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Notch signaling: simplicity in design, versatility in function

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Summary

Notch signaling is evolutionarily conserved and operates in many cell types and at various stages during development. Notch signaling must therefore be able to generate appropriate signaling outputs in a variety of cellular contexts. This need for versatility in Notch signaling is in apparent contrast to the simple molecular design of the core pathway. Here, we review recent studies in nematodes, *Drosophila* and vertebrate systems that begin to shed light on how versatility in Notch signaling output is generated, how signal strength is modulated, and how cross-talk between the Notch pathway and other intracellular signaling systems, such as the Wnt, hypoxia and BMP pathways, contributes to signaling diversity.

Key words: Cis-inhibition, Delta-like, Signaling diversity, Jagged, Notch, Notch intracellular domain

Introduction

Cells need to sense cues from their extracellular environment and integrate this information into appropriate developmental or physiological responses. Although there are a number of mechanisms that relay information from the exterior of the cell to the interior, a relatively small set of highly evolutionarily conserved signaling pathways stand out as playing particularly crucial roles in this transmission of information. In this roster of 'elite' intracellular signaling mechanisms are the Wnt pathway, the sonic hedgehog (Shh) pathway, the bone morphogenetic protein/transforming growth factor β (BMP/TGFβ) pathway, phosphatidylinositol 3-kinase/thymoma viral proto-oncogene (PI3K/AKT) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling, and, the subject of this review, the Notch signaling pathway. Each of these pathways converts information about the concentration of extracellular ligands into specific transcriptional responses in the nucleus. In most cases, the signaling mechanism consists of the 'core' signaling pathway, i.e. the minimal set of protein components required for transducing the signal, and a more elaborate set of 'auxiliary' proteins, which, in various ways, impinge upon the core pathway and modify the signal but are not intrinsically necessary for relaying the signal.

Among these highly conserved pathways (Gazave et al., 2009; Richards and Degnan, 2009), the Notch signaling pathway scores highly with regard to simplicity in molecular design, as it contains only a small number of core signaling components (Fig. 1). Despite this, Notch signaling affects cell differentiation decisions not only across a wide spectrum of metazoan species, but also across a broad range of cell types in a single organism and at different steps during cell lineage progression. The pleiotropic actions of Notch in different cell types and organs have recently been reviewed (Liu et al., 2010) and are summarized in Table 1. In keeping with its important role in many cell types, the mutation of Notch genes leads to diseases in various organs and tissues (Table 2). These studies highlight the fact that the Notch pathway must be able to elicit appropriate responses in many spatially and temporally distinct cell contexts.

In this review, we address the conundrum of how this functional diversity is compatible with the simplistic molecular design of the Notch signaling pathway. In particular, we focus on recent observations, in both vertebrate and invertebrate systems, that begin to shed light on how diversity is generated at different steps in the signal transduction pathway and how signal strength itself is modulated in Notch signaling output.

The core Notch pathway

The core Notch pathway has a simple molecular architecture (Fig. 1). The most extensively characterized signaling pathway initiated in response to Notch ligands is known as the canonical Notch signaling pathway. In canonical Notch signaling, a Notch transmembrane receptor interacts extracellularly with a canonical Notch transmembrane ligand on a contacting cell, initiating proteolytic cleavage of the receptor and the subsequent release of the Notch intracellular domain (Notch ICD or NICD) of the receptor. Notch ICD then translocates to the nucleus where it interacts with a CBF1/Suppressor of Hairless/LAG-1 (CSL) family DNA-binding protein {C promoter-binding factor (CBF1) is also known as recombination signal binding protein for immunoglobulin kappa J region (RBPJ-κ) or kappa-binding factor 2 (KBF2) in mammals, as Suppressor of Hairless [Su(H)] in flies and Longevity-assurance gene-1 (LAG-1) in *C. elegans*} and initiates the transcription of Notch target genes (Fig. 1). Non-canonical Notch signaling differs from canonical signaling in that it can be initiated by a non-canonical ligand, or may not require cleavage of the Notch receptor. Alternatively, in some forms of non-canonical signaling there is no involvement of CSL, which may reflect interactions with other signaling pathways upstream of the Notch ICD-CSL interaction. Non-canonical Notch signaling has recently been reviewed (D'Souza et al., 2010; Heitzler, 2010) and is outside the scope of this review. Here, we focus on the multitude of mechanisms that are utilized to generate diversity from the otherwise simple canonical Notch pathway.

A conspicuous feature of the core canonical Notch pathway is the lack of an amplification step during signal transduction; this is in contrast to most other pathways, which have integrated signal amplification steps, for example in the form of phosphorylation of one or more of the core pathway proteins. In addition, each activated Notch receptor molecule is consumed during signaling, yielding one NICD, suggesting that Notch signaling exhibits a stoichiometric relationship between signaling input and output and that signaling strength is important for generating the appropriate cellular response. In keeping with this line of reasoning, the Notch pathway is indeed very sensitive to gene dosage deviations, and

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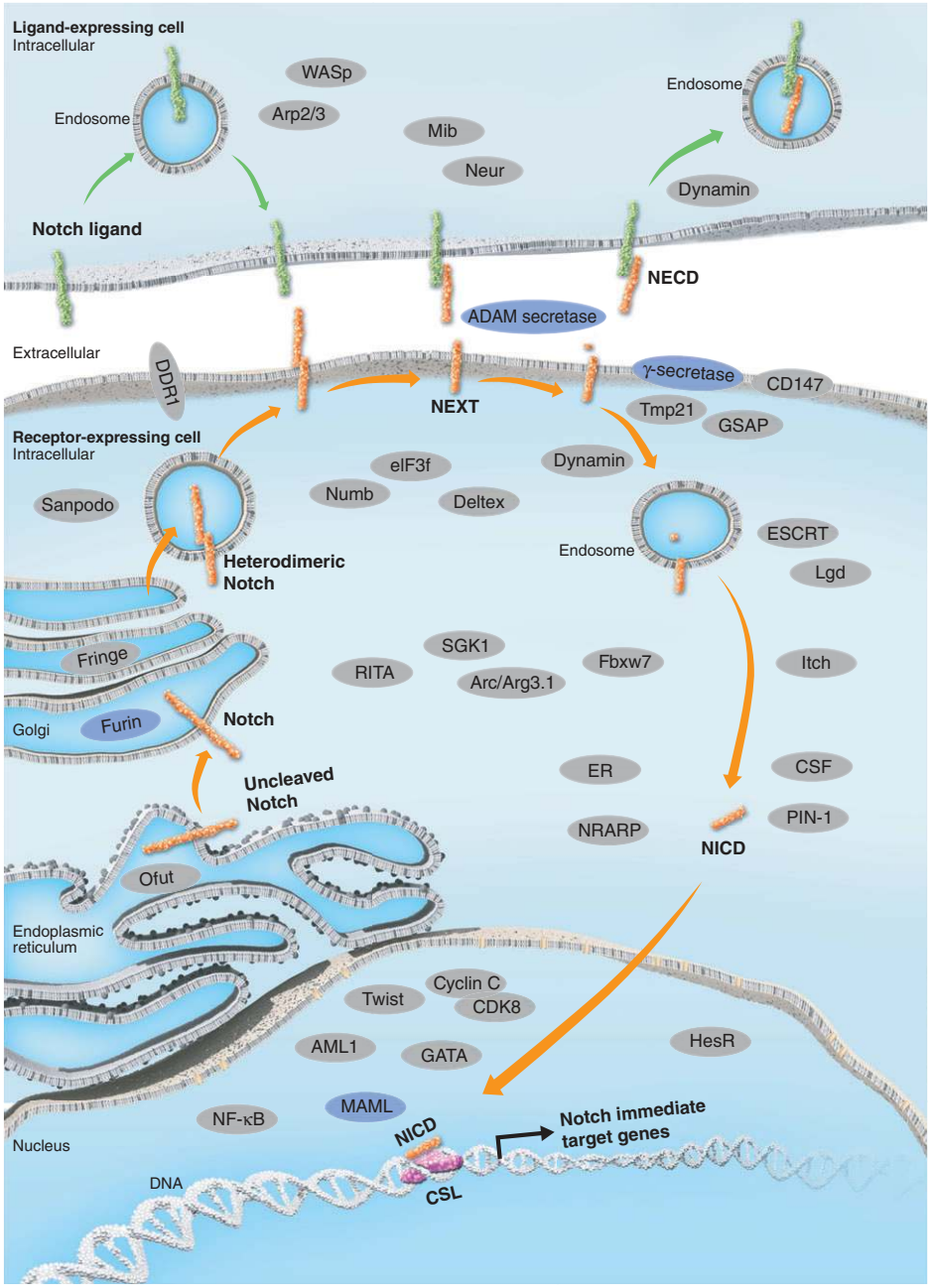

Fig. 1. The Notch pathway: simplicity and complexity in one. The core Notch pathway contains a limited set of components that form the signal-transmitting chain in the pathway: a ligand (green), a Notch receptor (orange) and the transcription factor CSL (pink). In addition, some components (furin, ADAM secretase, γ-secretase and MAML; blue ovals) are not part of conveying the signal but are nevertheless crucial for allowing the signal to be transmitted from one step to the next in the pathway. Briefly, the Notch receptor is synthesized as a single transmembrane receptor that is Furin-cleaved to yield a bipartite heterodimeric Notch receptor, which is expressed on the cell surface of a 'receptor-expressing' cell. This receptor can be activated at the plasma membrane by binding to Notch ligands on 'ligand-expressing' cells. This leads to the removal of the extracellular domain of Notch, which is then targeted for lysosomal degradation. The remaining portion of the receptor, termed the Notch extracellular truncated (NEXT) domain, undergoes sequential cleavage by ADAM secretases and γ-secretase as it becomes endocytosed, yielding the Notch intracellular domain (NICD). NICD then translocates to the nucleus where it binds the DNA-binding protein CSL (CBF1/Suppressor of Hairless/LAG-1) and activates the transcription of Notch target genes. This simple signaling pathway can be modified in a number of ways by a growing roster of auxiliary proteins (gray), which influence various stages of the transduction process and contribute to signal diversity. AML1, acute myeloid leukemia 1 (also known as RUNX1); DDR1, discoidin domain receptor family, member 1; NECD, Notch extracellular domain; RITA, RBP-J interacting and tubulin associated.

both haploinsufficiency and the presence of extra copies of the *Notch* gene in *Drosophila* result in aberrant phenotypes (Fanto and Mlodzik, 1999; Lyman and Yedvobnick, 1995; Mohr, 1919). Furthermore, mice haploinsufficient for *Notch1* display supernumerary hair cells in the inner ear (Zhang et al., 2000), whereas mice haploinsufficient for one of the Notch ligands (*Dll4*$^{+/-}$ mice) are embryonic lethal (Krebs et al., 2004). In human, haploinsufficiency of *NOTCH2* or jagged 1 (*JAG1*), which encodes a Notch ligand, is observed in Alagille syndrome (McDaniell et al., 2006), a broad-spectrum syndrome characterized by liver, heart and eye defects as well as vertebral malformations (Alagille et al., 1987; Alagille et al., 1975), and *NOTCH1* haploinsufficiency is also seen in aortic valve disease (Garg et al., 2006).

In addition to the components in the core pathway, a growing roster of auxiliary proteins has been shown to affect Notch signaling at various steps of the signal transduction pathway. Such auxiliary proteins range from intracellular proteins that affect ligand intracellular trafficking in the signal-sending cell, such as

Table 1. Notch signaling regulates numerous developmental processes

Organ/tissue	Processes regulated	References
Brain	Controls the balance between gliogenesis and neurogenesis; stem cell maintenance; apicobasal polarity of neuroepithelial cells	(Ohata et al., 2011) (reviewed by Tanigaki and Honjo, 2010)
Breast	During pregnancy: alveolar development, maintenance of luminal cell fate, prevention of uncontrolled basal cell proliferation	(Buono et al., 2006)
Craniofacial structures	Palate morphogenesis: loss of Notch signaling results in cleft palate, fusion of the tongue with the palatal shelves and other craniofacial defects; Alagille syndrome includes craniofacial defects; also involved in tooth development	Jag2 (Jiang et al., 1998), Jag2/Notch1 (Casey et al., 2006), Dll3/Notch1 (Loomes et al., 2007), Jag1 (Li et al., 1997), tooth development (Mitsiadis et al., 2005)
Ear	Defines the presumptive sensory epithelium, determines hair cell and supporting cell fates	CSL (Yamamoto et al., 2011), Jag1 (Kiernan et al., 2006) (reviewed by Cotanche and Kaiser, 2010)
Esophagus	Regulates esophageal epithelial homeostasis	(Ohashi et al., 2010)
Eye	Fiber cell differentiation in the lens/lens development	CSL/Notch1 (Rowan et al., 2008; Jia et al., 2007), Jag1 (Le et al., 2009)
Heart	Cardiac patterning, cardiomyocyte differentiation, valve development, ventricular trabeculation, outflow tract development	(Reviewed by MacGrogan et al., 2010)
Hematopoietic system (including immune and lymphatic systems)	Required for the second wave of hematopoiesis in development; controls the balance of B-cell versus T-cell development; maintenance of hematopoietic stem cells; maintenance of myeloid homeostasis	(Reviewed by Bigas et al., 2010)
Intestine	Controls proliferation and differentiation (including absorptive fate versus secretory fate choices)	(Reviewed by Heath, 2010)
Kidney	Notch2 defines cell fate of podocytes and proximal tubules	(Cheng et al., 2007)
Limbs	Apical ectodermal ridge (AER) formation and digit morphogenesis, especially regulation of apoptosis	Notch1/Notch2 (Pan et al., 2005), Notch1/Jag2 (Francis et al., 2005), Jag1 (McGlinn et al., 2005), Jag2 (Jiang et al., 1998), Hairy (Notch target gene) (Vasiliauskas et al., 2003)
Liver	Regulates ductal plate formation and intrahepatic bile duct morphogenesis in mice	Notch2 (Geisler et al., 2008; Zong et al., 2009), Notch2/Jag1 (Lozier et al., 2008), Jag1 (Li et al., 1997)
Lungs	Lateral inhibition between tracheal cells prevents extra cells from assuming the lead position during tracheal branching morphogenesis	(Ghabrial and Krasnow, 2006)
Muscle	Promotes transition of activated satellite cells to highly proliferative myogenic precursor cells and myoblasts; prevents myoblast differentiation into myotubes after injury	(Reviewed by Tsivitse, 2010)
Neural crest	Controls patterning of neural crest precursors for the outflow tract region of the heart; regulates the transition from Schwann cell precursor to Schwann cell, controls Schwann cell proliferation and inhibits myelination; controls melanocyte stem cell maintenance	(Reviewed by Jain et al., 2010; Mirsky et al., 2008; Schouwey and Beermann, 2008)
Pancreas	Specifies endocrine cell differentiation through lateral inhibition: endocrine lineage cells inhibit endocrine differentiation of their neighboring cells; maintains pancreatic endocrine precursor cells, inhibits terminal acinar cell differentiation; controls pancreatic epithelium branching and bud size	(Reviewed by Kim et al., 2010)
Pituitary	Regulates pituitary growth/proliferation, melanotrope specification and gonadotrope differentiation	Hes1 (Monahan et al., 2009; Raetzman et al., 2007), Notch2 (Raetzman et al., 2006) (reviewed by Davis et al., 2010)
Placenta	Controls fetal angiogenesis, maternal circulatory system development, spongiotrophoblast development	(Reviewed by Gasperowicz and Otto, 2008)
Prostate	Required for epithelial differentiation and growth; expressed by progenitors that are required for branching morphogenesis (Notch1); stromal survival [Notch2 and Delta-like 1 homolog (Dlk1)]	(Wang, X. D. et al., 2006; Wang et al., 2004; Orr et al., 2009)
Sex organs and germ cells	Maintenance of Leydig progenitor cells in testis; regulation of spermatogenesis; controls oocyte growth via actomyosin-dependent cytoplasmic streaming and oocyte cellularization	(Tang, H. et al., 2008; Hayashi et al., 2001; Nadarajan et al., 2009) (reviewed by Barsoum and Yao, 2010)
Skin	Regulates cell adhesion, control of proliferation, hair follicle or feather papillae differentiation and homeostasis	(Reviewed by Hayashi et al., 2001)

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Table 1. Continued

Organ/tissue	Processes regulated	References
Spine/spinal cord/somites	Somite segmentation through oscillation of genes	(Reviewed by Dunwoodie, 2009; Kageyama et al., 2010)
Spleen	Regulates generation of T lineage-restricted progenitors and marginal zone (MZ) B-cell development; controls homeostasis of $CD8^-$ dendritic cells in the spleen	(Reviewed by Yuan et al., 2010)
Stomach	Acts as a switch in choice between luminal and glandular cell fates	(Matsuda et al., 2005)
Thymus	Thymic morphogenesis, differentiation of gamma delta lineage T-cells	(Jiang et al., 1998)
Thyroid	Regulates the numbers of thyrocyte and C-cell progenitors and regulates differentiation and endocrine function of thyrocytes and C-cells	Hes1 (Carre et al., 2011)
Vasculature	Regulates arteriovenous specification and differentiation in endothelial cells and vascular smooth muscle cells; regulates blood vessel sprouting and branching	(Reviewed by Gridley, 2010)

Mind bomb (Mib) (Itoh et al., 2003) and Neuralized (Neur) (Yeh et al., 2001), to proteins that are important for regulating Notch ICD and CSL interactions, such as Mastermind-like (MAML) (Jeffries et al., 2002; Wu et al., 2002). Some of the most important auxiliary proteins are depicted in Fig. 1. In the following sections, we explore how modulations of the Notch pathway at different steps of signal transduction can contribute to the observed versatility in signaling output and to the modulation of signal strength.

Notch ligand-receptor interactions

In mammals, there are four Notch receptors (Notch1-4) and five canonical ligands of the Delta-Serrate-Lag (DSL) type [Jag1 and Jag2 and delta-like 1 (Dll1), Dll3 and Dll4] (reviewed by D'Souza et al., 2010). This generates a large number of receptor-ligand combinations, which could potentially generate distinct responses. There is, however, little evidence for differences in signaling output between particular receptor-ligand combinations, with the notable exception of Dll3, which is the most structurally divergent ligand and lacks an extracellular Delta and OSM-11-like protein (DOS) domain as well as lysine residues in the intracellular domain (Dunwoodie et al., 1997). Dll3 is incapable of activating Notch receptors in trans (Ladi et al., 2005) and is rarely, if ever, present at the cell surface (Chapman et al., 2011; Geffers et al., 2007).

The relative strength of receptor-ligand interactions, however, can be modulated by post-translational modifications of Notch receptors. The extracellular epidermal growth factor (EGF) repeats of Notch receptors can be modified by *O*-glucose or *O*-fucose additions, which are then subject to further modification (Stanley and Okajima, 2010). The addition of *O*-fucose to Notch receptors by protein *O*-fucosyltransferase 1 (Pofut1), which is not required for Notch receptor signal transduction per se (Okajima et al., 2008), is necessary for the subsequent glycosylation of Notch receptors by Fringe proteins (such as lunatic fringe, manic fringe and radical fringe in mammals). Fringe proteins can then add *N*-acetylglucosamine (GlcNAc) sugars to the *O*-fucose moiety. This glycosylation modulates the relative response of Notch receptors to ligands of the Delta versus Jagged/Serrate classes: Fringe potentiates interactions with Dll1 and reduces responsiveness to Jag1 (Hicks et al., 2000; Kato et al., 2010). The Fringe-mediated transcriptional changes reported thus far appear to be quantitative rather than qualitative in nature, i.e. the level of expression of the same set of downstream genes is modulated but the set of downstream genes that is activated or repressed is not changed, although this has not been systematically explored at a genome-wide level. Notch can also be glycosylated by the glycosyltransferase Rumi (Poglut1) (Acar et al., 2008; Fernandez-Valdivia et al., 2011) and by two enzymes of the human glycosyltransferase 8 family (Sethi et al., 2010). How the Notch receptor is modified by glycans is the subject of much research (Stanley and Okajima, 2010), and it will be interesting to see which modifications are required for basic Notch function and which confer ligand-specific effects. For example, a secreted Fringe protein, chondroitin sulfate synthase 1 (CHSY1), has recently been identified that appears to suppress Notch signaling; loss of function of *CHSY1* leads to hyperactivation of Notch signaling and Notch gain-of-function phenotypes (Tian et al., 2010).

The expression domains of Fringe genes frequently coincide with those of either Dll or Jag ligands, and it is likely that $Fringe^+/Jag^+$ domains and $Fringe^+/Dll^+$ domains have different effects on tissue organization and tissue domain boundaries. In situations in which a $Fringe^+/Jag^+$ domain is juxtaposed with a $Fringe^-/Dll^+$ domain, Notch signaling becomes localized to the interface between the two domains. For example, at the dorsoventral margin of the *Drosophila* wing, where Fringe is co-expressed with Jagged (Serrate) at the dorsal side, and Delta is expressed alone at the ventral side, Notch signaling is active in only the wing margin, as signaling in both the $Fringe^+/Jag^+$ and $Fringe^-/Delta^+$ domains is inhibited, and occurs only immediately across the domain boundary at the wing margin (Irvine and Wieschaus, 1994; Wu and Rao, 1999). Conversely, co-expression of Fringe with Dll1 but not with Jag1 results in Notch signaling both within the $Fringe^+/Delta^+$ and the $Fringe^-/Jag^+$ domains, but not at the domain boundary. This occurs, for example, in dorsoventral domains in the developing ventral spinal cord and is important for appropriate cell fate decisions and helps to insulate the domains from each other at the domain boundaries as the spinal cord develops (Marklund et al., 2010).

Restricting the distribution of Notch ligands and receptors to specific areas within cells can also contribute to signaling specificity, as it may allow only certain combinations of cells in a larger cellular cluster to engage in Notch signaling. This is observed in *Drosophila* sensory organ development, a model system that relies on Notch signaling to generate lateral inhibition

Table 2. Mutations in Notch signaling components result in developmental defects and diseases in humans

Gene	Diseases associated with mutated gene	References
DLL3	Spondylocostal dysostosis (axial skeleton segmentation disorder)	(Bonafe et al., 2003; Bulman et al., 2000; Turnpenny et al., 2003; Whittock et al., 2004)
JAG1	Alagille syndrome; patients with *JAG1* mutations display variable phenotypes in bile duct paucity, cardiac defects (including tetralogy of Fallot), posterior embryotoxon, spine defects (including butterfly vertebrae) and deafness	(Bauer et al., 2010; Colliton et al., 2001; Crosnier et al., 1999; Crosnier et al., 2001; Eldadah et al., 2001; Heritage et al., 2002; Heritage et al., 2000; Krantz et al., 1998; Krantz et al., 1999; Li et al., 1997; Oda et al., 2000; Oda et al., 1997; Raas-Rothschild et al., 2002; Ropke et al., 2003; Stankiewicz et al., 2001; Warthen et al., 2006)
LFNG	Spondylocostal dysostosis (axial skeleton segmentation and growth disorder)	(Sparrow et al., 2006)
MAML2	Mucoepidermoid carcinoma, secondary acute myeloid leukemia	(Conkright et al., 2003; Enlund et al., 2004; Tonon et al., 2003)
NOTCH1	T-ALL (T-cell acute lymphoblastic leukemia) Aortic valve disease	(Weng et al., 2004) (Garg, 2006)
NOTCH2	Alagille syndrome Hajdu-Cheney syndrome (progressive and severe bone resorption leading to osteoporosis)	(McDaniell et al., 2006) (Simpson et al., 2011)
NOTCH3	CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, a hereditary stroke disorder)	(Joutel et al., 1997a; Joutel et al., 2004; Joutel et al., 1997b; Oberstein et al., 1999)
NOTCH4	Debated involvement in schizophrenia	(Ivo et al., 2006; McGinnis et al., 2001; Sklar et al., 2001; Skol et al., 2003; Tochigi et al., 2004; Wang, Z. et al., 2006; Wei and Hemmings, 2000)

(the process whereby a cell adopts a particular fate and prevents its immediate neighbors from doing likewise) and which proceeds through a series of asymmetric cell divisions. The adult peripheral nervous mechanosensory system arises from the development of a single cell, the sensory organ precursor (SOP), which divides asymmetrically to produce a pIIa and a pIIb cell. Each of these cells also divides asymmetrically to produce a socket and a shaft cell (from the pIIa cell) and a glial cell and a pIIIb cell (from the pIIb cell). The pIIIb cell undergoes one more asymmetric division to produce a neuronal cell and a sheath cell (Wang and Chia, 2005). During SOP development, Delta is recycled in a Rab11-dependent manner (Emery et al., 2005) and is relocalized from the basolateral to the apical membrane in a process that requires Neur (Benhra et al., 2010), Actin-related protein 2/3 (Arp2/3) and Wiskott-Aldrich syndrome protein (WASp) (Rajan et al., 2009). This recycling exclusively juxtaposes Delta in the pIIb cell to the other Notch-expressing pIIa cell, providing the precise signal required for neuronal fate specification (Emery et al., 2005; Jafar-Nejad et al., 2005).

An alternative means to localize Notch activation is by positioning Notch ligands at cellular protrusions, such as filopodia, which leads to the activation of signaling some distance away from the signal-sending cell (Cohen, M. et al., 2010; De Joussineau et al., 2003), literally stretching the concept of cell-cell communication. Cellular motility can also generate specificity by providing a dynamic interaction between Notch ligands and receptors, thus influencing the duration of signaling. An example of this is seen in zebrafish *mikre oko* (*mok*) mutants, which are defective for the motor protein Dynactin 1. In these mutants, the pace of interkinetic movements within the neuroepithelium is altered and mutant neuroepithelial progenitor cells are therefore less exposed to active Notch signaling, resulting in premature cell cycle exit and overproduction of early-born retinal ganglion cells at the expense of later-born interneurons and glia (Del Bene et al., 2008).

In most cellular contexts, ligands are not uniquely expressed on the signal-sending cell and, vice versa, receptors are not expressed only on the signal-receiving cell. The cells therefore need to establish the direction in which signaling should occur, based sometimes on relatively small concentration differences of ligand and receptor. Directionality of Notch signaling stems, at least in part, from the fact that ligands activate receptors on contacting cells (trans-activation), but generally inhibit receptors expressed in the same cell (cis-inhibition) (de Celis and Bray, 1997; del Alamo et al., 2011; Micchelli et al., 1997; Miller et al., 2009; Sprinzak et al., 2010). Cis-inhibition has been reported to lead to a downregulation of Notch receptor at the cell surface (Matsuda and Chitnis, 2009; Perez et al., 2005), although this is not always seen (Fiuza et al., 2010), as well as to a cell-autonomous downregulation of Notch target genes. As discussed above, Dll3 might serve exclusively as a cis-inhibiting ligand, as it is incapable of activating receptors in trans (Ladi et al., 2005).

Progress has been made in unraveling how other ligands can expedite both trans-activating and cis-inhibitory activities. For example, the extracellular DSL-EGF 3 domain of Serrate is important for both trans-activation and cis-inhibition (Cordle et al., 2008), whereas mutations in the intracellular domain of Serrate affect trans-activation but not cis-inhibition (Glittenberg et al., 2006). However, it has also been shown that Notch ligand and receptor ICDs display competitive interactions. In endothelial cells, for example, Notch ICD can suppress the antiproliferative effect of Delta ICD (Kolev et al., 2005) and, conversely, the intracellular domain of Jag1 has been shown to suppress Notch ICD-induced transcription in COS cells (LaVoie and Selkoe, 2003). Cis-inhibition of the ligand by a Notch receptor can occur in the ligand-presenting cell (Becam et al., 2010), a process that is dependent on the Notch extracellular domain and which reduces the levels of cell-surface ligand available for transactivation of contacting cells.

In addition to the canonical ligands mentioned above, a multitude of non-canonical ligands (reviewed by D'Souza et al., 2010) can activate or inhibit Notch signaling. An interesting example of a non-canonical ligand is Delta-like homolog 1/2 (Dlk1/2), which is structurally similar to the Dll ligands but lacks a DSL domain. As such, it is believed to be incapable of transactivation and is thought to act through cis-inhibition by competing with trans-presented canonical ligands (Baladron et al., 2005). Recently, a model for trans-activation versus cis-inhibition has been proposed in which trans-activation occurs in a graded manner in response to increasing levels of ligand, whereas cis-inactivation occurs at a sharp threshold of Notch ligand co-expression, leading to an ultrasensitive switch that generates mutually exclusive sending (high ligand/low Notch) and receiving (low ligand/high Notch) signaling states (Sprinzak et al., 2010). This model remains to be tested in vivo.

Notch receptor processing

As a result of ligand activation, the Notch receptor is proteolytically processed. This is followed by the release of Notch ICD and its translocation to the nucleus. These processing and relocalization events are regulated at multiple steps, providing further opportunities for modulating Notch signaling. The binding of a Notch receptor to its ligand leads to removal of the Notch extracellular domain (NECD) and its trans-endocytosis into the ligand-expressing cell (Hansson et al., 2010; Nichols et al., 2007; Parks et al., 2000). The Notch receptor is cleaved repeatedly during its lifetime, first at site 1 (S1) by furin during its maturation (Logeat et al., 1998) and subsequently at site 2 (S2) and sites 3/4 (S3/S4) after trans-activation by a Notch ligand. The S2 cleavage is the key regulatory step in receptor activation and is executed by ADAM (a disintegrin and metalloprotease) proteases. Recently, structural analysis of the Notch receptor domain that harbors the S2 cleavage site has laid the ground for a model for Notch processing. In this model, the ligand pulls the receptor into a state in which the negative regulator region (NRR) of the receptor unfolds and exposes an ADAM cleavage site. Interestingly, different ADAMs have been implicated in this cleavage event (Brou et al., 2000; Canault et al., 2010; Tian et al., 2008; Tousseyn et al., 2009; van Tetering et al., 2009), and a recent report indicates that specific ADAM proteases may cleave Notch specifically in a ligand-dependent or -independent manner (Bozkulak and Weinmaster, 2009). The structural aspects of the cleavage process have been reviewed recently (Kovall and Blacklow, 2010) and will not be discussed further here.

The remaining membrane-tethered portion of Notch, termed the Notch extracellular truncation (NEXT), is then a substrate for regulated intramembrane proteolysis by the γ-secretase complex, a multi-subunit protease complex containing presenilin, nicastrin, presenilin enhancer 2 (Pen2) and anterior pharynx-defective 1 (Aph1) (Jorissen and De Strooper, 2010). It was previously assumed that S3 cleavage followed more or less constitutively in the wake of the regulatory S2 cleavage, but recent data indicate that the activity of γ-secretase is also regulated, both with regard to cleavage efficacy and the position of the cleavage site in the receptor. Emerging evidence suggests that γ-secretase complexes containing different presenilin (PS1 or PS2) subunits have different cleavage preferences for amyloid precursor protein (APP), and to what extent PS1- and PS2-containing complexes differ with regard to Notch processing in vivo largely remains to be explored (Jorissen and De Strooper, 2010). A recent report shows that nicastrin is dispensable for γ-secretase-mediated processing of Notch, but important for the stability of the γ-secretase complex (Zhao et al., 2010). Other proteins that modulate the function of the γ-secretase complex, such as CD147 (also known as BSG), transmembrane protein 21 (Tmp21, also known as Tmed10) and γ-secretase activating protein (GSAP, also known as Pion) (Chen et al., 2006; He et al., 2010; Zhou et al., 2006), have also been identified but the mechanistic basis for their differential effects on Notch versus other substrates awaits elucidation. Furthermore, S3 cleavage of Notch is heterogeneous with regard to the position of the cleavage site: Notch ICD fragments generated from S3 cleavage have either an N-terminal valine (Val) or an N-terminal serine/leucine (Ser/Leu), and Ser/Leu-NICD fragments have a shorter half-life than Val-NICD fragments (Tagami et al., 2008), which is likely to affect the duration of Notch signaling.

Notch processing is also controlled by estrogen receptor (ER) signaling, such that blockage of ER activity by tamoxifen increases Notch cleavage (Rizzo et al., 2008). Similarly, neuronal activity enhances Notch processing through the protein activity-regulated cytoskeleton-associated protein (Arc)/activity-regulated gene 3.1 protein homolog (Arg3.1) (Alberi et al., 2011), highlighting yet another way in which Notch processing, and hence Notch signaling, can be modulated.

Endocytosis and trafficking of processed Notch receptors

Endocytosis of the Notch receptor is an important step in the transmission of the Notch signal, and, although Notch receptors initially interact with components of the γ-secretase complex at the cell surface (Hansson et al., 2005), there are indications that the majority of cleavage occurs after internalization of the receptor by endocytosis (Vaccari et al., 2008), although there is also evidence to the contrary (Kaether et al., 2006; Sorensen and Conner, 2010; Tarassishin et al., 2004). It is thus possible that the localization of Notch cleavage is variable and constitutes another level of signal fine-tuning that is dependent on cell context (Tagami et al., 2008). Notch receptor endocytosis requires mono-ubiquitylation of the receptor at lysine 1749 (Gupta-Rossi et al., 2004), and, recently, this mono-ubiquitylation event has been shown to be followed by de-ubiquitylation mediated by elF3f, previously thought to solely constitute a subunit of translation initiation factor E74-like factor 3 (Elf3), which is required for Notch to be processed by γ-secretase (Moretti et al., 2010). The putative E3 ubiquitin ligase Deltex, which has been implicated in the regulation of Notch processing and internalization in several studies (Diederich et al., 1994; Hori et al., 2004; Matsuno et al., 1995; Wilkin et al., 2008; Yamada et al., 2011), serves as a bridging protein between elF3f and Notch in early endosomes (Moretti et al., 2010). Deltex has been described as both a positive (Fuwa et al., 2006; Matsuno et al., 1995; Matsuno et al., 2002; Wilkin et al., 2008) and a negative (Sestan et al., 1999; Mukherjee et al., 2005) regulator of Notch signaling, and Deltex appears to be required for Notch signaling in some, but not all, developmental processes in *Drosophila* (Fuwa et al., 2006). Likewise, loss of Deltex function does not always severely impinge on Notch-dependent processes, such as T-cell development, in the mouse (Lehar and Bevan, 2006). Perhaps some of the discrepancy can be explained by the recent suggestion that canonical Notch signaling and Deltex-activated Notch signaling are separate events that are activated in different endocytic compartments (Yamada et al., 2011).

Numb (which is found in both *Drosophila* and vertebrates) is an endocytic adaptor protein that, like its mammalian homolog Numb-like (found in vertebrates), acts as a suppressor of Notch

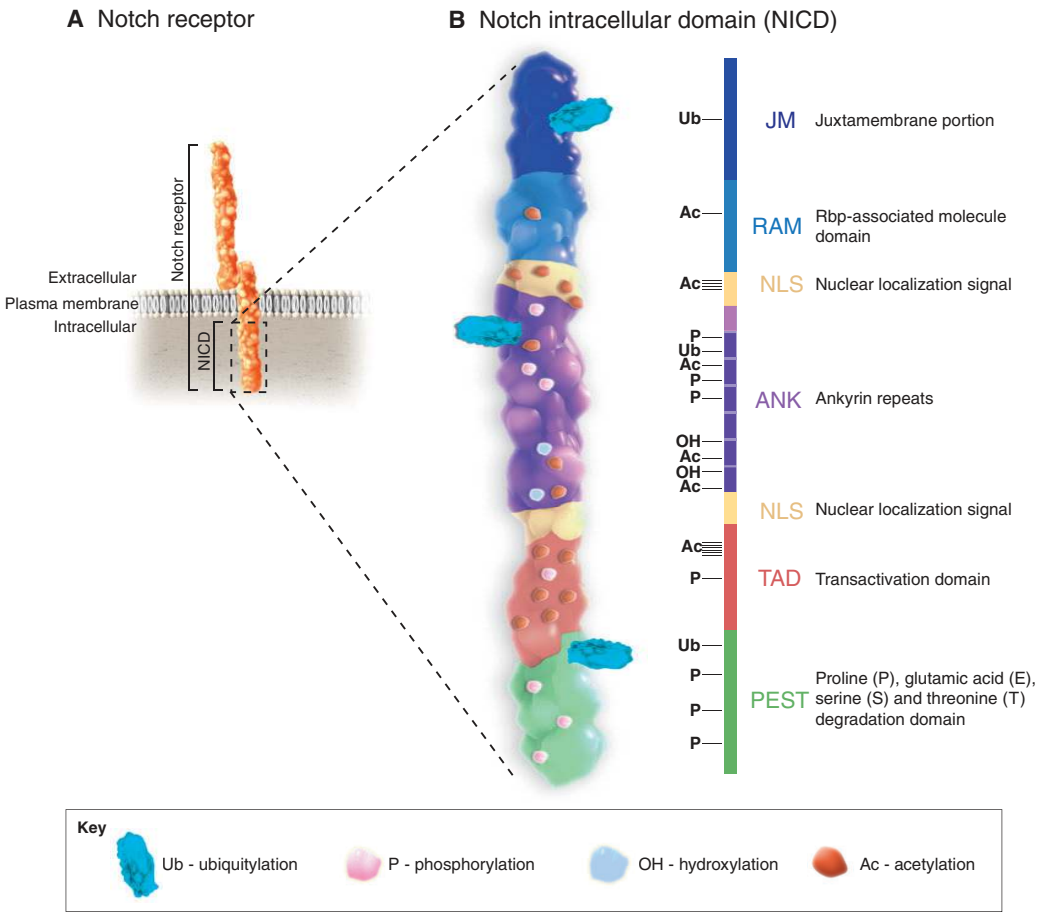

Fig. 2. Notch ICD: domain structure and post-translational modifications. (**A**) The Notch receptor is a heterodimeric transmembrane protein composed of an extracellular domain and a transmembrane domain that can be cleaved to yield the Notch intracellular domain (NICD). (**B**) The NICD is composed of several domains (JM, RAM, ANK, TAD and PEST), two nuclear localization signals and several ankyrin repeats. These various domains and motifs can be modified by phosphorylation, hydroxylation, ubiquitylation or acetylation to alter signaling through NICD. The specific proteins that mediate these modifications are described in the text.

signaling (Rhyu et al., 1994; Uemura et al., 1989; Zhong et al., 1997) (for reviews, see Cayouette and Raff, 2002; Gonczy, 2008). Mechanistically, Numb has been shown to recruit the E3 ubiquitin ligase itchy (Itch), the mammalian homolog of *Drosophila* Suppressor of deltex [Su(Dx)], to promote degradation of the Notch receptor (Beres et al., 2011) and to regulate post-endocytic sorting events for Notch (McGill et al., 2009). Numb differentially affects various Notch receptors, which might increase diversity in the signaling response, and a recent report indicates that Numb negatively regulates Notch1 and Notch2 receptors, but not Notch3, during myogenic differentiation (Beres et al., 2011). In human, six alternatively spliced *NUMB* isoforms have been characterized to date. The two most recently identified isoforms, NUMB5 and NUMB6, are less potent antagonists of Notch signaling (Karaczyn et al., 2010), although it remains to be established if the difference in biological effects among the different isoforms is strictly due to different effects on Notch, as Numb also interacts with other signaling proteins, such as p53 and Gli1, a Hedgehog pathway effector (Colaluca et al., 2008; Di Marcotullio et al., 2006). Sanpodo, a transmembrane protein so far found only in *Drosophila*, is an important regulator of Notch signaling and has also been shown to associate with Notch and Numb during asymmetric cell division (O'Connor-Giles and Skeath, 2003), where it augments Notch signaling in the absence of Numb but represses Notch signaling in the presence of Numb (Babaoglan et al., 2009). There is an emerging view that the relationship between Numb and Notch is not just unidirectional. Thus, in addition to negative Numb-mediated regulation of Notch, it has been shown that Notch can reciprocally influence Numb. High levels of Notch, for example, reduce Numb and Numb-like protein levels in cultured cells and in the developing chick CNS (Chapman et al., 2006), and Notch controls the expression of Numb, upregulating it in cells that have not inherited Numb during cell division but must express Numb to later repress Notch (Rebeiz et al., 2011).

After internalization by endocytosis, the intracellular trafficking of Notch receptors further modulates the Notch signal. Compromised sorting of Notch from early endocytic vesicles to multivesicular bodies (MVBs) or lysosomal compartments, as seen in endosomal sorting complex required for transport (ESCRT) and *lethal giant discs* [*lgd*; also known as *l(2)gd1*] mutants, respectively, leads to ectopic, ligand-independent activation of Notch signaling (Childress et al., 2006; Jaekel and Klein, 2006; Vaccari et al., 2008). Recently, studies of *Drosophila* SOPs revealed a specialized endocytic routing of Notch signaling that generates differential Notch signaling in the resulting daughter cells. This trafficking is mediated via SARA (Smad anchor for receptor activation) endosomes, which segregate specifically to one of the two daughter cells in the production of pIIa and pIIb cells during asymmetric SOP division (for a review, see Gonczy, 2008). During SOP mitosis, Delta and Notch are both internalized into SARA endosomes, which are then asymmetrically localized to the pIIa, but not to the pIIb, cell, resulting in the ligand-dependent appearance of Notch ICD in only the pIIa cell (Coumailleau et al., 2009). It is important to note that SARA itself is not required in this process.

The Notch intracellular domain – a well-decorated signaling hub

In the canonical Notch signaling pathway, the Notch ICD constitutes the 'business end' of the Notch receptor and, after localization to the nucleus, Notch ICD interacts with CSL to activate the transcription of downstream genes. The Notch ICD is composed of several domains (Fig. 2), including a Rbp-associated molecule (RAM) domain that mediates interactions with CSL, an ankyrin (ANK) repeat domain, a transcription activation domain (TAD) and a C-terminal PEST [rich in proline (P), glutamic acid (E), serine (S) and threonine (T)] degradation domain (Kovall and Blacklow, 2010). It is becoming increasingly clear that the Notch ICD is subject to a variety of post-translational modifications, including phosphorylation, ubiquitylation, hydroxylation and acetylation (Fig. 2).

Regulation of Notch ICD by phosphorylation

The Notch ICD is phosphorylated at several residues and by several kinases. Phosphorylation of Notch ICD by glycogen synthase kinase 3 β (GSK3β) occurs C-terminally to the ANK repeats and inhibits Notch2 ICD-mediated induction of genes such as hairy and enhancer of split 1 (*Hes1*) (Espinosa et al., 2003), but stabilizes Notch1 ICD (Foltz et al., 2002). Granulocyte colony stimulating factor (Csf) also phosphorylates Notch2 ICD, leading to its inactivation (Ingles-Esteve et al., 2001). The PEST domain of Notch ICD contains multiple phosphorylation sites, which are important for the control of Notch ICD stability and serve as triggers for subsequent ubiquitylation (see below). Furthermore, cyclin C/cyclin-dependent kinase (CDK) 8 phosphorylates Notch ICD, and this modification is important for both the activity and turnover of Notch ICD (Fryer et al., 2004).

Regulation of Notch ICD by ubiquitylation

The Notch ICD can also be ubiquitylated, for example by E3 ubiquitin ligases, and this modification regulates its half-life (for a review, see Le Bras et al., 2011). F-box and WD-40 domain protein 7 (Fbxw7; also known as Cdc4 and SEL10) can also ubiquitylate Notch ICD within its PEST domain, leading to the rapid degradation of Notch ICD (Fryer et al., 2004; Gupta-Rossi et al., 2001; Oberg et al., 2001; Wu et al., 2001). The activity of Notch1 ICD, but not that of Notch4 ICD, was enhanced by a dominant-negative form of Fbxw7 (Wu et al., 2001). In contrast to these findings, however, the analysis of *Fbxw7*$^{-/-}$ mice revealed that the levels of Notch4 ICD, but not those of the Notch1, 2 and 3 ICDs, were elevated following *Fbxw7* knockout (Tsunematsu et al., 2004), suggesting that the regulation of different Notch ICDs by Fbxw7 is likely to be complex. It has recently been shown that serum- and glucocorticoid-inducible kinase (SGK1) forms a trimeric complex with Notch ICD and Fbxw7, thereby enhancing Fbxw7-mediated Notch degradation (Mo et al., 2011). Functionally, Fbxw7 has been shown to be important in the control of stemness and neuronal fate versus glial differentiation in the developing brain (Matsumoto et al., 2011).

The importance of correctly controlling Notch ICD half-life and the role of Fbxw7 in this process is also underscored by the fact that *NOTCH1* and *FBXW7* mutations can be found in T-cell acute lymphoblastic leukemia (T-ALL) (Erbilgin et al., 2010; Malyukova et al., 2007). In T-ALL patients, gain-of-function mutations in *NOTCH1* are found in more than 50% of cases (Weng et al., 2004) and loss-of-function mutations in *FBXW7* have also been described (Malyukova et al., 2007; Mansour et al., 2009; O'Neil et al., 2007). The *NOTCH1* mutations are concentrated in the extracellular heterodimerization (HD) domain and the intracellular PEST domain; mutations in the HD domain enhance Notch cleavage, whereas those in the PEST domain make the NOTCH1 ICD more resistant to ubiquitylation and subsequent degradation (Weng et al., 2004). In keeping with this, T-ALL cell lines lacking functional FBXW7 display extended NOTCH1 ICD half-lives (Malyukova et al., 2007; Mansour et al., 2009; O'Neil et al., 2007). Mutations in the PEST domain of *NOTCH1* have also been found in non-small-cell lung cancer (Westhoff et al., 2009), suggesting that altered phosphorylation, ubiquitylation and degradation, and thus increased Notch signaling, can lead to cancer in several organs. Mutation or loss of *NUMB*, resulting in NOTCH gain of function, are likewise responsible for a large proportion of non-small-cell lung cancers (Westhoff et al., 2009), but it is not yet established whether mutations in *FBXW7* also appear in non-small-cell lung cancer. Other E3 ubiquitin ligases affecting Notch include Deltex, which in addition to its role in Notch intracellular trafficking ubiquitylates Notch ICD (Yamada et al., 2011), and Itch (Cornell et al., 1999; Qiu et al., 2000), which is required for Notch1 degradation in the absence of ligand (Chastagner et al., 2008). For a recent review on the role of ubiquitylation in Notch signaling, see Le Bras et al. (Le Bras et al., 2011).

There is an expanding list of other non-E3 ubiquitin ligase proteins that interact with Notch ICD and thereby might influence Notch signaling output (see Table 3). However, relatively little is known about many of these interactions, and a number of them have thus far only been observed under conditions of overexpression. It is therefore important to determine whether these interactions occur under physiological conditions in cells in vivo, and whether these interactions with Notch ICD occur when Notch ICD is free in the cytoplasm or nucleoplasm, or only when Notch ICD is present in the transactivating complex together with CSL.

Regulation of Notch ICD by hydroxylation

Hydroxylation is an additional, more recently discovered type of post-translational modification of Notch ICD. The asparagine hydroxylase factor-inhibiting HIF1α (FIH1, also known as HIF1AN), which also operates in the cellular hypoxic response (see below), hydroxylates Notch ICD at two residues (N1945 and N2012) (Coleman et al., 2007; Zheng et al., 2008). It is notable that the ICDs of Notch1, 2 and 3, but not that of Notch4, are hydroxylated by FIH1, and this might contribute to signaling diversity. In vitro data suggest that FIH1 negatively regulates Notch signaling, but the biological significance of the FIH1-mediated modifications is not fully understood, and mice targeted for FIH1 do not display an overt Notch gain-of-function phenotype (Zhang et al., 2010).

Regulation of Notch by acetylation

More recently, acetylation and deacetylation of the Notch ICD have been shown to contribute to fine-tuning Notch half-life and thus signaling in endothelial cells, where the deacetylase sirtuin 1 (Sirt1) has been identified as a key deacetylase in this process (Guarani et al., 2011).

Signaling diversity at the level of Notch ICD-mediated gene activation

The binding of Notch ICD to CSL, which is stabilized by MAML, and the subsequent activation of downstream genes by Notch ICD-CSL are central aspects of canonical Notch signaling (Kovall and Blacklow, 2010). The analysis of Notch-induced transcriptomes in

Table 3. Proteins that interact with the Notch ICD

Symbol	Protein	Interaction with Notch ICD	References
Apc	Adenomatous polyposis coli	Controls Notch trafficking	(Munoz-Descalzo et al., 2011)
Axin	Axin	Synergizes with Notch ICD to control β-catenin stability and controls trafficking of Notch ICD with Apc	(Hayward et al., 2006; Munoz-Descalzo et al., 2011)
CDK8	Cyclin-dependant kinase 8	Together with CycC phosphorylates Notch ICD to make it a substrate for ubiquitylation and degradation	(Fryer et al., 2004)
CSL/RBP-J	CBF1, Su(H) and LAG-1/Recombination signal binding protein for immunoglobulin kappa J region	Main canonical transcriptional co-factor for Notch ICD	(Tanigaki and Honjo, 2010)
Ctnnb1	β-catenin	Synergizes with Notch ICD/CSL on Notch target genes	(Hayward et al., 2005; Shimizu et al., 2008; Yamamizu et al., 2010)
CycC	Cyclin C	Together with CDK8 targets Notch ICD for phosphorylation to make it a substrate for ubiquitylation and degradation	(Fryer et al., 2004)
Dab	Disabled	Acts as link to Abl proteins in non-canonical Notch axon guidance	(Le Gall et al., 2008)
Dsh/Dvl	Dishevelled	Dvl controls ligand-independent Notch trafficking; inhibits canonical Notch signaling	(Axelrod et al., 1996; Munoz-Descalzo et al., 2010)
Dtx1-4	Deltex-1-4	Controls Notch ubiquitylation, processing and internalization	(Diederich et al., 1994; Hori et al., 2004; Matsuno et al., 1995; Wilkin et al., 2008; Yamada et al., 2011)
Fbxw7/Cdc4	F-box/WD repeat protein 7	Ubiquitylates Notch ICD, leading to its degradation	(Fryer et al., 2004; Gupta-Rossi et al., 2001; Oberg et al., 2001; Wu et al., 2001; Tsunematsu et al., 2004)
FIH	Factor inhibiting HIF1α	Hydroxylates Notch, represses Notch	(Coleman et al., 2007; Wilkins et al., 2009; Zheng et al., 2008)
GSK3β	Glycogen synthase kinase 3β	Phosphorylates Notch, which can lead to degradation or stabilization	(Espinosa et al., 2003; Foltz et al., 2002)
HIF1α	Hypoxia inducible factor 1, alpha subunit	Stabilizes Notch ICD and synergizes with it in transcription of Notch target genes	(Bertout et al., 2009; Gustafsson et al., 2005; Sahlgren et al., 2008)
Itch	Itchy, E3 ubiquitin protein ligase	Promotes ubiquitylation of Notch ICD	(Qiu et al., 2000)
Maml1/2	Mastermind-like 1/2	Co-activator for Notch ICD/CSL	(Bray and Bernard, 2010; McElhinny et al., 2008)
NF-κb	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	Notch ICD blocks NF-κb transcription of NF-κb target genes through binding to p50/cRel	(Wang et al., 2001)
		Notch ICD enhances NF-κb transcription of target genes by retaining NF-κb in the nucleus	(Shin et al., 2006)
Nrarp	Notch-regulated ankyrin repeat protein	Nrarp binds and inhibits Notch ICD/CSL	(Lamar et al., 2001; Yun and Bevan, 2003)
Numb	Numb homolog	Suppresses Notch signaling by recruiting E3 ubiquitin ligases to ubiquitylate Notch	(Beres et al., 2011; Rhyu et al., 1994; Uemura et al., 1989; Zhong et al., 1997)
		Controls Notch and Sanpodo trafficking during asymmetric cell division	(Hutterer and Knoblich, 2005; O'Connor-Giles and Skeath, 2003; Skeath and Doe, 1998; Tong et al., 2010)
p73α (TA)	Tumor protein p73 alpha (transactivating form)	Binds Notch ICD and inhibits Notch ICD/CSL-mediated transcription	(Hooper et al., 2006)
RITA/C12ORF52	RBP-J interacting and tubulin associated	Shuttles Notch ICD between the nucleus and cytoplasm on tubulin networks	(Wacker et al., 2011)
SMAD	Smad family members (homologs of Mothers against decapentaplegic)	Smads enhance Notch signaling, Notch fine-tunes signaling through Smads	(Blokzijl et al., 2003; Dahlqvist et al., 2003; Fu et al., 2009; Itoh et al., 2004; Sun et al., 2005; Tang et al., 2010)
SNW1/SKIP/NCOA-62	SNW domain-containing protein 1/Ski-interacting protein/Nuclear receptor co-activator NCoA-62	Can bind both Notch ICD and co-repressor SMRT, but these are mutually exclusive; forms multimers with Notch ICD and MAML, which then associates with CSL to activate transcription	(Vasquez-Del Carpio et al., 2011; Zhou et al., 2000)
Tacc3	Transforming, acidic coiled-coil containing protein 3	Binds Notch ICD and inhibits transcription from Notch target promoters; can be reversed by CSL overexpression	(Bargo et al., 2010)
Trio	Triple functional domain (PTPRF interacting)	A guanine nucleotide exchange factor (GEF) for Rho GTPases that acts as link to Abl proteins in non-canonical Notch axon guidance	(Le Gall et al., 2008)

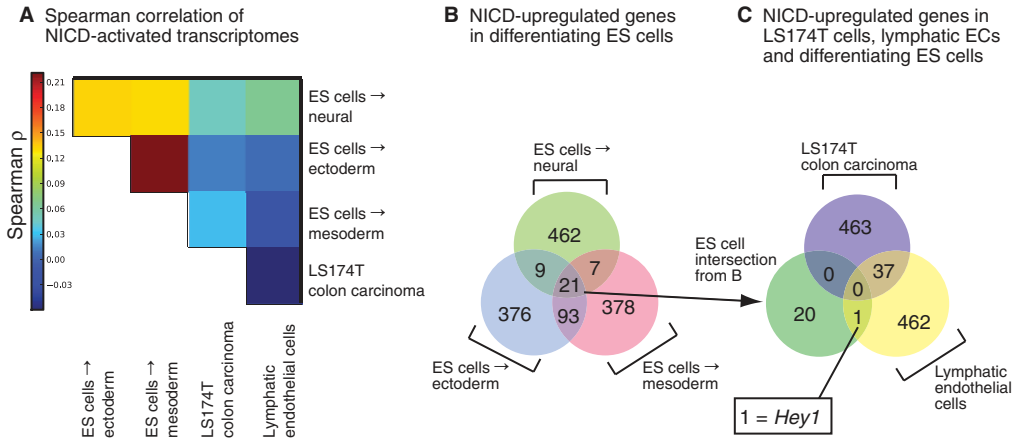

Fig. 3. Diversity in Notch ICD-induced transcriptomes. A comparison of genes upregulated by Notch1 ICD overexpression in different cell types [mouse embryonic stem (ES) cells undergoing ectodermal, mesodermal (Meier-Stiegen et al., 2010) or neural (Main et al., 2010) differentiation; the human colon carcinoma cell line LS174T (Okamoto et al., 2009); and human lymphatic endothelial cells (ECs) (unpublished, GSE20978)] reveals diversity in target gene activation. (**A**) Spearman correlation (ρ) of the five sets of transcriptomes following Notch1 ICD activation shows that the ES cell-derived transcriptomes are more similar to each other than to the colon carcinoma or lymphatic ECs, but that they demonstrate considerable diversity between them. (**B**) A comparison of the top 500 upregulated genes in ES cells undergoing ectodermal, mesodermal or neural induction and in response to Notch1 ICD activation. Twenty-one genes were found to be upregulated in all three differentiation paradigms (see Table S1 in the supplementary material). (**C**) The 21 genes upregulated in all three ES cell transcriptomes were compared with the top 500 upregulated genes in the colon carcinoma (LS174T) and lymphatic ECs. In this analysis, genes upregulated in all three situations were not identified, but *Hey1* was upregulated in all three ES cell transcriptomes and in the lymphatic ECs. Gene expression data for series GSE19074, GSE15268, GSE10136 and GSE20978 were downloaded from Gene Expression Omnibus (GEO). The microarray probe set annotations were converted into RefSeq transcript IDs, taking the average for cases with more than one probe set interrogating the same transcript. RefSeq transcripts for the human data were converted into mouse annotations using NCBI Entrez Gene. For each experiment independently, the relative expression difference for each gene between the Notch-induced and control samples was computed and transformed into $\log_2$ scale, averaging over replicates when available. These relative expression vectors (one per comparison) were used to compute Spearman correlations and to perform analyses of overlaps in the top 500 upregulated genes.

different cell types reveals a considerable diversity in the immediate downstream Notch response, which might be necessary for Notch to function in so many different cellular contexts. Genome-wide transcriptome studies in healthy or mutated T-cells (Chadwick et al., 2009; Dohda et al., 2007; Palomero et al., 2006; Weerkamp et al., 2006), mouse embryonic stem (ES) cells (Main et al., 2010; Meier-Stiegen et al., 2010), alveolar epithelial cells (Aoyagi-Ikeda et al., 2011), endometrial stromal cells (Mikhailik et al., 2009), C2C12 mouse myoblast cells (Buas et al., 2009) and *Drosophila* myogenic cells (Krejci et al., 2009) have unraveled distinct sets of Notch target genes with rather limited overlap of the transcriptomes. This is the case even when comparing transcriptome studies that were carried out with relatively similar modes and durations of Notch induction (summarized in Fig. 3). In addition to output diversity in different cell types, the Notch response changes during the cell cycle (for a review, see Kageyama et al., 2009) and throughout cell lineage progression, for example during T-cell development (for a review, see Radtke et al., 2010) and during neural differentiation of ES cells in vitro, when cyclin D1 is activated only at a specific temporal window during ES cell neural differentiation in vitro (Das et al., 2010).

Traditionally, hairy and enhancer of split-related (HESR) genes, which encode basic helix-loop-helix (bHLH) transcriptional repressors, have been considered key genes activated downstream of Notch signaling. HESR genes do indeed execute important aspects of Notch signaling, for example during tumor progression (Sethi et al., 2011; Wendorff et al., 2010), but it is becoming increasingly apparent that the immediate Notch transcriptome is larger, and that there are many genes activated in parallel with, rather than downstream of, the HESR genes. Challenging the view that HESR genes are always activated in response to Notch signaling, the microarray analyses performed for Fig. 3 revealed that only one HESR gene, hairy/enhancer-of-split related with YRPW motif 1 (*Hey1*), was upregulated in four of the five experiments, whereas *Hes5* was upregulated in the ES cell experiments (see Table S1 in the supplementary material) but was not similarly upregulated in colon carcinoma cells or in lymphatic endothelial cells. Thus, some genes are seen to be upregulated in a number of cell types, but no one gene can be identified as an 'obligatory' Notch target that will be upregulated in all cell types. Among the immediate Notch target genes, activated in parallel with HESR genes, are a number of 'high profile' genes such as *c-Myc* (Rao and Kadesch, 2003; Satoh et al., 2004; Weng et al., 2006), cyclin D1 (Cohen, B. et al., 2010; Ronchini and Capobianco, 2001; Satoh et al., 2004), cyclin D3 (Joshi et al., 2009), cyclin-dependent kinase 5 (*CDK5*) (Palomero et al., 2006), *p21* (Rangarajan et al., 2001), *Snail* (Sahlgren et al., 2008) and platelet-derived growth factor receptor beta (*PDGFR*β) (Jin et al., 2008; Morimoto et al., 2010).

The basis for the observed transcriptome diversity in different cell types is only partially understood. The conventional view holds that CSL is bound via CGTGGGAA motifs to target promoters and that it represses transcription when Notch is not activated. Upon Notch pathway activation, Notch ICD, together with MAML, then displaces co-repressors and brings co-activators to the Notch ICD-CSL complex, which leads to transcriptional activation of target genes. Certain genes, at least some in *Drosophila*, thus appear to be in a repressed state in the absence of Notch signaling (Bardin et

al., 2010; Castro et al., 2005; Koelzer and Klein, 2006), but it has also been shown that in *Drosophila*, the CSL homolog Su(H) is actively recruited to its binding sites by Notch ICD rather than being positioned there in the 'Notch-off' state (Krejci and Bray, 2007). In keeping with a more dynamic interaction between CSL and its cognate DNA-binding sites, the binding coefficient between CSL and DNA has been shown to be weaker than previously considered (Friedmann and Kovall, 2010), whereas the affinity of CSL for the RAM domain of Notch ICD is unchanged by DNA binding (Friedmann et al., 2008). As discussed below, studies that aim to identify the factors that modulate the affinity of the Notch ICD-CSL complex for distinct promoter sequences are beginning to contribute to our understanding of the complexity of Notch signaling output.

Given that different Notch receptors have at least partially distinct expression patterns in most tissues, diversity in the downstream response could be generated if the different Notch ICDs are capable of activating distinct sets of downstream genes. There is some evidence for target selectivity, and the configuration of CSL binding sites within Notch target genes, for example if they appear as monomers or dimers, influences the likelihood of recruiting Notch1 or Notch3 ICD, respectively (Ong et al., 2006). Interestingly, whereas Notch1 ICD performs well on paired CSL binding sites, Notch3 ICD activity is more amenable to binding CSL motifs adjacent to binding sites for zinc-finger transcription factors (Ong et al., 2006). The spacing of multimerized binding sites within target genes is also important for activation (for a review, see Bray and Bernard, 2010). The ability of Notch ICDs to form dimers might also influence the repertoire of activated genes by restricting the response to dimeric CSL binding sites (Cave et al., 2005), although structural analysis of the dimeric Notch ICD complex suggests that a flexibility in spacer length can be accommodated (Arnett et al., 2010). Recently, it has been proposed that Notch ICD multimerization is an initial step in forming the active transcriptional complex (Vasquez-Del Carpio et al., 2011). Based on the notion that different Notch ICDs may activate at least partially distinct transcriptomes, one might expect at least partially distinct biological functions for the various ICDs. Thus, Notch2 ICD, but not Notch1 ICD, promotes tumor growth in xenografts in a medulloblastoma model (Fan et al., 2004), and overexpression of Notch1 ICD or of Notch3 ICD signaling generate distinct phenotypes in pancreas (Apelqvist et al., 1999; Hald et al., 2003), whereas they appear to have more similar functions in adult CNS progenitor cells (Tanigaki et al., 2001). Furthermore, the expression of Notch3 ICD, but not that of Notch1 or 2 ICD, during embryonic CNS development results in the formation of invasive gliomas (Pierfelice et al., 2011).

Proteins encoded by genes activated immediately downstream of Notch can feed back on the Notch transcriptional response and, in this way, modulate the signaling output. This has been demonstrated for c-Myc, which, together with Notch ICD-CSL, activates a set of genes not activated by Notch ICD alone (Palomero et al., 2006). In smooth muscle cells, Hey1 and Hey2 are activated by Notch and subsequently negatively regulate Notch-mediated transcription by blocking Notch ICD-CSL binding to DNA (Tang, Y. et al., 2008), which might affect the duration of the Notch signaling response. Similarly, the immediate Notch target gene Notch-regulated ankyrin repeat protein (*Nrarp*) feeds back to negatively regulate Notch, and at the same time activates Wnt signaling by stabilizing the Lymphoid enhancer-binding factor 1 (LEF1) protein (Ishitani et al., 2005; Phng et al., 2009). By contrast, the Notch target gene *pin1* [*protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1*] positively reinforces Notch signaling by enhancing Notch receptor cleavage (Ishitani et al., 2005).

Cooperativity at the promoter level between Notch ICD-CSL and other transcription factors can also contribute to diversity in the Notch signaling output. Proneural bHLH proteins, for example, cooperate with Notch ICD-CSL in the regulation of HESR gene expression (Holmberg et al., 2008) and synergy between Notch ICD-CSL and GATA factors (Neves et al., 2007), NF-κB (Vilimas et al., 2007) and Twist (Bernard et al., 2010) has also been demonstrated. To what extent these genetic interactions require direct physical interactions between Notch ICD-CSL and the other factors remains to be established, but the spacing between the binding sites has, in some cases, been shown to be important (Swanson et al., 2010).

Despite the progress in this area, there are still unresolved questions as to how diversity is generated at the level of Notch ICD-CSL. It will be important to identify the factors that determine why CSL in some contexts remains bound to DNA in the absence of Notch and/or in other situations is recruited to DNA by Notch ICD. It also remains to be determined if the chromatin and epigenetic status can influence this choice. The establishment of genome-wide DNA-binding profiles for CSL and Notch ICD (through CSL) would be helpful in this regard, as would mapping studies that identify which co-repressors and co-activators are co-recruited in different cellular settings.

Generating diversity through interactions with other signaling mechanisms

Since the number of key cellular signaling mechanisms is rather small, it is becoming increasingly appreciated that signaling mechanisms do not operate in isolation but that they are integrated into signaling networks. Interactions can be divided into different categories based on their mode of interaction. First, one pathway can be epistatic over another pathway, for example by regulating the expression of key components of the other pathway, thus controlling the activity of the other pathway indirectly. Second, pathways can converge at the level of the promoters of downstream genes, such that transcriptional regulators, activated by two (or more) pathways, bind to distinct promoter elements and jointly control the level of expression of downstream genes. Third, a direct interaction between core components in the pathways can lead to complex regulatory events in both pathways. For Notch signaling, all three of the above categories of interaction are observed (Fig. 4), and to exemplify this we discuss recent advances in our understanding of how Notch intersects with Wnt signaling, TGFβ/BMP signaling and with the cellular hypoxic response.

Interactions with the Wnt pathway

Wnt signaling, like Notch signaling, is important for cellular differentiation and homeostasis in a number of tissues, and several nodes of Wnt-Notch signaling interactions have been identified. Wnt signaling upregulates *Jag1* transcription via β-catenin in the hair follicle (Estrach et al., 2006), increases *Dll4* transcription during vascular remodeling (Corada et al., 2010) and induces Notch2 expression in colorectal cancer cells (Ungerback et al., 2011). During somite differentiation, β1-integrin activity controls both Wnt and Notch signaling, and activation of both signaling mechanisms is required for activation of the downstream gene *cMESO1*/*mesp2* (Rallis et al., 2010). With regard to interaction between core components, Dishevelled (Dvl), an intracellular

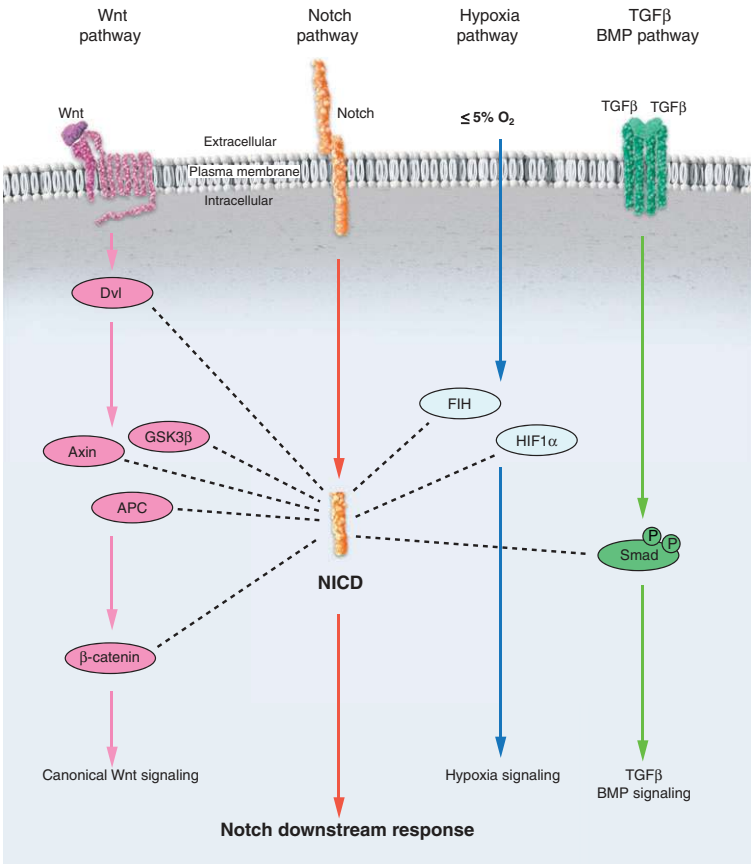

Fig. 4. Cross-talk between the Notch pathway and other signaling pathways. Key intracellular mediators of the Wnt, TGFβ/BMP and hypoxia pathways are depicted. Interactions between Notch ICD and key intracellular mediators in the other signaling mechanisms are indicated by dashed lines.

mediator of all Wnt signaling pathways described to date, binds to Notch ICD (Axelrod et al., 1996; Munoz-Descalzo et al., 2010), and interactions of Notch ICD with several components of the β-catenin destruction complex have also been described (Fig. 4). These include an interaction with Axin, which affects β-catenin stability (Hayward et al., 2006), the control of Notch trafficking by binding to Axin and adenomatous polyposis coli (APC) (Munoz-Descalzo et al., 2011), and GSK3β-mediated phosphorylation of Notch ICD (Espinosa et al., 2003; Foltz et al., 2002). Concomitantly, Notch controls the stability of Armadillo, the *Drosophila* homolog of β-catenin (Hayward et al., 2005; Munoz-Descalzo et al., 2011; Sanders et al., 2009).

Interactions between Notch and Wnt signaling are also context specific: β-catenin can bind Notch ICD in neural precursor cells (Shimizu et al., 2008) and can form complexes with Notch ICD-CSL on CSL binding sites in arterial cells, but it does not do so in venous endothelial cells (Yamamizu et al., 2010). Intriguingly, the Dll1 ICD has been shown to induce Wnt reporter activity and upregulate the expression of connective tissue growth factor (CTGF) (Bordonaro et al., 2011). MAML represents another nexus between Notch and Wnt signaling, and, in addition to its role in stabilizing Notch ICD-CSL interactions, MAML has now been shown to bind to both GSK3β (Saint Just Ribeiro et al., 2009) and β-catenin (Alves-Guerra et al., 2007). The binding of MAML to GSK3β (which is normally inhibited by active Wnt signaling) decreases MAML transcriptional activity (Saint Just Ribeiro et al., 2009), whereas MAML can act as a transcriptional co-activator for β-catenin, enhancing expression of the target genes cyclin D1 and *c-Myc* (Alves-Guerra et al., 2007). An unexpected level of cross-talk is also seen between soluble Frizzled-related proteins (sFRPs) and Notch signaling; sFRPs bind to ADAM10, downregulating its activity and thus inhibiting Notch signaling. This has consequences for retinal neurogenesis, a process known to be Notch dependent but Wnt independent (Esteve et al., 2011).

Interactions with TGFβ signaling pathways

Notch signaling also intersects with the TGFβ and BMP signaling pathways. In the canonical TGF signaling pathway, secreted dimeric cytokines, such as TGFβ, activin/inhibin and BMP, induce the assembly of a tetrameric complex of type I and type II transmembrane receptor serine/threonine kinases. Receptor II then phosphorylates and activates receptor I, which phosphorylates mothers against decapentaplegic (SMAD) transcription factors to activate transcription together with co-activators such as p300 (for a review, see Derynck and Zhang, 2003). TGFβ signaling also activates MAPK signaling cascades, RhoA-ROCK signaling and Ras signaling in a SMAD-independent manner (Derynck and Zhang, 2003).

A direct convergence between Notch and TGFβ/BMP signaling is evident in interactions of Notch ICD with SMADs (SMAD3 for TGFβ; SMAD1 for BMP) (Blokzijl et al., 2003; Dahlqvist et al., 2003; Itoh et al., 2004; Sun et al., 2005). During Notch-TGFβ cross-talk, TGFβ signaling enhances canonical Notch signaling, whereas the effect of Notch on TGFβ signaling is more multi-faceted. For example, Notch/TGFβ induction of Hey1 occurs at the expense of TGFβ-mediated induction of inhibitor of DNA binding 1 (Id1) (Itoh et al., 2004). TGFβ-mediated epithelial-to-mesenchymal transition (EMT) also requires functional Notch signaling in the developing heart (Timmerman et al., 2004) and in various epithelia (Niimi et al., 2007; Zavadil et al., 2004). During

endothelial-to-mesenchymal transition (EndMT) in cardiac cushion morphogenesis, Notch signaling represses SMAD1 and SMAD2 expression, but enhances *SMAD3* mRNA expression, and SMAD3 is recruited to both SMAD and CSL binding sites to orchestrate the downstream response (Fu et al., 2009). The interactions between Notch ICD and SMAD is receptor homolog-specific: Notch4 ICD, but not Notch1 or 2 ICD, was found to interact with phosphorylated SMAD2 and 3 in smooth muscle cells (Tang et al., 2010). Moreover, CSL co-immunoprecipitated with phosphorylated SMAD2 and 3 (Tang et al., 2010), supporting the notion that SMADs can be recruited to the Notch transcription complex. In cerebrovascular endothelial cells, SMADs bind to NICD and control the expression of N-cadherin by binding to CSL binding sites in the N-cadherin promoter (Li et al., 2011). Meanwhile, Dll1 ICD binds directly to SMADs and can occupy sequences within the *CTGF* promoter that contain SMAD binding elements (Bordonaro et al., 2011). During smooth muscle differentiation, TGFβ downregulates expression of Notch3 but upregulates Hes1 expression (Kennard et al., 2008), whereas in T-cells TGFβ requires active Notch signaling to induce a regulatory phenotype through Notch ICD/CSL/SMAD-mediated transcription of forkhead box P3 (*Foxp3*) (Samon et al., 2008).

Regulation of Notch signaling by hypoxia

A reduction in the level of oxygen activates the cellular hypoxic response, and Notch signaling is linked in several ways to the hypoxia pathway (Fig. 4). Certain aspects of the cellular hypoxic response, such as the control of myogenic differentiation, EMT and medulloblastoma precursor proliferation, require functional Notch signaling (Gustafsson et al., 2005; Pistollato et al., 2010; Sahlgren et al., 2008). Hypoxia also maintains a stem cell-like phenotype in colorectal tumor cells in a Notch-dependent manner (Yeung et al., 2011), and hypoxia resistance in *Drosophila*, acquired through genetic selection in low oxygen, can be overridden by blocking Notch signaling (Zhou et al., 2011). In pulmonary arterial hypertension, hypoxia upregulates Notch3 expression, which is important in disease development (Li et al., 2009). With regard to Notch and hypoxia cross-talk, hypoxia controls the expression of Notch ligands, and Dll1, Dll4 and Jag2 have been reported to be upregulated by low oxygen levels (Diez et al., 2007; Dong et al., 2011; Patel et al., 2005; Pietras et al., 2011; Sahlgren et al., 2008; Xing et al., 2011).

There are also genes that are synergistically controlled by both Notch and the cellular hypoxic response, and these contain binding sites for both Notch ICD and the key hypoxia transcriptional regulator hypoxia inducible factor 1 alpha (HIF1α) (Diez et al., 2007). Notch ICD has been shown to directly interact with two key components in the hypoxia pathway (Fig. 4): HIF1α and FIH. The binding of Notch ICD to HIF1α leads to the recruitment of HIF1α to Notch-responsive genes (Gustafsson et al., 2005; Sahlgren et al., 2008). Hypoxia also leads to the stabilization of Notch ICD (Bertout et al., 2009; Gustafsson et al., 2005; Sahlgren et al., 2008), but the underpinning mechanism for the increased Notch ICD half-life remains to be elucidated. In *Drosophila* crystal cells (a type of blood cell), Similar (Sima, encoded by the *Drosophila* ortholog of *Hif1α*), is expressed at high levels even in normoxia and activates Notch in a ligand-independent manner. Although this process does not result in the transcription of hypoxia target genes, it promotes hemocyte survival (Mukherjee et al., 2011). FIH serves as an asparagine hydroxylase not only for HIF1α, but also for Notch ICDs, with the exception of Notch4 ICD, as discussed in the previous section (Coleman et al., 2007; Wilkins et al., 2009; Zheng et al., 2008).

These examples illustrate that the signaling networks between Notch and other pathways are complex and that they are built on compound interactions between signaling pathways at multiple levels. The intersections are not only confined to Wnt, TGFβ/BMP and hypoxia, but are also elucidated for other pathways, such as the Shh pathway (Driver et al., 2008; Liu et al., 2003; Molnar et al., 2011; Morrow et al., 2009; Mukherjee et al., 2005) and NF-κB signaling (for a review, see Poellinger and Lendahl, 2008). A critical node of interaction with other signaling pathways appears to be the Notch ICD, but in some cases independent of any interaction with CSL. Signaling pathway cross-talk might, at least in part, underlie certain forms of non-canonical signaling, which require Notch ICD but not CSL [for a review of non-canonical Notch signaling, see Heitzler (Heitzler, 2010)].

Conclusions

In recent years, we have witnessed rapid progress in many fields of Notch research, both in identifying the cellular differentiation processes that are influenced by Notch signaling and in unraveling the molecular machinery that interprets cell context and converts this information into an appropriate signaling output. With these studies, we can begin to resolve the ostensible paradox of how simplicity in Notch pathway design is reconciled with the large number of cell fate decisions that are influenced by Notch. Importantly, these studies also highlight ways in which we can experimentally regulate Notch signaling in disease. In this area, sophisticated strategies have been developed to interfere with specific stages of Notch signaling, for example by developing MAML-interfering stapled α-helical peptides (Moellering et al., 2009) or antibodies that lock Notch receptors in the 'OFF state' (Aste-Amezaga et al., 2010; Wu et al., 2010). Unfortunately, the long-term use of the latter might still yield unwanted side effects (Yan et al., 2010), and a lesson from this is that, although the rapid advances in basic science can be converted into potential therapies, we still need to learn more about the finer details of the Notch pathway and how it specifically operates in different spatial and temporal cellular contexts.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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