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Notch and disease: A growing field

Angeliki Louvi[a,*] and **Spyros Artavanis-Tsakonas**[b,c,**]

[a]Departments of Neurosurgery and Neurobiology, Program on Neurogenetics, Yale School of Medicine, New Haven, CT, United States

[b]Department of Cell Biology, Harvard Medical School, Boston, MA, United States

[c]Collège de France, Paris, France

Abstract

Signals through the Notch receptors are used throughout development to control cellular fate choices. Our intention here is to provide an overview of the involvement of Notch signaling in human disease, which, keeping pace with the known biology of the pathway, manifests itself in a pleiotropic fashion. A pathway with such broad action in normal development, a profound involvement in the biology of adult stem cells and intricate and complex controls governing its activity, poses numerous challenges. We provide an overview of Notch related pathologies identified thus far and emphasize aspects that have been modeled in experimental systems in order to understand the underlying pathobiology and, hopefully, help the definition of rational therapeutic avenues.

Keywords

Notch; Pleiotropy; Dominant syndrome; CADASIL

1. Introduction

The Notch signaling pathway is one of the handful of fundamental mechanisms that define the cell signaling backbone of multi-cellular development by controlling cell fates and, consequently, morphogenesis. The outcome of signals transmitted by the Notch receptor, the central element of the pathway, is highly pleiotropic and, in a context specific manner, profoundly affects differentiation, proliferation and apoptotic events throughout development. Its fundamental influence in metazoan development is reflected by the fact that it affects a very broad, if not the entire, spectrum of developing tissues and organs, is intimately and rather generally associated with the maintenance and fate of stem cells, while aberrant signaling invariably leads to mutant phenotypes in every system examined. Thus, not surprisingly, Notch malfunction is associated with human disease and is increasingly valued and explored as a potentially important therapeutic target. Our intention here is to

*Corresponding author at: Department of Neurosurgery, Yale School of Medicine, New Haven, CT, United States. Tel.: +1 203 737 2457. **Corresponding author at: Department of Cell Biology, Harvard Medical School, Boston, MA, United States. Tel.: +1 617 432 7048. angeliki.louvi@yale.edu (A. Louvi), artavanis@cb.med.harvard.edu (S. Artavanis-Tsakonas).

provide an overview of the involvement of Notch in disease, which, keeping pace with the known biology of the pathway, manifests itself in a pleiotropic fashion. The highly pleiotropic action of Notch nevertheless serves what seems to be a rather simple and ubiquitous developmental logic: Notch signals control cellular fates and the segregation of lineages by linking the fate of one cell to that a neighbor, through the interaction of the Notch surface receptor expressed on one cell with membrane bound ligands expressed on the surface of an adjacent cell.

The central element of the pathway is the Notch receptor. It was first cloned and characterized in Drosophila, which has a single receptor [1]. The paradigmatic Drosophila Notch is composed of distinct domains that are essentially conserved across all species. Vertebrate Notch paralogues do display differences in primary sequence, which distinguish them from each other, and have overlapping, yet individual, expression profiles and developmental functions, even though likely interchangeable biochemical functions [2, 3]. The Drosophila Notch protein is approximately 2700 amino acids in length with a 1700-aa, extracellular, cysteine-rich domain harboring 36 tandem Epidermal Growth Factor (EGF)-like repeats [1]. The canonical signaling model has the Notch receptor being activated through a series of proteolytic events after it interacts with the ligands Delta (Dl) or Serrate (Ser) (also called Jagged in vertebrates) [3, 4]. The crucial cleavage event for signaling depends on γ-secretase and results in releasing the intracellular domain of Notch from the membrane. This allows it to translocate into the nucleus, where it directly participates in a core transcriptional complex together with the DNA binding protein Suppressor of Hairless [Su(H)] and the nuclear effector Mastermind, thereby activating the transcription of target genes [3, 4]. With the exception of γ -secretase, which was first implicated in Notch signaling through genetic analyses in *Caenorhabditis elegans* [5], all other core pathway elements were identified in Drosophila where they are represented by single genes. Vertebrates have multiple paralogues of each core element, including four Notch receptors (Notch 1, 2, 3 and 4) and multiple Delta and Jagged ligands.

Over the past few years, mostly through genetic – but now also genomic – approaches, we have come to realize that Notch signals can be attenuated by literally hundreds of genes, showing that the pathway is integrated in an astonishingly complex genetic circuitry, which can influence Notch signaling output [6–9]. Two additional levels of complexity are noteworthy, especially as we consider the involvement of Notch in disease. The first is related to the fact that the developmental outcome of Notch signals is intimately dependent on the “strength” of the signal. Development is exquisitely associated with Notch gene dosage: Notch defines one of the two known genes in Drosophila that are both haploinsufficient as well as triplo-mutant [2, 10, 11]. Thus, in most biological situations including disease [12], the outcome of Notch signals depends on quantitative parameters. Consequently, Notch signaling pathology may be the result of small, abnormal variation, either up or down, of the signal, as indeed we point out below. In addition however, especially as we consider the involvement of Notch in disease, it is relevant that the canonical Notch receptor signaling, which depends on the surface interaction between the receptor and its ligands, is not the only way that the receptor can be activated. Several recent studies from various laboratories including ours, have demonstrated the existence of

alternative receptor activation paths, especially as the receptor is sorted through endocytic compartments [13–15]. Our own studies, for example, demonstrated that a receptor bound towards lysosomal destruction can, under certain circumstances, be diverted into an activation path in a ligand independent fashion [16]. Thus, parameters that lead to such activation may potentially also contribute to disease.

Notch has become a sought after therapeutic target by academics as well as the pharmaceutical industry [17, 18], especially in view of its involvement in cancer, but a pathway with such broad action in normal development, a profound involvement in the biology of adult stem cells and intricate and complex controls governing its activity, poses numerous challenges. In this review we seek to provide an overview of Notch related pathologies identified thus far and emphasize aspects that have been modeled in experimental systems in order to understand the underlying pathobiology and, hopefully, help the definition of rational therapeutic avenues.

2. A short history of Notch in human disease

2.1. Notch as a classic oncogene

The very first association of altered Notch function with human disease came in 1991 with the identification of rare chromosomal rearrangements in human T lymphoblastic leukemias/lymphomas (T-ALL) involving what turned out to be a human ortholog of the *Drosophila* Notch receptor [19]. T-ALL is an aggressive cancer that preferentially affects children and adolescents and is commonly associated with acquired chromosomal translocations and other genetic or epigenetic abnormalities leading to aberrant expression of transcription factors. The translocation breakpoints in the original case of acute T-ALL were mapped within *NOTCH1* on chromosome 9 and the T-cell receptor β locus on chromosome 7, resulting in expression of a truncated, constitutively active Notch 1 receptor. This seminal work led to the cloning of the human Notch 1 cDNA and highlighted the remarkable sequence conservation between the human and the Drosophila Notch receptors, with amino acid sequences being overall 46% identical and 62% similar and with specific regions within the two sequences displaying even greater similarity. The authors' modest proposal that "the human Notch homolog functions in normal lymphoid development and, in rearranged form, may contribute to transformation or progression in *some* T cell neoplasms" [19] proved prophetic. The human Notch 1 receptor is now recognized to be essential for the normal development of T cell progenitors, required for the commitment of progenitors to T cell fate, as well as for the subsequent assembly of pre-T cell receptor complexes in immature thymocytes [20]. Even more importantly, it is now known that the growth of T-ALL cell lines that lack chromosomal translocations depend on Notch-transducing signals, and that more than half of T-ALL involve activating mutations of Notch 1 affecting the so called "homodimerization" or PEST domains [21], the latter being implicated in Notch protein turnover [3]. Aberrant activation of Notch 1 receptors is therefore central to the pathogenesis of T-ALL. The elucidation of the mechanisms by which Notch 1 mutations lead to pathology and the exploitation of Notch 1 as a therapeutic target in T-ALL are areas of active research and are discussed elsewhere in this issue (Aster and Fortini reviews). Suggesting a broader involvement of Notch in humans cancers, activating mutations in

Notch 2 have recently been discovered in a subset of diffuse large B-cell lymphomas, a subtype of mature B-cell lymphomas. These mutations lead to partial or complete deletion of the PEST domain, or a single amino acid substitution at the C-terminus of Notch2 protein. Some diffuse large B-cell lymphoma cases have also been found to harbor increased copies of the mutated Notch2 allele [22].

These findings confirmed the fact that Notch can act as an oncogene, supporting in vitro studies that indicated that the expression of the intracellular domain of Notch 1 or Notch 2 could drive focus formation in cultured mammalian cells. Tumor models in mice and other animal systems have extended these observations, associating abnormal Notch activity with solid tumors in addition to leukemias, linking all four Notch receptor paralogues to oncogenic events [23–26]. True to its context-dependent nature, it has been suggested that Notch functions as a tumor suppressor rather than an oncogene in skin tumor mouse models [27].

While Notch 1 is clearly a major oncogene in T-ALLs, the potential involvement of abnormal Notch signaling in other human cancers is far from established. It remains a remarkable fact that in spite of an ever-growing number of correlative studies linking Notch activity with almost all major solid tumors, searches for mutations in other cancers remains essentially unfruitful, notwithstanding a few suggestive reports [28] and an association in cutaneous squamous cell carcinomas with loss of function mutations in Notch 1 [29]. It is important to keep in mind, however, that there is a clear association between proliferative events and Notch activation in both a cell autonomous as well as a cell non-autonomous fashion. Based of models we have developed in mice [30, 31] involving the intestine and the mammary gland, Notch activation can clearly trigger proliferation, likely in synergy with other cellular activities, but this does not lead to cancer per se. Such hyper-proliferative states, however, can eventually lead to bona fide oncogenic events. As we have argued before, Notch signaling, presumably in synergy with other factors, may result in dramatically expanding cell populations, such as stem cells, that are prone to accumulating oncogenic mutations [30]. Thus Notch activation may have profound consequences for oncogenesis but may not be oncogenic per se. Such a role may not be readily addressed by targeting Notch pharmacologically.

2.2. Notch in hereditary pleiotropic disease

Two studies published in 1997 [32, 33] provided the first association of Notch signaling with *pleiotropic* human disease linking mutations in *JAGGED1*, which encodes a ligand for the Notch receptors, to Alagille syndrome (MIM 118450) [34], an autosomal dominant multisystem disorder of incomplete penetrance and variable expressivity with developmental abnormalities that involve many tissues and organs, including liver, skeleton, kidney, heart and face. The disorder has been traditionally defined by a paucity of intrahepatic bile ducts associated with clinical features that include chronic liver disease, cardiac disease, skeletal abnormalities, to list only the most common ones [35]. Again, it was the analysis of rare cytogenetic deletions in patients with Alagille syndrome that pointed to *JAGGED1*, and the identification of frameshift and splice site mutations confirmed the causative link [32, 33]. *JAGGED1* haploinsufficiency was proposed to underline Alagille syndrome [33],

highlighting for the first time in the context of human disease, that dosage of Notch signaling does not only manifest itself in Drosophila. The extraordinarily pleiotropic manifestation of the human *JAGGED1* mutations reflected in the wide spectrum of developmental abnormalities of Alagille syndrome patients was in concert with the broad developmental action of Notch, as it had become evident by that time by studies in *Drosophila*, but also from early studies in vertebrates already implicating Notch activity in a wide range of tissues and organs. Within a decade of the identification of *JAGGED1* mutations in the vast majority of patients, Alagille syndrome proved to be in reality a heterogeneous disorder of Notch signaling, with the discovery of premature termination and missense mutations in *NOTCH2* [36, 37], echoing observations in mice that *Notch 2*, perhaps not surprisingly given what we know about the genetic interactions between the ligand and the receptor in flies, is a genetic modifier of *Jagged1* haploinsufficiency in a model of Alagille syndrome [38]. The Alagille syndrome is discussed elsewhere in this issue (Spinner review).

2.3. Notch and the skeleton

Skeletal abnormalities of Alagille patients not withstanding, Notch pathway elements have been implicated in patterning the mammalian axial skeleton and long associated with skeletal disorders. The initial hint came from studies of a classic mouse mutant, *pudgy* [39], whose severe vertebral and rib deformities were found to be caused by a mutation in *Delta-like 3* (*Dll3*), encoding a Notch ligand [40]. These defects are similar to phenotypes of patients with spondylocostal dysostosis (SCD, MIM 277300), a group of vertebral malsegmentation syndromes with reduced stature resulting from axial skeletal defects, another paradigm of a Notch pathway disorder par excellence. The disease can be sporadic or familial, with both autosomal dominant and autosomal recessive modes of inheritance. The realization that autosomal recessive SCD maps to an interval on chromosome 19 that is homologous with a mouse region containing *Dll3*, lead to the identification of truncating and missense mutations in the human orthologue, *DLL3* [41, 42]. The underlying mechanism appears to involve Dll3-mediated cis-inhibition of Notch signaling, with mutant Dll3 targeting newly synthesized Notch 1 receptor for lysosomal degradation prior to its post-translational processing and cell surface presentation [43]. Inactivating mutations in *LUNATIC FRINGE*, encoding a glycosyl-transferase that modifies, and thus regulates, the Notch receptors, also cause severe SCD [44]. The mutations interfere with the sub-cellular localization of the protein, which appears to have lost its enzymatic activity [44]. Autosomal recessive SCD mutations have also been identified in *HES7*, encoding a basic helix-loop-helix transcriptional repressor of the Hairy-and-Enhancer-of-Split family; Hes7 is both a direct target of the pathway, and part of a negative feedback mechanism required to attenuate Notch signaling. A missense mutation that interferes with DNA binding and protein heterodimerization was identified in the DNA-binding domain [45], whereas two additional missense mutations were detected in a single family, with only compound heterozygotes being affected by SCD; at least one of these two mutations appears to disrupt DNA binding or protein heterodimerization [46]. Adding to the growing list of Notch pathway elements involved in SCD, mutations in *MESP2*, encoding another basic helix-loop-helix transcription factor that is a direct target of Notch signaling, cause both a rare form of SCD, as well as a related disorder, spondylothoracic dysostosis [47, 48]. Notch

pathway elements mutations in autosomal dominant forms of SCD are yet to be identified [49]. Needless to say, deletion of these genes in the mouse produces phenotypes with similar vertebral defects to those observed in human congenital syndromes (reviewed in [50]).

2.4. Notch in metabolic bone disease

If 'traditional' gene discovery efforts were not fruitful enough to implicate Notch in pathogenic conditions, enter exome sequencing in 2011, which like other genomic approaches such as deep sequencing, transcriptional profiling, offer a new level of resolution and hence means for phenotype/genotype correlations. Thus, *NOTCH2* has been identified as the culprit in Hajdu–Cheney syndrome (MIM 102500) [51, 52], a rare, mostly sporadic, but with autosomal dominant inheritance in a handful of families, multisystem disorder of the connective tissues, characterized by severe and progressive focal bone loss, generalized osteoporosis and variable craniofacial abnormalities and renal cysts. The mutations cluster in the last exon of *NOTCH2*, and are predicted to lead to premature termination, disrupting or even eliminating the PEST domain, and possibly causing elevated Notch signaling [53–55]. Analogous *NOTCH2* mutations are also present in another very rare syndrome, Serpentine fibula polycystic kidney syndrome (MIM 600330) that shares many similarities with Hajdu–Cheney syndrome, suggesting that perhaps the two may be related [56, 57]. These findings are significant as they have wider implications beyond the small group of patients with these rare syndromes given that they support a broader role of Notch signaling in metabolic bone disease. Consistent with this, a recent genome-wide association study that identified *JAGGED1* as a candidate gene for bone mineral density regulation and a potential risk factor for fracture [58]. Moreover, analysis of mice lacking *Notch1* and *Notch2* in limb skeletogenic mesenchyme, develop a complex age-related bone phenotype: in adolescence, they have increased bone mass, but develop osteopenia as they age, implicating Notch signaling in bone homeostasis reflecting its role in osteoblast-lineage cells [59]. Accordingly, constitutive activation of Notch 1 signaling in osteoblasts causes severe osteosclerosis, a consequence of increased proliferation of immature osteoblasts, and conversely, loss of Notch activity in osteoblasts (in mice without presenilins, and hence, γ-secretase activity) results in late-onset, age-related osteoporosis [60]. The underlying cause seems to be the misregulation of Notch targets including osteoblastic transcription factors (e.g. Runx2) and cell cycle proteins.

2.5. Notch in cardiovascular disease

Given the broad, pleiotropic and context-dependent action of Notch signaling, its involvement in human congenital cardiovascular disease is not surprising, in view of a plethora of mouse genetic analyses demonstrating the importance of the Notch pathway in various aspects of cardiovascular development, with many mutants displaying either lethal cardiovascular defects or vascular abnormalities (see [61, 62, 119] for a detailed review of Notch signaling in cardiac development and disease). *NOTCH1* nonsense and frameshift mutations have been identified in familial and sporadic forms of aortic valve disease [63, 64], which is characterized by structural defects of the aortic valve and high rate of valve calcification in adulthood. The underlying mechanism here may also be related (see above) to deregulation of Notch-mediated repression of Runx2, a transcription factor critical for osteoblast cell fate [63]. Furthermore, cardiovascular anomalies are among the most

common features of Alagille syndrome [65], and *JAGGED1* mutations have been found in patients with isolated congenital heart defects, including tetralogy of Fallot or pulmonic stenosis [66–68], thus implicating Notch more broadly in cardiovascular disease.

2.6. Notch in cerebrovascular disease

2.6.1. Ischemic stroke and vascular dementia—The identification in 1996 of dominant mutations in *NOTCH3* in patients with CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) [69], the most common monogenic form of ischemic stroke, linked for the first time Notch signaling pathway in hereditary disease. To date, more than 500 families with the disease have been identified worldwide, with close to unique 200 mutations reported. The overall prevalence is unknown (1/50,000 but likely underdiagnosed), and in addition to the hereditary forms rare sporadic cases have been reported [70, 71], as have homozygous patients with phenotypes not different from heterozygotes [72, 73]. The clinical presentation of the syndrome varies, and includes subcortical ischemic events, cognitive impairment and dementia, migraine with aura, mood disturbances and apathy [74]. Currently, there is no treatment of proven efficacy. The arteriopathy affects mainly the small penetrating cerebral and leptomeningeal arteries and is characterized by thickening of the arterial wall and prominent morphological alterations on vascular smooth muscle cells and pericytes [74–76]. A specific, and indeed pathognomonic, ultrastructural feature of the disease is the presence of granular osmiophilic material (a.k.a. GOM) close to the cell surface of smooth muscle cells and pericytes in brain and skin arteries [76–79]; these deposits are mostly extracellular and of variable morphology, size, shape, and osmiophilic density. Their detection by electron microscopy in skin biopsies represents a highly reliable diagnostic method [80]. Their composition, however, had remained enigmatic until recently, when direct proteomic analysis of blood vessels from post-mortem brains of CADASIL patients identified for the first time components of the GOM, including the proteins clusterin and collagen 18 alpha 1/endostatin [81].

An extraordinary feature of CADASIL is the commonality of the mutations in the Notch 3 receptor causing the disease. They are highly stereotypical, affecting exclusively the extracellular domain and occur in exons 2–24, encoding the EGF-like repeats, with a strong clustering in exons 3 and 4 [82]. More than 95% of the mutations are missense, whereas the remaining are small in-frame deletions or splice site mutations. Importantly, all mutations lead to an odd number of cysteine residues within the affected EGF-like repeat.

Elucidating the functional, structural and cell biological consequences of extracellular Notch 3 receptor mutations underlying CADASIL is indeed a challenging biological problem. Perhaps surprisingly, given that the mutations were identified more than 15 years ago, the jury is still out, despite considerable effort from many groups. In spite of numerous hypotheses on the nature of the CADASIL-associated Notch 3 mutations [83–86], their impact on receptor function, as well as the molecular link to the pathophysiology of the syndrome, remain poorly understood. Early attempts using in vitro assays are difficult to compare as they were based on heterologous experimental systems [87–91]. More recently, it was found that at least certain CADASIL mutations (R133C, C183R and C455R) enhance

the formation of higher order multimers of the Notch 3 extracellular domain, thus inviting proposals that the mutations are in fact neomorphic, i.e. conferring a novel function to the mutant receptor [92]. Subsequently, mutant Notch 3 truncated peptides (containing EGF-like repeats 1–5 and harboring the R133C or the C183R mutations) transiently overexpressed in HEK293 cells and collected from conditioned media, were shown to aggregate, as they also did when mixed with wild type peptides [93]. These observations are interesting, but should be interpreted with caution, as they involve only a small portion of the Notch 3 extracellular domain. On the other hand, overexpression of full-length wild type or mutant (R133C, C185R) Notch 3 receptor in HEK293 cells resulted in increase of the expression of ER-resident protein folding chaperones. It was further observed that the mutant receptors form dot-like cytoplasmic aggregates in the perinuclear region more readily than wild type counterparts, accumulate in the ER and appear to be highly resistant to degradation by the ERAD system. Cells expressing mutant Notch 3 were markedly sensitive to proteasome inhibition, leading to cytotoxicity associated with accumulation of mutant Notch [94]. Although suggestive of the possible cell biological consequences of the CADASIL-linked Notch 3 mutations, such results from the cell culture system must be corroborated in vivo.

2.6.2. The Notch 3 knockout(s)—Modeling CADASIL in mice and more generally using mice to understand Notch 3 biology has been the approach of choice to explore Notch 3 function.

The first Notch 3 loss of function mouse model examined, which lacks EGF-like repeats 8–12 [95] and is likely to be a functional null, was found to have defects in tail arteries that were attributed to impaired differentiation and maturation of vascular smooth muscle cells [96]. A second knockout model showed some defects in the thymus [97], while yet another, similarly viable and fertile [98], did not show any abnormalities in brain vessels or the aorta [99], but had reduced levels of the Notch 3 target PDGFRβ in tail arterial smooth muscle cells [100]. More importantly, these Notch 3 mutants displayed a striking susceptibility to ischemic stroke upon challenge, a phenotype that could be rescued by directing the expression of wild type Notch 3 specifically in smooth muscle cells, thus establishing an unambiguous link between Notch 3 function in vessels and susceptibility to ischemia [99]. Molecular profiling of brain-derived smooth muscle cells from this model revealed significant functional differences between knockout and control cells, including downregulation of genes implicated in muscle contraction, and variable misregulation of genes involved in cell structure and motility, and in muscle and mesoderm development [99]. The susceptibility to stroke upon challenge uncovered in this study defined an assay, albeit quite involved, to examine and compare the functionality of CADASIL-linked mutations in vivo (see below).

2.6.3. Mouse models of CADASIL: lessons from knock-ins and transgenics—Several attempts to model the disease and gain insight into Notch 3 (patho)biology, have been reported over the years [101]. Intuitively, a knock-in approach seemed best, as it does not disturb gene dosage, an essential parameter of Notch signaling, but proved however not very informative. The first knock-in mouse harboring the R142C mutation (the mouse equivalent of the common human mutation R141C) in the endogenous Notch 3 locus had no

discernible phenotype, despite an exhaustive analysis [102]. With the benefit of hindsight (see below), this may be explained by the fact that in order for phenotypic consequences to occur, higher than endogenous levels of expression of the mutant receptor may indeed be necessary, in the context of this organism. Another possible explanation may be that the neurological and histopathological abnormalities only manifest in old age and with incomplete penetrance, as suggested by a more recent knock-in mouse harboring the R170C mutation [103], necessitating large numbers of animals to be analyzed. These latest knock-in mice did indeed develop age-dependent GOM-like deposits in brain arteries and peripheral arteriopathy (but no evidence of accumulation of the Notch 3 extracellular domain, a frequently reported finding in transgenic mice overexpressing mutant receptors); a few also developed some histopathological brain abnormalities and motor defects [103].

In addition to the knock-in models, various strains of transgenic mice have been reported [81, 104–107]. We discuss here in more detail models that take into account quantitative aspects of Notch, while keeping in mind that Notch signaling depends on (and thus can be affected by) feedback loops that may further complicate the analysis. These models differ both in design and in choice of mutations. On one hand, traditional approaches placing human Notch 3 transgenes (wild type or harboring CADASIL-linked mutations) under the control of the smooth muscle cell specific SM22a promoter or PAC transgenesis, that enables a transgene – in this case the rat *Notch 3* locus – to utilize its cognate regulatory sequences, are both influenced by insertion site and transgene copy number, and necessitate careful screening to identify transgenic lines that express the wild type or mutant receptors at similar levels [105–107]. These considerations aside, such models do indeed recapitulate several aspects of the disease, as detailed below. On the other hand, mice engineered to express conditionally the human Notch 3 (again, either wild type, or harboring CADASIL mutations) from the ROSA26 locus following SM22a-Cre mediated excision of a stop cassette, thus ensuring comparable and tightly controlled levels of expression, also develop disease-linked phenotypes [81]. So far analyzed are R90C, a recurrent mutation located within the mutational hotspot region encompassing EGF-like repeats 2–5 and associated with a classical CADASIL phenotype in patients [105]; R169C, also in the mutational hotspot [107]; C428S, located in the ligand-binding domain of the receptor [106]; and finally, C455R and R1031C, respectively mapping to the ligand-binding domain, and to the 26th EGF-like repeat, and importantly associated with distinct clinical phenotypes in two large Colombian families, the former being apparently more severe with the median age of onset of stroke preceding by more than two decades that of individuals carrying the latter [81, 108].

Given the different experimental designs, these transgenic mice are not directly comparable, but in general, it appears that high levels of transgene overexpression (about 4-fold over the endogenous expression level) are indeed required to recapitulate a broad spectrum of CADASIL features [107]. All models develop osmiophilic deposits whose similarity with the human GOMs remains to be proven, as well as vascular smooth muscle cell abnormalities [81, 105–107]; however, extensive cerebral white matter damage is only seen in aged mice with significant levels of transgene overexpression [107].

Among these transgenic mice, two classes can be compared directly and thus can give some insight into the functionality of the mutations in question. The first comprises mice expressing the R90C or C428S mutations (or the wild type sequence) under the control of the SM22a promoter, at about physiological levels [105, 106]: the R90C mutant receptor appears to be equally potent as the wild type in rescuing the arterial defects of *Notch3* mutant mice, suggesting it remains functional and does not exhibit dominant/negative activity, further retaining the ability to elicit Notch 3-mediated RBP-Jk transcription in brain arteries of TP1-nLacZ reporter mice (these mice [109, 110] harbor a lacZ reporter transgene linked to 12 RBP-Jk binding motifs upstream from a minimal promoter) [105]. In contrast, the C428S mutant receptor appears to have lost wild type activity in similar assays, failing to rescue the arterial defects of the *Notch3* mutants, and exhibiting instead mild dominant/ negative activity [106].

The second group comprises mice expressing identical levels of either the C455R or the R1031C mutant receptors (or the wild type) in vascular smooth muscle cells [81]. When assessed in the in vivo assay mentioned above [99] for their ability to rescue the stroke susceptibility phenotype of the *Notch3* mutants, both behaved as hypomorphic receptors: the C455 mutant receptor failed to rescue the stroke susceptibility phenotype of the *Notch 3* mutants, whereas the R1031 receptor rescues the phenotype only in young, but not in aged, *Notch 3* mutants, therefore uncovering age-dependent phenotypes and suggesting that the two mutant receptors are of different strength, and remarkably reflect the severity of the equivalent mutations in CADASIL patients [81]. Complementing the in vivo observations, both mutants behave as partial loss-of-function receptors in a newly developed cell-based assay designed to address receptor activity in a quantitative manner [81].

Taken together, these observations suggest that the impact of the different mutations on the activity of the Notch 3 receptor vary. A common denominator is the age-dependent appearance of phenotypes, to some extent correlating with human phenotypes. At present, notwithstanding the quantitative nature or the level of sensitivity of the available assays, it is not unreasonable to entertain the hypothesis that the CADASIL-linked mutations have hypomorphic receptor activities but retain neomorphic attributes, e.g. GOM formation, which manifest themselves in an age dependent manner. More experimentation needs to be carried out in order to examine the generality of this conclusion but if true, one could imagine that a therapeutic avenue may not be geared towards the elimination of the neomorphic phenotypes, which may conceivably be the consequence of Notch receptor hypomorphism, but rather towards preventing their appearance in the first place.

3. What next?

The involvement of the Notch pathway in human disease is firmly established. In addition to the paradigms discussed above, representing causal links between Notch pathway mutations and human disease, examples exist in the literature sketching other possible associations. For example an association between Notch and multiple sclerosis (MS) has been suggested. MS is an inflammatory demyelinating disease of the central nervous system that results form destruction of the protective myelin sheaths that surround and protect axons, and is thought to be mediated by an immune attack directed against myelin-producing oligodendrocytes.

While Notch has been clearly implicated in the control of oligodendrocyte differentiation and myelination [111, 112], and hence conceivably implicated in MS, the data presented thus far may be intriguing but far from conclusive [113–115]. Notch has also recently been associated with tuberous sclerosis, a dominantly inherited multisystem disease characterized by the growth of multiple benign tumors that develop in many organs, caused by inactivating mutations in TSC1 and TSC2, whose normal function is to inhibit the activation of the mammalian target of rapamycin signaling. TSC loss appears to be associated with upregulation of Notch signaling [116, 117], however again in this case, the disease link with Notch activation is quite opaque [118].

There is no doubt in our minds, however, that the arena encompassing Notch pathologies will be enlarged as the search for the molecular underpinnings of human pathologies goes forward. The fundamental nature of the biology influenced by or dependent on Notch signals is so broad, that predicting that more pathologies will be associated with Notch does not require particular insight. However the great pleiotropy of Notch presents also a great challenge, as modulating its activity for therapeutic purposes will be highly dependent on the cellular context and quantitative aspects of signal modulation, carrying thus implicitly the danger of unacceptable toxicities. Nevertheless it is clear that such challenges can only be addressed through a deep understanding of the Notch biology, keeping thus many of us busy for years to come.

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Abbreviations

EGF	epidermal growth factor
T-ALL	T cell acute lymphoblastic leukemia
MIM	Mendelian Inheritance in Man
SCD	spondylocostal dysostosis
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
GOM	granular osmiophilic material
ER	endoplasmic reticulum
ERAD	endoplasmic-reticulum-associated protein degradation
MS	multiple sclerosis
PAC	P1 artificial chromosome

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