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REVIEW

Notch Signaling: From the Outside In

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1. Introduction

Development makes reiterative use of a surprisingly small set of essential molecular signals: the Wingless (Wg/Wnt), Hedgehog (Hh), Transforming Growth Factor-β (TGF-β), Receptor Tyrosine Kinase/Phosphotase (RTK/P) and Notch pathways (Gerhart, 1999). These five molecular archetypes act both individually and coordinately to interpret and transmit extrinsic signals as distinct cellular transcriptional responses. They mediate the establishment of polarity and body axes, coordinate pattern formation, and ultimately choreograph the morphogenesis of individual tissues. Recent advances have elucidated both the biochemical mechanisms regulating receptor activation and the molecular participants forming the intracellular signaling cascades of each of these pathways.

A number of recent reviews have provided insight into the ever-expanding efforts directed at understanding Notch biology (Angerer and Angerer, 1999; Annaert and De Strooper, 1999; Artavanis-Tsakonas *et al.*, 1999; Baker, 2000; Beatus and Lendahl, 1998; Bertrand *et al.*, 2000; Bray, 1998a,b; Chan and Jan, 1998, 1999; Deftos and Bevan, 2000; Greenwald, 1998; Gridley, 1997; Hoyne *et al.*, 2000; Joutel and Tournier-Lasserve, 1998; Kimble *et al.*, 1998; Lewis, 1998; Miele and Osborne, 1999; Panin and Irvine, 1998; Perron and Harris, 2000; Robey, 1999; Rooke and Xu, 1998; Saito and Watanabe, 1998; Selkoe, 2000; Weinmaster, 1997, 2000). In this review we summarize the efforts of many groups that, over the past decade, contributed to the discovery that a novel signaling paradigm, Regulated Intramembrane Proteolysis (RIP), controls Notch receptor activation. In addition to Notch, the RIP paradigm has recently been shown to impact a number of other proteins (Brown *et al.*, 2000). RIP utilizes "dual address" proteins which function at two discrete subcellular locations. The full-length "receptor" is first held at a docking site where, in response to stimulus/ligand, it undergoes intramembranous proteolysis to release a subdomain that then translocates to a second site of action, typically the nucleus. In the case of Notch, ligand binding is now believed to initiate a proteolytic cascade that culminates in release of the Notch IntraCellular Domain (NICD) and concomitant activation of immediate downstream target genes. Here we present a comprehensive overview of the latest developments in Notch biology with special emphasis on the biochemistry of signal transduction by Notch.

2. The Notch Signaling Pathway—Brief Overview

Notch loci, first described in *Drosophila* nearly a century ago (for a historical perspective see Artavanis-Tsakonas *et al.*, 1999; Wu and Rao, 1999), encode large (~2500 amino acids) Type I transmembrane receptors that have been conserved throughout evolution (Fig. 1). Later, Notch was identified as a "neurogenic" gene (Poulson, 1937); in *Drosophila*, Notch mutants display a hyperplasia of the nervous system at the expense of epidermal tissue. Notch receptors are activated by Type I transmembrane ligands, known collectively as DSL (*Delta*, *Serrate*, and *Lag 2*) proteins, and are therefore proposed to receive short-range signals between directly apposed cells. Interactions between Notch and either of its two ligands in *Drosophila*, Delta or Serrate, can be differentially modulated by the glycosyltransferase Fringe (for review, see Wu and Rao, 1999). Recently, this modulation has been suggested to stem from Fringe-mediated elongation of specific *O*-linked fucose residues within the extracellular epidermal growth factor-like repeats of Notch (Bruckner *et al.*, 2000; Moloney *et al.*, 2000). Ligand-mediated Notch activation ultimately leads to the conversion of CSL (CBF1, Su(H), Lag-1) proteins from repressors to transcriptional activators, and subsequent up-regulation of downstream targets (e.g., HES, Hairy/Enhancer of Split genes). This "core" Notch signaling pathway is depicted in Fig. 1.

Notch signaling has been classically described as directing equivalent cells (or "equivalence groups"), each expressing both ligand and receptor, to acquire the proper cell fates during development. Detailed studies of this type of "lateral signaling" have implicated Notch in inhibition of "default" primary cell fates, thus allowing expression of secondary

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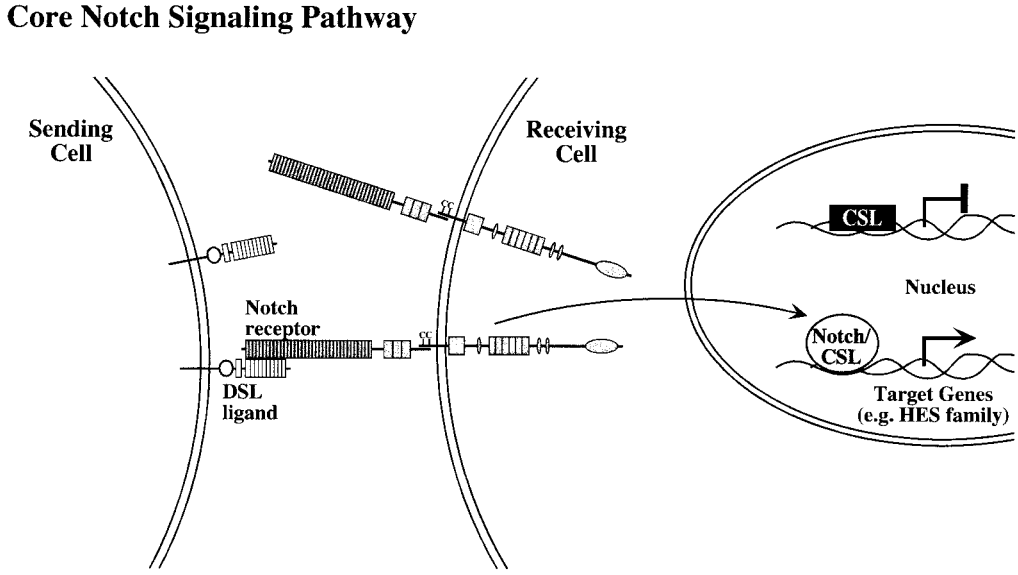

FIG. 1. Core Notch signaling pathway. The four core elements of the Notch signaling system are diagrammed. These include the Notch receptor, DSL (Delta, Serrate, Lag-2) ligands, CSL (CBF1, Suppressor of hairless, Lag-1) transcriptional cofactors, and target genes such as the HES (Hairy/Enhancer of Split) family of basic helix-loop-helix transcriptional regulators. Upon binding ligand the Notch signaling converts CSL from a transcriptional repressor to a transcriptional activator. The current model proposes that this conversion is via direct protein-protein interactions between the Notch intracellular domain and the CSL (see text for further details).

fate pathways (reviewed in Beatus and Lendahl, 1998; Greenwald and Rubin, 1992; Kopan and Turner, 1996; Lewis, 1998; Muskavitch, 1994; Rooke and Xu, 1998; Sawamoto and Okano, 1996; Schlosser and Northcutt, 2000; Simpson, 1990; Tiedemann *et al.*, 1998). Notch also participates in multiple developmental processes as a source of "inductive signaling" between nonequivalent cells. In these cases the "signaling" cells (ligand-expressing) and neighboring "receiving" cells (receptor-expressing) are clearly demarcated. Maintenance of germline proliferation in *C. elegans*, and boundary formation at the dorsal/ventral wing margin of *Drosophila* are two well-described examples of this type of signaling paradigm (for review, see Bray, 1998b; Greenwald, 1998; Kimble and Simpson, 1997). Mounting evidence suggests that Notch signaling, in addition to inhibiting cell fates, can serve to promote cell fates directly. Recent evidence for Notch-mediated cell fate "promotion" includes ectopic upregulation of "master control genes," *vestigial* (*vg*), *eyeless* (*ey*), and *Distal-less* (*Dll*), in imaginal discs expressing an activated Notch construct (N^{act}). Strikingly, early ectopic expression of N^{act} leads to homeotic transformations appropriate to the master control gene affected (Kurata *et al.*, 2000). In addition, it has recently been suggested that Notch can directly specify glial cell fates rather than simply inhibit neuronal differentiation (Furukawa *et al.*, 2000; Gaiano *et al.*, 2000; Morrison *et al.*, 2000); however, direct proof of this proposal awaits identification of Notch-regulated factors required for glial differentiation.

3. Biochemistry of Notch Signaling: Proteolysis-Mediated Activation

Over the past decade researchers have begun to piece together components of the regulatory circuitry governing the Notch signaling pathway (Artavanis-Tsakonas *et al.*, 1999; Chan and Jan, 1998, 1999). Early structure/function analyses established discrete roles for the extracellular and intracellular domains of the Notch receptor (for review, see Greenwald, 1994; Kimble *et al.*, 1998). As with most cell surface receptors, the intracellular domain was found to have signal-transducing capacities while the extracellular domain bound ligand and served to regulate signaling by inhibiting activity in the absence of ligand binding. However, unlike other surface receptors, mounting evidence suggested that the intracellular domain of Notch might directly function within the nucleus as a transcriptional adapter protein (see below). The identification of a putative nuclear localizing signal (NLS) in the cytoplasmic sequence of Notch homologs (Stifani *et al.*, 1992) was quickly followed by demonstrations of nuclear localization for truncated, nonmembrane-tethered, intracellular Notch constructs (collectively referred to here as N^{IC}; Fortini *et al.*, 1993; Lieber *et al.*, 1993; Struhl *et al.*, 1993). Additionally, constitutive gain of function phenotypes were obtained when N^{IC} was expressed in flies and worms devoid of endogenous Notch/lin-12 activity (Lieber *et al.*, 1993; Roehl and Kimble, 1993; Struhl *et al.*, 1993).

A key observation for the course of later studies came from ectopic overexpression studies of extracellularly deleted constructs in *Xenopus* and *Drosophila*. Based on the presumed product of human Notch chromosomal translocations associated with neoplastic transformation (Ellisen *et al.*, 1991; Jhappan *et al.*, 1992; Robbins *et al.*, 1992), deletion constructs were made that had the majority of the extracellular domain removed (these type of constructs will be collectively referred to as $N^{\Delta E}$; Coffman *et al.*, 1993; Rebay *et al.*, 1993). $N^{\Delta E}$ constructs differ from N^{IC} in that signal peptide and transmembrane domain have been retained; thus the intracellular domain is tethered to the plasma membrane. When ectopically overexpressed, $N^{\Delta E}$ was interpreted as having a dominant gain of function activity resulting in a presumed delay of differentiation in frogs (Coffman *et al.*, 1993) and "a complex array of dominant phenotypes" associated with Notch activation in flies (Rebay *et al.*, 1993). In addition, side by side comparison of N^{IC} and $N^{\Delta E}$ transgenic flies showed "indistinguishable" effects on eye development (Fortini *et al.*, 1993). Thus, both N^{IC} and $N^{\Delta E}$ performed as constitutively active proteins, which is in keeping with the interpretation that neoplastic transformations from $N^{\Delta E}$-type chromosomal translocations indicated an activated phenotype (Ellisen *et al.*, 1991). An activation mechanism for Notch was proposed that would explain why both N^{IC} and $N^{\Delta E}$ act equivalently (Lieber *et al.*, 1993; Stifani *et al.*, 1992; Struhl *et al.*, 1993); in the words of Lieber *et al.*, "a segment of Notch might normally enter and function in the nucleus after cleavage of the transmembrane protein." Despite an obvious conflict—the C-terminus of $N^{\Delta E}$ constructs was localized to the cell membrane and never seen in the nucleus (Fortini *et al.*, 1993), and endogenous Notch could not be detected in the nucleus under any circumstances during fly development—the cleavage model of Notch activation proved correct.

The first test of the dual address hypothesis came when an attenuated mouse $N1^{IC}$ fragment failed to inhibit myoblast differentiation when the NLS sequences were removed (Kopan *et al.*, 1994). Restoring nuclear entry of this NLS-deleted $N1^{IC}$ ($N1^{IC\Delta NLS}$) by addition of an exogenous NLS also restored its activity in the myogenic assay. This established that $N1^{IC\Delta NLS}$ was functional and that its site of action was indeed in the nucleus. In the next few years cell culture assays verified the constitutive activity of $N^{\Delta E}$ and N^{IC} moieties; both constructs activate appropriate reporter constructs, inhibit myogenic and neurogenic differentiation, and impact T cell development (Aster *et al.*, 1994; Jarriault *et al.*, 1995; Kopan *et al.*, 1994; Nye *et al.*, 1994; Pear *et al.*, 1996; Shawber *et al.*, 1996). Biochemical analysis of $N1^{\Delta E\,6myc}$ (mouse Notch1ΔE with a hexameric myc tag replacing the C-terminal 348 amino acids) was conducted to resolve the apparent paradox of constitutive Notch activity at two cellular locations—in the nucleus and at the cell membrane (Kopan *et al.*, 1996). This study presented evidence that although the N-terminus of $N1^{\Delta E\,6myc}$ was present outside the cell, the C-terminus could be localized within the nucleus of *Xenopus* embryos and 3T3 fibroblasts. Smaller N-terminally truncated polypeptides are produced from $N^{\Delta E}$ by two distinct and mutually independent mechanisms. First, it was noted that $N1^{\Delta E\,6myc}$ could undergo alternative translations from an internal methionine in the transmembrane domain. Mutating this methionine to a valine (M1727V) eliminated the product of alternative initiation and revealed a second, proteolytic fragment. The proteolytic fragment was later purified and found to be the result of proteolysis between glycine 1743 and valine 1744 of mouse Notch1 (Schroeter *et al.*, 1998). This cleavage site has been termed site 3 (or S3, Fig. 1) and is presumed to lie within the transmembrane domain, although this has yet to be rigorously demonstrated (see below). Schroeter *et al.* went on to show that this cleavage event occurs post-Golgi and that single point mutations of valine 1744 partially inhibit S3 processing and concomitantly reduce the signaling activity of $N1^{\Delta E}$ while having no effect on the signaling efficacy of processing independent $N1^{IC}$ V1744 mutant constructs. In addition, ligand binding to transfected Notch in cell culture was shown to induce S3 cleavage and, again, efficient processing was dependent on V1744 (Schroeter *et al.*, 1998). Concurrent with these efforts, investigators of Notch signaling in Drosophila revealed an *in vivo* ligand-dependent nuclear translocation, presumably via proteolysis, of intracellular Notch (Lecourtois and Schweisguth, 1998; Struhl and Adachi, 1998). It was also demonstrated biochemically that modulation in the expression level of the Notch ligand Delta resulted in similar modulations in NICD production (Kidd *et al.*, 1998).

These experiments demonstrated that ligand-dependent proteolysis can occur *in vivo* and *in vitro*. Recently a requirement for proteolysis at S3 for Notch1 signaling was demonstrated in the mouse; a processing impaired Notch1 allele with a single point mutation at the S3 site (V1744G) was homologously "knocked-in" to the Notch1 locus. Strikingly, the V1744G mutation results in an embryonic lethal phenotype in homozygous mice (Huppert *et al.*, 2000), that occurs within the same period as Notch1 null mice (~e10; Conlon *et al.*, 1995; Swiatek *et al.*, 1994). As expected from a mutation that reduced but did not eliminate NICD production detailed analysis of these mice revealed that the V1744G mutants are hypomorphic. Finally, a biochemical comparison of the four mouse Notch homologs (N1–N4) has shown that all four undergo an S3-like cleavage event (M. T. Saxena and R. Kopan, unpublished observations). Taken as a whole, these experiments lead us to conclude that proteolysis at S3 is a conserved signaling mechanism that is required for CSL-mediated Notch signaling.

4. Notch Is Not Alone—Regulated Intramembrane Proteolysis and the Emergence of Candidate Proteases

Several proteins, in addition to Notch, respond to ligand binding or other stimuli by undergoing unidentified conformational or localization changes which promote, through a proteolytic cascade, the release of part of these "dual

address" proteins. This phenomenon, called regulated intramembranous proteolysis (RIP), is a recently appreciated signaling paradigm whereby transmembrane-bound proteins are released from the cell surface by proteolytic cleavage within the membrane (recently reviewed in Brown *et al.*, 2000). Another definitive example of RIP is the sterol regulatory element binding protein (SREBP; Brown and Goldstein, 1997; Brown *et al.*, 2000) which regulates cholesterol metabolism. In addition, studies involving cleavage of the amyloid precursor protein (APP) to produce the amyloid beta (Aβ) peptide provides an example of a disease process (Alzheimer's disease) which is profoundly linked to intramembranous proteolysis (Hardy and Israel, 1999).

What enzymes catalyze intramembranous cleavage of Notch? The best candidates to date are the presenilin genes (PS), independently linked to familial Alzheimer's disease (Sherrington *et al.*, 1995, 1996), and regulation of the Notch signaling pathway (Levitan and Greenwald, 1995). Notch and APP are both believed to be proteolytically cleaved within their transmembrane domains (at the S3 and γ-secretase sites, respectively) by an unidentified proteinase(s), termed γ-secretase(s). PS must act at or upstream of S3 cleavage and NICD release, because N^{IC} proteins are epistatic to mutations in the *C. elegans* PS homolog sel-12; mutations in sel-12 are incapable of suppressing the constitutive activity of N^{IC}, but do abbrogate the activity of dominant gain of function alleles that are membrane tethered (Levitan and Greenwald, 1998). PS and Notch have been shown to physically interact early in the secretory pathway and appear to remain associated thereafter, as they are found in a complex at the cell membrane (Ray *et al.*, 1999a,b). In an effort to define the level at which PS genes impact the Notch pathway, primary neuronal and fibroblastic cells derived from wild-type and PS1-deficient animals were transfected with $N^{\Delta E\ M1727V}$. The results showed that S3 cleavage is dramatically reduced in the absence of PS1 (De Strooper *et al.*, 1999). This is consistent with findings that Aβ production is reduced in the absence of PS1 (De Strooper *et al.*, 1998) and suggests that PS gene functions are required for the intramembranous processing events, perhaps as γ-secretase(s) (Wolfe *et al.*, 1999a,b,c). In support of this, animals from three phyla which are deficient in all PS activity phenocopy Notch null animals (Donoviel *et al.*, 1999; Herreman *et al.*, 1999; Li and Greenwald, 1997; Struhl and Greenwald, 1999; Westlund *et al.*, 1999; Ye *et al.*, 1999). Moreover, three different inhibitors of γ-secretase activity have recently been shown to bind PS proteins directly (Esler *et al.*, 2000; Li *et al.*, 2000a; Seiffert *et al.*, 2000) providing the best evidence to date that PS proteins function as unique intramembranous enzymes. Although definitive proof awaits a demonstration that the PS proteins function as proteases in an *in vitro* system the recent finding that presenilin proteins share a conserved catalytic center with the bacterial type 4 prepilin peptidases (C. Haas, personal communication) strongly argues that the catalytic center of γ-secretase lies within the presenilin protein. Most recently, a novel member of the high molecular weight presenilin-containing complex, wherein γ-secretase activity resides (Li *et al.*, 2000b), has been identified. The evidence suggests that this factor, nicastrin (APH-2 in *C. elegans*), may be an integral component of a putative multimeric complex (the "secretasome") required for intramembrane proteolysis of both APP and Notch (Yu *et al.*, 2000). Investigation of APH-2 function in *C. elegans* previously established it as a novel member of the Notch signaling pathway; however, chimeric analysis suggested that APH-2 can act non-cell autonomously in either the signaling or receiving cell (Goutte *et al.*, 2000), a result potentially in conflict with the secretasome proposal (for recent reviews on intramembrane proteolysis, see Brown *et al.*, 2000; Kopan and Goate, 2000).

5. Ectodomain Shedding and Notch: New Twists with Old Knives

Since Notch activation requires presenilin-dependent intramembranous proteolysis, how does ligand binding to the Notch extracellular domain regulate this step? Structure/function analyses of Notch mutants and deletion constructs in flies and worms suggest that the extracellular domain functions to repress Notch signaling in the absence of ligand (Greenwald, 1994; Kimble *et al.*, 1998). This negative control region is likely located somewhere between the LNR and the transmembrane domain (Kimble *et al.*, 1998; Lieber *et al.*, 1993). Results from three groups using different approaches have recently shed light on the mechanism of this regulation.

First, the existence of a ligand-induced, "ectodomain shedding-like" cleavage event has been described (Brou *et al.*, 2000; Mumm *et al.*, 2000). Cleavage occurs at an extracellular site (S2), between Ala^{1710} and Val^{1711} residues, approximately 12 amino acids outside the transmembrane domain. The resultant carboxy product of S2 cleavage is called NEXT (for Notch EXtracellular Truncation). The data suggest metalloproteases are responsible for S2 cleavage; the metalloprotease TACE/ADAM17 was shown to cleave Notch *in vitro* at the S2 site (Brou *et al.*, 2000) and zinc chelators block S2 cleavage in culture (Mumm *et al.*, 2000). However Sup-17/Kuzbanian/ADAM10, a metalloprotease genetically linked to Notch signaling (Rooke *et al.*, 1996; Sotillos *et al.*, 1997; Wen *et al.*, 1997), is not required for S1, S2, or S3 cleavage (Mumm *et al.*, 2000). These data suggest that a ligand-induced proteolytic cascade activates Notch1: inhibitor studies demonstrated that NEXT is a proteolytic intermediate required for the generation of NICD (Mumm *et al.*, 2000). Notch undergoes Furin-convertase-type cleavage in the secretory pathway at a site called S1 (site 1, Fig. 1), the cleaved fragments held together by a calcium coordinated bond (Rand *et al.*, 2000). Dissolving this bond by calcium chelation also results in activation via S3 cleavage (Rand *et al.*, 2000). Ligand binding might therefore overcome "juxtamembrane" repression of Notch intramembrane proteolysis (and thus signaling) by simply removing the

negative control region. This work and additional experimental evidence support the existence of a shared regulatory mechanism among Notch, SREBP, and APP (Chan and Jan, 1999; De Strooper *et al.,* 1999; Hardy and Israel, 1999), with extracellular proteolysis serving to facilitate proteolysis at the intramembranous site to release NICD.

Evidence that S2 cleavage truly results in "ectodomain shedding" has come from studies in *Drosophila*. A comparison of temperature-sensitive *shibire* (Grigliatti *et al.,* 1973) and temperature-sensitive *Notch* mutants (Shellenbarger and Mohler, 1975, 1978) noted a similarity in their phenotypes and suggested that *shibire* has a "neurogenic" phenotype (Poodry, 1990). *shibire* is the *Drosophila* homolog of vertebrate dynamin, a GTPase required for clathrin-mediated endocytosis (Damke *et al.,* 1994; Herskovits *et al.,* 1993; Kosaka and Ikeda, 1983). Subsequent investigation demonstrated that Notch signaling requires dynamin/shibire-mediated endocytosis, in both signaling cells (ligand-expressing) and receiving cells (Notch-expressing), during sense organ development in *Drosophila* (Seugnet *et al.,* 1997). A recent study provides further insight demonstrating that the Notch extracellular domain is "*trans*-endocytosed" into ligand (Delta)-expressing cells at sites of Notch activation where ligand-presenting and receptor-presenting cells are clearly demarcated, the developing eye and wing imaginal discs (Parks *et al.,* 2000). Importantly, they find that an endocytosis-defective class of ligand mutants function as loss-of-function alleles, presumably due to their inability to *trans*-endocytose Notch (Parks *et al.,* 2000). Taken together these results have led to the hypothesis that ligand-mediated Notch activation requires an endocytosis-driven, stress-based conformational change that exposes the S2 site to cleavage and leads to the cascade of proteolytic activation (Mumm *et al.,* 2000; Parks *et al.,* 2000; Fig. 2).

6. Dual Address Protein: Notch Is a Transmembrane Receptor; NICD Is a Nuclear Adapter Regulating CSL-Mediated Transcription

The results discussed above established that NICD is released by proteolysis and that its release is required for Notch activity in cell culture (Schroeter *et al.,* 1998) and *in vivo* (Huppert *et al.,* 2000). Early efforts demonstrated that Notch activity was required in the nucleus (Kopan *et al.,* 1994; Struhl and Adachi, 1998) where NICD binds directly to CSL proteins, through the Notch RAM domain (amino acids 1748–1810; Tamura *et al.,* 1995), and the Ankyrin repeats (Kato *et al.,* 1997; Kurooka *et al.,* 1998; Roehl and Kimble, 1993). The coupled proteins can be extracted from vertebrate nuclei in cell culture and the complex is capable of binding DNA (Jarriault *et al.,* 1995). However, CSL/Notch interactions were also detected in the cytoplasm of Drosophila cells in culture (Fortini and Artavanis-Tsakonas, 1994; Kidd *et al.,* 1998) spurring a debate as to the actual site of Notch action *in vivo:* in the nucleus (Kopan *et al.,* 1994; Lieber *et al.,* 1993; Struhl *et al.,* 1993) or elsewhere (Artavanis-Tsakonas *et al.,* 1999; Aster *et al.,* 1997, and see Section 4). Nuclear Notch has recently been observed in differentiated neurons *in vivo* (Ahmad *et al.,* 1995; Redmond *et al.,* 2000; Sestan *et al.,* 1999) and Notch activation by ligand was shown to result in nuclear immunoreactivity in neurons in cell culture (Sestan *et al.,* 1999). However, evidence for nuclear Notch in tissues where Notch signaling is active during development is still lacking. A number of studies have identified nuclear cofactors capable of binding intracellular Notch (see below); one trivial explanation could be that antibody recognition is disrupted by such interactions, thereby masking detection. The difficulty in identifying nuclear Notch in most cells undergoing Notch signaling was partially explained by the demonstration that NICD acts at a nuclear concentration below the threshold of current detection techniques (Huppert *et al.,* 2000; Schroeter *et al.,* 1998). Therefore, only small amounts of NICD may be normally produced in cells receiving Notch signal. How could low amounts of nuclear NICD produce the transcriptional output required for early developmental decisions? The answer to this central question remains elusive, but a flurry of recent publications produce the clearest picture yet of the players and events that enable Notch signaling to orchestrate a developmental switch in the nucleus.

a. The Corepressor Complex

In the absence of activation by Notch, it has been demonstrated that CSL acts as a transcriptional repressor (Dou *et al.,* 1994; Hsieh and Hayward, 1995). Recent publications offer an increasingly detailed view of how Notch and its viral mimic, the EBNA2 protein (Sakai *et al.,* 1998; Strobl *et al.,* 2000), affect transcription. CSL binds to at least two corepressor complexes: the SMRT/NcoR/histone deacetylase 1 (HDAC1) (Kao *et al.,* 1998) and CIR/HDAC2/SAP30 complexes (Hsieh *et al.,* 1999; Zhou *et al.,* 2000b). A third corepressor, KyoT2, (Taniguchi *et al.,* 1998) has also been identified (Taniguchi *et al.,* 1998). In all cases, intracellular Notch can antagonize the CSL/corepressor interaction (Hsieh *et al.,* 1999; Zhou *et al.,* 2000a,b, Fig. 3A). The involvement of the corepressor complex in CSL^{CBF1}-mediated repression ($RBP_{J\kappa}$ and CBF1 are two different names for the same CSL protein, reflecting the cloning history) was further substantiated by demonstrating that a CSL mutant ($CBF1^{EEF233AAA}$), which lost interaction with both the SMRT and the CIR proteins, fails to repress transcription (Hsieh *et al.,* 1999; Zhou *et al.,* 2000a,b).

If CSL acts as a transcriptional repressor, removal of CSL protein, or its binding sites in target promoters, should result in activation of these repressed targets. This prediction was first demonstrated for the adenovirus pIX promoter. Here, promoter-bound CSL directly associates with TFIIA and TFIID (members of the basal transcription machinery), preventing transcription from progressing (Olave *et al.,* 1998). Deletion of the CSL-binding site or their

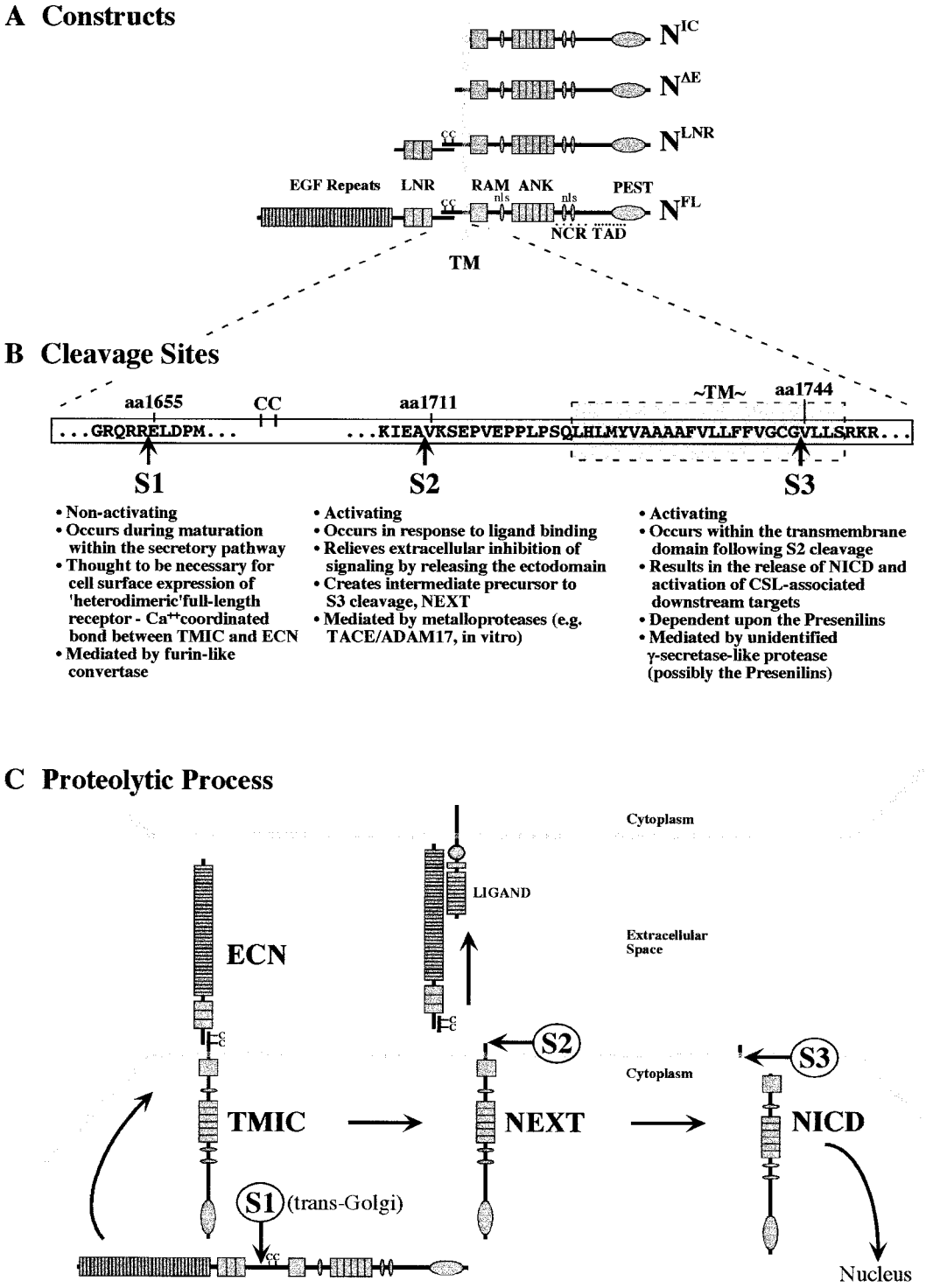

FIG. 2. Diagram of Notch proteins and the proteolytic cascade of Notch activation. (A) Schematic of artificial Notch plasmids referred to in the text: N^{IC}, Notch Intracellular construct; $N^{\Delta E}$ Notch Deleted Extracellular construct; N^{LNR}, Notch Lin/Notch Repeat construct; N^{FL}, Notch Full-Length construct. Note that N^{IC} and $N^{\Delta E}$ constructs can vary considerably between individual laboratories. Constructs which mimic natural proteolytic products (e.g., $N^{IC\ V1744}$, see below) have been employed more recently. Conserved domains are denoted above the full-length Notch diagram. Abbreviations: EGF, Epidermal Growth Factor-like repeats; LNR, Lin/Notch Repeat domain; RAM, RAM23 domain; nls, nuclear localizing signals; ANK, CDC10/Ankyrin repeat domain; PEST, a region rich in proline (P), glutamine (E), serine (S),

location upstream from a Sp1-binding site restores transcriptional activity, indicating that this viral promoter utilizes CSL only for repression (Olave *et al.*, 1998). In more complex promoters, CSL protein is required for both repression and activation. In *Drosophila*, both Notch and CSL are normally required together to activate the gene *single-minded* (*sim*) in the midline (Morel and Schweisguth, 2000). Loss of $CSL^{Su(H)}$ results in partial misexpression of *sim*, a phenomenon previously interpreted to indicate CSL-independent signaling by Notch (Lecourtois and Schweisguth, 1995; Rusconi and Corbin, 1999). However, removal of the CSL-binding site from the *sim* promoter is sufficient to induce ectopic *sim* expression pattern in a Notch-independent manner (Morel and Schweisguth, 2000). These experiments demonstrated the consequence of loss of repression by CSL *in vivo*, validating results accumulating from cell culture work. Moreover, transcription of $CSL^{Su(H)}$ itself has recently been shown to be regulated by the repressor activity of $CSL^{Su(H)}$ in one particular cell, the bristle cell of the external sensory organ. A complex 3′ enhancer element was identified in the *Drosophila* $CSL^{Su(H)}$ locus, containing multiple CSL-binding sites (S. Barolo and J. W. Posakony, personal communication). Interestingly, this element (termed ASE, for Autoregulatory Socket Enhancer) is responsible for preventing accumulation of $CSL^{Su(H)}$ via auto-repression. Point mutations disrupting these sites result in loss of repression, elevated $CSL^{Su(H)}$ in bristle precursor cells, and conversion of the bristle cell to a socket cell, its sibling cell fate.

Strong support of a direct role for Notch in transcription stems from experiments reported by Kuroda *et al.* These authors demonstrated that 1 h after addition of ligand-expressing cells, a 7-fold increase was detected in the accumulation of the Hes-1 mRNA in Notch-expressing cells, reaching 20-fold in 2 h. This robust increase was only detected in the presence of cycloheximide, a protein translation inhibitor. A modest, biphasic, 2.5-fold increase was observed when protein translation was not inhibited (Kuroda *et al.*, 1999). These observations indicate that *Hes-1* is a direct target of Notch and point to the existence of a negative feedback loop by some Notch targets (Hes-1 and possibly Deltex), (Deftos and Bevan, 2000). However, the molecular mechanism by which Notch antagonizes CSL-mediated repression remains mysterious. How can undetectable amounts of nuclear NICD displace several ubiquitous transcriptional repressors from DNA-bound CSL?

b. The Intermediate Complex

A recent discovery offers important insight into NICD-mediated transition of CSL from a repressor to an activator complex. Using CSL^{CBF1} as bait in a yeast two-hybrid screen, Zhou and co-workers identified the Ski interacting protein (SKIP) as a member of the CSL/corepressor complex (Zhou *et al.*, 2000a,b). cSki is known to participate in TGFβ/SMAD2/MAD and nuclear receptor-mediated repression as a member of the corepressor complex (Akiyoshi *et al.*, 1999; Hammond *et al.*, 1998; Luo *et al.*, 1999; Nomura *et al.*, 1999; Baudino *et al.*, 1998; Tagami *et al.*, 1998). SKIP was identified as a protein involved in derepression of this complex (Baudino *et al.*, 1998; Dahl *et al.*, 1998; Tagami *et al.*, 1998).

SKIP, and its conserved orthologs in *Drosophila* and *C. elegans*, interact with CSL at a domain distant and distinct from the CSL/SMRT interaction site. In fusion with Gal4, SKIP mediates repression (as does CSL) most likely through interaction with the SMRT/HDAC complex. However, SKIP is also able to interact with N^{IC} under the same conditions. The SKIP interaction interface on Notch is revealing: point mutations located in the fourth ankyrin repeat that have been shown to disrupt Notch activity, M1 and M2 (Jarriault *et al.*, 1995; Kopan *et al.*, 1994), also abolish SKIP interaction. Under the conditions used by Zhou, M2 mutants retained the ability to interact with CSL^{CBF1} but did not bind SKIP (Zhou *et al.*, 2000b). Most importantly, while SMRT competes with Notch for CSL binding, SKIP promotes Notch/CSL interactions. For example, a Notch construct with a deletion of the RAM23 domain binds CSL weakly (Kato *et al.*, 1997) or not at all (Zhou *et al.*, 2000b); addition of SKIP recruits this same construct to CSL (Zhou *et al.*, 2000b). The binding of SMRT or Notch to SKIP is mutually exclusive; Skip/CSL can form

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and threonine (T) residues; TM, transmembrane region. Also included are two roughly mapped regions recently identified as potentially important signaling domains, NCR, Notch Cytokine Response region, and TAD, Trans-Activating Domain. (B) The amino acid sequence of Mouse Notch1 encompassing each known cleavage site is shown within the open rectangle. Precise sites of S1, S2, and S3 cleavage (site one, etc.) are denoted by arrows and the amino acid number of the respective C-terminal ends. The location and function of these processing sites may not be conserved in all species; for instance, it is unclear whether S1 occurs in flies or whether it is absolutely required for Notch receptor maturation. The shaded gray rectangle represents an approximated transmembrane domain with a 24 amino acid span (note that a 21aa span could place the S3 site at the cytosolic interface of the transmembrane domain). A brief description of the known functions and putative proteases responsible for each processing event is given. (C) A diagram of the proteolysis-mediated model of ligand-induced Notch activation. Upon ligand-binding the heterodimeric receptor is believed to undergo an endocytosis-driven conformational change which exposes S2 to proteolysis. The resultant C-terminal product, NEXT, undergoes constitutive S3 intramembranous proteolysis resulting in translocation of NICD and activation of the Notch transcriptional response. Abbreviations: ECN, ExtraCellular Notch; TMIC, TransMembrane and IntraCellular domain (also referred to as p120); NEXT, Notch ExtraCellular Truncation; NICD, Notch IntraCellular Domain.

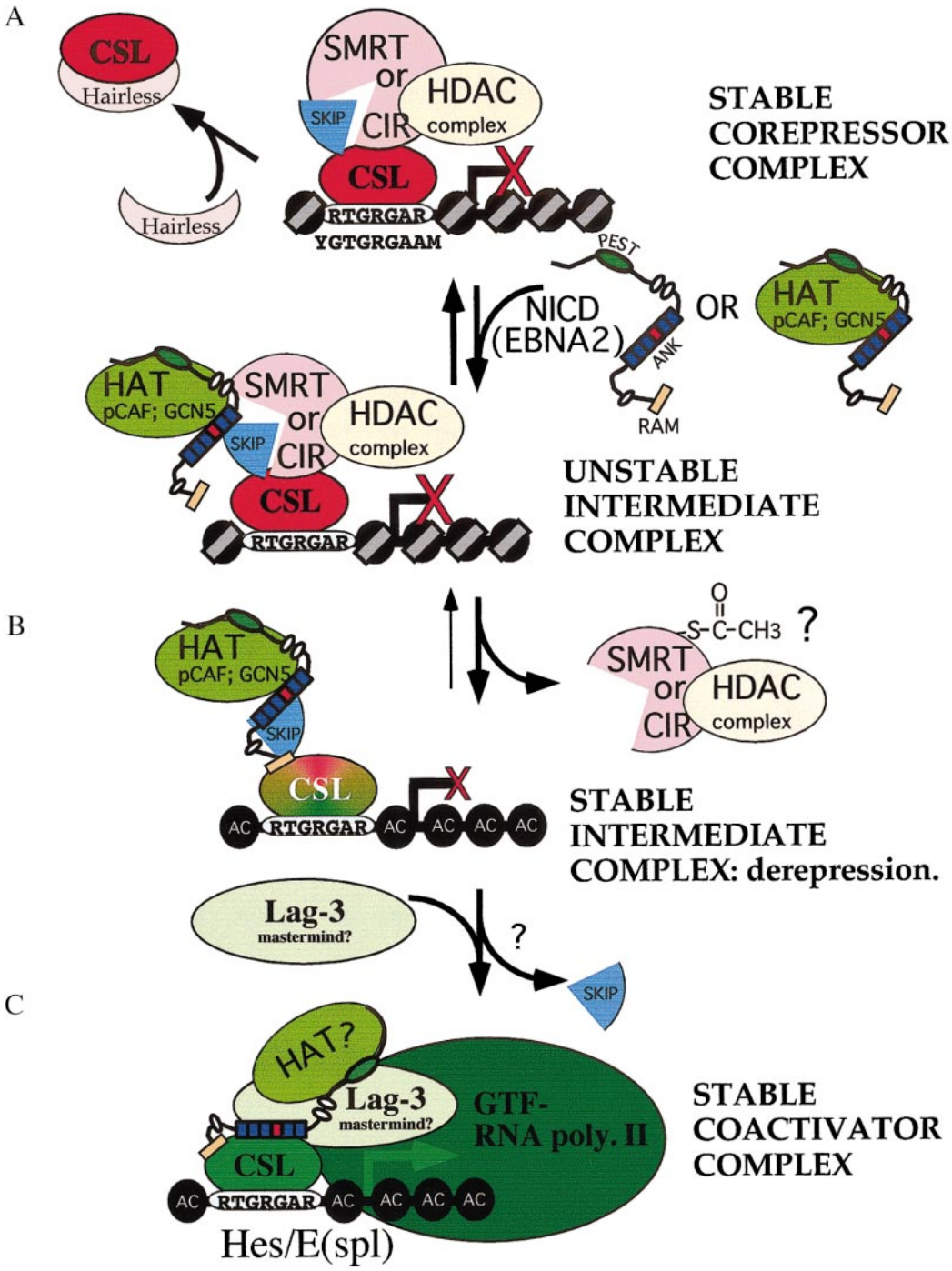

FIG. 3. Model for Notch-mediated conversion of CSL. Diagram of hypothetical mechanism by which Notch mediates conversion of the CSL corepressor complex to a transcriptional coactivator complex (see text for further details). RTGRGAR and YGTGRGAAM are low-affinity and high-affinity consensus CSL-binding sites, respectively (Nellesen *et al.*, 1999). Abbreviations: SMRT, Silencing Mediator of Retinoid and Thyroid hormone receptors; CIR, CBF1 Interacting coRepressor; SKIP, Ski-related Protein; HDAC, Histone Deacetylase; HAT, Histone Acetylase; EBNA2, Epstein Barr virus Nuclear Antigen; AC, Acetylated histones. Other abbreviations are given in the text and preceding figures.

a triprotein complex with either Notch or SMRT but not with both (Fig. 3). Importantly, the authors demonstrate that even at 4-fold SMRT excess, Notch remained associated with SKIP/CSL. Thus, Zhou *et al.* illuminate a possible mechanism by which low levels of NICD in the nucleus may convert CSL into a transcriptional activator. SKIP, which is a permanent resident of the CSL repressor complex, seems to facilitate NICD binding by providing an interface to which NICD may have a higher affinity than SMRT. Since the binding of SMRT and NICD to SKIP is mutually exclusive, NICD recruitment to the complex may destabilize the SMRT/CSL interaction leading to the conversion from SMRT-corepressor complex to an intermediate, NICD-containing complex (Fig. 3B). Whether NICD concentration is higher near Notch-regulated promoters remains to be determined.

Additional members of this intermediate complex were revealed recently by Kurooka and Honjo. Using mammalian two-hybrid assays, they have demonstrated an interaction between Notch and two conserved histone acetylases (HATs), PCAF and GCN5 (Kurooka and Honjo, 2000). Both the Ankyrin repeats (amino acids 1810–2097) and the TAD region (amino acids 2194–2398) of Notch are necessary to mediate the interaction with the amino-terminus of PCAF and GCN5, thus recruiting these HATs to the Notch/CSL complex. The HAT/Notch/CSL complex would acetylate histones, increasing the likelihood for the formation of an open chromatin and an active transcription complex (Struhl, 1999). Another speculative possibility is that HATs acetylate SMRT, catalytically promoting Notch/CSL interactions. This would provide another mechanism whereby low levels of Notch could displace ubiquitous corepressors. It should be noted that a truncated version of fly $Notch^{IC}$ from which the TAD equivalent region was removed behaved *in vivo* in an identical fashion to the complete NICD protein (Tomlinson and Struhl, 1999). Thus, questions persist regarding the relevance of the HAT/TAD interaction for all systems.

c. The Coactivator Complex

The HAT/Notch/CSL complex is, however, only part of the story. PCAF and GCN5 were fused with the DNA binding domain of yeast Ga14 and tested for their transcriptional prowess on the ga14 promoter in cultured cells. Intriguingly, neither HAT-Ga14 construct was sufficient to induce transcription while NICD-Ga14 showed robust induction (Kurooka and Honjo, 2000). Using a different strategy, Petcherski and Kimble identified a new constituent of the coactivation complex. These authors modified a yeast two-hybrid screen to identify proteins that interact with the intracellular domain of the Notch ortholog Glp-1, but only in the presence of CSL^{LAG-1}. They identified only one protein (cloned six separate times) that met this criteria. The protein is novel, nuclear, and glutamine-rich. RNAi-mediated interference of this gene led to a phenotype identical to a complete loss of Notch (lin-12 and glp-1) activity in *C. elegans*, hence the name Lag-3 (Lin and Glp-3, Petcherski and Kimble, 2000a; or Sel-8, Doyle *et al.*, 2000). Lag-3 now joins an elite group of genes in *C. elegans* whose absence prevents Notch signaling; the others include a Delta-like ligand (Lag-2) and CSL (Lag-1). Using the same strategy these authors show that in *Drosophila* and vertebrates, *mastermind* provides the same function as *lag-3* (Petcherski and Kimble, 2000b), thus providing a possible mechanistic explanation for the previously observed epistatic relationship between Notch and mastermind (Helms *et al.*, 1999; Schuldt and Brand, 1999).

Further experiments revealed the molecular mechanism by which lag-3 may mediate Notch signaling. *In vitro* experiments determined that Lag-3 interacts weakly with $N^{IC\ Glp1}$; however, the interaction is strongly enhanced by the addition of CSL^{Lag-1}. Using a temperature-sensitive mutation Petcherski and Kimble established that the fourth Ankyrin repeat, the very same region identified as a SKIP interaction interface, mediates N^{IC}/Lag-3 interactions (Petcherski and Kimble, 2000a). Finally, unlike the HATs, LexA/Lag-3 fusion proteins are strong transcriptional activators. LAG-3 could therefore be a transcriptional activator in *C. elegans*, with the glutamine-rich domain serving as the primary activation domain or acting cooperatively with the Notch TAD domain. It is not clear if the coactivator complex still contains SKIP, and it is possible that NICD recruits Lag-3 to the HAT complex. The exact composition of, and the sequence of events leading to, the coactivator complex remain to be elucidated. However, a speculative model consistent with all these recent publications is presented in Fig. 3.

d. Termination of Notch Signaling

After NICD acts in the nucleus of mitotic cells, it must be removed as the daughters will often again rely on Notch signaling to determine their fate (Kopan, 1999). It has been recently demonstrated that the proteasome plays a role in Notch signaling (Schweisguth, 1999) as do the E3 ubiquitin ligases Suppressor of Deltex (Su(Dx); Cornell *et al.*, 1999; Fostier *et al.*, 1998) and Sel-10 (Hubbard *et al.*, 1997). While one mode of proteasome action could be to degrade Notch effectors such as Tramtrack (ttk) (Campos-Ortega, 1996; Dong *et al.*, 1999) NICD itself was recently shown to be a target for ubiquitinition by a Su(Dx) homolog in vertebrates, Itch (Qiu *et al.*, 2000).

7. Alternative Modes of Notch Signaling?

Recent evidence suggests that more surprises may be in store for Notch signaling. As previously suggested by misexpression of *sim* (see above, Lecourtois and Schweisguth, 1995), some Notch signaling may occur in a CSL-independent manner; expression of a dominant negative Su(H) affects only certain sublineages of Notch/Numb-dependent cell fates during bristle development in *Drosoph-*

ila (Wang *et al.*, 1997), and *Su(H)* mutant phenotypes differ from *Notch* mutants in the wing, the eye, and the embryo of *Drosophila* (Arias, 1998; Brennan *et al.*, 1999a,b, 1997; Ligoxygakis *et al.*, 1998; Rusconi and Corbin, 1999; Zecchini *et al.*, 1999). Similar evidence has been presented for vertebrate B cell and muscle development (Ordentlich *et al.*, 1998; Shawber *et al.*, 1996). Interestingly, this signaling may also be independent of DSL ligands or proteolysis (Brennan *et al.*, 1999a, 1997; Rusconi and Corbin, 1999).

Several possible mechanisms by which this function of Notch is accomplished have been proposed. Genetic experiments in *Drosophila* lead to the hypothesis that Notch represses Wingless function at a stage preceding cell fate specification (Brennan *et al.*, 1999a, 1997, 1999c; Rusconi and Corbin, 1999). Wg may repress *Notch* function through a subdomain containing EGF repeats 17–19 and/or 24–26 (Brennan *et al.*, 1999c) to prevent the formation of an equivalence group. Further support for this possibility comes from recent biochemical studies demonstrating that Wg and Notch physically interact (Wesley, 1999) without producing a NICD equivalent (Wesley and Saez, 2000). Notch also interacts with the Wg signal transducer, Dishevelled (Axelrod *et al.*, 1996). It remains to be determined what the significance of Wg binding to Notch is. Interestingly, the same region of Notch mediates binding of other proteins, including Fringe (Ju *et al.*, 2000; Wesley and Saez, 2000). Moreover, while some speculate that Notch represses Wg activity (Brennan *et al.*, 1999a) others find that Wg and Notch interact to activate specific targets (Wesley, 1999). Finally, recent evidence suggests that some CSL "independent" activity of Notch is due to CSL derepression. Another alternative for the core pathway may be modulation of kinase activity, for example, Jun kinase (JNK), by Notch (Ordentlich *et al.*, 1998; Zecchini *et al.*, 1999). Clearly, modulation of *Notch* during development is complex, and the relationship of these factors to the "core" *Notch* pathway or to novel signaling pathways remains to be clarified. Finally, $CSL^{Su(H)}$ has recently been shown to auto-activate itself in a Notch-independent manner, maintaining its own expression via the 3′ ASE in the socket cell of the adult bristle of *Drosophila* (S. Barolo and J. W. Posakony, personal communication), the very same elements required for CSL-mediated repression in its sister cell, the bristle precursor. Though a special case, this observation reminds us that detailed knowledge of Notch/CSL-regulated promoters is essential for complete understanding of the pathway.

8. Future Directions

Despite benefiting in recent years from an increasingly collaborative, multidisciplinary effort many questions remain in the Notch field. Structural information regarding Notch and its interacting proteins remains scarce. The oligomeric state of Notch and the molecules that are associated with it at the cell surface are unknown, nor is it clear what oligomerization changes if any are induced by ligand binding from within the same cell (*cis* interactions) or from another cell (*trans* interactions). Another area lagging behind is the elucidation of the molecular mechanisms of known genetic modifiers of Notch signaling. While some recent progress in this area has been made (e.g., Fringe; Ju *et al.*, 2000; Bruckner *et al.*, 2000; Moloney *et al.*, 2000; Numb; Verdi *et al.*, 1999; Wakamatsu *et al.*, 1999; and mastermind; Petcherski and Kimble, 2000b) the role of proteins such as Deltex, big brain, pecanex, scabrous, sanpodo, and neuralized remains elusive. Another unresolved issue is the existence of "soluble" ligands. Soluble forms of DSL ligands are capable of binding to Notch (Shimizu *et al.*, 1999) but have been reported to either antagonize (Hukriede and Fleming, 1997; Sun and Artavanis-Tsakonas, 1997) or promote (Han *et al.*, 2000; Li *et al.*, 1998; Qi *et al.*, 1999; Sestan *et al.*, 1999; Wang *et al.*, 1998) Notch signaling. Also, the degree of functional redundancy of the four vertebrate Notch proteins and their role in the adult vertebrate is only beginning to be investigated. Perhaps the biggest challenge remaining is complete integration, at the biochemical level, of the genetic interactions between the Notch, Wingless, and Ras pathways. If the last few years are any indication, future research will no doubt continue to produce many surprising and intriguing results.

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