Furthermore, satellite cells were discovered to consist of a heterogeneous population of stem cells and committed progenitors. Notch3 expression was particularly elevated in the former cell population [176]. It was additionally shown that Notch signaling is important in the early phase of response after injury, and a temporal switch from Notch to Wnt signaling in the later stage of regeneration was critical for progenitor cells to differentiate [177].

Interestingly, modulating Notch activity can restore a decrease in regenerative capacity in aging muscle. In aged muscle, decreased Delta1 expression in satellite cells correlated with an age-dependent decrease in its regenerative capacity [178]. However, this was rescued by increasing Delta1 expression levels, suggesting that regenerative capacity is not intrinsically impaired in older satellite cells. Furthermore, an *in vivo* study using parabiotic pairing between young and old mice demonstrated that some systemic cues could increase Delta1 expression and activate Notch signaling in aged muscle [179]. One interesting caveat of this study was that this effect was not muscle-specific; hepatocyte proliferation was also enhanced. Wnt3a, TGF β , and testosterone have emerged as candidate systemic factors although the molecular mechanisms underlying their effects still remain to be elucidated [180, 181].

In summary these studies suggest that the reduction of Notch signaling by γ -secretase inhibition could mimic the age-dependent loss of regenerative capacity in adult muscle. Simultaneous introduction of systemic factors that maintain satellite cell regenerative capacity may be a useful therapeutic strategy to antagonize the undesirable effects of γ -secretase inhibition in adult muscle and possibly other organs. However, further investigation is still needed to identify these factors to be able to precisely manipulate the dose of Notch signaling within the safe therapeutic window.

Notch signaling and human diseases

Given its role in regulating many fundamental processes such as cell proliferation, differentiation and apoptosis in a variety of tissues, the Notch signaling pathway is highly regulated. Accordingly, misregulation or misexpression of Notch pathway components has been linked to cancer (e.g., T-ALL) and adult-onset diseases (e.g., CADASIL and multiple sclerosis). Aberrant Notch signaling has also been associated with several developmental syndromes but these are beyond the scope of this review [182].

Notch and T-ALL

As Notch signaling is required for multiple stages of T-cell development, it is not surprising that the human *Notch1* gene was discovered for its involvement in T-ALL, an aggressive blood cancer resulting from malignant transformation of T-cell precursors [27, 183-187]. A t(7;9) chromosomal translocation in a small subset (~1%) of T-ALL cases leads to expression of an extracellular domain-truncated, constitutively active form of Notch1 [183]. A more general role for Notch signaling in human T-ALL was recognized when activating Notch1 mutations were subsequently found in >50% of T-ALL cases [27]. These activating Notch1 mutations are clustered in two regions, the extracellular heterodimerization domain (HD) and the C-terminal PEST domain. The HD mutations found in T-ALL either destabilize Notch heterodimers or displace the S2 site to the outside of NRR, thereby leading to ligand-independent Notch signaling [5, 188, 189]. Partial or complete loss of the PEST domain increases NICD half-life, thus enhancing Notch signaling [189, 190]. Notably, mutations in HD and the PEST domain were also found in *cis* in the same *NOTCH1* allele; reporter gene assays confirmed the synergistic effect of these two groups of mutations [27]. Recently, Sulis et al found in a subset of T-ALLs a novel type of mutation resulting in extracellular juxtamembrane expansions (JMEs) [191]. These mutations markedly enhance Notch signaling in a ligand-independent manner, but do not affect the stability of Notch heterodimers and still require prior S2 cleavage [191]. It is still unclear how JME mutations affect S2 site cleavage.

While the association of activating Notch mutations with T-ALL is clear, the downstream targets of Notch signaling and how they relate to the pathogenesis are far from being completely understood. A large body of evidence indicates that the oncogenic transcription factor Myc is a direct Notch target in T-ALL [192-194]. Recently, accumulating evidence also suggests that Notch signaling induces cell transformation via multiple signal pathways, including NF- κ B [195], PI3K-AKT [196], and the NFAT signaling pathways [197].

The discovery of activating Notch mutations in more than 50% of cases of human T-ALL provides a rationale for Notch as a therapeutic target. Indeed, GSIs were proposed as potential drugs for T-ALL [27, 93]. However, the application of GSIs in T-ALL has been hampered by the limited anti-leukemic effects of GSIs [27] and severe gastrointestinal toxicity due to the inhibition of Notch signaling in the gut, as discussed earlier [90, 91, 93]. Still, GSIs appear to sensitize T-ALL cells to chemotherapy [192, 198] and a recent report showed that the combination therapy of GSIs and glucocorticoids improved the anti-leukemic effects and reduced GSI-induced intestinal toxicity [184]. It was suggested that inhibition of Notch signaling by GSIs leads to upregulation of glucocorticoid receptor expression presumably via the transcriptional repressor HES1, whereas glucocorticoid treatment diminishes GSI-induced intestinal toxicity by upregulation of Ccnd2 [94, 184]. Although the molecular mechanisms involved remain to be elucidated, the combination therapy of GSIs and glucocorticoids may provide a promising strategy to target T-ALL.

Notch and CADASIL

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is the most common form of hereditary adult-onset stroke

and vascular dementia that is caused by mutations in *Notch3* [199-201]. The Notch3 receptor is required for arterial identity and maturation of vascular smooth muscle cells (vSMCs) [202] and *Notch3* is predominantly expressed in vSMCs in adults [203]. The key pathological features of CADASIL include the loss of vSMCs and the accumulation of Notch3 extracellular domains (ECDs) in the plasma membranes of vSMCs [200, 203]. To date, more than 170 mutations in *Notch3* have been identified to be linked with CADASIL from different pedigrees [204]. These mutations span exons 2-24, with most clustering in exons 3 and 4, which encode EGF repeats 2-5. Most are missense mutations and in some cases short in-frame deletions. Strikingly, almost all CADASIL-causing mutations hitherto described result in loss or gain of 1, or rarely 3, cysteines in 1 of the 34 EGF repeats in Notch3 [200]. Normally, each EGF repeat contains 6 cysteines, whereas all *Notch3* mutations in CADASIL lead to an odd number of cysteines in the affected repeat.

Although the association of *Notch3* mutations with CADASIL has been well-established, the underlying molecular mechanisms remain unclear. Similar to AD, both loss- and gain-of-function models have been hypothesized for CADASIL [205, 206]. It is possible that the unpaired cysteines may lead to a misfolded Notch3 ECD, which may compromise Notch3 signaling by affecting trafficking, post-translational modifications, S1 cleavage and/or ligand binding [207], and ultimately affecting vSMC maturation and small artery function. However, *in vitro* downstream reporter gene assays show no significant differences between wild-type and most CADASIL-associated mutant Notch3 receptors [208, 209]. The only exceptions are mutations that map to the ligand-binding domain, which lead to decreased activity in reporter gene assays and are associated with an earlier onset of the disease [210]. In addition, although a complete loss of Notch3 leads to structural and functional alterations in small arteries as well as increased susceptibility to stroke in mice, it does not lead to CADASIL-like symptoms [202, 211, 212]. Therefore, a loss of Notch3 function alone cannot cause this disease. Consistent with this idea, disease-associated mutant Notch3 receptors are functional *in vivo* and can rescue the arterial defects in Notch3 knockout mice [213]. Alternatively, mutant Notch3 with unpaired cysteines may gain some novel disease-causing functions. Several lines of evidence favor this gain-of-function model. For instance, compared to wild-type Notch3, CADASIL-causing mutants are more prone to aggregation [214] and accumulate in the membranes of vSMCs [203]. Additionally, CADASIL-associated Notch3 mutants can accumulate in the endoplasmic reticulum and impair cell proliferation [215]. Interestingly, transgenic mice that express higher levels of mutant Notch3 (above a certain threshold) develop several main pathological features of CADASIL [216]. Therefore, the current data favor a neomorphic effect of CADASIL mutations. How Notch3 ECD mutations and protein aggregation specifically alter vSMC function and lead to the disease still need to be elucidated. It is unclear whether they act by affecting normal Notch signaling processes and/or other cellular pathways. Regardless, these studies have revealed a role for normal Notch signaling in the maintenance of vSMCs and small artery functions.

Notch and Multiple sclerosis

The Notch signaling pathway has also been implicated in multiple sclerosis (MS), a central nervous system (CNS) disorder characterized by chronic inflammation, demyelination of

axons as well as damage to axons and oligodendrocytes [207, 217-219]. A key feature of MS is the impaired differentiation of oligodendrocyte precursor cells (OPCs) into myelin-forming oligodendrocytes [220].

Canonical Notch signaling prevents oligodendrocyte maturation and myelin formation by blocking OPC differentiation in the developing CNS [221, 222]. It was therefore speculated that canonical Notch signaling might be activated in MS lesions to prevent OPCs from differentiating into myelin-forming oligodendrocytes [223]. Consistent with this idea, Notch1, Jagged1 and the downstream target Hes5 are expressed in active MS lesions [224]. Other studies show that Notch1 and Jagged1 ligand are also expressed in remyelinated lesions [225, 226] although the functional significance is unclear. Notably, recent *in vivo* data showed that Olig1-Cre-mediated loss of Notch1 in OPCs led to accelerated OPC differentiation and remyelination in demyelinating lesions [227], indicating a role for Notch signaling in preventing OPC differentiation in MS lesions.

Non-canonical Notch signaling pathways are also involved in MS. Under physiological conditions, Notch is activated by the non-canonical ligand F3/contactin to promote OPC differentiation and upregulate myelin-related protein MAG [11, 228]. Interestingly, during the repair of MS lesions, while F3/contactin activates Notch signaling in OPCs and releases NICD via γ -secretase cleavage, the NICD molecule is trapped in the cytoplasm, rather than translocating into the nucleus, which is required for myelinogenesis [229]. Normally, NICD nuclear translocation is mediated by the nuclear transporter importin- β [230]. However, in demyelinated lesions, an inhibitor of nuclear importin- β , TAT-interacting protein TIP30, is markedly upregulated and forms aggregates with nuclear importin- β , thereby blocking NICD nuclear translocation [229].

Collectively, both canonical and non-canonical Notch signaling pathways appear to be involved in the pathogenesis of MS. It should be noted that unlike in T-ALL and CADASIL, where a major role for Notch signaling is well established, the Notch pathway might be just one of several signaling pathways to be involved in MS [220, 231]. Interestingly, pharmacological inhibition of Notch signaling with GSIs had beneficial effects in the mouse experimental autoimmune encephalomyelitis model of MS and therefore, warrants further study [232-234].

Conclusions and Perspectives

Notch signaling plays a critical role in various tissues and is used reiteratively at all stages of development, and during adult tissue renewal. It is clear that different functional thresholds of Notch signaling exist for various contexts. The central role of proteolysis (by both ADAMs and γ -secretase) in the Notch signaling mechanism underlies the major concern towards the use of both α -secretase agonists and GSIs in the chronic treatment of AD. On the other hand, GSIs have emerged as possible drugs for treatment of several types of cancers involving Notch. Regardless of clinical application, the studies summarized here provide a framework for organ systems that might be especially sensitive to chronic reduction or enhancement in Notch activity. Paralog-specific functions will have to also be addressed in future studies. Even strategies that avoid simple γ -secretase inhibition (e.g., γ -

secretase modulators, BACE inhibitors, targeting specific γ -secretase complexes) will need to be extensively characterized in various self-renewing systems. Combination therapies could be promising.

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Figure 1.

Overview of the Notch signaling pathway.

(a) Schematic depiction of mammalian Notch receptors and DSL ligands. Notch receptors are large single-pass Type I membrane proteins. The extracellular domain has 29-36 epidermal growth factor (EGF) repeats, followed by the conserved Lin12-Notch repeats (LNR: light green) and a heterodimerization domain (HD). The LNR and HD domains constitute the Negative regulatory region (NRR). The single transmembrane domain (TMD: pink) is followed by the RBPjk association module (RAM) domain (purple), nuclear localization sequences (NLSs: blue), 7 ankyrin repeats (ANK) (light blue), and a transactivation domain containing a C-terminal proline/glutamic acid/serine threonine-rich motifs (PEST: gray). The major Notch ligands are also Type I membrane proteins that have an N-terminal Delta/Serrate/LAG-2 (DSL: light pink) domain and multiple EGF motifs (light blue). The ligands can be further classified based on the presence or absence of the Cysteine-rich (CR; purple) and of the Delta and OSM-11-like protein (DOS; lavender) domains.

(b) Canonical Notch signaling pathway. Notch receptors are expressed at the plasma membrane as intramolecular heterodimers, held together by non-covalent interactions within the heterodimerization domain (HD). The receptors are activated by binding to ligands presented by a neighboring signal-sending cell. Ligand endocytosis is thought to generate the force needed to unfold the NRR (and perhaps dissociate the HD) to expose S2 to ADAM cleavage. Ectodomain shedding generates the membrane-anchored intermediate NEXT (Notch extracellular truncation), which is subsequently cleaved by γ -secretase progressively within the TMD to release the Notch intracellular domain (NICD) and the N β peptides. γ -secretase cleavage of Notch can occur at the cell surface or in an endosomal compartment. Once released, NICD translocates to the nucleus, associates with DNA-binding protein CSL and the transcriptional coactivator Mastermind to activate transcription. Some Notch ligands also go through sequential cleavages by ADAM proteases and γ -secretase. ADAM-mediated shedding of ligand ectodomains has been shown to modulate the strength and timing of Notch pathway activity. The role of the γ -secretase cleavage of ligands and released ligand intracellular domain fragments is still unclear.

(c) TMD region of mouse Notch1, showing cleavage sites and products. Notch is cleaved by an ADAM protease at the S2 site. After ectodomain shedding, γ -secretase cleaves the Notch TMD, starting at the S3 site. Multiple scissile bonds can be cleaved to release different NICD molecules with varying N-terminal residues. Only NICD-V (blue) is resistant to N-end rule degradation and is likely the major biologically functional species. TMD proteolysis then proceeds toward S4 until the N β peptides can be released.

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Figure 2.

Notch signaling is utilized in multiple organs in the adult for cell renewal and tissue maintenance.

(a) In the adult intestine, there are two types of adult intestinal stem cells (ISCs): Bmi+ quiescent and Lgr5+ proliferating ISCs (both in pink). They produce transit amplifying (TA) cells (light orange), and differentiate into four types of cells: absorptive enterocyte (light blue), secretory goblet (green), enteroendocrine (blue), and Paneth (purple) cells. Notch signaling promotes (blue arrow) proliferation of the adult ISCs and determines binary cell fate decisions between absorptive and secretory cells.

(b) During adult hematopoiesis, Notch signaling promotes T cell lineage differentiation and regulates multiple other differentiation steps. It is still controversial whether Notch signaling regulates proliferation of hematopoietic stem cells (HSCs: pink) in the adult. Leukemia/lymphoma related factor (LRF) is a negative regulator of Notch signaling in the bone marrow.

(c) In the adult skin, Notch signaling promotes spinous cell fate. Ablation of Notch signaling leads to tumorigenesis through non-cell autonomous effects. It also leads to secretion of thymic stromal lymphopoietin (TSLP), which can eventually lead to atopic dermatitis and asthma in the adult.

(d) Adult neurogenesis takes place in two regions of the brain, the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ). In the SVZ, Notch signaling promotes cell proliferation in the adult neural stem cells (NSCs, pink, marked by glial fibrillary acidic protein (GFAP), glutamate-aspartate transporter (GLAST), or Nestin) and inhibits differentiation toward transit amplifying (TA) cells (light blue). Notch signaling maintains quiescence of ependymal cells (ciliated cells), which produce astrocyte (pink) and neuroblast (blue) upon stroke/injury. In the SGZ, Notch signaling promotes proliferation of adult NSCs (pink) and inhibits entry from NSCs to TA cells and/or cell cycle exit. Notch signaling could also promote survival and maturation of neurons and modulate neurogenesis in mature neurons.

(e) In adult muscle, Notch signaling is activated upon injury in satellite cells (light blue), which are the adult muscle stem cells, and promotes proliferation to produce myogenic

progenitors (pink). Asymmetric localization of Notch inhibitor Numb during cell divisions results in two daughter cells: myoblast (blue) and a myogenic progenitor.

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