Critical Reviews in Biochemistry and Molecular Biology, 41:339–385, 2006

ISSN: 1040-9238 print / 1549-7798 online
DOI: 10.1080/10409230600914344

informa
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Signal Integration During Development: Mechanisms of EGFR and Notch Pathway Function and Cross-Talk

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ABSTRACT Metazoan development relies on a highly regulated network of interactions between conserved signal transduction pathways to coordinate all aspects of cell fate specification, differentiation, and growth. In this review, we discuss the intricate interplay between the epidermal growth factor receptor (EGFR; Drosophila EGFR/DER) and the Notch signaling pathways as a paradigm for signal integration during development. First, we describe the current state of understanding of the molecular architecture of the EGFR and Notch signaling pathways that has resulted from synergistic studies in vertebrate, invertebrate, and cultured cell model systems. Then, focusing specifically on the Drosophila eye, we discuss how cooperative, sequential, and antagonistic relationships between these pathways mediate the spatially and temporally regulated processes that generate this sensory organ. The common themes underlying the coordination of the EGFR and Notch pathways appear to be broadly conserved and should, therefore, be directly applicable to elucidating mechanisms of information integration and signaling specificity in vertebrate systems.

KEYWORDS drosophila, epidermal growth factor receptor, notch, signal tranduction, eye development

INTRODUCTION

Development in metazoans provides a fruitful backdrop in which to study how a limited collection of signaling networks accurately regulates a diverse array of cellular events, including cell fate specification, morphogenesis, proliferation, differentiation, polarity establishment, programmed cell death, and motility. Somehow, the spatial and temporal mechanisms that coordinate the function of these pathways must impart specificity. A major part of the answer, which we are just beginning to decipher, is that rather than functioning as completely independent and insulated modules, signaling pathways interface in intricate ways to create a web of specific interactions that the cell integrates and interprets in a spatially and temporally appropriate manner (Sundaram & Han, 1996; Tan & Kim, 1999; Voas & Rebay, 2004; Shilo, 2005; Sundaram, 2005).

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The evolutionarily conserved epidermal growth factor receptor (EGFR) pathway and the Notch pathway represent two such signal transduction mechanisms. Recent studies have emphasized that the interaction between the EGFR and Notch pathways is intimate and critical during developmental processes in metazoa (Shilo, 2005; Sundaram, 2005). The reports of EGFR and Notch signaling interactions are storied, but the molecular mechanisms underlying the cross-talk and signal integration between these two pathways are just beginning to be revealed (Sundaram, 2005).

In the following review, our goal is to compare and contrast the various types of relationships reported between the EGFR and Notch signaling pathway in *Drosophila melanogaster*. In this system, EGFR and Notch may interact antagonistically or cooperatively over specific time and space. First, incorporating results from both genetic and biochemical analysis, we will describe the biochemical and molecular interactions involved within the individual EGFR and Notch signaling pathways. Then, we focus on the interaction of these pathways in the context of progressive development of the Drosophila eye, a well characterized, genetically tractable system to study cell-cell signaling integration and cross-talk.

THE EPIDERMAL GROWTH FACTOR RECEPTOR PATHWAY

The EGFR signaling pathway was described initially through cooperative genetic analyses in *Drosophila melanogaster* and *Caenorhabditis elegans* and biochemical studies in mammalian cell culture (Wassarman *et al.*, 1995; Perrimon & Perkins, 1997; Voas & Rebay, 2004; Shilo, 2005). In Drosophila, the EGFR pathway is used reiteratively throughout development, contributing to processes as diverse as adult abdominal dorsoventral patterning, wing vein determination, and oogenesis (Shilo, 2003; Shilo, 2005). In the following sections, we review the signaling machinery that drives the EGFR pathway in Drosophila. In particular, we highlight the mechanisms that convert extracellular signals into transcriptional responses. It is important to note that in presenting a broad overview of EGFR signaling, we have incorporated information from multiple contexts and have down-played the tissue-to-tissue variations in the signaling machinery.

DER Ligands

Signal transduction mechanisms require that molecular cues initiated at the cell surface be relayed to the nucleus. In the EGFR signaling pathway, these signals take the form of small protein ligands. There are at least eight different EGFR ligands in mammals, which include EGF, transforming growth factor-α (TGF-α), and the Neuregulins (Riese & Stern, 1998). EGFR ligands are synthesized as transmembrane precursors where they either function as membrane-anchored proteins for juxtacrine signaling or are cleaved proteolytically to release a soluble ligand (Riese & Stern, 1998). Four activating ligands and one inhibitory ligand have been identified for the *Drosophila* epidermal growth factor receptor (DER) (Figure 1A) (Shilo, 2003): (1) the Neuregulin Vein; (2) the TGF-α ligands Spitz, Keren, and Gurken; and (3) the repressive ligand Argos.

The Neuregulin Vein Ligand

Vein (Vn), a Neuregulin-class secreted ligand with a weak activation capacity, is utilized in tissues that require low EGFR activation (Schnepp *et al.*, 1996; Shilo, 2003). Neuregulins have an immunoglobulin-like (Ig) domain, multiple potential glycosylation sites,

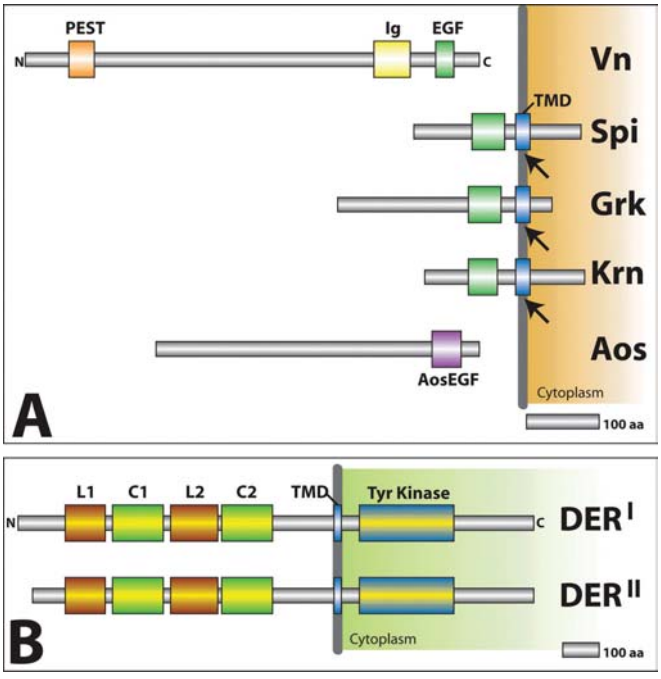

FIGURE 1 **The Drosophila epidermal growth factor (EGF) Receptor (DER) and its ligands. (*A*) DER has five ligands. Spi, Grk, and Krn are cleaved for secretion at their transmembrane domain (TMD; *arrow*). (*B*) The two different isoforms of DER differ at their N-termini. See text for domain details. Adapted from Shilo, 2003.**

and a cysteine-rich EGF domain (Garratt *et al.*, 2000). The Ig domain of Vn may facilitate protein-protein interaction through dimerization or interactions with other extracellular proteins (Figure 1A) (Schnepp *et al.*, 1996). Developmentally, Vn is necessary during embryogenesis for the patterning of the ventral ectoderm and specification of muscle precursors (Shilo, 2003). The role of Vn during eye development is less clear. Although it is not required genetically, Vn may stimulate DER signaling in the contexts of cell proliferation and survival, and possibly differentiation (Spencer *et al.*, 1998). Because Vn does not appear to play a major role in the eye (Shilo, 2003), it will not be considered further in this review. Rather, the ensuing discussion will focus on Spi, the DER ligand that has a key role during eye development.

The TGF-α Family of DER Ligands

The primary DER ligand, Spi, is responsible for activation of the DER pathway in most tissues during Drosophila development, beginning in oogenesis and continuing through adult male spermatogenesis (Shilo, 2003). Spi, like the other two TGF-α family ligands, Keren (Krn) and Gurken (Grk), is generated as a transmembrane precursor with an N-terminal signal peptide, an EGF domain in the extracellular region, a transmembrane domain and a cytosolic C-terminus (Figure 1A) (Rutledge *et al.*, 1992; Neuman-Silberberg & Schupbach, 1993; Kumar *et al.*, 1995; Reich & Shilo, 2002). In the past several years, the mechanisms by which Spi is processed have begun to be elucidated (Klambt, 2000; Klambt, 2002; Shilo, 2003). Based on strong genetic interactions with DER pathway components, *Rhomboid* (*Rho*) and *Star* (*S*), which encode novel transmembrane proteins, were initially proposed to facilitate DER signaling via cell-surface processing and presentation of the Spi ligand (Sturtevant *et al.*, 1993). More recently, Rho and S were found to mediate processing of Spi within the cell (Figure 2) (Bang & Kintner, 2000; Lee *et al.*, 2001; Urban *et al.*, 2001; Tsruya *et al.*, 2002). *Rho* encodes a seven-transmembrane protein, while *S* encodes a Type II transmembrane protein; each has a cytoplasmic N-terminus and a lumenal C-terminus (Bier *et al.*, 1990; Kolodkin *et al.*, 1994; Lee *et al.*, 2001).

Biochemical and cell culture experiments showed that S colocalizes with Spi, such that S translocates the Spi precursor protein from the endoplasmic reticulum (ER) to the Golgi apparatus by either blocking

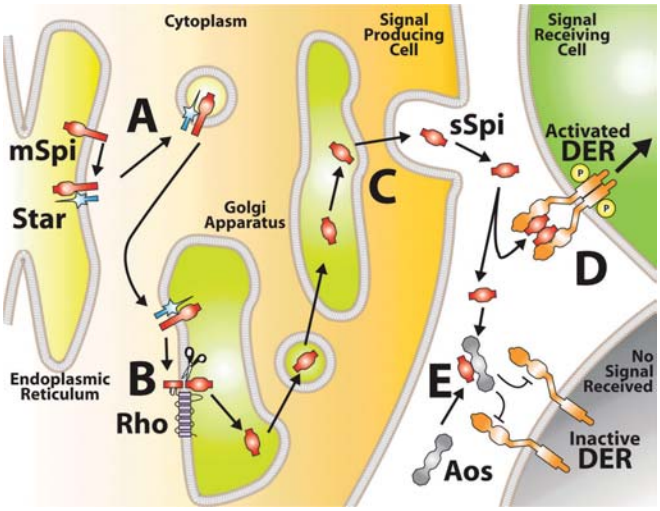

FIGURE 2 **Spi ligand processing, DER activation, and Spi-inhibition by Aos. (*A*) Membrane-bound Spitz (mSpi) is transported to the Golgi by Star, (*B*) cleaved by Rhomboid (Rho) and (*C*) secreted, where it (*D*) activates DER or (*E*) is sequestered by Aos, preventing DER activation. Adapted from Shilo, 2003.**

an ER retention signal in Spi or actively exporting Spi to the Golgi (Figure 2) (Lee *et al.*, 2001). The lumenal domain of S mediates this trafficking through interaction with the lumenal domain of the Spi precursor (Lee *et al.*, 2001). Following translocation, Spi and S sequentially bind Rho (Tsruya *et al.*, 2002) and Spi is then cleaved within its transmembrane domain in a Rho dependent manner (Figure 2) (Lee *et al.*, 2001; Urban *et al.*, 2001; Tsruya *et al.*, 2002; Urban & Freeman, 2002). Once cleaved, the mature Spi ligand is transported from the Golgi and out of the cell to bind its cognate receptor.

Rho encodes a novel intramembrane serine protease whose catalytic activity is required for Spi cleavage at the motif ASIASGA (Lee *et al.*, 2001; Urban *et al.*, 2001; Urban & Freeman, 2003). Rho is specifically sensitive to serine protease inhibitors, supporting its placement within the serine protease superfamily (Urban *et al.*, 2001). Rho is well conserved across species, indicating that other Rhomboid proteins may also have proteolytic function (Urban *et al.*, 2001; Urban & Freeman, 2002; Urban *et al.*, 2002b). Indeed, the other three Drosophila rhomboids (Rho2-4) are functional proteases, although unlike Rho, they do not require S-mediated translocation for their function (Urban *et al.*, 2002a). The existence of multiple rhomboids suggests there may be tissue-specific regulation of proteolytic activation of DER ligands. Interestingly, the other DER ligands Grk and Krn are also activated by S-mediated

translocation and Rho proteolytic cleavage (Reich & Shilo, 2002; Urban *et al.*, 2002a).

However in vertebrates, Rho-mediated cleavage of TGF-α ligands appears not to be the predominant mechanism of EGFR activation. For example, although the vertebrate rhomboid $p100^{hRho}$ can associate with TGF-α ligands in cultured mammalian cells, the relevance of the interaction to TGF-α processing *in vivo* remains unclear (Nakagawa *et al.*, 2005). Rather, mounting evidence suggests that other proteases, such as the tumor necrosis factor-α converting enzyme (TACE)/A disintegrin and metalloproteinase (ADAM) 17, mediate TGF-α cleavage and ectodomain secretion (Peschon *et al.*, 1998; Killar *et al.*, 1999; Merlos-Suarez *et al.*, 2001; Black, 2002; Sunnarborg *et al.*, 2002; Hinkle *et al.*, 2003; Lee *et al.*, 2003; Hinkle *et al.*, 2004; Juanes *et al.*, 2005). In summary, the ER-Golgi translocation and proteolytic cleavage of Spi by the transmembrane proteins S and Rho, while obligate in Drosophila, may reflect a specialized mechanism of EGFR ligand processing and presentation.

The Repressive 'Ligand' Argos

DER signal transduction is negatively regulated at the ligand-receptor level. The gene that encodes the DER regulator protein Argos (Aos) was initially identified based on lethal mutations that disrupted development of the Drosophila eye (Freeman *et al.*, 1992). Molecular cloning revealed that *aos* encodes a novel secreted protein with a cysteine-rich region similar to an EGF repeat (Figure 1A) (Freeman *et al.*, 1992). Genetic analysis and cell culture experiments led to the hypothesis and eventual demonstration that this protein functions antagonistically to the DER pathway (Freeman *et al.*, 1992; Freeman, 1994a; Sawamoto *et al.*, 1994; Schweitzer *et al.*, 1995; Sawamoto *et al.*, 1996). Interestingly, high levels of DER signaling potentiate *aos* expression, suggesting the importance of a negative feedback loop to attenuate high levels of signaling output (Golembo *et al.*, 1996; Wasserman & Freeman, 1998).

Structure-function analysis shows that the Aos atypical EGF domain, the N-terminal EGF-flanking regions, and the C-terminus, are all critical for Aos' inhibitory ability (Figure 1A) (Howes *et al.*, 1998). In domain-swap experiments in which the EGF domain of the DER activator ligands Vn or Spi was replaced with the Aos atypical EGF domain, the Aos EGF domain was sufficient to convert Vn or Spi into an inhibitor (Schnepp *et al.*, 1998; Jin *et al.*, 2000). These experiments show that the Aos EGF domain is essential for abrogation of DER signaling.

There are conflicting reports that describe the mechanism by which Aos represses DER signaling. Initial experiments in Drosophila S2 cultured cells exposed to the activating ligand Spi demonstrated that Aos expression reduced DER phosphorylation levels (Schweitzer *et al.*, 1995), indicating that Spi and Aos are mutually antagonistic in their regulation of DER. However, in the presence of overexpressed DER, expression of Aos was sufficient to repress DER activation, suggesting Aos may act independent of Spi (Schweitzer *et al.*, 1995). Subsequent co-immunoprecipitation (co-IP) experiments showed that DER and Aos could interact and that the extracellular domain of DER and the EGF/C-terminal region of Aos were necessary both for the interaction and for DER repression (Jin *et al.*, 2000; Vinos & Freeman, 2000). Cross-linking experiments also suggested that Aos-DER interaction prevented DER dimerization, thereby blocking activation (Jin *et al.*, 2000). Further, when DER and Spi were expressed along with increasing amounts of Aos, co-IP of DER and Spi was reduced. Thus, it was concluded that Aos competes with Spi for binding to DER (Jin *et al.*, 2000).

Recently, the model that arose from the above Aos/Spi/DER analysis has been challenged (Figure 2E). Biochemical studies using surface plasmon resonance (SPR), analytical ultracentrifugation, and cell culture experiments demonstrated that Aos did not bind DER to inhibit signaling. Rather, Aos bound to Spi with a 1:1 stoichiometry and sequestered Spi from DER, suggesting Aos functions as a 'ligand sink' to bind soluble Spi (Klein *et al.*, 2004). In contrast to an earlier report, Aos did not disrupt bound Spi-DER complexes, according to order-of-addition experiments performed in cell culture. However, formation of Spi-Aos complexes prior to addition or expression of DER, led to the failure of Spi to bind receptor (Vinos & Freeman, 2000; Klein *et al.*, 2004). The model of ligand sequestration by Aos follows with *in vivo* observations, where Aos does not function over a long distance, but acts at a short range to negatively regulate Spitz activity (Figure 2E) (Klein *et al.*, 2004; Reeves *et al.*, 2005). In conclusion, the secreted protein Aos inhibits DER signaling at the ligand-receptor level, not by acting directly as an antagonistic ligand, but likely by sequestering activating-ligand away from the DER receptor (Figure 2E).

DER: The Drosophila EGF Receptor

EGFR/ErbB proteins belong to a subset of the receptor tyrosine kinase (RTK) superfamily that serves as receptors for growth factors, differentiation factors, and metabolic stimulation factors (van der Geer *et al.*, 1994). EGFR/ErbB was identified as a viral oncogene (*v-ErbB*) in the genome of the avian erythroblastosis virus (AEV), transduced from the chicken genome's *Egfr* gene, *c-ErbB* (Roussel *et al.*, 1979; Vennstrom & Bishop, 1982; Downward *et al.*, 1984). Since its early characterization in oncogenesis, EGFR signaling was found to play fundamental roles in proliferation, differentiation, and development (Olayioye *et al.*, 2000).

The Drosophila genome encodes a single locus of the EGFR/ErbB family: *Drosophila* epidermal growth factor receptor (*DER*) (Livneh *et al.*, 1985). *C. elegans* also possesses a single EGFR: LET-23 (Aroian *et al.*, 1990). Humans, on the other hand, have four EGFRs: ErbB1/EGFR/HER1, HER2/Neu, ErbB3/HER3, and ErbB4/HER4 (Klapper *et al.*, 2000; Olayioye *et al.*, 2000). *DER* was isolated and cloned in a screen to identify homologs of chicken *v-ErbB* in the Drosophila genome (Livneh *et al.*, 1985). The *DER* locus encodes alternatively-spliced isoforms whose encoded proteins (isoform I and II) differ at their amino terminus (Figure 1B) (Schejter *et al.*, 1986). These two isoforms may have distinct functions. For example, a constitutively-activated form of isoform I, but not of isoform II, was shown to be sufficient to induce ectopic differentiation in the developing Drosophila eye (Kumar & Moses, 2001b). The nature of the difference between the two isoforms is still unclear and remains to be elucidated.

DER, like other RTKs, possesses three domains that facilitate intracellular transduction of signals initiated at the cell surface: a glycosylated extracellular ligand-binding domain, a single hydrophobic Type I transmembrane domain, and a cytoplasmic tyrosine kinase catalytic domain (Figure 1B) (Ullrich & Schlessinger, 1990; van der Geer *et al.*, 1994; Bazley & Gullick, 2005). DER belongs to Subclass I of the RTK family, characterized by a pair of ligand-binding domains (L1 and L2) and Cys-rich repeat sequences (C1 and C2) located in its extracellular domain (Ullrich & Schlessinger, 1990). While conservation in the kinase and ligand-binding domains is too strong to allow classification of DER within the EGFR family, comparision of the Asn-linked glycosylation sites indicates DER is more similar to HER1 than to Neu (Livneh *et al.*, 1985; Schejter *et al.*, 1986). Specifically, DER shares twelve Asn with HER1, but only nine with Neu (Schejter *et al.*, 1986). These sites may prove important for function, because N-linked glycosylation was reported to be essential for proper EGFR plasma membrane translocation, dimerization, ligand-binding specificity, and activation (Soderquist & Carpenter, 1984; Slieker & Lane, 1985; Olson & Lane, 1989; Tsuda *et al.*, 2000; Whitson *et al.*, 2005).

It is interesting that Drosophila and *C. elegans* each only have a single EGFR. In mammals, EGFRs are not only able to homodimerize but also to heterodimerize with other EGFRs (Schlessinger, 2000). Heterodimerization of receptors permits receptor diversity and specificity of function, signaling level, and response (Schlessinger, 2000). For example, in some contexts, heterodimerization may result in stronger signaling than achieved from homodimeric receptor pairs (Moghal & Sternberg, 1999). This begs the question of whether or not this type of diversity is required in *Drosophila* or *C. elegans*. If variation is necessary, then diversity at the receptor level may occur with variable N-linked glycosylation, with a repertoire of ligands, or with the differential localization or targeting of the receptor (Moghal & Sternberg, 1999; Whitson *et al.*, 2005).

The stoichiometry of ligand-to-receptor binding is varied across classes of receptors. As a member of the RTK Subclass I, each DER protein binds to a monomeric ligand (Ullrich & Schlessinger, 1990). Binding of ligand to the DER extracellular domain stimulates conformational alteration and receptor dimerization (Figure 3B) (Schlessinger, 1988). According to biophysical studies, binding of monomeric ligand with monomeric receptor forms a 1:1 EGF:EGFR dimer that facilitates heterotetramerization. That is, a 2:2 EGF:EGFR ratio is required for activation of the cytoplasmic domain (Lemmon *et al.*, 1997). Dimerization stabilizes protein interactions, promotes juxtaposition of the cytoplasmic domains, and induces a conformational change that leads to activation of its kinase function (Figure 3B). The activated kinase domain then leads to trans- and auto-phosphorylation of tyrosine residues on the cytoplasmic tail of the receptor (Schlessinger, 1988; van der Geer *et al.*, 1994; Zhang *et al.*, 2006). Activation of the DER dimer facilitates its ability to catalyze phosphorylation of cytoplasmic substrates and/or to recruit phosphotyrosine binding (PTB) or Src-homology 2 (SH2) domain-containing proteins that have increased affinity for activated receptor

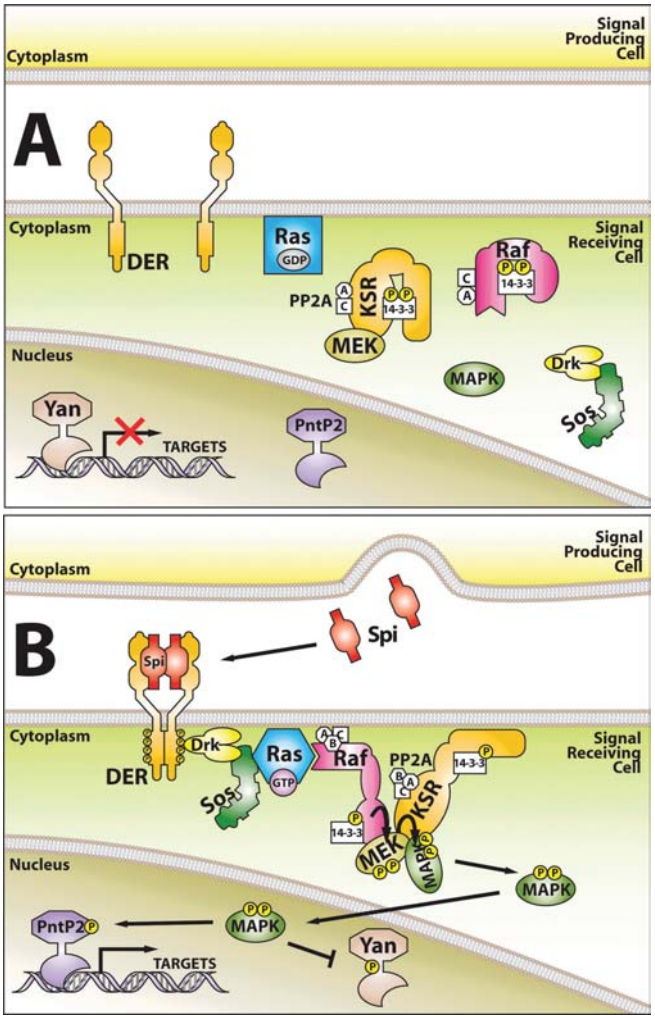

FIGURE 3 **The DER signaling pathway. (*A*) In non-stimulated cells, Ras exists in a GDP-bound, inactive state. 14-3-3 binds to phosphorylated Raf and Ksr on two Ser residues, retaining each in the cytoplasm. (*B*) DER activation by Spi leads to its activated, GTP-bound state. PP2A dephosphorylates Raf and Ksr and displaces 14-3-3. This permits Raf and Ksr to re-localize with Ras near the membrane to activate the kinase cascade. Adapted from Raabe & Rapp, 2003.**

(Figure 3B) (Mohammadi *et al.*, 1993; van der Geer *et al.*, 1994; Pawson & Scott, 1997; Sherrill, 1997). In summary, DER phosphorylation following ligand reception initiates nucleation of subsequent cytosolic signaling complexes.

Activation of Ras

The primary consequence of EGFR activation in Drosophila is assembly of a membrane-localized protein complex that recruits and activates Ras (Figure 3B). Specific tyrosine phosphorylation of DER promotes docking of the cytoplasmic adaptor protein Downstream of Receptor Kinase (Drk), the homolog of mammalian Grb2 and *C. elegans* SEM-5, via its single SH2 domain (Figure 3B). Drk converts this phosphotyrosine input to a signaling output by serving as the cornerstone for the protein complex that ultimately recruits and activates Ras (Pawson *et al.*, 2001). Drk is a flexible protein with two Src-homology 3 (SH3) domains flanking the phosphotyrosine-binding SH2 domain (Olivier *et al.*, 1993; Yuzawa *et al.*, 2001). These SH3 domains bind to proline-rich motifs in a separate set of proteins, thus building a protein complex around activated receptors (Ren *et al.*, 1993; Pawson & Scott, 1997). Drk complexes constitutively with the guanine nucleotide exchange factor (GEF) Son of sevenless (Sos) by binding to its C-terminal Pro-rich region (Figure 3B) (Simon *et al.*, 1991; Buday & Downward, 1993; Olivier *et al.*, 1993; Schlessinger, 1993; Hunter, 2000). Recruitment of the Drk-Sos complex to activated DER promotes the association of Sos with Ras (Figure 3B).

Ras is a proto-oncogene localized to the cytoplasmic side of the plasma membrane by farnesylation and palmitoylation (Neuman-Silberberg *et al.*, 1984; Simon *et al.*, 1991; Magee & Marshall, 1999). It serves as a guanine nucleotide-binding protein that cycles between inactive GDP-containing and active GTP-containing conformations (Figure 3). GEFs such as Son-of-sevenless (Sos) facilitate the exchange of GDP to GTP, thereby activating Ras. Providing balance, GTPase activating proteins (GAPs) promote the hydrolysis of GTP to GDP by Ras, thereby returning it to the inactive conformation (McCormick, 1994). This biochemical cycle allows Ras to operate as an 'on/off' switch. Amino acid substitutions, like the Gly12Val (Ras^{V12}) mutation, that stabilize the GTP-bound state, create a constitutive 'on' state (Barbacid, 1987). *In vivo* genetic studies have taken advantage of targeted transgenic expression of activated Ras^{V12} to dissect the mechanisms of signaling flow through the pathway in many different developmental contexts (Firth *et al.*, 2005).

Structural studies demonstrate that Ras conformation is dynamic, particularly in two loop regions known as Switch 1 and Switch 2. Sos tightly binds the Switch 2 region of Ras, induces conformational changes, and activates Ras by opening up a nucleotide-binding site in the Switch 1 region, facilitating the exchange of guanine nucleotides (Boriack-Sjodin *et al.*, 1998; Hall *et al.*, 2001). Following nucleotide exchange, Sos is released from Ras. Interestingly, it was demonstrated that the process of nucleotide exchange is potentiated through positive-feedback mechanisms whereby allosteric binding of Ras-GTP to the Ras Exchanger Motif domain of

Sos forms a ternary complex and increases Sos-mediated nucleotide exchange of a separate Ras protein (Margarit *et al.*, 2003).

Activation of the Raf/MEK/MAPK Cascade

The activation of Ras switches 'on' a series, or cascade, of protein kinases, such that as one kinase is phosphorylated, it subsequently phosphorylates the next. This culminates in phosphorylation and activation of the mitogen activated protein kinase (MAPK) (Figure 3B). The kinase cascade is initiated as activated Ras binds to the proto-oncogene Raf with high-affinity, potentiating its catalytic activity as a serine/threonine kinase (Figure 3B) (Chong *et al.*, 2003). Raf contains several conserved domains: a Ras-binding domain (RBD), a Cys-rich domain (CRD), a Ser/Thr-rich region, and a catalytic kinase domain (Chong *et al.*, 2003). The RBD is required for translocation of Raf from the cytosol to the cell membrane where it associates with Ras. The CRD, which encodes a zinc-finger domain, is required for activation of Raf and also plays a role in binding to Ras (Avruch *et al.*, 2001). Phosphorylation of Raf at the Ser/Thr-region and the catalytic domain may subject Raf to autoregulation or regulation by separate kinases; certain phosphorylation events may promote Raf function while others may antagonize its function (Kolch, 2000; Avruch *et al.*, 2001; Chong *et al.*, 2003).

Physical interaction with Ras is hypothesized to elicit an activated Raf transition state by relieving intramolecular inhibitory regulation (Figure 3A) (Kolch, 2000; Avruch *et al.*, 2001). In resting cells, Raf exists in an inactive state such that its C-terminus negatively inhibits the kinase domain. This is mediated through serine phosphorylation by protein kinase A (PKA) and phospho-binding-protein 14-3–3 interaction (Figure 3A) (Avruch *et al.*, 2001; Dhillon *et al.*, 2002). Activation of signaling relieves autoinhibition through protein phosphatase 2A (PP2A) mediated dephosphorylation, which displaces 14-3-3 and allows the RBD and CRD of Raf to interact with Ras near the plasma membrane (Figure 3B) (Jaumot & Hancock, 2001). Raf is then phosphorylated at a separate serine residue by the Pak3 kinase. This prevents reassociation and autoinhibition by allowing 14-3-3 to take up residence at these newly phosphorylated sites to maintain stably-active Raf (Figure 3B) (Avruch *et al.*, 2001).

Following activation, Raf binds via its kinase domain to the MAP/ERK kinase (MEK)/Downstream of Raf1 (Dsor1) and activates MEK by dual phosphorylation of Ser or Thr residues within an activation loop (Figure 3B) (Crews *et al.*, 1992; Crews & Erikson, 1992; Huang *et al.*, 1993; Macdonald *et al.*, 1993; Tsuda *et al.*, 1993; Seger & Krebs, 1995; Pearson *et al.*, 2001; Kolch, 2005). MEK is a dual-specificity kinase able to phosphorylate Ser, Thr, as well as Tyr residues (Huang *et al.*, 1993; Seger & Krebs, 1995; Pearson *et al.*, 2001). Activated MEK binds with its N-terminal docking site to MAPK and phosphorylates it first on Tyr and then on Thr residues located within MAPK's flexible activation loop (Figure 3B) (Zhang *et al.*, 1994; Zhang *et al.*, 1995; Pearson *et al.*, 2001; Tanoue & Nishida, 2003). Dual phosphorylation is necessary for the activity of MAPK (Pearson *et al.*, 2001).

How is the activation and function of such a three-kinase signaling module regulated and coordinated? Recent studies have demonstrated the importance of scaffolding in this context. Scaffolding of the MAPK cascade is mediated, at least in part, by the kinase suppressor of Ras (Ksr) protein, first isolated in a Drosophila genetic screen as an effector of Ras signaling (Figure 3B) (Therrien *et al.*, 1995, 1996). Ksr facilitates assembly and activity of the Raf, MEK, and MAPK kinase cascade by mediating the individual protein-protein interactions and by localizing the complex to an appropriate subcellular site (Figure 3) (Schaeffer & Weber, 1999; Raabe & Rapp, 2003). The complex choreography underlying these events remains an area of active research and its further discussion is beyond the scope of this review (Morrison & Davis, 2003; Raabe & Rapp, 2003; Kolch, 2005).

Regulation of Nuclear Targets via Activated MAPK

MAPK completes the transfer of information from cell surface to the nucleus by re-localizing from the cytoplasm to the nucleus upon activation. Translocation of activated, di-phosphorylated MAPK (dpMAPK) into the nucleus may occur by multiple means including passive diffusion through the nuclear pore, interaction with the nuclear pore complex, association and co-shuttling with other nuclear-targeted proteins, or direct importin-mediated transport (Khokhlatchev *et al.*, 1998; Cyert, 2001; Lorenzen *et al.*, 2001; Baker *et al.*, 2002; Kondoh *et al.*, 2005). In Drosophila,

Moleskin (Msk) and Ketel, the homologs of Importin-7 and Importin-β, respectively, are required for nuclear import of dpMAPK (Lorenzen *et al.*, 2001). Recently, it was identified that Msk developmentally modulates the hold of dpMAPK in the cytoplasm or movement of dpMAPK into the nucleus (Figure 4B) (Marenda *et al.*, 2006; Vrailas *et al.*, 2006; Vrailas & Moses, 2006). Thus, regulation of dpMAPK nuclear import appears critical in affecting output from the DER signaling pathway.

Nuclear dpMAPK phosphorylates specific target proteins, including two Drosophila Ets transcription factors relevant to our discussion of DER signaling: Pointed-P2 (PntP2) and Yan/Anterior open (Aop) (Figure 4B) (O'Neill *et al.*, 1994). PntP2 is the homolog of the human Ets-1 transcription factor and functions as a transcriptional activator when phosphorylated by dpMAPK (Figure 4B) (Watson *et al.*, 1985). Yan is the homolog of the human Tel1 protein and represses target genes in the absence of dpMAPK (Golub *et al.*, 1994). Both PntP2 and Yan contain an Ets DNA-binding domain and compete for access to promoter regions of common downstream transcriptional targets, with level and duration of activity of these two key DER pathway effectors determining the net response to pathway stimulation.

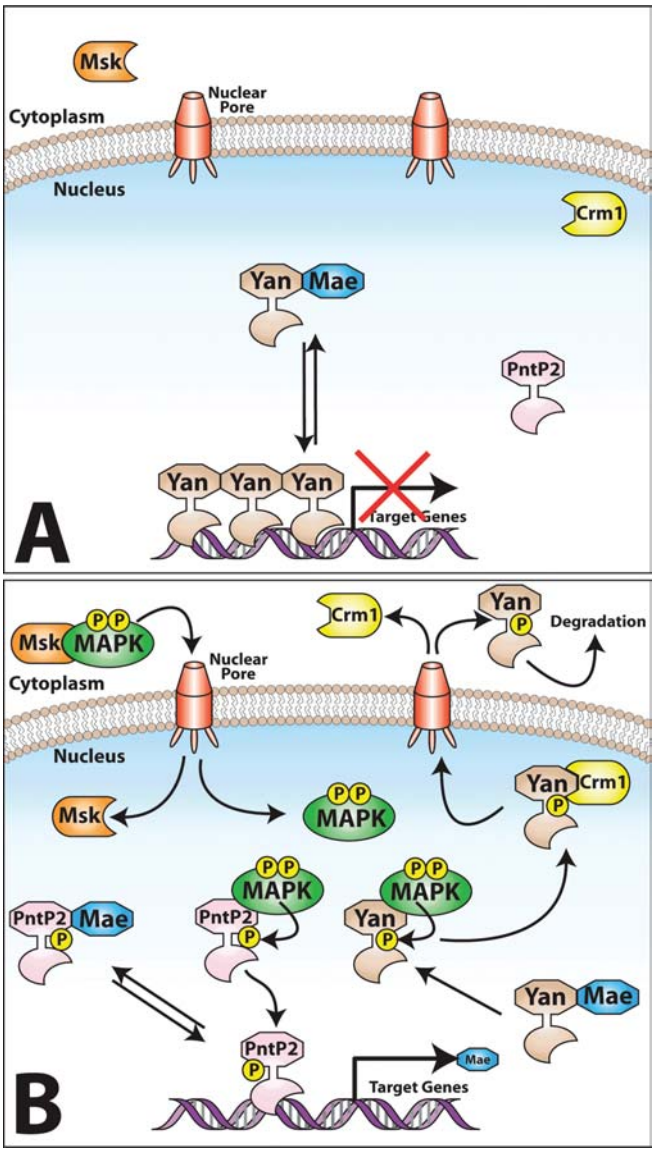

FIGURE 4 **Regulation of the Ets transcriptional regulators Yan and Pnt. (*A*) In non-stimulated cells, Yan polymers repress target gene transcription. (*B*) In stimulated cells, dpMAPK translocates into the nucleus promoting PntP2 activation of target gene transcription and export of Yan from the nucleus. Mae mediates Yan depolymerization and PntP2 activity. Adapted from Song *et al.*, 2005; Qiao *et al.*, 2006.**

Work from multiple labs, combining both *in vitro* and *in vivo* experimental approaches, has led to a model in which Yan protein localization is dynamically regulated by protein-protein associations, phosphorylation, nuclear export and stability (Figure 4) (Rebay & Rubin, 1995; Tootle *et al.*, 2003; Qiao *et al.*, 2004; Song *et al.*, 2005). At its N-terminus, Yan contains a Sterile α Motif (SAM), found in a subset of Ets proteins, which mediates homotypic and heterotypic protein-protein interactions and is required for transcriptional repression. *In vitro,* Yan self-associates to form head-to-tail polymers through its SAM domain (Figure 4A) (Qiao *et al.*, 2004). These polymers can be disrupted upon binding to Modulator of the activity of Ets (Mae)/Ets domain lacking (Edl), a SAM domain-containing protein that lacks an Ets DNA-binding domain (Figure 4) (Baker *et al.*, 2001; Yamada *et al.*, 2003; Qiao *et al.*, 2004; Song *et al.*, 2005).

Although the polymerization model awaits *in vivo* validation, the prediction is that in unstimulated cells, Yan polymers would be favored in an equilibrium with depolymerized Yan monomers bound to Mae, leading to transcriptional repression of target genes (Figure 4A) (Song *et al.*, 2005). Upon Ras activation and subsequent stimulation of dpMAPK, Yan S127 is phosphorylated (Rebay & Rubin, 1995). This breaks up the polymers, facilitates association with the exportin, Crm1, and effectively displaces Mae from depolymerized Yan (Tootle *et al.*, 2003; Song *et al.*, 2005). Yan is then exported from the nucleus to the cytoplasm where it is proposed to be degraded (Figure 4B) (Rebay & Rubin, 1995; Tootle *et al.*, 2003). This results in derepression of transcriptional targets and allows the cell to respond appropriately to the initial activating signal. Interestingly, among the validated transcriptional targets of Yan is *mae* itself

(Vivekanand *et al.*, 2004). In this context, accumulation of Mae has been suggested to promote a feedback loop resulting in further depolymerization, phosphorylation by dpMAPK, and export of Yan (Figure 4B) (Song *et al.*, 2005).

In contrast to its role in triggering the dismantlement of Yan-mediated repression, dpMAPK-mediated phosphorylation of PntP2 converts it into a potent activator (Figure 4B). Like Yan, PntP2 bears a SAM domain. Interestingly, the SAM domain of PntP2 contains a docking site for dpMAPK similar to that observed in mammalian Ets-1 and Ets-2 (Seidel & Graves, 2002). Recently, it was shown that mutation of amino acids within this putative docking site leads to a reduction or loss in PntP2 phosphorylation and transcriptional activity, suggesting that dpMAPK docks at the SAM interface to phosphorylate and activate PntP2 (Figure 4B) (Qiao *et al.*, 2006). Like Yan, the PntP2 SAM domain can be bound by Mae (Tootle *et al.*, 2003; Yamada *et al.*, 2003). *In vitro* kinase assays support the hypothesis that the SAM-mediated Mae-PntP2 association interferes with PntP2 phosphorylation by dpMAPK, thereby downregulating the transcriptional activation ability of PntP2 (Qiao *et al.*, 2006). As mentioned above, *mae* is a transcriptional target of PntP2 and Yan (Vivekanand *et al.*, 2004). Thus, activation of *mae* by activated PntP2 leads to a negative feedback loop whereby Mae protein occludes the phosphorylation and activation of PntP2 (Figure 4B).

THE NOTCH SIGNALING PATHWAY IN DROSOPHILA

The Notch signaling pathway, like the DER pathway, is one of several fundamental and evolutionarily conserved mechanisms utilized in metazoans to control cell fate decisions. Notably, cell-cell signaling through the Notch pathway is involved in diverse processes including neurogenesis, proliferation, polarity/axes establishment, and morphogenesis (Artavanis-Tsakonas *et al.*, 1999; Mumm & Kopan, 2000; Kopan, 2002; Portin, 2002; Baron, 2003; Louvi & Artavanis-Tsakonas, 2006). The pathway has been characterized as simple and complex: 'simple' in that it does not require secondary messengers, but 'complex' because of the intricacies of how the relatively few components transduce the signal to the nucleus (Kopan, 2002). The general framework of the pathway is such that signaling is initiated when ligand binds the Notch receptor. Subsequently, Notch is proteolytically cleaved to release its intracellular domain. The Notch intracellular domain then translocates into the nucleus to activate target genes by binding to a DNA-bound transcription factor. Like our presentation of the EGFR pathway, the discussion of the Notch pathway presented here represents a broad overview of information derived from various contexts. Thus, it is important to note that tissue-to-tissue differences in deployment of the signaling machinery are not emphasized.

Structure of Notch and its Ligands

Notch signaling occurs predominantly *via* cell-cell contacts that allow the extracellular domain of a ligand in a signal-producing cell to interact directly with the extracellular domain of Notch on an adjacent signal-receiving cell (Fehon *et al.*, 1990; Rebay *et al.*, 1991). In addition to *trans* ligand-receptor interactions, where ligand from the signal-producing cell binds receptor of the signal-receiving cell, it has been observed that ligand and receptor produced within the same cell may autonomously bind. Although not entirely understood, this *cis* interaction of Notch and ligand is proposed to reduce signaling output of the pathway (Sakamoto *et al.*, 2002; Schweisguth, 2004).

In *Drosophila*, ligands for the Notch (N) receptor are encoded by two single-pass transmembrane proteins: Delta (Dl) and Serrate (Ser) (Figure 5A) (Vassin *et al.*, 1987; Kopczynski *et al.*, 1988; Fleming *et al.*, 1990; Artavanis-Tsakonas *et al.*, 1999). Mammals possess five Notch ligands. These include proteins similar to Dl–Delta-like-1, -3, and -4 (DLL1/3/4)–as well as Ser-like proteins–Jagged-1 and -2 (Jag1/2) (Radtke & Raj, 2003). *Dl* and *Ser* encode structurally similar Type I transmembrane proteins with EGF-like homology in their extracellular domains. Dl contains nine continuous EGF-like motifs, a single transmembrane domain and a short cytosolic tail (Figure 5A) (Vassin *et al.*, 1987; Kopczynski *et al.*, 1988). Ser is somewhat larger, with 14 copies of the EGF-like repeat interspersed with short linker sequences in its extracellular domain (Figure 5A) (Fleming *et al.*, 1990). The overall structure of the mammalian DLL1/3/4 proteins is similar to Drosophila Dl, however, DLL3 bears only six EGF-like repeats (Radtke & Raj, 2003). The mammalian Jagged1/2 proteins are similar to Ser, but contain 17 EGF-like repeats with fewer intervening linker sequences (Radtke & Raj, 2003).

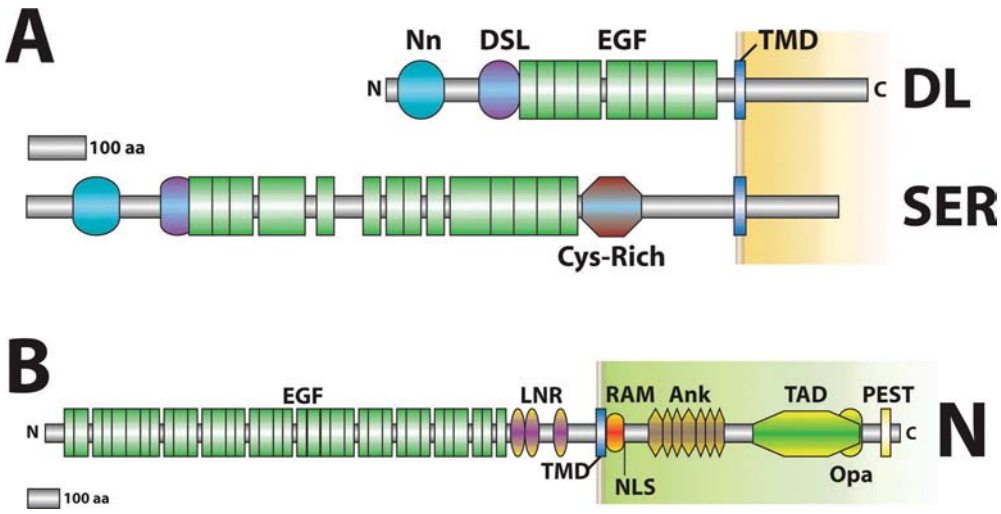

FIGURE 5 **Notch and its ligands. (*A*) Dl and Ser have similar N-termini with a Notch-ligand N-terminus (Nn) and a Delta-Serrate-LAG-2 (DSL) domain. Ser differs by having a Cys-rich domain that follows the EGF repeats. (*B*) Notch encodes a large EGF repeat-rich receptor. See text for domain details.**

While there is a single Notch protein encoded by the Drosophila genome, the mammalian genome encodes four Notch receptors, Notch1–4 (Radtke & Raj, 2003). The Drosophila *N* locus encodes a 2,703 amino acid single-pass transmembrane receptor protein of ~300 kDa (Figure 5B) (Wharton *et al.*, 1985a; Kidd *et al.*, 1986). At its N-terminal extracellular domain, Notch, has 36 EGF-like tandem repeats, followed by three highly-conserved Cys-rich LIN-12/Notch Repeats (LNRs) (Lieber *et al.*, 1993). Within its cytoplasmic domain, Notch has an RBP-Jκ association module (RAM), a single nuclear localization signal (NLS), seven ankyrin (Ank) repeats, a transcriptional activation domain (TAD) that contains a glutamine-rich Opa repeat, and Pro-Glu-Ser-Thr (PEST) motifs (Figure 5B) (Wharton *et al.*, 1985b; Breeden & Nasmyth, 1987; Bork, 1993; Jarriault *et al.*, 1995; Tamura *et al.*, 1995; Aster *et al.*, 1997; Kidd *et al.*, 1998; Kurooka *et al.*, 1998; Zweifel & Barrick, 2001). The mammalian Notch receptors differ slightly within their extracellular and cytoplasmic domains. In their ectodomains, Notch1–4 proteins contain 36, 36, 34, and 29 EGF repeats, respectively. The cytoplasmic TADs also vary between the receptors, such that Notch1 has a strong but Notch2 has a weak TAD. This is in marked contrast to Notch 3 and 4, which each lack a TAD (Radtke & Raj, 2003). Despite these variations, the high degree of structural and functional conservation of the Notch receptor and its ligands between *Drosophila* and mammals has permitted integration of a broad range of genetic and biochemical investigations into a unifying model of Notch signaling presented below.

Notch is Processed to Generate Heterodimeric Receptor

Notch proteins are synthesized as precursors that are cleaved during transport to the cell surface, generating N^{TM} transmembrane-bound intracellular and N^{EC} extracellular polypeptides (Logeat *et al.*, 1998). Cleavage, at the site termed S1, leaves only 12 amino acids exposed to the cell surface of the N^{TM} fragment (Logeat *et al.*, 1998). Once cleaved, the products heterodimerize via the hydrophobic N^{EC}-C-terminal domain to function as a mature receptor (Logeat *et al.*, 1998; Sanchez-Irizarry *et al.*, 2004). Heterodimerization is non-covalent and dependent on Ca^{2+} levels (Rand *et al.*, 2000). Cleavage of newly synthesized Notch proteins occurs by the catalytic action of Furin-like Convertases in the *trans*-Golgi network (Logeat *et al.*, 1998). Proprotein convertases, such as the Furins, function as 'master switches' in the activation of growth factors, neuropeptides, receptors, enzymes, glycoproteins, and toxins (Seidah & Prat, 2002).

The requirement of Notch processing is different between Drosophila and vertebrates. In mammals, convertase-processing of Notch is constitutive and required for signaling (Logeat *et al.*, 1998). However, in *Drosophila*, the membrane predominantly has N in its full-length, uncleaved form. Cleavage does proceed at low levels but is not required for N signaling function (Kidd & Lieber, 2002). Therefore, post-translational cleavage of Notch expression is essentially absent in Drosophila. However, the subsequent

cleavages, described in the following sections in which we discuss the regulation of and responses to ligand-receptor binding, are fundamental to Notch signaling in both invertebrates and vertebrates.

Notch Discriminates Between Ligands Through Differential Glycosylation

Following protein synthesis, Notch receptors are modified by the addition of sugar moieties. For example, Notch undergoes *N*-glycosylation at Asn residues, *O*-linked glucosylation on Ser residues and *O*-linked fucosylation on Ser or Thr residues. These reactions are catalyzed by different sets of enzymes. *O*-glucose and *O*-fucose may be additionally elongated by a set of glycosyltransferases. The function of *O*-glucosylation is unclear, but *O*-fucosylation and its subsequent elongation plays a critical role in ligand-receptor interactions in Notch signaling (Figure 6) (Haltiwanger, 2002; Haltiwanger & Stanley, 2002; Haines & Irvine, 2003).

O-glucosylation and *O*-fucosylation of Notch occur on Ser or Thr residues found in its repetitive extracellular EGF domains (Figure 6) (Wang *et al.*, 1996; Wang & Spellman, 1998; Moloney *et al.*, 2000; Wang *et al.*, 2001). *In vivo* studies suggest that *O*-fucosyltransferase (*O*fut1) activity is developmentally regulated and required for Notch signaling (Okajima & Irvine, 2002). Specifically knockdown of *Ofut1* via RNA interference (RNAi) in cell culture led to a reduction of Notch *O*-fucosylation, impaired its interaction with ligand, and reduced signaling output from the pathway (Okajima & Irvine, 2002; Okajima *et al.*, 2003). *In vivo* overexpression of *O*fut1 antagonized lateral inhibition and inductive signaling (described later in this review) and led to differential signaling, such that Ser-N signaling was increased, but Dl-N signaling was inhibited (Okajima *et al.*, 2003). Addition of *O*-fucosylation sites within the EGF-like repeats may result in ectopic Notch signaling (Li *et al.*, 2003b). Together, these observations suggest that *O*-fucosylation regulates Notch signaling by influencing ligand-receptor interactions.

O-fucosylated residues of Notch are further glycosylated by the β-1,3-*N*-acetylglucosaminyltransferase enzyme, Fringe (Figure 6) (Irvine & Wieschaus, 1994; Haines & Irvine, 2003). Fng-mediated glycosylation potentiates Dl-N binding but inhibits Ser-N binding (Bruckner *et al.*, 2000; Lei *et al.*, 2003). Interestingly, the EGF-like repeats found in the Notch ligands are also *O*-fucosylated and then Fng-dependently glycosylated

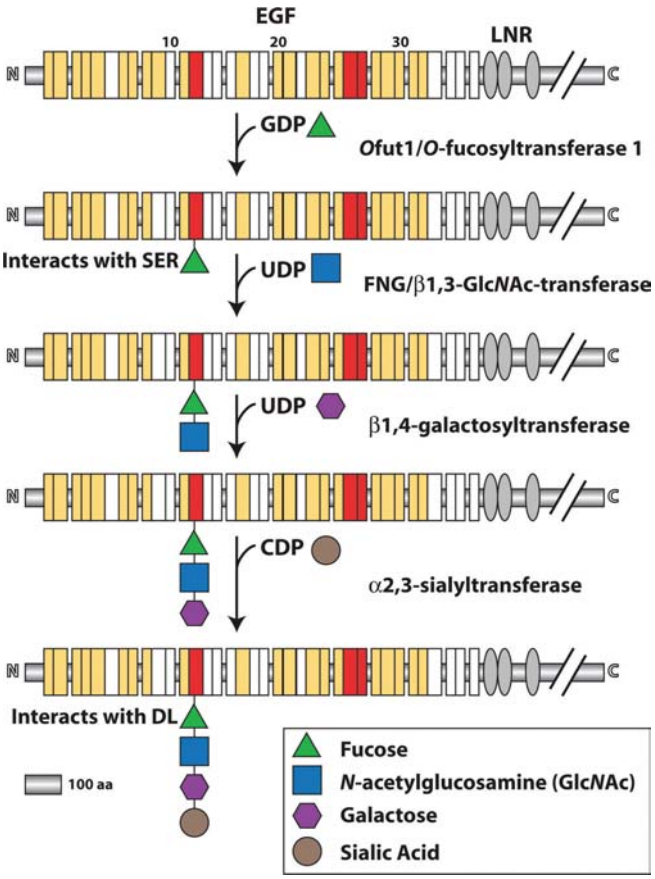

FIGURE 6 **Differential sugar modification of Notch affects ligand-binding. *O*-fucosylation by *O*fut1 promotes binding of Ser. Dl interacts with Notch that has been glycosylated by Fng and further modified with galactose and sialic acid. Yellow:Potential EGF repeats for *O*-fucosylation. *Red*:EGF Repeat 12, 26, and 27 are highly conserved. Repeat 12 *O*-fucosylation mediates Dl-N signaling activation, whereas *O*-fucosylation on Repeats 26 and 27 have distinct roles, see following reference for more information:Rampal *et al.*, 2005. Adapted from Okajima & Irvine, 2002; Haines & Irvine, 2003.**

(Panin *et al.*, 2002). Glycosylation of the ligands adds another level of complexity, regulation, and specificity in ligand-receptor interactions. In perspective, Fng activity is developmentally regulated and is only required for a subset of Notch signaling events (Haines & Irvine, 2003). It functions in inductive signaling events, but not during lateral inhibition events, each of which are described later in this review (Irvine & Vogt, 1997; Irvine & Rauskolb, 2001; Haines & Irvine, 2003).

Notch-Ligand Interaction Results in Extracellular Truncation

To initiate cell-cell communication, the ligand, Dl or Ser, binds to the extracellular domain of N *via* its own extracellular EGF repeats (Fehon *et al.*, 1990; Rebay

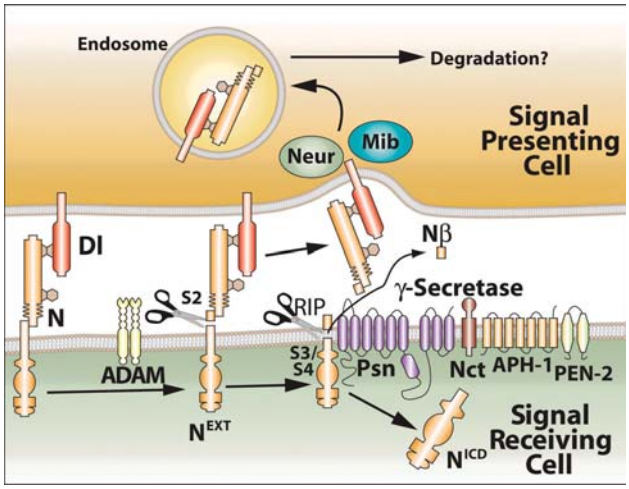

FIGURE 7 **Notch proteolytic cleavages. Following binding of ligand, Notch is proteolytically cleaved on its extracellular side at site S2 by ADAM proteases. Neur and Mib promote transendocytosis of Dl and N^{EC}. N^{EXT} is then cleaved at sites S3 and S4 by the γ-secretase complex (Psn/Nct/APH-1/PEN-2), releasing N^{ICD} into the cytoplasm and secreting the small Nβ peptide. Adapted from Radtke *et al.*, 2005.**

et al., 1991). This leads to highly coordinated and regulated proteolytic cleavage of both the ligand and the receptor. As described below, these proteolytic cleavages are essential for Notch signaling (Figure 7) (Selkoe & Kopan, 2003).

As mentioned above, the first proteolytic cleavage of Notch at site S1 occurs while the precursor protein transits through the Golgi to the plasma membrane, prior to interacting with ligand. Ligand-receptor binding triggers the second cleavage, S2, performed by proteases of the ADAM family (Figure 7). The Drosophila genome encodes five ADAM proteases: TACE/ADAM17, Kuzbanian (Kuz)/ADAM10, Kuzbanian-like (Kul)/ADAM10, Meltrin/Meltrin α, and Mind-meld (Mmd)/Meltrin α (Sapir *et al.*, 2005). TACE cleaves Notch extracellularly near the transmembrane domain to generate the Notch extracellular truncation (N^{EXT}) product (Brou *et al.*, 2000; Mumm & Kopan, 2000). The N^{EXT}-generating cleavage promotes subsequent proteolytic cleavages by removing inhibitory extracellular sequences surrounding the S2 region, including the three LNR repeats (Figure 5B,7) (Mumm *et al.*, 2000; Sanchez-Irizarry *et al.*, 2004).

Additional observations suggest that other ADAM proteases may be involved in Notch signaling events. Kuz was genetically and molecularly implicated in the cleavage processing of the S2 site (Pan & Rubin, 1997; Sotillos *et al.*, 1997; Lieber *et al.*, 2002). In Drosophila, Kuz was shown to be necessary and sufficient for S2 cleavage *in vivo* and *in vitro* (Lieber *et al.*, 2002). In contrast, TACE, not Kuz, appears both necessary and sufficient for the S2 cleavage of Notch in mammals (Brou *et al.*, 2000) whereas in *C. elegans*, ADM-4/TACE and SUP-17/Kuz were reported to function redundantly (Jarriault & Greenwald, 2005). TACE is not required, but is sufficient for S2 cleavage in Drosophila (Lieber *et al.*, 2002).

Interestingly, recent studies suggest that ADAM metalloproteases may also have a broader role in processing ligands. For example, Kuz was shown to cleave Dl, as well as Jag/Ser, independent of ligand-receptor interactions (Qi *et al.*, 1999; LaVoie & Selkoe, 2003; Six *et al.*, 2003). This requirement for ligand proteolytic processing conflicts with reports that suggest that Kuz activity is required exclusively in the signal-receiving cell (Sotillos *et al.*, 1997; Wen *et al.*, 1997; Klein, 2002). The ligands are further processed by the γ-secretase enzyme which is also necessary for release of the intracellular membrane of Notch, as will be described in the following section (LaVoie & Selkoe, 2003; Six *et al.*, 2003). The biological activity of the ligand cleavage products is under debate. One model indicates that the Dl proteolytic event may release its extracellular domain (Dl^{EC}) to function as a secreted activating ligand (Qi *et al.*, 1999). In contrast, another model implicates Kuzbanian-mediated proteolysis of ligand as a signal-downregulating event to yield non-functional ligand (Mishra-Gorur *et al.*, 2002). Recently, another ADAM protease, Kul, has been reported to function with Kuz to mediate *Dl* downregulation to promote unidirectional Notch signaling (Sapir *et al.*, 2005).

Regardless of whether the Dl^{EC} and Ser/Jag^{EC} products serve as active ligands, the intracellular products of γ-secretase activity, Dl^{IC} and Ser/Jag^{IC}, may themselves play a functional role. One model suggests that ligand cleavage results in localization of the intracellular domains to the nucleus to mediate bi-directional signaling events by facilitating the expression of target genes (Bland *et al.*, 2003; LaVoie & Selkoe, 2003; Six *et al.*, 2003). In addition, these studies demonstrate that such bi-directional signaling events may be antagonistic to one another (LaVoie & Selkoe, 2003). In summary, despite the ongoing controversies, it is clear that extensive proteolytic processing of both ligand and receptor occurs *in vivo* and that future work will be required to resolve the biological outcome of these events.

Endocytosis Regulates Ligand-Receptor Interactions in the Notch Pathway

Notch signaling requires release of the Notch intracellular domain into the cytoplasm. However, amino acid sequences that lie in the ectodomain adjacent to the plasma membrane may create steric or conformational hindrance to intramembrane proteolytic events that release Notch (Figure 7) (Sanchez-Irizarry *et al.*, 2004). In recent years, a model has arisen such that relief of inhibition is mediated by endocytic events that involve both ligand and Notch (Le Borgne & Schweisguth, 2003a; Gupta-Rossi *et al.*, 2004; Le Borgne *et al.*, 2005; Pitsouli & Delidakis, 2005; Wilkin & Baron, 2005; Chitnis, 2006). Upon interaction with Dl or Ser, the ligand-bound cleaved Notch extracellular domain (N^{ECD}) is transendocytosed into the signal-producing cell (Figure 7) (Parks *et al.*, 2000). The pulling force produced from the endocytic event creates a conformational change and/or relief of steric hindrance of Notch, thus providing access for TACE to cleave at S2 and permitting subsequent Notch cleavage steps by γ-secretase (Parks *et al.*, 2000).

Endocytosis of ligand is regulated by the activity of the E3 ubiquitin ligase enzymes Neuralized (Neur) and Mind bomb (Mib) (Figure 7) (Portin & Rantanen, 1991; Haddon *et al.*, 1998; Deblandre *et al.*, 2001; Lai *et al.*, 2001; Yeh *et al.*, 2001; Itoh *et al.*, 2003; Lai *et al.*, 2005; Wang & Struhl, 2005). Although Dl and Ser ligands can be polyubiquitylated and degraded (Deblandre *et al.*, 2001; Lai *et al.*, 2001), in the context of endocytic trafficking, mono- or multi-ubiquitylation is more likely involved (Haglund *et al.*, 2003; Le Borgne *et al.*, 2005). Such a model remains speculative because the ubiquitylation state (mono- *versus* multi-) of endocytosed Dl or Ser has not yet been elucidated. Also, it is not clear how the proteasome and the endocytic machinery differentiate between the various states of ubiquitylated proteins (Le Borgne *et al.*, 2005). In certain cases, this may be facilitated by the activities of the epsin Liquid Facets (Lqf) and the de-ubiquitylating enzyme Fat facets (Faf) that regulate ligand endocytosis (Chen *et al.*, 2002; Overstreet *et al.*, 2004).

In addition to the ligand-receptor pulling mechanism for activating Notch, other, not necessarily mutually exclusive, models for Notch activation have been proposed (Gupta-Rossi *et al.*, 2004; Le Borgne *et al.*, 2005). For example, one model suggests that ligand is produced in an inactive form that must be endocytosed and post-translationally modified prior to being recycled back to the plasma membrane to bind to the Notch receptor (Wang & Struhl, 2004). Alternatively, endocytosed ligand may lead to sorting and lysosomal degradation or to packaging into secreted exosome vesicles that deliver active ligand (Mishra-Gorur *et al.*, 2002; Le Borgne & Schweisguth, 2003a). The details of these rather novel and debated models are beyond the scope of this discussion and are best reviewed in the following references (Le Borgne & Schweisguth, 2003a; Schweisguth, 2004; Le Borgne *et al.*, 2005; Le Borgne, 2006). It seems likely that the current abundance of models that describe the processing and vesicular transport of ligand and receptor stems from the inherent mechanistic diversity and complexity of the Notch pathway signaling, and that many, if not all, of the currently envisioned paradigms reflect biologically relevant means of regulation used in different contexts.

Intramembrane Cleavage by γ-Secretase Releases Intracellular Notch

Ligand-induced ectodomain shedding to generate N^{EXT} permits subsequent proteolytic events to occur within the transmembrane domain of Notch, liberating intracellular Notch (N^{ICD}) (Selkoe & Kopan, 2003). Early studies showed the importance of N^{ICD} in nuclear localization and transcriptional activation (Greenwald, 1994; Kimble *et al.*, 1998). Expression of N^{ICD} or Notch proteins whose extracellular domain was deleted ($N^{\Delta E}$) resulted in constitutive signaling output (Lieber *et al.*, 1993; Rebay *et al.*, 1993; Roehl & Kimble, 1993; Struhl *et al.*, 1993).

Regulated intramembrane proteolysis (RIP) is catalyzed by γ-secretase activity, originally characterized in the processing of the amyloid β-protein precursor (APP) to generate amyloid β-protein (Aβ) and defective in cases of Alzheimer's disease (Selkoe & Kopan, 2003). γ-secretase activity is attributed to the activity of the core protein, Presenilin (Psn), a nine-pass transmembrane protein, that acts in a multi-enzyme protease unit (Figure 7) (De Strooper, 2003; Selkoe & Kopan, 2003; Iwatsubo, 2004; Periz & Fortini, 2004; Laudon *et al.*, 2005). Presenilin physically interacts with Notch and is crucial for the release of N^{ICD} (De Strooper *et al.*, 1999; Ray *et al.*, 1999a; Ray *et al.*, 1999b; Struhl &

Greenwald, 1999; Ye *et al.*, 1999). Loss of *Psn in vivo* produces a phenotype similar to *Notch* loss-of-function mutants, emphasizing its critical role in the pathway (Ye *et al.*, 1999). Drosophila Psn is active in a homodimeric conformation, whereas the mammalian paralogs Psn-1 and -2 operate as heterodimers (Selkoe & Kopan, 2003; Cervantes *et al.*, 2004).

γ-Secretase is composed of multiple factors that constitute the holoenzyme (Figure 7). In addition to Psn, the proteolytic enzyme includes Nicastrin (Nct), a large Type I glycoprotein that binds homodimeric Psn and co-traffics to the plasma membrane via the secretory pathway (Goutte *et al.*, 2000; Yu *et al.*, 2000). Like Psn, Nct is required for liberation of N^{ICD} and loss of Nct results in reduced γ-secretase activity (Chung & Struhl, 2001; Hu *et al.*, 2002; Lopez-Schier & St Johnston, 2002). Initially described in *C. elegans*, further factors that regulate the formation of the γ-secretase holoenzyme include APH-1, a putative seven-pass transmembrane protein, and PEN-2, a small two-pass transmembrane protein (Figure 7) (Francis *et al.*, 2002; Goutte *et al.*, 2002). Although their activities remain unclear, Nct, APH-1, and PEN-2 facilitate the formation and maintenance of the Psn homodimer (Selkoe & Kopan, 2003).

γ-Secretase cleaves Notch within its transmembrane domain. Although it has not been shown with Drosophila Notch, murine Notch-1 is cleaved by γ-secretase at two sites, S3 and S4 (Figure 7) (Ray *et al.*, 1999a, 1999b). Proteolysis at these sites occurs sequentially such that S4 cleavage is dependent on S3 cleavage (Chandu *et al.*, 2006). The S3 cleavage leads to the release of N^{ICD}, whereas the S3-S4 cleavage leads to release of a small hydrophobic Nβ peptide (Figure 7) (Moehlmann *et al.*, 2002; Okochi *et al.*, 2002). Recent reports indicate that the Nβ peptide product is secreted, although its function remains to be elucidated (Okochi *et al.*, 2006).

Like its ligands, Notch receptor endocytosis may be required for signaling events. Monoubiquitylation and clathrin-dependent endocytosis of Notch is required for γ-secretase activity in signal-receiving cells (Gupta-Rossi *et al.*, 2004). It is unclear whether Notch is cleaved at the plasma membrane or within an intracellular endocytic compartment. One model suggests that following S2-cleavage, N is endocytosed, and that subsequent S3/4 cleavage occurs within lipid rafts where active γ-secretase has been observed (Gupta-Rossi *et al.*, 2004; Pasternak *et al.*, 2004; Vetrivel *et al.*, 2004; Le Borgne *et al.*, 2005). However, the data do not rule-out models in which proteolysis occurs at the plasma membrane (Struhl & Adachi, 2000).

Recent work suggests that endocytosis may also lead to downregulation of Notch via lysosomal degradation (Le Borgne, 2006). Notch is targeted for endocytosis by the membrane-associated protein Numb and the AP2 clathrin-adaptor complex subunit protein α-Adaptin (Guo *et al.*, 1996; Berdnik *et al.*, 2002). Degradation may also be mediated by a set of E3 ubiquitin ligases. For example, Numb may interact with the E3 ubiquitin ligases Itch, Nedd4 or Suppressor of Deltex (Su[Dx]) to promote ubiquitylation, endocytosis, and degradation of Notch (Cornell *et al.*, 1999; Qiu *et al.*, 2000; Mazaleyrat *et al.*, 2003; McGill & McGlade, 2003; Sakata *et al.*, 2004; Wilkin *et al.*, 2004; Shaye & Greenwald, 2005). Adding another layer of complexity, rather than promoting N degradation as is most common for E3 ligases, Deltex prevents it from entering the degradative pathway, possibly through an endosomal sorting mechanism (Hori *et al.*, 2004). Determining the validity of these models remains an active area of research, and additional information regarding the endocytic events that govern Notch can be found in the following references (Le Borgne & Schweisguth, 2003a; Schweisguth, 2004; Le Borgne *et al.*, 2005; Le Borgne, 2006).

Transcriptional Repression in the Absence of Signaling

The primary nuclear effector of Notch signaling in Drosophila is the bifunctional transcriptional regulator Suppressor of Hairless (Su[H]) (Figure 8A) (Schweisguth & Posakony, 1992; Lai, 2002b). Its requirement in Notch signaling is well documented, both genetically and biochemically (Schweisguth & Posakony, 1992; Fortini & Artavanis-Tsakonas, 1994; Bailey & Posakony, 1995; Furukawa *et al.*, 1995; Lecourtois & Schweisguth, 1995). In the absence of Notch pathway activation, Su(H) functions as a transcriptional repressor (Figure 8A), whereas in the presence of active Notch signaling, Su(H) becomes a transcriptional activator (Figure 8B) (Dou *et al.*, 1994; Waltzer *et al.*, 1995; Hsieh *et al.*, 1996). This dual role allows Su(H) to exert opposing effects on the same target genes in different contexts. For example, during specification of the embryonic mesectoderm, Su(H) acts as a downstream effector of Notch signaling to activate expression of the gene *singleminded* (*sim*), while simultaneously repressing

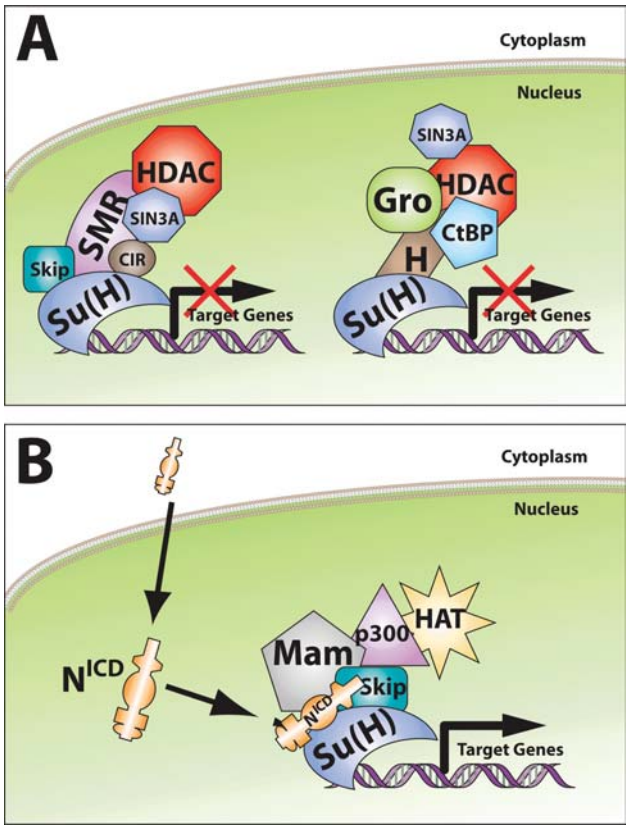

FIGURE 8 **Su(H) mediates transcriptional repression and activation. (*A*) In unstimulated cells, Su(H) represses target genes mediated by a (i) Smr or a (ii) H/Gro repressor complex. (*B*) Stimulation of the pathway promotes conversion of Su(H) into an activator by N^{ICD} and Mam, recruiting an activator complex. Adapted from Lai, 2002b.**

sim expression in the adjacent neuroectoderm independent of Notch (Klein *et al.*, 2000; Morel & Schweisguth, 2000).

In the pathway 'off' state, Su(H) exists in a repressor complex. In vertebrates, the Su(H) ortholog, Recombination signal sequence-binding protein Jκ (RBP-Jκ)/C-promoter Binding Factor 1 (CBF-1), mediates transcriptional repression by binding to a variety of co-repressors including silencing mediator for retinoid and thyroid receptor (SMRT), nuclear receptor co-repressor (NCoR) (Kao *et al.*, 1998), CBF-interacting repressor (CIR) and KyoT2 (Taniguchi *et al.*, 1998; Hsieh *et al.*, 1999; Qin *et al.*, 2004). Each of these co-repressors regulate repression with RBP-Jκ/CBF-1 in a large complex that includes Sin3A, SAP18, SAP30, RbAp46/48, Sharp/Mint, and histone deacetylases (HDACs) (Lai, 2002b; Oswald *et al.*, 2002; Kuroda *et al.*, 2003).

In Drosophila, Su(H) interacts with the SMRT homolog, Smrter (Smr), and chromatin regulators to facilitate transcriptional repression of target genes (Figure 8A) (Tsai *et al.*, 1999; Tsuda *et al.*, 2002). In a distinct complex, Su(H) also mediates transcriptional repression through the co-repressor adaptor protein Hairless (H) (Figure 8A) (Schweisguth & Posakony, 1994; Bang *et al.*, 1995). With genetic loss of *H*, Notch signaling targets are ectopically activated (Barolo *et al.*, 2000; Furriols & Bray, 2000; Klein *et al.*, 2000; Morel *et al.*, 2001). H functions by binding to DNA-bound Su(H) along with the global co-repressors C-terminal binding protein (CtBP) and Groucho (Gro) (Barolo *et al.*, 2002). Recent studies indicate that H-binding to CtBP and Gro, in combination, is required to exert transcriptional repression of Notch signaling targets (Nagel *et al.*, 2005). Like the mammalian CBF-1/RBP-Jκ repression complexes, HDACs are recruited to maintain a repressed state by the Drosophila Su(H)/H/CtBP/Gro complex (Lai, 2002b).

Transactivation of Target Genes Follows Proteolytic Cleavage

Proteolytic activation of Notch leads to activation of target genes of the pathway. Following liberation of N^{ICD} through proteolytic cleavage, the N^{ICD} is directed to the nucleus via its NLS motif (Figure 8B) (Lieber *et al.*, 1993; Hsieh *et al.*, 1996). Once in the nucleus, N^{ICD} interacts directly with Su(H) through its RAM and Ank repeat domains (Roehl & Kimble, 1993; Kato *et al.*, 1997; Kurooka *et al.*, 1998). N^{ICD} binding to Su(H) displaces the co-repressor complexes and switches Su(H) from a repressor to an activator (Mumm & Kopan, 2000; Lubman *et al.*, 2004).

The conversion of Su(H) from a repressor to an activator is mediated, not only by N^{ICD}, but also by co-activators (Figure 8B). This is facilitated by the bifunctional cofactor Ski-interacting protein (Skip), which is constitutively bound to Su(H). In the absence of activated Notch, Skip is bound to the SMRT co-repressor complex (Zhou *et al.*, 2000a, 2000b; Leong *et al.*, 2004). However, Skip has a greater affinity for N^{ICD} than SMRT, and because binding is mutually exclusive, the presence of N^{ICD} competes SMRT and associated co-repressors away from Skip and Su(H) (Zhou *et al.*, 2000b; Leong *et al.*, 2004). The N^{ICD}/Su(H)/Skip complex recruits co-activators such as the histone acetyl transferase (HAT) enzymes CBP/p300, PCAF, and GCN5 which all interact with N^{ICD} at its Ank repeats and TAD (Figure 8B) (Kurooka & Honjo, 2000; Wallberg *et al.*, 2002; Leong *et al.*, 2004).

Transactivation by N^{ICD}/Su(H) also requires the recruitment of Mastermind (Mam) (Figure 8B) (Portin & Rantanen, 1991; Petcherski & Kimble, 2000b; Petcherski & Kimble, 2000a; Wu *et al.*, 2000; Fryer *et al.*, 2002; Wu & Griffin, 2004; Wilson & Kovall, 2006). *mam* encodes a Gln-rich nuclear protein that contains an N-terminal Basic domain required to bind N^{ICD} Ank repeats, an N-terminal Acidic I domain that functions in p300 interactions, and a C-terminal Acidic II domain (Wu & Griffin, 2004). Recruitment of Mam completes the transcriptional complex that activates expression of N^{ICD}/Su(H) target genes (Wallberg *et al.*, 2002). Recently, the crystal structure of the N/Su(H)/Mam ternary complex was solved suggesting that conversion of Su(H) from repressor to activator occurs via a conformational change induced by cooperative interaction of Su(H) with N^{ICD} and Mam (Barrick & Kopan, 2006; Nam *et al.*, 2006; Wilson & Kovall, 2006).

Several genes have been characterized as transcriptional targets of the Notch pathway. These include the Enhancer of split complex (*E[spl]-C*) that consists of thirteen Notch-responsive genes (Knust *et al.*, 1987a, 1987b; Jennings *et al.*, 1994): seven basic helix-loop-helix (bHLH) transcription factors (E[spl]-mβ, -mγ, -mδ, -m3, -m5, -m7, and -m8), four Bearded (Brd) family protein (E[spl]-mα, -m2, -m4, and -m6), the co-repressor Gro, and the Kazal-family protease inhibitor E(spl)-m1 (Preiss *et al.*, 1988; Klambt *et al.*, 1989; Delidakis & Artavanis-Tsakonas, 1992; Knust *et al.*, 1992; Paroush *et al.*, 1994; Wurmbach *et al.*, 1999; Lai *et al.*, 2000).

The E(spl)-C encodes two families of transcriptional regulators. The E(spl)-bHLH class represses the transcription of proneural genes of the Achaete-Scute complex (AS-C) (Oellers *et al.*, 1994). Little is known about the E(spl)-Brd class, although, Brd family proteins encoded by the Brd complex (Brd-C) locus were recently found to interfere with the interaction of Neur and Dl, thereby inhibiting Dl endocytosis and activation (Lai *et al.*, 2000; Bardin & Schweisguth, 2006). Thus, it is possibile that the Brd family proteins within the E(spl)-C, like the Brd-C proteins, may exert negative feedback on the Notch signaling pathway by antagonizing ligand activation.

In summary, we have described how the binding of ligand leads to the proteolytic release of activated N^{ICD}, conversion of Su(H) from a repressor to an activator, and transcription of target genes such as the E(spl)-C. In the following section, the different general contexts for which Notch signaling is used will be presented.

Context-Specific Variations in Notch Signaling

Numerous developmental processes require Notch signaling (Bray, 1998; Lai, 2004). However, the Notch signaling machinery is not utilized in exactly the same way in all contexts; instead, variations in the basic events described in the previous section are used to elicit an array of context-specific cell-to-cell signals. Three major contexts requiring Notch signaling will be discussed below: lateral inhibition, binary cell fate decisions, and boundary formation (Figure 9).

Lateral inhibition occurs when one cell is selected from a group of equivalent precursors and subsequently signals to its neighbors to prevent them from adopting that same fate (Figure 9A). In the context of Notch signaling during Drosophila embryonic neurogenesis, the process starts with a field of homogeneous and equipotent cells, called the neurogenic region. These cells initially express ligand and receptor equally and have the ability to signal to one another (Wigglesworth, 1940; Moscoso del Prado & Garcia-Bellido, 1984; Doe & Goodman, 1985; Bray, 1998; Artavanis-Tsakonas *et al.*, 1999; Lai, 2004). Expression of transcription factors of the bHLH family that include Achaete (Ac), Scute (Sc), Lethal of Scute (L'sc), and Asense (Ase) (Achaete-Scute complex/[AS-C]), referred to collectively as the proneural genes and initially expressed throughout the neurogenic region, becomes refined to smaller proneural clusters from which a single neuroblast will be selected (Figure 9A) (Skeath & Carroll, 1994). Dl and N expression is initially uniform within the proneural cluster, but an elevation of Dl in a single cell, the presumptive neuroblast, leads to the activation of Notch in the surrounding cells. Notch signaling activates the *E(spl)-C* genes whose encoded products repress expression of the proneural *AS-C* genes, resulting in downregulation of Dl, a downstream transcriptional target, and leading to molecular inhibition in Notch-activated cells (Figure 9A). In turn, increased Dl expression in the presumptive neuroblast cell results in decreased Notch activation in the presumptive neuroblast, leading to reduced E(spl)-C expression and de-repression of the AS-C (Figure 9A) (Singson *et al.*, 1994). Recently, it was observed that Su(H)'s repressive function assures the downregulation of E(spl)-C in this process (Castro *et al.*,

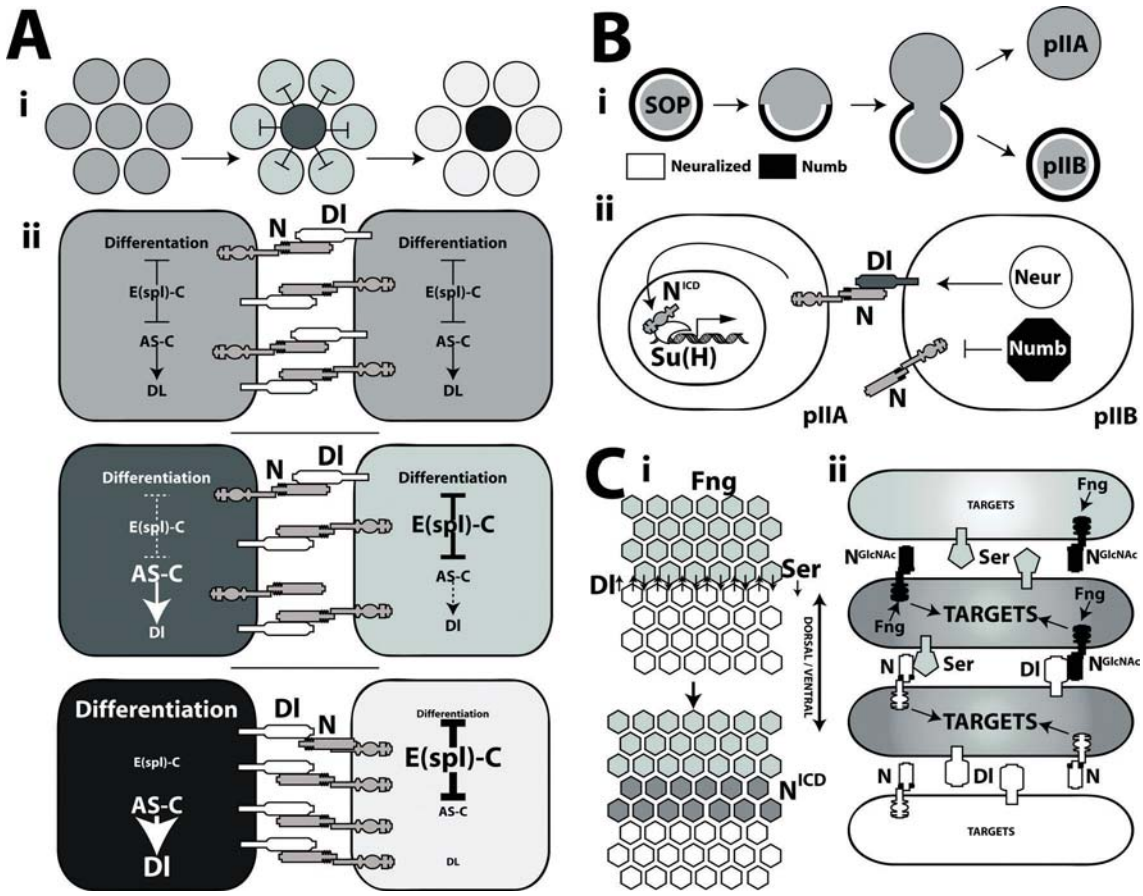

FIGURE 9 **Three modes of Notch signaling function. (*A*) In lateral inhibition, (i) initially, cells in a field have equal potential, but stochastically or through a slight bias, one cell adopts a specific fate, *e.g.*, a neuroblast (black cell), while inhibiting surrounding cells from taking on that same fate. (ii) This change is due to differential AS-C expression to activate Dl in the neuroblast versus upregulation of E(spl)-C in the surrounding cells to repress Dl expression. (*B*) During the determination of binary cell fates from an SOP, (i) a pair of cells have a bias towards one cell fate due to differential segregation of Neur and Numb components into pIIB. (ii) Neuralized promotes Dl-N transendocytosis in pIIB, while Numb inhibits activation of Notch. (*C*) During wing compartment formation, (i) Asymmetric ligand expression, dorsal Ser and ventral Dl along the dorsal-ventral midline, along with asymmetric dorsal Fng expression (light shading) results in high activated Notch expression (dark shading) and formation of a dorsal-ventral boundary. (ii) Due to asymmetric Fng-mediated Notch *O*-glycosylation, cells at the boundary have high Notch activity because of both Dl-N and Ser-N target activation. Adapted from Beatus and Lendahl, 1998; Haines and Irvine, 2003; Lai, 2004.**

2005). Thus, at the conclusion of this process, lateral inhibition focuses ligand expression within a single cell that will adopt a neural fate, while the surrounding cells with active Notch signaling will become non-neuronal, epidermal cells (Figure 9A) (Beatus & Lendahl, 1998). This inhibitory process is proposed to occur at a local level such that cell fates are determined through direct contact and short-range signaling between cells expressing proneural and neurogenic genes (Artavanis-Tsakonas *et al.*, 1999).

The specification of a single precursor from within the proneural cluster was initially proposed to be a stochastic decision (Simpson, 1997; Beatus & Lendahl, 1998). However, it was subsequently observed that the SOP always occupies the same position within the cluster, suggesting that the expression of the proneural genes is somehow 'pre-patterned' to create a slight bias for selection of the SOP (Cubas *et al.*, 1991; Culi & Modolell, 1998). Although the notion of pre-patterning provides a satisfying explanation for the regularly spaced arrangement of SOPs, the mechanisms that bias the SOP for selection remain unclear. Once a bias is determined, maintainance of the selection becomes important. For example, the simple model whereby cells that surround the single SOP are inhibited from adopting a neural fate goes hand-in-hand with a model whereby the SOP is affirmed, or 'wins,' its cell fate. It is observed that once pre-patterned, the proneural *AS-C* genes play an active role in selecting a single precursor. That is, the proneural genes expressed in the precursor provide positive

feedback to regulate themselves in *cis* and to upregulate other proneural target genes in *trans* to overcome the repression of those genes repressed by the E(spl)-C (Skeath & Carroll, 1994; Nakao & Campos-Ortega, 1996; Culi & Modolell, 1998; Gibert & Simpson, 2003).

The choice of binary cell fate decisions during asymmetric cell divisions may also be regulated by the Notch signaling pathway (Figure 9B). This special directional inhibitory signaling mechanism differs from lateral inhibition. In these specialized cell divisions, a sensory organ precursor (SOP) cell divides to produce two daughter cells. These daughter cells are each capable of sending and receiving Notch signaling, however, rather than being equipotent, these cells have bias and directionality in their signaling interactions as influenced by determinants that were asymmetrically allocated between the daughter cells during cell division. This leads to adoption of differential sibling fates such that one cell expresses signal, whereas the other receives signal from the Notch receptor (Figure 9B) (Bray, 1998; Lai, 2004).

This type of Notch inhibitory signaling occurs in the course of binary cell fate decisions following the SOP choice during bristle cell development (Roegiers & Jan, 2004). The SOP undergoes an asymmetric cell division such that Numb and Neur are segregated to the pIIb cell but not the pIIa cell (Figure 9B) (Uemura *et al.*, 1989; Rhyu *et al.*, 1994; Guo *et al.*, 1996; Spana & Doe, 1996). Neur promotes Dl activation while Numb antagonizes Notch activation in pIIb (Lai & Rubin, 2001; Le Borgne & Schweisguth, 2003b). This leads to an asymmetry of ligand and receptor: pIIb expresses ligand and pIIa expresses a competent receptor (Figure 9B). The asymmetry results in activation of the Notch pathway in pIIa, activation of E(spl)-C, and inhibition of proneural genes (Gigliani *et al.*, 1996; Heitzler *et al.*, 1996). The pIIb divides asymmetrically to give forth sheath and neuronal cells (along with a glial precursor cell in the gliogenic SOP lineage); the pIIa divides asymmetrically to yield hair shaft cell and socket cell progeny (Figure 9B) (Jan & Jan, 1995; Van De Bor & Giangrande, 2001). These cells together form a sensory unit in the adult fly. The bristle sensory unit is, therefore, a product of lateral inhibition generated by the SOP that in turn yields two different lineages of cells as a result of binary cell fate decisions mediated by the Notch pathway.

In contrast to inhibitory Notch signaling, inductive Notch signaling promotes rather than represses a given cell fate in the signal-receiving cells by activating transcriptional targets (Bray, 1998; Lai, 2004). Like the binary cell fate decision, inductive Notch signaling proceeds among cells that have a biased, rather than an equivalent, potential (Lai, 2004).

The inductive process is well characterized in the development of the dorsal-ventral boundary in the Drosophila wing (Figure 9C) (Rauskolb *et al.*, 1999). In the developing wing tissue, Fng and Ser are expressed in the dorsal compartment whereas Dl is expressed in the ventral part (Figure 9C) (Doherty *et al.*, 1996; Rauskolb *et al.*, 1999). Since Fng inhibits Ser's ability to interact with Notch, dorsal cells may only signal to dorsoventral boundary cells. These boundary cells receive signal from ventrally-expressed Dl as well as dorsally-expressed Ser. Thus, the dorsoventral boundary cells have a sharp activation of Notch (Figure 9C). This leads to induction of the cell fate for the wing boundary through the induction, rather than the inhibition, of the genes *wingless* (*wg*), *vestigial* (*vg*), and others (Fleming *et al.*, 1997; Panin *et al.*, 1997; Rauskolb *et al.*, 1999; Wu & Rao, 1999; Lai, 2004).

In conclusion, the three general mechanisms for Notch signaling–lateral inhibition, binary cell fate decision and boundary formation–outlined above will be an important reference for our discussion of DER and Notch signaling pathway interactions below.

INTERPLAY OF THE DER AND NOTCH SIGNALING PATHWAYS

Relationships Between DER and Notch Signaling During Development

Three main themes will run through our discussion of the mechanisms by which DER and Notch relate: consequence, time, and space. First, the outcome, or consequence, of DER and Notch signaling is highly context specific. Mutual antagonism is perhaps the most common relationship in EGFR-Notch interactions, although in some situations the pathways cooperate to potentiate each other's signaling activities. Second, understanding the temporal integration of DER and Notch signaling, that is whether the two pathways operate sequentially or simultaneously and how that affects overall output, is a critical element that we are only beginning to have the tools to address at single cell resolution in an *in vivo* system. Third, the spatial coordination of DER and Notch signal transduction across a field of developing cells provides another key

determinant of output specificity. For example, activation of DER and Notch may occur within the same cell, or in adjacent cells, and depending on context, these differences lead to profound distinctions in how the information is processed. In the remainder of this review, we will compare and contrast the various signaling interactions between DER and Notch during the temporal course of *Drosophila* eye development.

The Drosophila Eye

Drosophila provides a superb genetically tractable system to study developmental signal transduction using genetic, biochemical, cellular, genomic, and molecular approaches. In particular, the evolutionarily conserved signaling circuitries that produce the precise stereotyped patterning of the Drosophila compound eye have proven an excellent backdrop for studying cell-cell communication, especially with respect to signaling integration and cross-talk (Voas & Rebay, 2004). Importantly, many of the discoveries emanating from investigations of signaling mechanisms in the fly eye have proven to be broadly conserved.

The Drosophila compound eye is composed of about 750 repetitive subunits, termed ommatidia, which are arranged symmetrically with respect to the horizontal equator line to form a precise neurocrystalline lattice (Figure 10B). The cellular architecture of an ommatidium enables it to focus light from its individual visual field and to transmit the information to the brain via the axonal projections of the photoreceptors (Ready *et al.*, 1976; Tomlinson & Ready, 1987). Each mature ommatidium contains eight neuronal photoreceptors (R1–8) arranged in a stereotypical trapezoidal pattern (Figure 10C). The non-neuronal cells consist of four lens-secreting cone cells, two primary (1°) pigment cells, and a hexagonal lattice of secondary/tertiary (2°/3°) pigment cells with interspersed sensory bristle cells (Ready *et al.*, 1976; Tomlinson & Ready, 1987). Cells within the lattice are specified in a regulated and reproducible manner (Ready *et al.*, 1976; Tomlinson & Ready, 1987). Deviations from the precise development of the eye by loss or gain of cell types, *e.g.*, through genetic mutation or transgene expression, lead to obvious defects. Characterization of the molecular basis behind these defects has resulted in elucidation of the genes and pathways required for proper development of the eye. In the ensuing discussion, we have selected several cell fate specification and morphogenetic events as the backdrop for considering the various modes of DER-Notch pathway crosstalk and integration. Our goal is not to provide a comprehensive view of eye development *per se*, but rather to discuss the conserved signaling paradigms that have emerged from these studies.

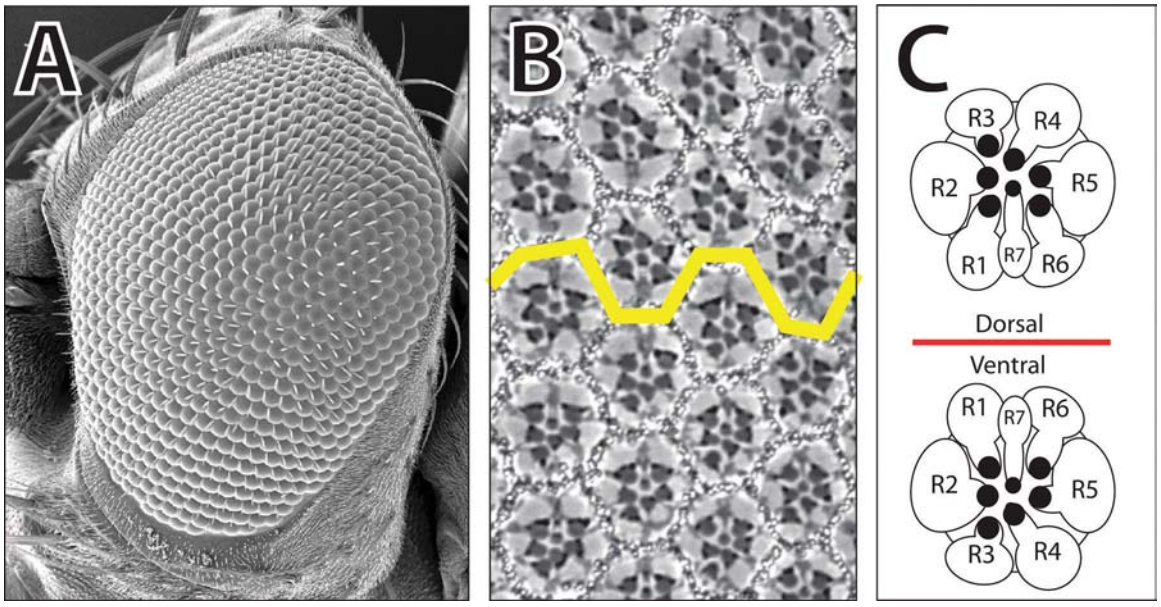

FIGURE 10 **The Drosophila eye. (*A*) Scanning electron micrograph of the adult compound eye. (B) Tangential section and cartoon of the adult eye showing the hexagonal ommatidia and regular pigment cell lattice. The dark-stained light-sensing photoreceptor organelles, or rhabdomeres, are arranged in a trapezoidal pattern. R1-7 are shown in this section; R8 is located at lower section levels. (*B*, *C*) Ommatidia are arranged symmetrically with respect to the equator, such that R3 points up in the dorsal half, and points down in the ventral half.**

DER and Notch Regulate the Retinal Determination Gene Network

The eye and antenna arise from a columnar epithelial primordium, known as the eye-antennal imaginal disc (Figure 11B). Set aside as small clusters of cells in the early embryo, imaginal disc epithelia undergo extensive proliferation and patterning during larval and pupal stages, and eventually give rise to the adult structures during metamorphosis (Cohen, 1993). During early larval stages, the eye-antennal disc grows through unpatterned, asynchronous cell division (Wolff & Ready, 1993). Within this epithelium, specification of the eye field occurs through the actions of the retinal determination gene network (RDGN) (Figure 11A) (Pappu & Mardon, 2004; Silver & Rebay, 2005), a group of transcription factors that function as master regulators or eye selector factors.

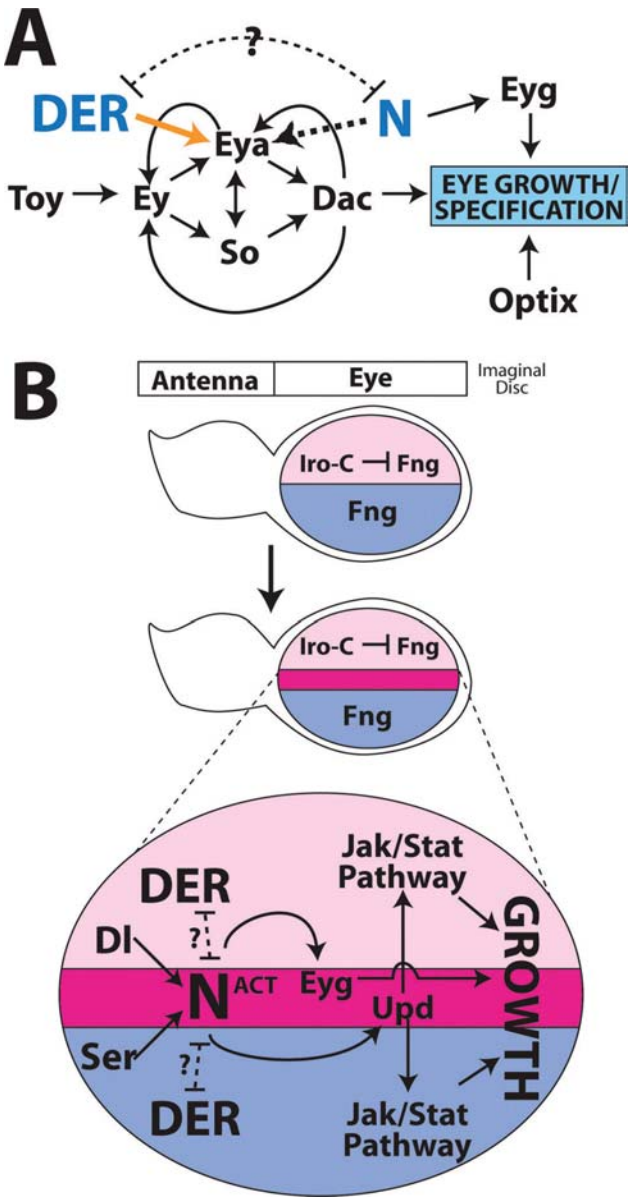

FIGURE 11 **Regulation of early eye development by Notch and DER. (*A*) Eye growth and specification are controlled by the Retinal Determination Gene Network. Dark solids lines indicate transcriptional regulation. Notch indirectly activates Eya and may mutually antagonize DER (dotted lines). DER activates Eya through phosphorylation via MAPK (orange line). (*B*) The Iroquois Complex (Iro-C) inhibits Fng in the dorsal half of the early eye imaginal disc. Differential ligand expression of Dl (dorsal) and Ser (ventral) along with asymmetric Fng expression (ventral) mediates dorsal-ventral boundary formation where activated Notch (N^{ICD}) is expressed, resulting in disc growth via Eyg and Upd. Adapted from Cavodeassi *et al.*, 2001.**

Proteins within the RDGN are expressed in a hierarchial manner whereby the Pax6 transcription factor Twin of eyeless (Toy) activates another Pax6 member Eyeless (Ey). Ey potentiates expression of Sine oculis (So), Eyes absent (Eya), and, subsequently, Dachshund (Dac). As the name suggests, these regulators function as a *network*: the hierarchy does not solely run in a linear fashion, but, rather, factors downstream initiate feedback loops to activate other members within the group. In addition, other factors such as Optix and the Pax6 member Eyegone (Eyg) play independent but necessary roles in specifying the eye field (Figure 11A) (Pappu & Mardon, 2004; Dominguez & Casares, 2005; Silver & Rebay, 2005).

The DER and Notch pathways both play critical roles during early eye development. Early reports postulated that these two pathways antagonize each other in the specification of the eye field within the eye-antennal disc anlagen in the latter half of the second larval instar (Kurata *et al.*, 2000; Kumar & Moses, 2001a). However, a more recent report supports a model whereby Notch and DER are not involved in specification, but, rather, specification occurs earlier during the first half of the second instar larval stage, independent of Notch and DER (Kenyon *et al.*, 2003). At this time, the eye selector genes Ey and Toy retract from their ubiquitous expression in the eye-antennal disc to a region that includes the posterior two-thirds of the eye-antennal disc, where they inhibit the expression of the antennal marker *Distalless* (*Dll*) (Kenyon *et al.*, 2003). The signaling events that initiate the re-distribution of Ey and Toy expression remain to be elucidated.

The Notch signaling pathway promotes proliferation within the eye disc over a period of several days that spans from late first to the early third larval instar (Kenyon *et al.*, 2003; Dominguez *et al.*, 2004; Dominguez & Casares, 2005). Although expressed

throughout the eye disc, Notch activation is restricted to the dorsoventral equator line through Notch's inductive boundary formation activities that are regulated by Fng in the ventral half of the eye disc and the Fng-inhibiting Iroquois protein complex in the dorsal compartment (Figure 11B) (Cho & Choi, 1998; Dominguez & de Celis, 1998; Papayannopoulos *et al.*, 1998; Cavodeassi *et al.*, 1999; Yang *et al.*, 1999; Dominguez & Casares, 2005). From here, the Notch pathway induces proliferation of the eye disc by establishing an organizing center required for eye growth (Dominguez & de Celis, 1998; Cavodeassi *et al.*, 2001; Kenyon *et al.*, 2003; Reynolds-Kenneally & Mlodzik, 2005). This organizing center requires Notch as well as Eyg to promote growth of the eye disc. The Jak/Stat pathway ligand Unpaired (Upd) is a downstream component of the organizing center that acts over long distance to promote cell proliferation over the entire eye field (Figure 11B) (Chao *et al.*, 2004; Reynolds-Kenneally & Mlodzik, 2005). It remains to be elucidated whether *upd* is a direct transcriptional target of the Notch signaling pathway during the proliferative process. In addition, Notch has been reported to mediate proliferation by promoting the G1/S transition by activating genes such as *dE2F1*, which encodes the Drosophila homolog of the vertebrate cell-cycle regulatory transcription factor E2F (Baonza & Freeman, 2005). Whether this mechanism contributes to early proliferation in the eye disc remains an open question.

The Notch pathway also plays a positive role with respect to early eye development through the regulation of the RDGN gene *eyg*. Because *eyg* is dispensible for the specification of the eye field, in this context, Notch again positively regulates eye growth, not specification (Figure 11B) (Dominguez *et al.*, 2004). It is not yet understood if the regulation of *eyg* occurs through direct transcriptional activation by N/Su(H), but analysis in conditional *Notch* mutants along with mosaic mutant analysis of *Su(H)* and *Ser* loss-of-function alleles implicate this to be the case. In support of this hypothesis, Eyg protein was found to be coincidently expressed with the E(spl)-C proteins (Dominguez *et al.*, 2004).

The observation that eye specification occurs earlier than had been previously reported and the insight that Notch regulates eye disc proliferation does not address the observation that the Notch and DER pathways antagonize each other during the second larval instar (Kurata *et al.*, 2000; Kumar & Moses, 2001a; Kenyon *et al.*, 2003). Does DER signaling still play a role at this stage? These observations implicate DER signaling in antagonizing the proliferation mediated by Notch. This would be surprising because the DER pathway is considered to be a key *positive* regulator of eye development with respect to proliferation and differentiation (Freeman, 1996). Furthermore, the DER pathway positively regulates the activity of the RDGN through MAPK phosphorylation of Eya (Figure 11A) (Hsiao *et al.*, 2001). Reconciliation of these observations can be achieved by proposing that the DER pathway functions in temporally distinct manners during the development of the eye: it may antagonize early Notch-mediated proliferation, but later promote proliferation and other aspects of eye development. If such a model is true, then it will be important to determine how regulation of DER specificity is achieved.

DER and Notch at the Morphogenetic Furrow

Following eye field specification, determination and differentiation occurs in a dramatic regulated fashion across the eye imaginal disc. During the late larval and early pupal stages, an indentation in the eye disc, known as the morphogenetic furrow (MF), is initiated from the posterior and progresses anteriorly in a wave that leaves in its wake differentiating cells (Figure 12) (Wolff & Ready, 1991). Overtly undifferentiated cells randomly proliferate anterior to the MF, whereas posterior to the MF, ommatidial clusters start to form as cells that make up each ommatidium are recruited (Wolff & Ready, 1993). Five domains of gene expression have been defined in the developing eye disc to categorize the complex series of events that underly ommatidial development (Figure 12). The MF lies within Zone III. Ahead of the MF are two domains of transcription factor expression, Zones I and II, where the early RDGN proteins–Toy, Ey, Eyg, Optix are expressed. Other RDGN proteins, such as Eya and So, are expressed in Zones II-V (Bessa *et al.*, 2002; Pappu & Mardon, 2004).

The MF is initiated by the coordination of several signaling pathways (Figure 12A) (Treisman & Heberlein, 1998). Beginning at the posterior of the eye disc, the hedgehog (Hh) signal is expressed in differentiating photoreceptor neurons. Hh is necessary and sufficient for MF formation (Ma *et al.*, 1993; Heberlein & Moses, 1995). Hh induces both long-range signaling by the

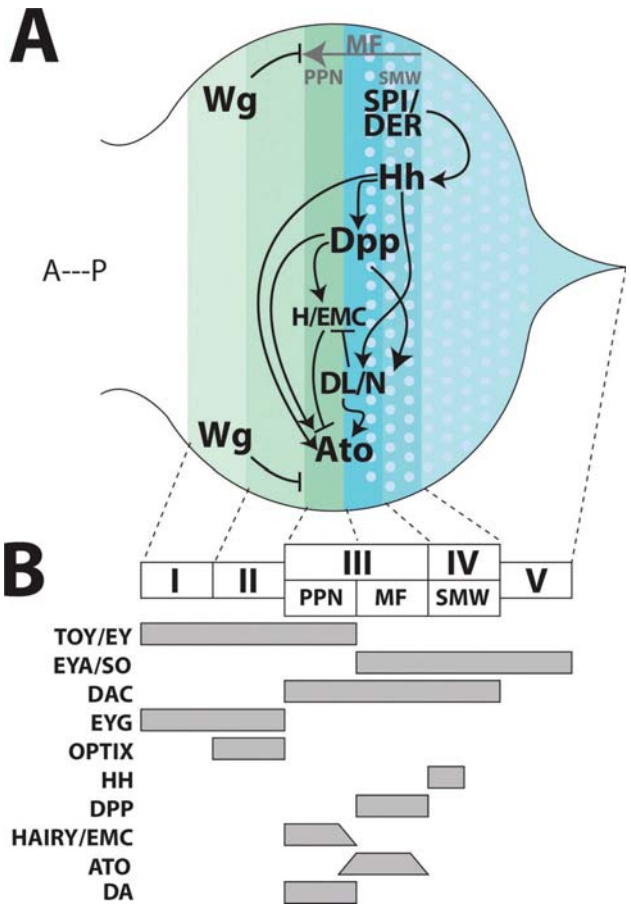

FIGURE 12 **Morphogenetic Furrow (MF) progression. (*A*) DER from differentiating cells activates the Hh pathway to initiate and re-initiate the MF. Hh, Dpp, and Notch synergize to activate Ato expression in region III. Expression domains of regulators of the eye disc are indicated in (*B*). Adapted from Silver and Rebay, 2005.**

secreted protein Decapentaplegic (Dpp), the Drosophila transforming growth factor-β (TGF-β) ortholog, and the pre-patterned expression of the basic helix-loop-helix (bHLH) transcription factor Atonal (Ato) (Jarman *et al.*, 1994, 1995; Baker *et al.*, 1996; Greenwood & Struhl, 1999). Since Hh drives the progressive movement of the MF wave, cells that receive Hh will begin differentiation, and then express and send Hh to anterior cells (Figure 12A) (Ma *et al.*, 1993; Heberlein & Moses, 1995; Rogers *et al.*, 2005).

Dpp signals to cells anterior to the MF, in Zone II, to upregulate both Ato and the repressors Hairy (h) and Extramacrochaetae (Emc), which are needed to establish a pre-proneural (PPN) state (Figure 12) (Brown *et al.*, 1995; Greenwood & Struhl, 1999; Fu & Baker, 2003). Hairy regulates bHLH proteins through transcriptional repression; Emc represses bHLH proteins through protein-protein interaction (Ellis *et al.*, 1990; Garrell & Modolell, 1990; Van Doren *et al.*, 1992). Hairy and Emc provide negative feedback with respect to the Hh pathway to repress Ato transcription (Brown *et al.*, 1995). Additionally, the Wg signaling pathway provides negative regulation of the MF, preventing MF initiation at the lateral margins of the disc (Figure 12A) (Ma *et al.*, 1993; Treisman *et al.*, 1997).

The DER and Notch signaling pathways are both required for MF initiation upstream of the Hh and Dpp pathways, but downstream of Wg (Kumar & Moses, 2001b). In contrast to their antagonistic role in early eye development, described above, DER and Notch function cooperatively to promote the formation of the MF. Through the use of conditional mutants and tissue- and temporal-specific overexpression of activated DER pathway or dominant-negative Notch pathway components, the requirement of both of these pathways was identified. DER is required at two times for proper MF establishment: (1) before initiation of the MF (birth) and (2) for propagation of new ommatidial columns along the disc's lateral margins (reincarnation) (Kumar & Moses, 2001b). Notch is required for the reincarnation step of MF generation, where *dpp* function is required to promote differentiation along the margins to permit the MF to move anteriorly (Wiersdorff *et al.*, 1996; Chanut & Heberlein, 1997; Treisman & Heberlein, 1998). Epistasis experiments suggested that DER acts upstream of Notch (Kumar & Moses, 2001b) and upstream of Hh signaling during MF birth. DER and Notch also function upstream of Dpp during MF reincarnation (Kumar & Moses, 2001b). It remains to be elucidated how the DER and Notch pathways relate to each other at a molecular level. Do the two pathways interact directly or do they simply converge to promote MF initiation?

Maintenance of the wave-like progression of the MF, where cells at or behind the MF signal Hh to cells ahead of the furrow in a cyclical manner, requires a mechanism that propagates the Hh signal forward. The observation that Hh protein expression is spatially coincident with where the DER pathway is activated suggests that DER signaling might provide this function (Figure 12A) (Kumar *et al.*, 2003; Rogers *et al.*, 2005). The transcriptional activator Pointed (Pnt), a key effector of the DER pathway, binds to a set of four Ets-binding sites located in an enhancer element that drives *hh* expression in the developing photoreceptors behind the MF (Rogers *et al.*, 2005). *In vivo* experiments show that loss of Pnt leads to downregulation of *hh*-enhancer reporter expression, strongly suggesting that Hh is activated by the DER

pathway (Rogers *et al.*, 2005). Thus, DER-activated expression of Hh induces progressive differentiation of photoreceptors, in turn leading to additional DER signaling and providing a positive feedback loop for continued forward propagation of the MF (Rogers *et al.*, 2005).

The Notch pathway acts downstream of Hh signaling to promote proneural activity *via* inductive regulation of the bHLH transcription factor Ato (Figure 12A) (Jarman *et al.*, 1994, 1995; Baker & Yu, 1997; Baker, 2000; Frankfort & Mardon, 2002; Hsiung & Moses, 2002). As aforementioned, Hairy and Emc negatively regulate *ato* expression in the PPN state (Brown *et al.*, 1995) but are themselves negatively regulated by Notch signaling (Figure 12) (Baonza *et al.*, 2001). During the PPN period, *ato* is also directly repressed by the Notch pathway effector Su(H) (Li & Baker, 2001). Dl-mediated activation of the Notch pathway converts Su(H) to an activator with respect to *ato* transcription and also potentiates expression of the E(spl)-C, which downregulates the Hairy and Emc mediated repression of *ato*. The ensuing upregulation and accumulation of Ato immediately ahead of the MF, a process termed 'proneural enhancement,' promotes subsequent neural differentiation (Figure 12A, 13A) (Baker & Yu, 1997; Baker, 2000; Baonza *et al.*, 2001; Li & Baker, 2001; Frankfort & Mardon, 2002). Because of its positive function in regulating proneural activity, Notch signaling is considered to function in an inductive signaling context during these events (Nagel & Preiss, 1999; Baonza & Freeman, 2001).

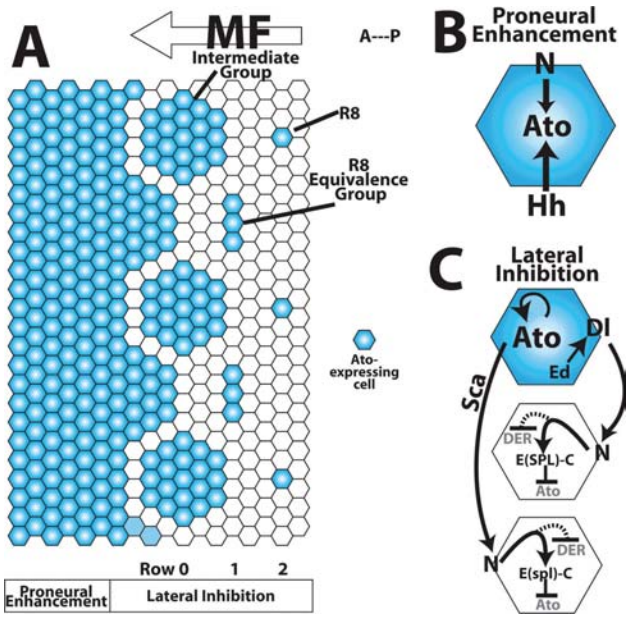

FIGURE 13 **Founder cell specification. (*A*) Ato expression (blue) is upregulated in cells anterior to the MF via proneural enhancement (*B*). Lateral inhibition (*C*) refines Ato expression to a single Ato expressing cell that will be specified to be the R8 founder cell (*A*). Adapted from Frankfort and Mardon, 2002.**

Although both DER and Notch act positively in the MF, the two pathways may not directly cooperate, but rather may function in a precise temporal sequence to promote the MF. That is, Notch signaling promotes proneural enhancement ahead of the MF leading to neural differentiation and DER signaling in presumptive photoreceptors, which upregulates the Hh signal to initiate a new round of proneural development (Baonza *et al.*, 2001; Rogers *et al.*, 2005). Therefore, Notch and DER activity in the MF, like Hh, is cyclical, allowing neural differentiation to sweep across the eye field.

Notch and DER in R8 Founder Cell Determination

The proneural protein Ato is essential for ommatidial formation (Jarman *et al.*, 1995). Ato forms heteromultimers with another bHLH protein Daughterless (Da) (Jarman *et al.*, 1993). These Ato-Da complexes are proposed to act as sequence-specific DNA-binding complexes to mediate the transcription of target genes. Ato is initially expressed broadly at the MF, but posteriorly, becomes restricted to a group of 20 cells known as intermediate groups or rosettes (Jarman *et al.*, 1995; Brown *et al.*, 1996; Baker & Yu, 1997).

Prior to intermediate group restriction, *ato* expression is under the transcriptional activation of Hh and Notch (Figure 12A, 13) (Baonza *et al.*, 2001). Through differential regulation of transcriptional enhancer sites, *ato* switches to a mode of autoregulation to promote restriction of cells into intermediate groups (Figure 13A,C) (Baker *et al.*, 1996; Dokucu *et al.*, 1996; Sun *et al.*, 1998). This signifies a critical switch whereby Ato is no longer activated by Notch, but instead is repressed by Notch activity in the context of lateral inhibition.

Thus, the Notch pathway transitions from a mode of inductive proneural enhancement to that of lateral inhibition (Figure 13) (Baker & Yu, 1997). Ato expression, which correlates with competence to become founder cells, is refined in two steps: first, Ato is restricted to two to three cells termed the R8 equivalence group, and second, Ato expression is finally focused to a single R8 photoreceptor founder cell (Figure 13A) (Jarman *et al.*, 1994, 1995; Dokucu *et al.*, 1996; Baker & Yu, 1997). The process whereby Ato is restricted to a single founder

cell within the intermediate group requires lateral inhibition through Notch signaling. As we described earlier for embryonic neurogenesis, strong Dl activity becomes restricted first to the equivalence group and then to the presumptive R8 cell and activates the N/Su(H)/E(spl)-C cascade which represses *ato* in surrounding cells (Figure 13C) (Lee *et al.*, 1996; Li & Baker, 2001). Successful lateral inhibition is important for producing the regular, evenly-spaced, alternating groups of cells that comprise the ommatidial units (Artavanis Tsakonas *et al.*, 1999; Baker, 2000; Frankfort & Mardon, 2002; Hsiung & Moses, 2002).

Lateral inhibition is also facilitated by the fibrinogen-related dimeric secreted glycoprotein Scabrous (Sca) (Figure 13C) (Baker *et al.*, 1990; Mlodzik *et al.*, 1990a; Baker & Zitron, 1995; Lee *et al.*, 1996). In contrast to localized lateral inhibition, inhibitory events mediated by Sca occur over greater distances. Importantly, as R8 cells are spaced further apart than cells seen in SOP formation, Sca function may provide a mechanism by which cell spacing over greater distances is achieved. Sca is activated downstream of Ato in the equivalence groups and then in the presumptive R8 from where it is secreted to the surrounding cells over multiple cell diameters. There, Sca interacts with Notch to single-out the R8 cell by facilitating the activation of the Notch pathway several cell-diameters away from the Sca-secreting cell (Baker & Zitron, 1995; Lee *et al.*, 1996). This effectively broadens the inhibitory strength of the Ato-producing R8 cell to inhibit *ato* expression in its neighbors. Secreted Sca is received by surrounding cells and endocytosed along with inactive Notch receptors within these cells (Chou & Chien, 2002; Li *et al.*, 2003a). Within the endosomes Sca somehow activates Notch through direct interaction, perhaps by protecting Notch from endocytic degradation (Powell *et al.*, 2001; Li *et al.*, 2003a). It would be interesting to determine whether endocytic targeting is affected and/or whether interactions with the E3 ubiquitin ligase Deltex are relevant to Notch endocytic sorting and activation in this context.

Initially, DER was proposed to play a role in the specification of R8, because the expression of the hypermorphic *DER* allele *Ellipse* (*Elp*) leads to changes in ommatidial pre-cluster formation and loss of R8 differentiation (Baker & Rubin, 1989). In order to determine DER's requirement in R8 specification, mitotic recombination was induced to generate and compare *DER null* mutant clones with wildtype control clones (Xu & Rubin, 1993; Dominguez *et al.*, 1998). Since DER^{null} homozygosity only yielded very small clones, likely due to a requirement for DER in cell proliferation and/or survival, the clones were induced in a *Minute* mutant background (Dominguez *et al.*, 1998; Lesokhin *et al.*, 1999; Baonza *et al.*, 2001; Yang & Baker, 2001). In the *Minute* system, all cells, except those that are homozygous for DER^{null}, are heterozygous for a dominant growth-impeding mutation (Ferrus, 1975; Morata & Ripoll, 1975). This provides cells in the DER^{null} clone a competitive growth advantage. In this *Minute* background, R8s still develop in DER^{null} clones, suggesting that *DER* does not have a role in R8 specification (Dominguez *et al.*, 1998; Lesokhin *et al.*, 1999; Baonza *et al.*, 2001; Yang & Baker, 2001). This conclusion was further confirmed when R8 specification was demonstrated in eye discs from flies carrying the temperature-sensitive DER^{tsla} allele and reared at the restrictive temperature. Loss-of-function clones of *Ras* similarly underscored the dispensability of DER pathway signaling for R8 specification (Spencer *et al.*, 1998; Yang & Baker, 2001).

As we have mentioned above, ommatidial spacing and the focusing of Ato to a single R8 is essential for the regular cell complement and pattern in the adult eye. While the Notch pathway and Sca function are clearly needed, there is much debate concerning the requirement for DER in R8 spacing. Arguing for a role, DER^{null} and *Ras* mutant clones generated in a *Minute* background have aberrant and perturbed R8 spacing as immunohistochemically measured with Ato expression and R8-specific markers (Dominguez *et al.*, 1998; Lesokhin *et al.*, 1999; Baonza *et al.*, 2001; Yang & Baker, 2001). A subsequent study, however, raised into question the interpretation of these experiments and argued against a role for DER in R8 spacing, based on the finding that $Minute^{+}$ clones could produce a non-cell autonomous effect on Ato expression and R8 spacing, even in control clones (Rodrigues *et al.*, 2005). In contrast, the investigators who conducted the DER^{null} studies (Baonza *et al.*, 2001) have not observed such non-autonomous *Minute*-induced spacing defects in mutant clones of numerous other genes, but have observed spacing defects in non-*Minute* clones of various components of the DER pathway (A. Baonza and M. Freeman, personal communication). This suggests that the surprising finding by Rodrigues and colleagues (2005) likely reflects a problem with the particular genetic background rather than a general problem with the commonly utilized *Minute* technique (M. Freeman, personal

communication). Slightly more difficult to interpret, and further fomenting the debate, is the finding that at the restrictive temperature, DER^{tsla} mutant clones initially maintain proper R8 spacing, but later develop R8 spacing defects, a result that may indicate a role in maintenance rather than initiation of R8 spacing (Kumar *et al.*, 1998, 2003; Rodrigues *et al.*, 2005). Relevant to the interpretation of this experiment is the nature of the DER^{tsla} allele, in particular whether or not it represents a true genetic null. The DER^{tsla} allele carries an apparently critical substitution in the ligand-binding domain and has been reported to behave as a *null* at the restrictive temperature based on reduced protein stability, loss of dpMAPK, loss of phosphotyrosine signal, and loss of cell surface localization in Drosophila cultured cells (Kumar *et al.*, 1998; Rodrigues *et al.*, 2005). However, it remains formally possible that *in vivo*, DER^{tsla} retains sufficient function, perhaps via binding to the alternate ligand Keren, to promote R8 spacing. Although definitive resolution of the debate will require further experimentation, it seems clear that if DER signaling contributes to initial R8 spacing, then significantly lower levels of pathway activation are required in this context than in many others during eye development.

An alternate model regarding DER's role in R8 spacing is that fine-tuning through reduction or repression in level of DER signaling is important for proper R8 spacing. The prediction in this case is that even if DER signaling is not required for R8 spacing, overexpression of activated DER components should disrupt R8 spacing, as has in fact been seen with ectopic Ras^{V12} expression (Spencer *et al.*, 1998). Insight into the possible mechanism of DER repression derives from mutants for *echinoid* (*ed*), a gene that encodes a cell adhesion molecule. Ed is a component of adherens junctions and cooperates with DE-cadherin to mediate cell adhesion (Wei *et al.*, 2005). Loss of *ed* leads to twinning of R8s at the MF. DER signaling activities, including dpMAPK levels, are upregulated in *ed* mutants suggesting that Ed functions to repress DER signaling (Figure 13C) (Bai *et al.*, 2001; Islam *et al.*, 2003; Rawlins *et al.*, 2003b; Spencer & Cagan, 2003).

Interestingly, Ed cooperates with the Notch signaling pathway. *ed* mutants have similar phenotypes to that of Notch signaling mutants and synergize with mutants of this pathway. Together, Ed and Notch were reported to regulate the efficient development of SOPs and to antagonize DER signaling during embryonic and wing neurogenesis (Ahmed *et al.*, 2003; Chandra *et al.*, 2003; Escudero *et al.*, 2003; Islam *et al.*, 2003; Rawlins *et al.*, 2003a). One model suggests that Ed potentiates Dl-N signaling by promoting Dl endocytosis in signal-producing cells (see earlier discussion concerning Notch ligand endocytosis). Although it has not yet been observed during eye development, perhaps Notch and Ed cooperate to antagonize DER signaling during R8 cell spacing (Figure 13C).

While the role of DER in R8 founder cell formation remains uncertain, it is clear that the Ras/MAPK pathway is active at some level in the intermediate groups and in the cells immediately posterior. For example, in wild-type eye discs, dpMAPK accumulation in the intermediate groups is dependent upon signaling through DER (Kumar *et al.*, 1998; Lesokhin *et al.*, 1999; Baonza *et al.*, 2001; Yang & Baker, 2001; Kumar *et al.*, 2003; Rodrigues *et al.*, 2005). DER^{null} clones and DER^{tsla} clones lack dpMAPK in these clusters. In DER^{tsla} clones, dpMAPK re-appears two hours later, whereas in DER^{null} clones, there is no reappearance (Kumar *et al.*, 1998; Lesokhin *et al.*, 1999; Baonza *et al.*, 2001; Yang & Baker, 2001; Rodrigues *et al.*, 2005). However the functional significance of elevated dpMAPK expression in the intermediate groups has been a topic of debate.

Those arguing for a role for DER in the spacing of R8, have posited that the DER pathway plays an active role in lateral inhibition alongside the Notch pathway and Sca, based on the reduction of Ato expression and subsequent loss of photoreceptors observed in either the gain-of-function DER^{Elp} allele or upon ectopic expression of Rhomboid (Baker & Rubin, 1989; Zak & Shilo, 1992; Dokucu *et al.*, 1996; Dominguez *et al.*, 1998; Spencer *et al.*, 1998; Chen & Chien, 1999; Lesokhin *et al.*, 1999; Baonza *et al.*, 2001; Yang & Baker, 2001). The DER pathway is hypothesized to inhibit Ato and affect R8 spacing in two ways: *via* activation of the secreted DER antagonist Argos (Aos), and/or *via* the activation of the homeodomain transcription factor rough (Ro) (Saint *et al.*, 1988). Initially, it was shown that the DER^{Elp} phenotype was dependent on functional *aos* to exert its effect (Lesokhin *et al.*, 1999). Aos ectopic overexpression was also shown to lead to the loss of Ato and aberrant R8 spacing, suggesting that Aos might be a lateral inhibition signal (Spencer *et al.*, 1998). In large aos^{null} mutant clones, R8 spacing is perturbed non-cell autonomously, confirming Aos' spacing function (Yang & Baker, 2001). The DER pathway may also regulate spacing–at least indirectly–through

Ro, a homeodomain protein that represses Ato expression and R8-identity in the R2/5 equivalence group (Dokucu *et al.*, 1996). Supporting this idea, Ro expression is abolished in DER^{null} mutant clones (Dominguez *et al.*, 1998). Together these data suggest that DER may play a parallel role with Notch lateral inhibition to focus R8 to a single cell within the proneural clusters.

In contrast, the competing model argues that the DER pathway does not regulate R8 spacing (Kumar *et al.*, 1998, 2003; Rodrigues *et al.*, 2005). The major tenet of this argument is that the high expression of dpMAPK within the intermediate group does not lead to expression of downstream target genes because dpMAPK in these cells is prevented from entering the nucleus to phosphorylate target transcriptional regulators. Rather, dpMAPK is in a state of 'cytoplasmic hold' within the intermediate groups, as evidenced by immunohistological studies that localize dpMAPK primarily to the cytoplasm of these cells (Kumar *et al.*, 1998, 2003; Rodrigues *et al.*, 2005). A system of tagged MAPK proteins to monitor MAPK translocation into the nucleus suggested that cytoplasmic sequestration of dpMAPK in the intermediate group is relevant biologically (Kumar *et al.*, 2003). That is, if MAPK is forced into the nucleus in these cells, patterning is perturbed (Kumar *et al.*, 2003). Therefore, dpMAPK localization appears to be developmentally regulated, begging the question of the mechanism behind the cytoplasmic hold. A prime candidate is the importin Msk that is required for import of dpMAPK into the nucleus and whose expression levels appear to be dynamically regulated in the intermediate group region (Lorenzen *et al.*, 2001; Marenda *et al.*, 2006; Vrailas *et al.*, 2006; Vrailas & Moses, 2006). In the intermediate group, Msk is present at such low abundance that signaling from the DER/Ras pathway is blocked. Posterior to these groups, where Msk is no longer limiting, dpMAPK is escorted efficiently into the nucleus where it phosphorylates key transcriptional effectors (Vrailas *et al.*, 2006; Vrailas & Moses, 2006).

One caveat to the above model is that although dpMAPK is not permitted to enter the nucleus in the cluster, it is possible that cytoplasmic dpMAPK could phosphorylate substrates that would then transduce the signal into the nucleus. The identification of dpMAPK cytoplasmic substrates through biochemical analyses of cytplasmic extracts may offer insight into this question. Nevertheless, the debate concerning DER^{null} *Minute* experiments has left open the question whether DER functions during R8 spacing. In order to capitalize on DER^{null} allele analysis, it would be interesting to determine if a variation of the *Minute* technique that uses eye-specific overexpression of the pro-apoptotic gene *head involution defective* (*hid*) may overcome the problems outlined above. In this system, cells that carry the wild-type *DER* allele undergo programmed cell death, consequently generating a tissue that is completely null for *DER* (Stowers & Schwarz, 1999).

DER and Notch Are Required for Cell Cycle Regulation During Eye Formation

Cell proliferation is tightly regulated during the development of the eye, particularly in the region of the MF where cell division transitions from asynchrony to synchronous G1 arrest (Ready *et al.*, 1976; Wolff & Ready, 1991; Thomas *et al.*, 1994). Ahead of the MF in Zone I, cells divide asynchronously. In Zone II, adjacent to the MF, cells become increasingly synchronous with an increase in mitosis, leading to G1 arrest in the MF/Zone III (Thomas *et al.*, 1994; Heberlein & Moses, 1995). Photoreceptor specification initiates with recruitment of the R8 founder cell followed by pair-wise recruitment of R2/5 and R3/4 (Figure 14) (Ready *et al.*, 1976). In Zone IV, undifferentiated cells that lie between these five cell clusters return to the cell cycle for a synchronous and terminal cell division leading into Zone V (Thomas *et al.*, 1994). This return to mitosis is referred to as the 'second mitotic wave' (SMW; Figure 14) (Thomas *et al.*, 1994).

The Notch and DER pathways cooperate to positively regulate progression of the SMW, although they influence distinct phases of the cell cycle (Figure 14) (Firth *et al.*, 2000; Baker & Yu, 2001; Baonza & Freeman, 2005; Thomas, 2005). Clonal analysis of Notch mutant tissue indicates that mitosis and DNA synthesis is reduced, as measured by phosphohistone H3 (PH3) staining and BrdU incorporation, respectively (Baonza & Freeman, 2005). These observations suggest that Notch is required for the entry into S phase. This requirement is dependent on signaling from N's ligand *Dl* and also requires Su(H) (Firth *et al.*, 2000; Baonza & Freeman, 2005). Mechanistically, the Notch pathway regulates the G1/S transition by activating transcription of dE2F1 and Cyclin A (CycA). E2Fs comprise a family of transcriptional activators important for S phase entry,

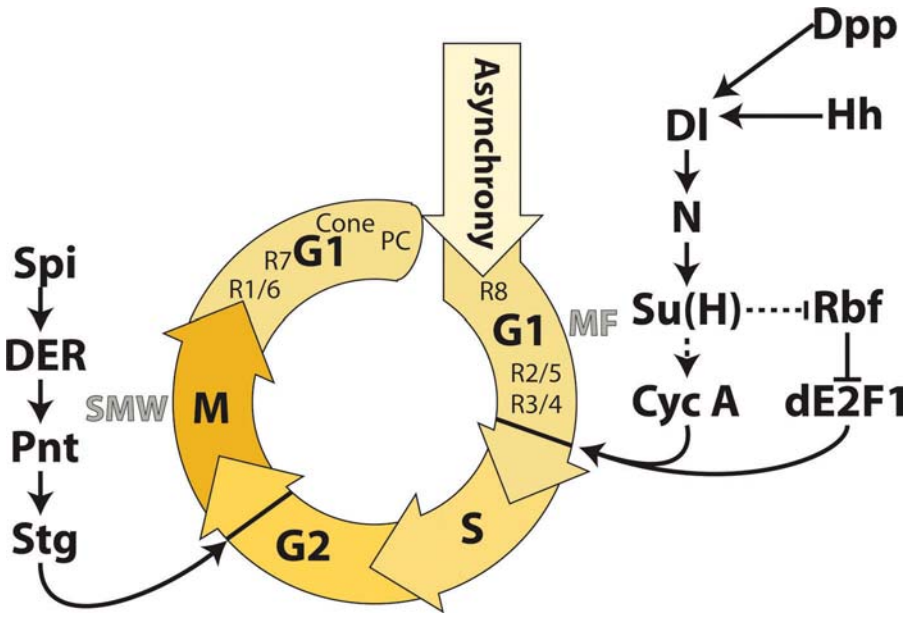

FIGURE 14 **The Second Mitotic Wave. Following recruitment of the first five photoreceptors, unspecified cells return to the cell cycle via Notch-mediated G1/S and DER-mediated G2/M transitions. Cells recruited into the ommatidium during this process are indicated within the arrows (PC = pigment cells). Adapted from Baonza and Freeman, 2005.**

while CycA, although typically involved in M phase, here plays a special role in G1/S transition (Figure 14B) (Baonza & Freeman, 2005).

The other cell division checkpoint for the progression of the SMW occurs at the G2/M transition. Genetic analyses have revealed that G2/M progression is dependent on DER activation with a requirement for the transforming growth factor-α (TGF-α) ligand Spitz (Spi) (Baker & Yu, 2001). The Cdc25 phosphatase String (Stg) is downstream of DER signaling and is required for entry into mitosis (Figure 14) (Baker & Yu, 2001). Spi and DER mutant clone tissue is defective in Zone IV mitoses, measured by phosphohistone H3 staining and accumulation of the G2-cyclin CycB (Baker, 2001). Conversely, overexpression of activated DER or Ras is sufficient to promote mitoses in Zone IV (Baker & Yu, 2001).

How are the developing photoreceptors in the five cell cluster prevented from responding to the signaling events that drive the SMW? R8, the "oldest" neuron in the cluster, is maintained in G1, independent of Notch and DER signaling (Baker, 2001; Yang & Baker, 2003), presumably because it has achieved a level of differentiation that prevents it from responding to or requiring such signals. Notch is known to be active in R2/5/3/4, but the differentiating cells do not return to the cell cycle (Baker *et al.*, 1996; Dokucu *et al.*, 1996). Therefore, there must be a mechanism that prevents R2/5/3/4 from entering S phase again. One possibility is that the DER pathway provides this function. Supporting this idea, in the absence of the DER pathway member *Son of sevenless* (*Sos*), R2/5/3/4 re-enter the cell cycle. This effect is suppressed in cells mutant for both *Sos* and *Su(H)* (Firth *et al.*, 2000). The molecular basis for DER's antagonism of Notch in maintaining G1 arrest in R2/5/3/4 remains to be elucidated.

It has been recently noted that although R2/5 and R3/4 are specified soon after R8, they do not begin to differentiate, as measured by expression of neuronal markers, until several hours later (Yang & Baker, 2006). Interestingly, the timing of this short delay, or hold on differentiation, corresponds exactly to when the unspecified cells progress through the SMW. These observations suggest that the differentiative block might 'protect' the specified photoreceptors from the pro-mitotic signaling events driving the SMW. This inhibition of differentiation is independent of cell cycle progression, but rather is dependent on the relationship of the Notch and DER pathways (Yang & Baker, 2006). DER signaling is required not only for the specification of R2/5 and R3/4 but also for their differentiation (Freeman, 1996; Yang & Baker, 2006). However, Notch signaling inhibits the differentiation potentiated by DER in this region as evidenced by the premature differentiation of R2/5 and R3/4 that occurs in N^{ts} mutants (Yang & Baker, 2006).

In summary, the Notch and DER pathways have distinct roles in the progression of the SMW. In the undifferentiated cells, the two pathways cooperate to promote the SMW, although they act sequentially at distinct points in the cell cycle, whereas in differentiating neurons, they act antagonistically to maintain G1 arrest in the photoreceptors and to block inappropriate differentiation in the cycling cells. Exactly how such context specificity is achieved remains an important area of future investigation.

Combinatorial Factors in the Determination and Differentiation of Ommatidial Cell Types

The DER pathway is required for the sequential recruitment and patterning of most cell types within an ommatidium, except for the R8 founder cell and interommatidial bristle cells (Tomlinson & Ready, 1987; Wolff & Ready, 1993; Freeman, 1996; Dominguez *et al.*, 1998; Frankfort *et al.*, 2004). Following R8 specification, DER signals repeatedly to recruit the remaining ommatidiatial cells in a stereotyped sequence: first, the remaining seven photoreceptors in a pair-wise manner–R2 and 5, R3 and R4, R1 and R6, and finally R7; second, the four cone cells; third, the primary (1°) pigment cells; and, lastly, the secondary (2°) and tertiary (3°) pigment cells (Freeman, 1997). Each round of specification relies on secretion of Spitz ligand from the differentiating cells to activate DER signaling in adjacent uncommitted cells, thereby recruiting the next cell type into the ommatidium.

The reiterative use of DER signaling to determine distinct cell fates in an ommatidium has provided an ideal context in which to study the question of specificity, namely how repeated use of the same signaling machinery can elicit distinct outcomes over time and space. For example, with respect to photoreceptors, the distinction between cell types is important for the expression of rhodopsin (rh) subclasses, which in turn is important for the visual discrimination capability of the animal. The outer photoreceptors (R2/5, R3/4, and R1/6) express rhodopsin rh1, whereas the inner photoreceptors express specific combinations of rh3, rh4, rh5, and rh6 (Cook & Desplan, 2001; Cook *et al.*, 2003). In order to meet this need for specificity, the DER pathway works in-concert with other signaling pathways, especially the Notch pathway, along with a suite of prepatterned transcriptional regulators to mediate specification of cell types in the developing disc (Freeman, 1997; Mollereau *et al.*, 2001; Voas & Rebay, 2004; Sundaram, 2005). Therefore, a combinatorial code integrates DER-mediated inputs, pre-patterned determinants, and cell-type specific differentiation factors to regulate the identity of ommatidial cell types (Freeman, 1997; Kumar & Moses, 1997; Hayashi & Saigo, 2001). The ensuing discussion will focus on the contributions of DER/Notch-specific patterning events to specific aspects of this combinatorial code. A complete review of combinatorial signaling in the eye, with consideration of the recruitment of each cell type and related patterning determinants, can be found in the following reference (Voas & Rebay, 2004).

Planar Polarity Regulates R3 *Versus* R4 Identity

Following recruitment of photoreceptors R8, R2, and R5, secretion of the DER ligand Spi from these newly specified cells recruits the presumptive R3/4 cells into a five-cell rosette cluster (Tomlinson & Ready, 1987; Wolff & Ready, 1993; Freeman, 1994b; Freeman, 1996). Expression of the homeotic gene Spalt (Sal), the orphan nuclear-receptor Seven-up (Svp) and the Helix-turn-helix (HTH) transcription factor Pipsqueak (Psq) further defines the R3/R4 pair combinatorially with the DER pathway (Mlodzik *et al.*, 1990b; Begemann *et al.*, 1995; Kramer *et al.*, 1995; Domingos *et al.*, 2004). Below we discuss how refinement of R3/4 identity occurs through Notch-mediated mechanisms that regulate planar cell polarity (PCP) across the eye disc.

The adult photoreceptors lie in a trapezoidal configuration arranged in two chiral forms to generate mirror-image symmetry across the DV boundary, or equator (Figure 10B, C) (Tomlinson & Ready, 1987). Initially, R3 and R4 are positioned in parallel to the anterior/posterior axis of the eye disc, *i.e.*, orthogonal to the equator, with R3 positioned closer to the equator, and R4 closer to the margins of the eye disc (Figure 15A). Later during morphogenesis, the ommatidia rotate 90° to assume the trapezoidal orientation seen in adult ommatidia, such that the R3 points 'up' in the dorsal half or 'down' in the ventral half of the eye (Figure 15A) (Tomlinson & Ready, 1987; Cooper & Bray, 1999).

The Notch and DER pathways, along with the Frizzled (Fz) pathway, play distinct roles in coordinating the polarity and orientation of the ommatidia (Dominguez & de Celis, 1998; Irvine, 1999; Wu & Rao, 1999;

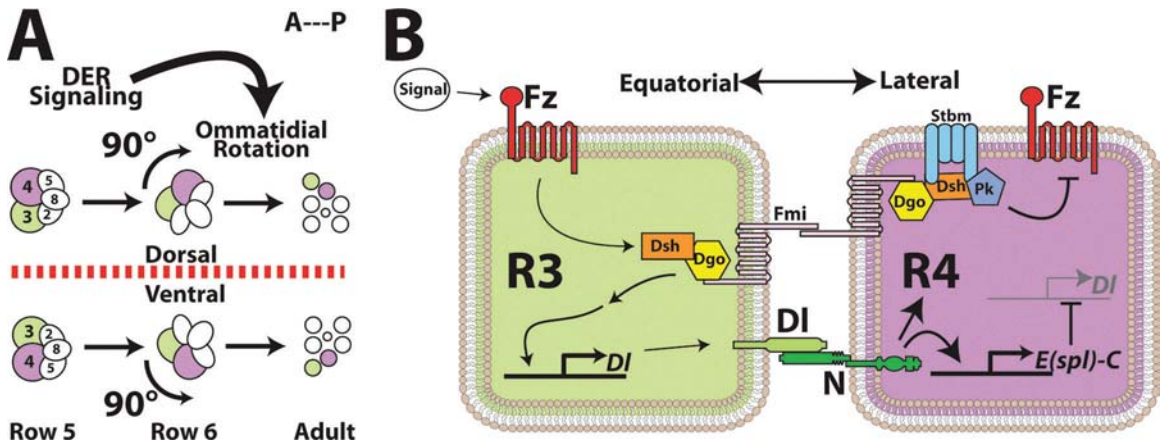

FIGURE 15 **Planar cell polarity and ommatidial rotation. (*A*) At the five-cell cluster stage, R3 and R4 begin to have distinct cell fates. DER signaling mediates ommatidial rotation with respect to the equator. (*B*) R3 receives signal from the equator bound by Fz. Dsh, bound in a complex with the Ank-repeat protein Diego (Dgo) and the atypical cadherin Flamingo (Fmi), transduces the signal to activate *Dl* expression. Dl signals from R3 to R4 to activate the Notch pathway, R4 fate, and E(spl)-C repression of *Dl*. R4 Fz-Dsh signal transduction is inhibited by a complex that includes the four-pass transmembrane protein Strabismus (Stbm) and the LIM-interaction domain protein Prickle (Pk). Adapted from Strutt and Strutt, 2003.**

McNeill, 2002; Wolff, 2003). Fz, a serpentine receptor-like transmembrane protein best known for its role in the canonical Wg/Armadillo (Arm)/β-catenin (β-Cat) signaling pathway, also controls planar organization via an alternate pathway transduced through the cytoplasmic protein Dishevelled (Dsh) (Vinson *et al.*, 1989; Zheng *et al.*, 1995; Mlodzik, 1999; Adler & Lee, 2001; McNeill, 2002; Strutt, 2003). In the eye disc prior to ommatidial rotation, the presumptive R3 cell, distinguished from R4 by its more proximal position relative to the equator, receives a signal from the equator that activates the Fz pathway (Figure 15B) (Zheng *et al.*, 1995; Fanto *et al.*, 1998; Fanto & Mlodzik, 1999). Transduction of the polarizing signal by Fz leads to activation of Dl in R3, which in turn signals to the presumptive R4. Notch activation, indicated by induction of E(spl)-mδ transcription, promotes commitment to the R4 fate (Cooper & Bray, 1999; Fanto & Mlodzik, 1999). In the absence of Notch signaling, such as in *Notch* or *Dl* mosaic mutant clones, R4 adopts the R3 fate, leading to ommatidial polarity defects.

Once Fz and Notch signaling have distinguished the R3 and R4 fates, the DER pathway facilitates the subsequent rotation that orients the ommatidia to their correct position (Figure 15A). Mutations in the DER pathway, including those in *pnt*, *Ras*, the DER ligand *spi*, and the signaling inhibitor *aos*, exhibit PCP defects (Brown & Freeman, 2003; Gaengel & Mlodzik, 2003; Strutt & Strutt, 2003; Wolff, 2003). Careful analysis of these mutants suggested that the DER pathway may be involved in two processes: during initial events that establish PCP and/or at latter stages of ommatidial rotation. One model suggests that DER pathway mutations might interfere with Fz rotational cues due to the presence of too few or many photoreceptors with decreased or increased DER signaling, respectively (Strutt, 2003). Alternatively, or in addition to involvement during the initial events that establish PCP, the DER pathway may work at later steps to regulate DE-cadherin and myosin II during the rotation process (Brown & Freeman, 2003; Gaengel & Mlodzik, 2003). Thus, the Notch and DER pathways act sequentially in a tightly orchestrated temporal sequence of events to establish ommatidial PCP whereby Notch regulates R3/4 cell fate decisions and DER regulates ommatidial rotation.

R7 Develops Through Orchestrated Signaling by DER, Sev, and Notch

The determination of the final photoreceptor recruited to the ommatidium, R7, has been well characterized and provides one of the best understood examples of how integration of signals from multiple pathways results in specific cellular outcomes (Nagaraj & Banerjee, 2004). Historically, many of the initial discoveries that defined the components of the RTK/Ras/MAPK pathway, were identified through characterization of the signaling mechanism initiated by interaction of the membrane-bound ligand Bride-of-Sevenless (Boss), expressed on the surface of R8, with the RTK sevenless (Sev), found on R7 (Figure 16) (Dickson & Hafen, 1993). The subject of this discussion will be the pioneering

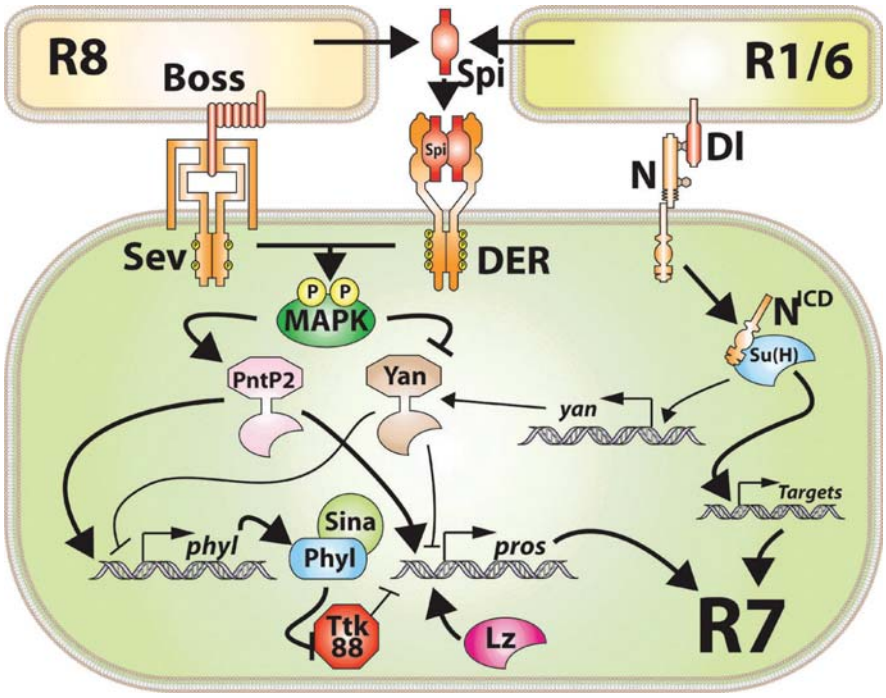

FIGURE 16 **Combinatorial signaling in R7. The Boss/Sev, Spi/DER and Dl/Notch signaling pathways promote R7 cell fate.**

insights derived from investigations into how Sev signaling is integrated with both the DER and Notch pathways to specify a unique R7 photoreceptor neuron. As we have emphasized throughout this review, an important question in developmental signal transduction is how specific cellular outcomes are achieved in response to reiterative use of common signaling pathways. Study of R7 development in the fly eye has provided one of the first definitive answers and the paradigms defined in this system are likely to be broadly relevant to other developmental contexts.

The *sev* mutation was first identified as a defect in which the R7 photoreceptor was missing and its precursor had adopted a non-neuronal cone cell fate (Tomlinson & Ready, 1986). *sev* was cloned and identified to encode an RTK that is expressed in R3/4, R1/6, and R7, but is only required for the development of R7 (Banerjee *et al.*, 1987a, 1987b; Hafen *et al.*, 1987; Tomlinson & Ready, 1987; Basler & Hafen, 1988; Simon *et al.*, 1989). The restricted requirement for Sev in R7 was revealed when the Sev ligand Boss was shown to regulate R7 development (Reinke & Zipursky, 1988). Boss is a seven-pass transmembrane ligand with a large extracellular domain that is localized specifically in R8 to restrict Boss-Sev signaling to induce R7 (Reinke & Zipursky, 1988; Hart *et al.*, 1990; Van Vactor *et al.*, 1991).

Boss binds to Sev and is trans-endocytosed by Sev into the R7 cell (Kramer *et al.*, 1991; Cagan *et al.*, 1992). Like DER, ligand-receptor interaction leads to RTK activation via trans- and auto-phosphorylation on tyrosine residues (Figure 16) (Hart *et al.*, 1990; Fortini *et al.*, 1992). The signaling machinery downstream of Sev is identical to that recruited by DER activation, and, in fact, the Ras/MAPK signaling cassette was initially characterized in Drosophila through genetic investigations of Sev-mediated control of R7 development (Simon *et al.*, 1991; Dickson *et al.*, 1992; Fortini *et al.*, 1992; Olivier *et al.*, 1993; Simon *et al.*, 1993; Biggs *et al.*, 1994; Brunner *et al.*, 1994a; Rebay & Rubin, 1995; Gebelein *et al.*, 2004).

If Sev and DER both feed into the same signaling pathway and regulate the same target genes, then why are both required for the proper determination of R7? (Brunner *et al.*, 1994b; Diaz-Benjumea & Hafen, 1994; Freeman, 1996). The answer appears to be that a significantly higher level of Ras/MAPK activation is required to overcome repressive mechanisms specific to R7 (Figure 16). Supporting this idea, overexpression of one RTK has been shown to compensate for loss of the other. For example, if DER is overexpressed in *sev* mutants, then proper R7 specification is restored (Freeman, 1996).

Elevated levels of RTK signaling appear necessary to overcome two parallel mechanisms that negatively regulate R7 determination. The first, mediated by the Ets family repressor Yan, is not unique to R7 (Figure 16).

Although different cell fate specification events in the eye all require Ras/MAPK signaling to dismantle Yan-mediated repression of differentiation, the specific target genes involved are likely to be cell-type specific. Interestingly, regulation of *yan* expression appears to be an important point of cross-talk between the DER and Notch signaling pathways in this context (Rohrbaugh *et al.*, 2002). Genetic interactions between *yan* and *Su(H)* or *Notch* provided the impetus for *in vitro* and *in vivo* studies that demonstrated a direct role for Su(H) in activating *yan* transcription in response to Notch activation (Rohrbaugh *et al.*, 2002). Therefore, Notch promotes transcription of *yan* and thereby helps to establish a specific threshold for DER responsiveness during the precisely choreographed events of ommatidial assembly (Figure 16). To allow each cell fate to be correctly specified, Ras/MAPK signaling must exceed this threshold.

The second repressive mechanism involves the Zn-finger transcriptional repressor Tramtrack88 (Ttk88) (Xiong & Montell, 1993; Lai *et al.*, 1996; Lai *et al.*, 1997). Ttk88 blocks expression of specific neural fate promoting genes in the presumptive R7, most notably the homeodomain transcription factor *prospero* (*pros*) (Figure 16) (Kauffmann *et al.*, 1996; Xu *et al.*, 2000). Expression of *pros* and other Ttk88 targets is derepressed by RTK pathway activation through expression of its target *phyllopod* (*phyl*) (Chang *et al.*, 1995; Dickson *et al.*, 1995; Lai *et al.*, 1996, 1997; Xu *et al.*, 2000). Phyl mediates degradation of Ttk88 through interaction with the R7 specific factor Seven in absentia (Sina)–a protein that interacts with a ubiquitin-conjugating enzyme for proteasome degradation (Figure 16) (Li *et al.*, 1997; Tang *et al.*, 1997). Interestingly, and relevant to the discussion below, *pros* is also directly repressed by Yan, suggesting that coordinated remodeling of transcriptional complexes in response to upstream signaling events is central to proper R7 specification (Figure 16).

The mechanisms by which the DER, Sev, and Notch pathways function in the development of R7 is one of the best understood examples of signal integration and combinatorial cell fate specification during development. The DER and Sev pathways play both direct and indirect roles in the transcriptional activation of *pros* in R7. Indirectly, as mentioned above, signaling through these RTK pathways dismantles both Yan and Ttk88-mediated repression of *pros*. Acting more directly, the RTK pathway effector Pnt activates *pros* expression (Figure 16) (Xu *et al.*, 2000). Furthermore, adjacent to the four Ets-binding sites, the Runt/AML1/CBFA1-like transcription factor Lozenge (Lz), binds to two Lz-binding sites in the same *pros* enhancer (Figure 16) (Daga *et al.*, 1996; Flores *et al.*, 1998; Xu *et al.*, 2000). Thus, a combination of transcription factors is required for the activation of *pros*.

The Notch pathway has a critical role in the development of R7 (Figure 16). Activation of Notch signaling in R7 can be followed molecularly by onset of expression of a transgene containing an enhancer-reporter transcriptional fusion element for the N/Su(H)-target E(spl)-mδ (Cooper *et al.*, 2000). The genetic requirement for Notch was demonstrated when it was observed that a hypomorphic allele N^{spl} or temperature-sensitive *Notch* (N^{ts}) mutants exhibited transformation of R7 cells to outer photoreceptor cells when placed at the restrictive temperature (Van Vactor *et al.*, 1991; Cooper *et al.*, 2000). Expression of a dominant repressor of Notch signal transduction (*sev-Su[H]enR*) in R1/6–7 similarly resulted in R7 to outer photoreceptor transformation (Tomlinson & Struhl, 2001). Conversely, targeted overexpression of activated Notch (N^{act}) in cells that include the outer photoreceptors, instructed these cells to adopt R7 characteristics (Cooper *et al.*, 2000). Notch's ability to inductively potentiate R7 development is dependent on expression of the Notch ligand Dl in R1/6 (Figure 16) (Cooper & Bray, 2000; Tomlinson & Struhl, 2001). Therefore, the DER, Sev, and Notch pathways appear to cooperate in specifying the correct cell fate of the R7 photoreceptor. Moreover, the combinatorial control by DER and Notch suggests that target genes may have enhancer/promoter elements that may be bound by transcriptional effectors of both pathways. Such a model has been formally proven in the context of cone cell development, as described below.

The DER and Notch Pathways Determine Cone Cell Fate

The lens for each ommatidium is secreted by four cone cells that rely on DER signaling from the neighboring R cells for the determination of their fate (Ready *et al.*, 1976; Tomlinson & Ready, 1987; Freeman, 1996). Analogous to the case of *prospero* expression in the R7 neuron, expression of *dPax2*, the Drosophila homolog of the vertebrate *Pax2* gene and a marker of cone cell fate, is regulated through combinatorial transcriptional control of the lz^{spa} minimal enhancer (*SME*) by Lozenge

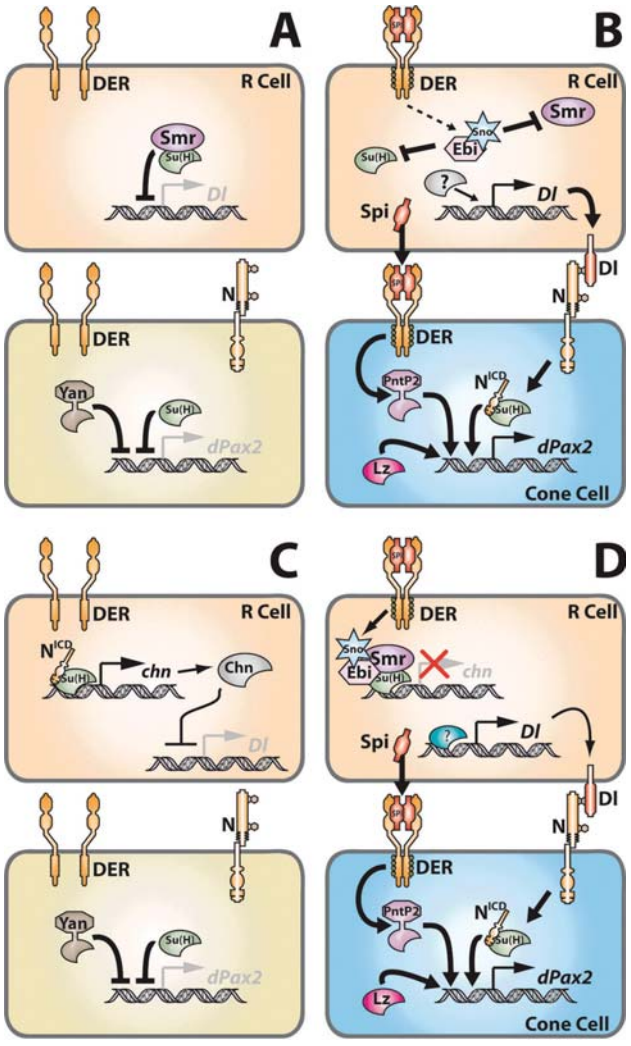

FIGURE 17 **DER and Notch inductive signaling in cone cells. In an initial model, (*A*) in unstimulated R cells, transcriptional repression of *Dl* through a Su(H)/Smr complex occurs in presumptive R cells. (*B*) DER activation in the R cell activates Ebi/Sno inhibition of the Su(H) repressor complex, permitting inductive signaling by Dl/N and Spi/DER to cone cells to activate *dPax2*. Adapted from Lai, 2002a. The model was then revised to propose that (*C*) in unstimulated R cells, repression of *Dl* occurs through Chn. (*D*) *chn* expression is repressed by a Sno/Ebi/Su(H)/Smr complex. Adapted from Tsuda *et al.*, 2006.**

(Lz), the DER effectors Yan and PntP2, and the Notch pathway transcription factor Su(H) (Figure 17) (Daga *et al.*, 1996; Flores *et al.*, 2000). Thus, insufficient DER or Notch signaling results in loss of *dPax2* expression and elimination of cone cells (Fu & Noll, 1997; Fu *et al.*, 1998; Flores *et al.*, 2000).

Cone cell development is dependent on inductive signaling through both the DER and Notch pathways. Spi and Dl are released from the photoreceptor (R) cells to activate signaling in the presumptive cone cells (Tsuda *et al.*, 2002, 2006). Inductive Notch signaling is mediated by Ebi and the novel protein Strawberry notch (Sno) (Figure 17B, D) (Tsuda *et al.*, 2002, 2006). Ebi is the homolog of transducin β-like 1 (TBL1), a conserved F-box/WD-40-repeat-containing protein that functions in co-repressor complexes but has recently been identified to convert transcriptional cofactors between repressive and stimulative states (Dong *et al.*, 1999; Guenther *et al.*, 2000; Yoon *et al.*, 2003). Ebi and Sno influence the Notch pathway by regulating in R cells the transcription of mRNA encoding the Notch ligand Dl that is received by the cone cells (Figure 17B, D) (Tsuda *et al.*, 2002, 2006).

A model has evolved for Dl regulation in R cells based on the following set of observations (Figure 17): first, Ebi, Sno, and the DER pathway positively regulate Dl expression in R cells (Tsuda *et al.*, 2002, 2006); second, the bifunctional transcription factor Su(H) switches between its role as a repressor and an activator (Tsuda *et al.*, 2002, 2006); and third, Ebi and Sno interact with a repressor complex that contains Su(H), the Drosophila SMRT homolog Smrter (Smr), and HDACs (see discussion on Notch transcriptional repression) (Tsuda *et al.*, 2002; Tsuda *et al.*, 2006).

The model initially suggested that Dl transcription was inhibited by a Su(H)/Smr complex in R cells prior to inductive signaling (Figure 17A, B) (Lai, 2002a; Tsuda *et al.*, 2002). In support of this argument, Dl protein is greatly overexpressed in *Su(H)* mutant eye clones (Tsuda *et al.*, 2002). Overexpression studies identified the DER pathway as acting in concert with Sno and Ebi to antagonize the Su(H)/Smr complex. The outcome of these interactions was proposed to derepress Su(H)/Smr mediated transcriptional inhibition of *Dl* (Tsuda *et al.*, 2002). The mechanism by which the DER pathway cooperates with Sno and Ebi–transcriptional, post-transcriptional, direct, or parallel–is unclear, but the relationship was supported further by loss-of-function mutant analysis (Tsuda *et al.*, 2002). On the other hand, the disruption of the Su(H)/Smr repressor complex was inferred from protein localization studies. In wild-type eye discs, Smr is localized predominantly to the cytoplasm, whereas in *ebi* or *sno* mutant eye discs, Smr is mostly nuclear. Thus, Ebi and Sno were proposed to promote Smr nuclear export and downregulation of Smr by the proteasome (Tsuda *et al.*, 2002). Further, the ability of investigators to co-IP Sno, Ebi, Su(H), and Smr indicated the formation of a stable disruption complex.

The above model concluded that *Dl* is regulated downstream of Su(H). However, it was unclear whether Su(H) represses *Dl* transcription directly through DNA-binding or indirectly through an intermediary. In seeking to identify how *Dl* was regulated, the model described above was revised. Specifically, new insight into the mechanistic context by which Su(H) functions to repress *Dl* led to the suggestion that in R cells prior to *Dl* expression, Su(H) functions as an *activator* of transcription in a complex with activated $Notch^{ICD}$ (Figure 17C) (Tsuda *et al.*, 2006). However $Notch^{ICD}$/Su(H) does not regulate *Dl* directly, but rather potentiates the expression of Charlatan (Chn), a homolog of the vertebrate Neuronal Restricted Silencing transcription Factor (NRSF/REST), a well characterized transcriptional repressor (Kania *et al.*, 1995; Jones & Meech, 1999). Supporting this, in *Su(H)* mutant eye discs, *chn* expression was downregulated. Further, Su(H) was biochemically shown to bind to the Chn promoter by EMSA at degenerate Su(H)-binding sites (Tsuda *et al.*, 2006). Activation by $Notch^{ICD}$/Su(H) leads to Chn's repression of *Dl* transcription through interaction with its cofactor dCoREST. Thus Dl expression is elevated in *chn* mutants and the *Dl* promoter contains two chn-binding elements (CBEs) which suggests direct transcriptional regulation (Tsuda *et al.*, 2006). This revised model of *Dl* repression is still consistent with a role of Su(H) to antagonize *Dl*, as described in the earlier model. In order for *Dl* expression to be de-repressed, N^{ICD}/Su(H)-mediated activation of *chn* must be countered (Figure 17D). DER pathway activation leads Ebi (and presumably Sno) to form a complex with Su(H) and Smr to generate a *repressor* complex that inhibits *chn* expression through binding of the *chn* promoter. The N^{ICD} complex is then swapped for the Ebi/Smr complex, resulting in repression of *chn* transcription. Binding of each of the Su(H)-containing complexes to the DNA is mutually exclusive as measured through ChIP (Tsuda *et al.*, 2006). These data are consistent with the formation of a Sno/Ebi/Su(H)/Smr complex that acts to transcriptionally repress *chn*, subsequently leading to *Dl* derepression. Interestingly, this contrasts with the earlier hypothesis that this complex was disrupted in transcriptional repression capability. That is, Su(H) is now thought to switch from a transcriptional activator to a repressor–a contrast from the previous model where Su(H) repressor activity is disrupted by Ebi/Sno. This new model, on the other hand, does not address the earlier result that Sno/Ebi disrupts Smr localization–identified initially as a marker for the interruption of the Su(H)/Smr complex. Furthermore, the investigators did not relate this earlier result to propose a biological function of Smr protein re-localization seen in wildtype compared to *ebi* or *sno* mutant eye discs (Tsuda *et al.*, 2002). A study of the genetic interactions between *smr* and *ebi/sno* may clarify these questions.

In the above evolving model, the ensuing production of Dl from the R cells activates inductive Notch signaling in the presumptive cone cells. Due to reiterative DER signaling, Spi is also secreted from the R cells to activate the DER signaling pathway in the cone cells. This potentiates PntP2 transcriptional activity and occludes Yan transcriptional repression. Activated PntP2, N^{ICD}/Su(H), and Lz all converge to induce *dPax2* expression via the *SME* enhancer element, providing a clear mechanistic explanation for how DER and Notch signaling are integrated to regulate cone cell development (Figure 17B, D) (Flores *et al.*, 2000; Lai, 2002a; Tsuda *et al.*, 2002, 2006). Moreover, the complex nature between DER and Notch signaling is underscored through their opposition in R cells and their cooperation in the adjacent cone cells.

DER, Notch, Programmed Cell Death and the Development of Pigment Cells

Following cone cell recruitment, the DER and Notch signaling pathways are integral in the development of the pigment cells. Each ommatidium contains two 1° pigment cells surrounded by a hexagonal lattice of 2° and 3° pigment cells and bristle cells that are shared between ommatidia (Ready *et al.*, 1976; Higashijima *et al.*, 1992; Wolff & Ready, 1993). Thus, each ommatidium effectively has six 2° cells, three 3° cells, and three bristles (Miller & Cagan, 1998). Following R and cone cell determination, the pigment cells and the sensory bristle cells are determined from the undifferentiated pool of cells remaining in the eye discs. As previously discussed in the case of the photoreceptors, determination of accessory cell fates also relies on sequential rounds of DER signaling, with earlier specified cell types providing the secreted Spitz ligand. Here, we will discuss a different role for DER and Notch signaling, namely how they regulate survival versus apoptosis decisions during the final stages of ommatidial patterning.

Cell death is essential during development to pattern and specify mature structures (Steller & Grether, 1994;

Rusconi *et al.*, 2000; Brachmann & Cagan, 2003; Hay *et al.*, 2004; Twomey & McCarthy, 2005). Late in pupal development, after the final pigment cells have been recruited to the ommatidia, the remaining undifferentiated interommatidial cells undergo regulated programmed cell death (PCD) (Ready *et al.*, 1976; Wolff & Ready, 1991; Hay *et al.*, 1994). Failure to eliminate these surplus cells perturbs the ommatidial lattice structure and leads to a malformed adult eye. Again, the Notch and DER pathways play pivotal and spatially restricted roles in this final event (Rusconi *et al.*, 2000; Baker, 2001).

The role of the Notch pathway in promoting cell death in this context was first suggested based on the finding that temperature-sensitive N^{ts} mutants shifted to the restrictive temperature at late stages of eye development have supernumerary 2° pigment cells, a defect resulting from loss of PCD (Cagan & Ready, 1989). In a subsequent experiment, laser ablation of 1° cells caused the opposite phenotype, namely an increase in PCD and loss of 2° and 3° cells (Miller & Cagan, 1998). These results suggested that the 1° cells provide a signal that represses PCD and/or promotes survival of cells within the 2°/3° equivalence group, or interommatidial precursor cells (IPCs) (Miller & Cagan, 1998).

The DER pathway feeds directly into this process of cell survival/death of IPCs by antagonizing PCD (Miller & Cagan, 1998). The DER pathway has been characterized as important for cell survival in many contexts throughout Drosophila development (Hay *et al.*, 1994, 1995; Kurada & White, 1998; Sawamoto *et al.*, 1998; Baker, 2001; Yu *et al.*, 2002). Mechanistically, DER activation leads to MAPK-mediated phosphorylation and downregulation of the pro-apoptotic protein Hid, thereby preventing PCD (Bergmann *et al.*, 1998; Kurada & White, 1998). Supporting such a role late in eye development, expression of constitutively-activated Ras^{V12} at the time of pigment cell formation abrogates cell death. Similarly, loss of *DER* in the IPCs leads to loss of 2°/3° pigment cells and increased death (Freeman, 1996; Miller & Cagan, 1998). Signaling by the ligand Spi is thought to be derived from the 1° and cone cells, but this hypothesis is still in need of testing (Miller & Cagan, 1998). However, genetic epistasis analysis suggests that although Spi plays a role in survival, it may not provide the specific survival signal that effectively antagonizes Notch-mediated PCD of IPCs (Yu *et al.*, 2002). Rather, Notch indirectly promotes PCD by overriding and antagonizing the downstream DER survival pathway (Yu *et al.*, 2002).

If Notch promotes PCD and DER inhibits it, how is the 'winner' determined? More specifically, how does Notch signaling override the DER survival signal in the undifferentiated IPCs but not in the specified pigment cells? The answers to such questions remain unclear. One possible answer might involve Aos, the inhibitory DER ligand (Sawamoto *et al.*, 1994, 1998). If Notch signaling activated Aos expression, this would be sufficient to dampen DER output, resulting in death.

Additional mechanistic insights may derive from analysis of mutations in other genes that alter the death/survival balance in the pupal eye, particularly with respect to how they interface with the DER and Notch pathways. One such example is *klumpfuss* (*klu*), a gene encoding a zinc finger transcription factor, whose loss leads to supernumerary 2° pigment cells (Rusconi *et al.*, 2004). Klu, normally expressed in the IPCs, is necessary and sufficient for PCD of these cells (Rusconi *et al.*, 2004). Dominant genetic interactions between *klu* mutations and hypermorphic DER^{Elp} or Ras^{V12} suggest Klu opposes the DER pathway to promote PCD in the retina. (Rusconi *et al.*, 2004). Furthermore, loss of *klu* in the retina induces greater activation of dpMAPK in the DER pathway, demonstrating molecularly the dampening of the signal (Rusconi *et al.*, 2004). It will be interesting to determine how Klu functionally inhibits the DER pathway and whether this activity is regulated by the Notch pathway.

CONCLUDING REMARKS

We have only touched upon the signaling responsibilities for DER and Notch pathway interactions during metazoan development, but it is apparent from this discussion that these two signaling networks play an integral role in modulating each other's activities. Although we have not discussed the interactions between DER and Notch outside of the eye, the common themes regarding how the two pathways are coordinated in time and space appear to be broadly conserved and directly applicable to many other developmental contexts (Diaz-Benjumea & Garcia-Bellido, 1990; Diaz-Benjumea & Hafen, 1994; zur Lage & Jarman, 1999; Jordan *et al.*, 2000; Carmena *et al.*, 2002; Chandra *et al.*, 2003; Escudero *et al.*, 2003; Islam *et al.*, 2003; Kojima, 2004; zur Lage *et al.*, 2004; Schober *et al.*, 2005;

Walters *et al.*, 2005). Furthermore, the high degree of evolutionary conservation of the EGFR and Notch signaling pathways means that the highly regulated and choreographed interactions we have described in the fly eye, are relevant to understanding how antagonism, cooperation, and coordination of EGFR and Notch contribute to vertebrate development, and by extension, how improper integration contributes to the development of human disease (Sundaram, 2005).

Future progress in elucidating further the role of these two pathways will derive both from continuing the types of experiments that have led us to our current level of understanding and from implementing novel methodologies now available in the post-genomic era. For example, genetic screens have been pivotal in identifying novel factors, co-factors, or effectors that modulate the response of DER and Notch signaling in specific developmental contexts (Diaz-Benjumea & Garcia-Bellido, 1990; Price *et al.*, 1997). These forward genetic techniques are not yet completely saturated, and are likely to continue to yield important discoveries well into the future. Continued emphasis on elucidating the biochemical and molecular nature of the DER/Notch interaction mechanisms suggested by the genetics will also remain a top priority. Simultaneously, incorporating functional genomic approaches such as global expression profiling and high-throughput RNAi screens to identify additional nodes of pathway crosstalk promises to shed new light into the intricacies underlying these two signaling pathways (Lum *et al.*, 2003; Wheeler *et al.*, 2004; DasGupta *et al.*, 2005; Jordan *et al.*, 2005; Nybakken *et al.*, 2005; Wheeler *et al.*, 2005).

ACKNOWLEDGMENTS

We apologize to those whose work was not cited. We thank Jennifer C. Jemc and Matthew Freeman for helpful comments on the manuscript and to all members of the Rebay Lab for stimulating discussions. D.B.D. was supported by a National Science Foundation Graduate Research Fellowship. This work was supported in part by National Institutes of Health grant R01 EY12549 to I.R.

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Editor: John York