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SPATIOTEMPORAL TUNING OF NOTCH SIGNALING DURING DEVELOPMENT

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DEDICATION

To my parents, Maria G. Alban Suarez and Julio J. Miranda More, for being the brightest lights illuminating my universe. Their lifelong guidance has taught me that, with passion and dedication, no star in heaven is beyond reach.

Y a mi tía Nelly Bayona, quien en vida me enseñó que, aunque el camino sea difícil, si te tomas tiempo para disfrutar las pequeñas alegrías del día a día, nada podrá impedir que llegues a la meta. Tía, con tu increíble energía y amorosa sonrisa siempre impulsaste mi carrera. Que quede escrito aquí que jamás te olvidaré.

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ABSTRACT

The highly conserved Notch signaling pathway governs a plethora of cellular processes and developmental transitions. Unlike other cell surface receptors that rely on elaborate cytoplasmic cascades to adjust pathway activity, Notch receptors transmit messages directly to the nucleus. Despite this deceptively straightforward pathway architecture, a variety of mechanisms exists to amplify or limit Notch inductive inputs, thereby ensuring precise pathway output and appropriate biological functions. This dissertation investigates three developmentally relevant mechanisms of Notch pathway regulation. One operates intracellularly, affecting both ligand-exposed and unexposed cells (Chapter 2), another relies on dynamic ligand-receptor interactions between neighboring cells (Chapter 3), and the third effectively activates the pathway in the absence of ligand induction (Chapter 4).

Using the *Drosophila melanogaster* wing and S2 cells, I first show how the Abelson kinase and the E3 ubiquitin ligases Su(dx) and Nedd4Lo cooperatively regulate the passage of Notch through late endosomes/multivesicular bodies (LEs/MVBs). Whether as part of the same pathway or as parallel inputs, these proteins divert Notch from the membrane into the lumen of LEs, a subcellular domain that restricts signaling. I discuss how this regulatory network modulates Notch activity in the developing pupal wing epithelium. Second, I uncover how a Delta-Notch feedback mechanism continuously refines noisy Notch activity to establish vein-intervein boundaries in the pupal wing. Through genetic manipulations, I demonstrate that this feedback is robust, uses multi-step sequential intercellular interactions, and can be activated anywhere in the tissue under inappropriate levels of signaling. Finally, I provide evidence that low levels of Notch signaling actively repress vein fate within intervein domains, where cells are distant from a significant ligand source. Genetic mosaic analyses confirm that intervein cells

sustain pathway activation in a Delta/Serrate-independent manner. This represents the first report of a developmental role for ligand-independent Notch signaling in non-circulating cells.

In conclusion, this work provides critical insights into how intracellular and intercellular inputs are integrated to achieve precise spatiotemporal Notch activation during wing vein patterning. The interplay between local cell-autonomous mechanisms and longer-range cell-cell interactions is essential to establishing vein/intervein tissue domains and boundaries. While local regulatory events can drive fast-scale adjustments to Notch induction within individual cells, intercellular Delta-Notch feedback interactions coordinate signaling across longer distances, refining pathway deployment and tissue patterns over time. Together, these complementary modes of regulation optimize Notch activity, ensuring the precision and robustness of its cellular and developmental functions.

CHAPTER 1

INTRODUCTION

Metazoan development exhibits remarkable reproducibility and tolerance to perturbation. The robust construction of complex organisms persists amidst challenges posed by intrinsic and extrinsic factors. On one hand, developmental processes entail intricate cellular and molecular interactions across multiple spatiotemporal scales, each capable of amplifying variabilities. On the other hand, developing organisms endure fluctuations in temperature, nutrient availability, and other environmental variables. Nevertheless, developing cells consistently produce reliably patterned and fully functional adult structures.

Precise organ formation requires meticulously coordinated cellular behaviors, which are orchestrated through cell-cell signaling events. At the molecular level, these interactions usually involve a ligand molecule expressed by a signal-sending cell and a receptor protein expressed on a signal-receiving cell (Perrimon et al., 2012). Upon binding, the receptor transduces the signal intracellularly through cytoplasmic relay factors, ultimately regulating nuclear events such as target gene transcription. Through the Heidelberg genetic screen and subsequent studies, researchers have identified the primary signaling networks that mediate intercellular communication during development (Nüsslein-Volhard & Wieschaus, 1980; Nüsslein-Volhard et al., 1984; Jürgens et al., 1984; Wieschaus et al., 1984; Wieschaus & Nüsslein-Volhard, 2016). These pathways include the Epidermal Growth Factor Receptor (EGFR), Bone Morphogenetic Protein (BMP), Wnt, Hedgehog and Notch. While significant knowledge now exists regarding core signaling components and their roles in specific developmental processes, much remains unknown about how each pathway is finely tuned to achieve proper biological output.

In particular, this dissertation focuses on the Notch signaling pathway, named after the eponymous transmembrane receptor. Notch is characterized by a unique pathway architecture, as it lacks a traditional cytoplasmic relay system. Instead, this receptor functions as a direct transcriptional effector upon activation. Since cytoplasmic cascades typically offer multiple regulatory nodes to adjust pathway output, Notch relies on alternative mechanisms to modulate its signaling activity. In this context, using a prominent system for developmental studies, the *Drosophila melanogaster* wing vein pattern, my PhD work has investigated two different regulatory schemes that optimize Notch activity.

In this introductory chapter, I provide a brief overview of fruit fly wing development, with a focus on the key signaling networks involved in vein patterning, and in particular Notch. Using historical context, I also delve into the biology of the Notch pathway, as well as some of its roles in development, homeostasis and disease. In addition, I review regulatory approaches currently known to fine-tune Notch activity. Despite this chapter's subdivision into sections, I present these topics in an integrated manner, reflecting the longstanding and interconnected relationship between Notch signaling and fly wing development.

1.1 Introduction to *Drosophila* wing development

Drosophila's life cycle

For over a century, the fruit fly *Drosophila melanogaster*, a small insect measuring approximately 3mm in length and 2mm in width, has been extensively employed as a model organism for biological research. *Drosophila* undergoes holometabolous development, involving

complex metamorphosis through four distinct stages: embryo, larva, pupa, and adult (Allocca et al., 2018) (Fig. 1.1). Following fertilization and zygote formation, embryonic development occurs within the egg membrane. The embryo gives rise to a larva, which feeds and grows through 3 instar stages until it undergoes metamorphosis into a pupa. Finally, the pupa matures into an adult, also known as an imago.

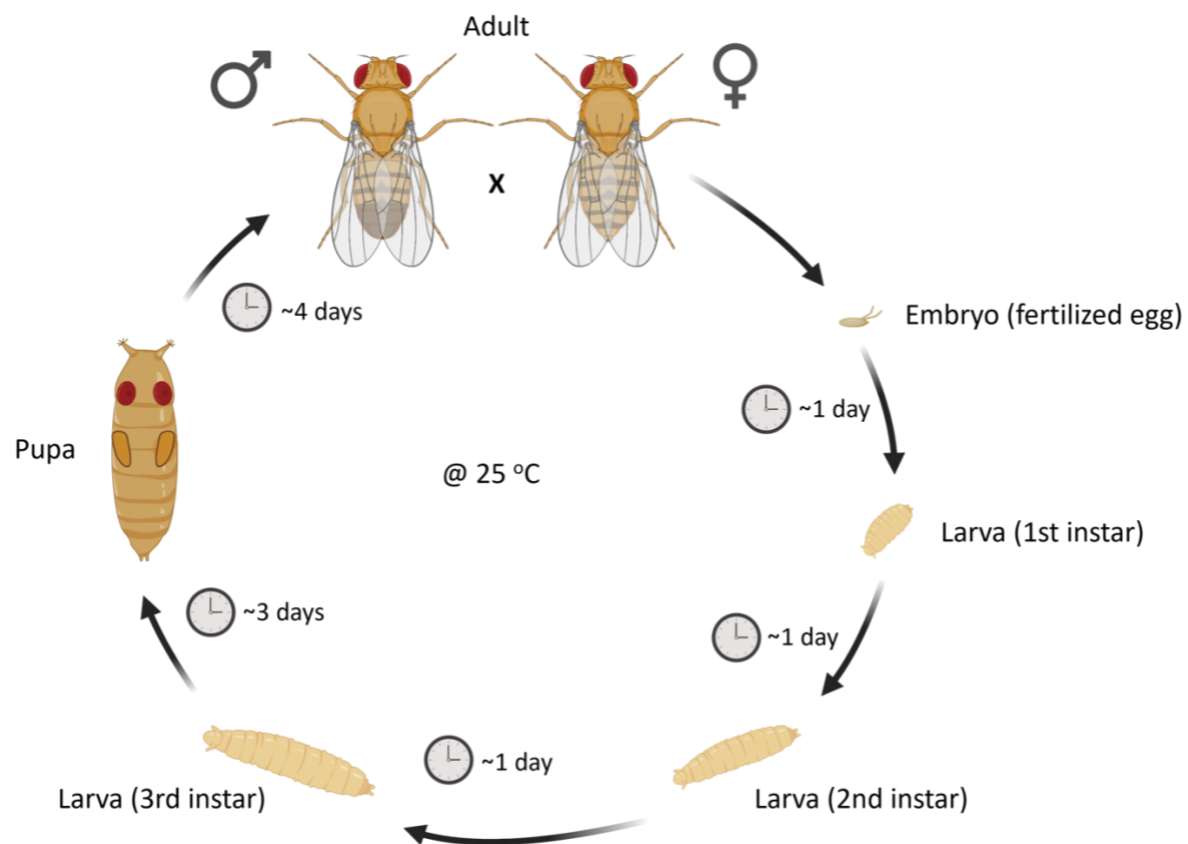


Figure 1.1. The *Drosophila melanogaster*'s life cycle.

The schematic shows the approximate duration of the embryonic, larval instar (first, second and third) and pupal stages at 25°C. Adult flies eclose after 10-12 days of development. Made with BioRender.

Embryonic origin of the wings

The adult anatomy of the fruit fly consists of a central body, composed of a head, thorax and abdomen, and various appendages such as wings, legs and antennae. Appendages are outward extensions of an animal's body used for movement, feeding, or exploring their environment. In *Drosophila*, these appendages originate from imaginal discs, epithelial sac-like structures specified in the embryo that grow and are patterned during larval and pupal development, ultimately forming the imago. Over several decades, genetic and developmental studies in the fruit fly have greatly advanced our understanding of the mechanisms governing appendage formation (Blair, 1995; Ruiz-Losada et al., 2018). Notably, the signaling pathways and genetic factors identified in *Drosophila* often play analogous roles in the development of appendages in vertebrates.

The two adult wings develop from the left and right wing imaginal discs, which arise from invaginations of the lateral epidermis in the second thoracic parasegment (T2) during embryonic stages 11–13 (Bate & Martinez Arias, 1991; B. Cohen et al., 1993; Requena et al., 2017). These discs are easily identifiable within the larval body cavity and play a crucial role in the development of the wing and adjacent structures in the adult fly body (reviewed in Tripathi & Irvine, 2022) (Fig. 1.2). Detailed coverage of these structures follow later in this section.

Around embryonic stage 11, a small group of cells is specified as the thoracic imaginal disc primordia (TP) along a dorsal-ventral (D/V) stripe of Wingless (Wg) expression near the anterior-posterior (A/P) boundary of T2. TP cells are characterized by the expression of the transcription factor Distal-less (Dll) and contribute to the development of both leg and wing discs (S. M. Cohen, 1990; B. Cohen et al., 1993; Requena et al., 2017; Tripathi & Irvine, 2022) (Fig.

1.3). Around stages 12–13, wing disc primordia, also known as dorsal primordia (DP), become identifiable by the expression of the transcription factors Snail (Sna) and Vestigial (Vg) in the most dorsal portion of the TP. The rest of the TP cells constitute the leg primordia, also known as the ventral primordia (VP). Around embryonic stage 14, the T2 imaginal disc primordia undergo physical separation as the wing primordia cells migrate dorsally.

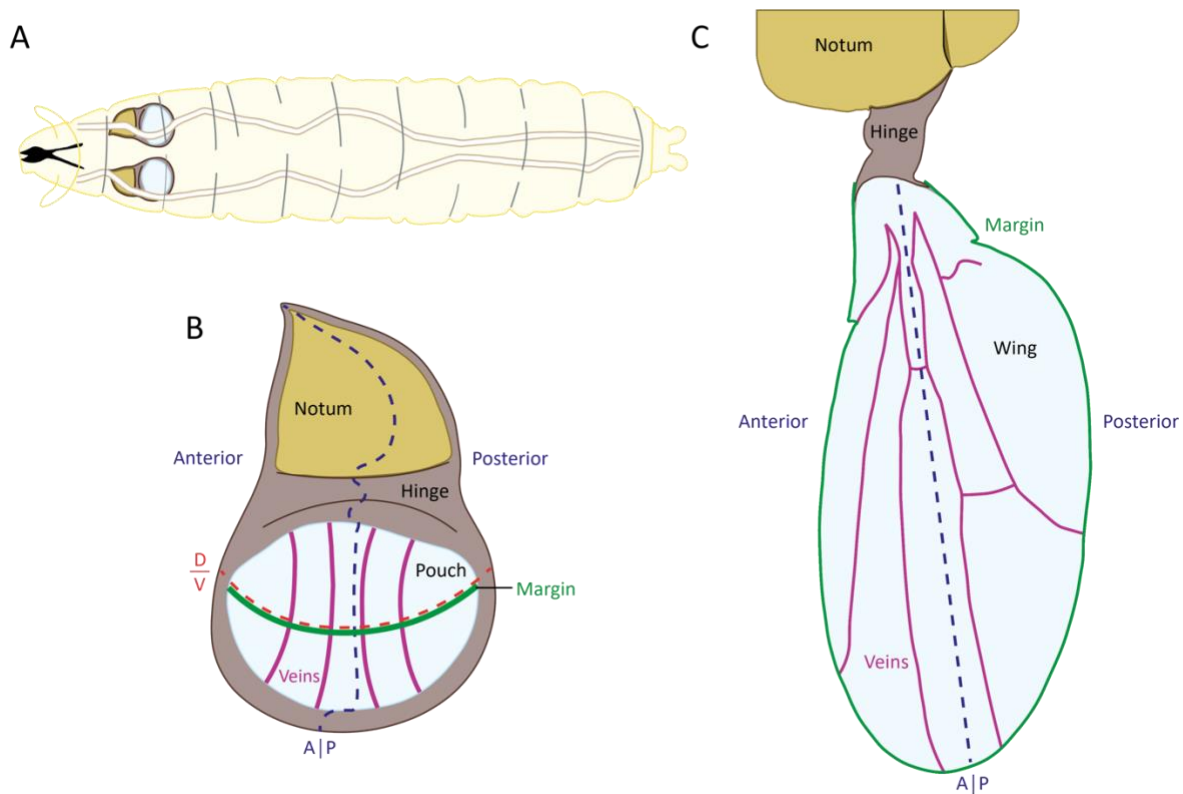


Figure 1.2. The *Drosophila* wing disc.

(A) Schematic of the third instar larva with the approximate location of the wing discs indicated. (B) Schematic of a late third instar wing disc, depicting the approximate locations of the notum (cream), hinge (brown), and pouch/wing (light blue), along with the anterior-posterior (A/P) and dorsal-ventral (D/V) boundaries (blue and orange dashed lines, respectively), precursor vein structures (purple), and margin (green). (C) Schematic of adult wing, hinge, half notum, veins and margin, marked as in (B) to illustrate relationship between wing disc and adult derivatives. Adapted from Tripathi & Irvine, 2022.

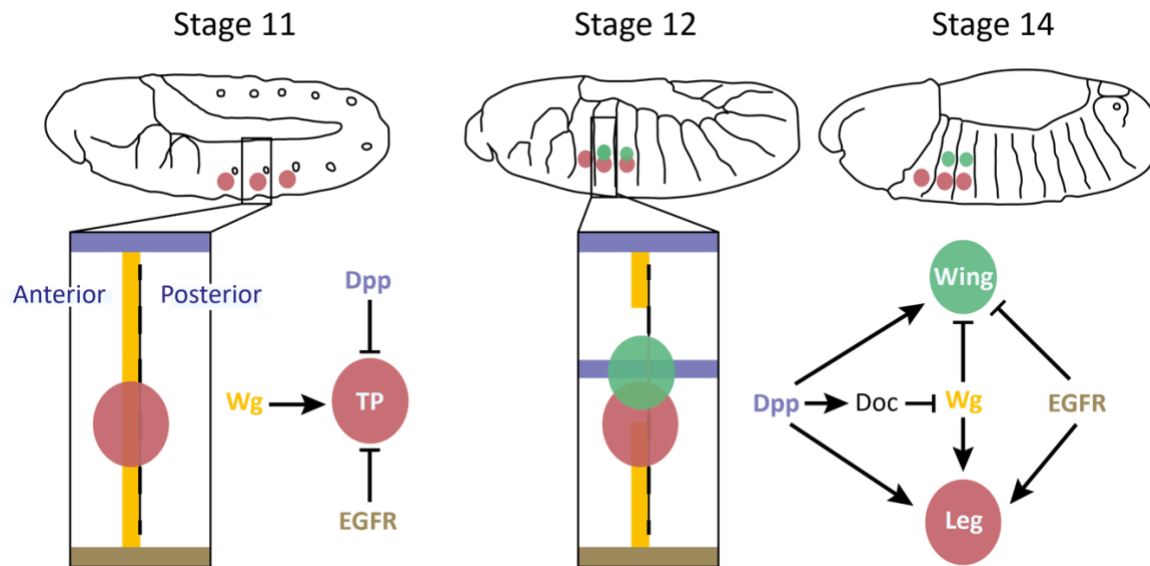


Figure 1.3. Embryonic origin of the wing disc.

The schematics illustrate the approximate embryonic locations of thoracic imaginal disc primordia (above) and the signaling inputs controlling their specification (below), based on Requena et al, 2017. At stage 11, the formation of the thoracic primordia (TP, red oval) is promoted by Wg (yellow line) signaling near the anterior-posterior (A/P) compartment boundary (dashed line) and is suppressed in more dorsal and ventral cells by Dpp and EGFR signaling, respectively. At stage 12, the wing primordia begin to form, incorporating cells from both the TP and adjacent dorsal cells (green). The Wg stripe is interrupted by Doc-mediated repression, while Dpp promotes and Wg and EGFR signaling inhibit the formation of the wing primordia. By stage 14, the wing primordia migrate dorsally, separating from the leg primordia. Adapted from Tripathi & Irvine, 2022.

The specification of the wing disc primordium is guided by key developmental factors identified both in the Heidelberg screen and subsequent studies (Wieschaus & Nüsslein-Volhard, 2016; Hrycaj & Wellik, 2016). Hox genes impart segmental identity to embryonic cells, with Antennapedia (Antp) promoting T2 segmental identity (reviewed in Rosales-Vega et al., 2024). The formation of thoracic and wing primordia is suppressed in anterior segments by Sex combs

reduced (Scr) and in posterior segments by Ultrabithorax (Ubx), abdominal A (abdA), and Abdominal B (AbdB) (Vachon et al., 1992; Carroll et al., 1995; Gebelein et al., 2002; Uhl et al., 2016; Requena et al., 2017). Within the relevant segments, the Wnt, BMP and EGFR signaling pathways dictate the positioning of the thoracic primordia along the A/P and dorsal-ventral D/V axes, while also driving their segregation into leg and wing primordia (S. M. Cohen, 1990; B. Cohen et al., 1993; Goto & Hayashi, 1997; Kubota et al., 2000, 2003; Hamaguchi et al., 2004; Requena et al., 2017). Along the A/P boundary of T2, the *Drosophila* Wnt ligand Wg is expressed, whereas expression of the BMP ligand Decapentaplegic (Dpp) and active EGFR signaling occur at the dorsal and ventral edges of the segment, respectively (Fig. 1.3).

Initially, around stage 11, Wg is crucial for inducing Distal-less (Dll) expression in TP cells (B. Cohen et al., 1993; S. M. Cohen, 1990), and the lateral positioning of the TP is regulated by repression from Dpp signaling in dorsal cells and EGFR signaling in ventral cells (Goto & Hayashi, 1997). As development progresses into stages 12-14, Dpp begins to be expressed in a lateral stripe just dorsal to the TP, promoting the fate of the wing primordium while also influencing proximal leg development to a lesser extent. Conversely, EGFR signaling from ventral cells suppresses wing primordium fate while enhancing leg fate. Dpp signaling also locally inhibits Wg expression through the upregulation of Dorsocross (Doc) transcription factors, which results in reduced Wg signaling levels that favor the specification of the wing disc over the leg disc fate (Kubota et al., 2003; Requena et al., 2017). Thus, the coordinated interplay of these positional signals directs the specification of two bilaterally symmetric clusters comprising approximately 25-30 embryonic cells as wing disc primordia (Tripathi & Irvine, 2022). During larval development, extensive proliferation increases initial cell numbers by over 1,000-fold (McClure & Schubiger, 2005; Martín et al., 2009).

Cellular and regional organization of the wing disc

The early stage of the wing disc features a flat sac composed of cuboidal epithelial cells oriented with their apical sides facing the lumen (Fig. 1.4). As the wing disc grows, cells on one side flatten to create a thin squamous epithelium known as the peripodial membrane or peripodial epithelium (PE). Cells on the opposite side, conversely, elongate apico-basally forming a columnar epithelium termed the disc proper (DP), where most of the wing disc's growth takes place. Towards the periphery of the wing disc, there is a transition zone where cells are roughly cuboidal in shape. In addition, besides epithelial cells, smaller numbers of other cell types, including neural cells, adult muscle precursors (AMPs), and tracheal cells, are also present.

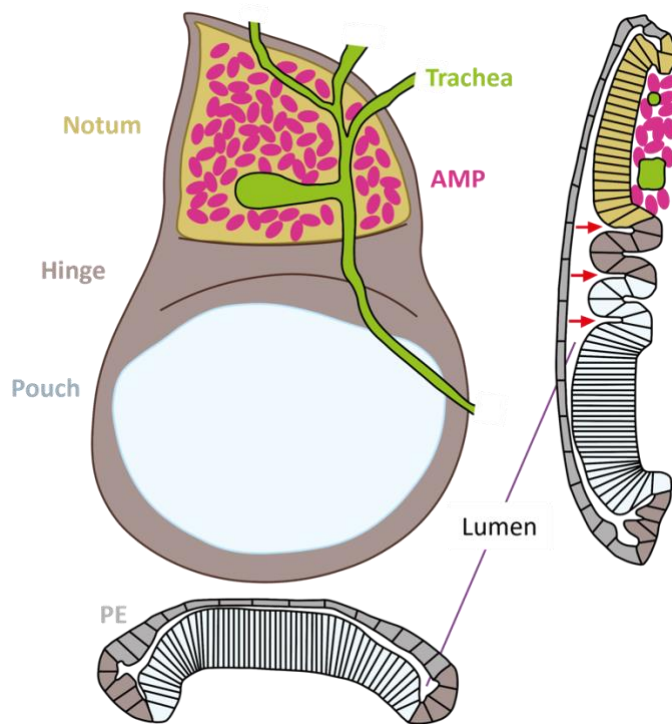


Figure 1.4. Cellular and regional organization of the wing disc.

Schematics depicting a horizontal view of the wing disc and cross-sections through the length and width of the disc illustrate the various cell types: squamous peripodial epithelium (PE) (gray), columnar cells of the wing (light blue), hinge (brown), notum (cream), as well as adult muscle precursors (AMP) (pink) and tracheal cells (green) underlying the notum. Red arrows indicate the hinge–notum fold (top), hinge–hinge fold (middle), and hinge–pouch fold (bottom). Adapted from Tripathi & Irvine, 2022.

By the end of the third larval stage, the wing disc exhibits a distinct regional organization that facilitates the differentiation of consistent adult structures (Figs. 1.2 and 1.4). The third instar wing disc is characterized by three main regions: (1) the wing pouch, an oval-shaped area responsible for forming the wing blade; (2) the wing hinge, a folded region contributing to structures at the wing base; and (3) the notal region or notum, which predominantly develops into the dorsal thoracic region of the fly. The specific fates for different regions of the wing disc are determined through a process of gradual refinement as development progresses (reviewed in detail in Tripathi & Irvine, 2022).

Signaling-dependent compartmentalization of the wing disc

Starting with an initial division of the wing disc into broad regions, further subdivisions occur as specific patterns of transcription factors, signaling molecules, and their targets become expressed. Development of the wing disc relies on its segmentation into anterior-posterior (A/P) and dorsal-ventral (D/V) compartments (Fig. 1.5), and cell communication within and between these compartments establishes signaling centers that direct subsequent wing patterning and growth (Blair, 2007; Tripathi & Irvine, 2022). Importantly, tissue compartments were first

identified in the *Drosophila* wing and have since been recognized in various invertebrate and vertebrate systems (Irvine & Rauskolb, 2001; Dahmann et al., 2011).

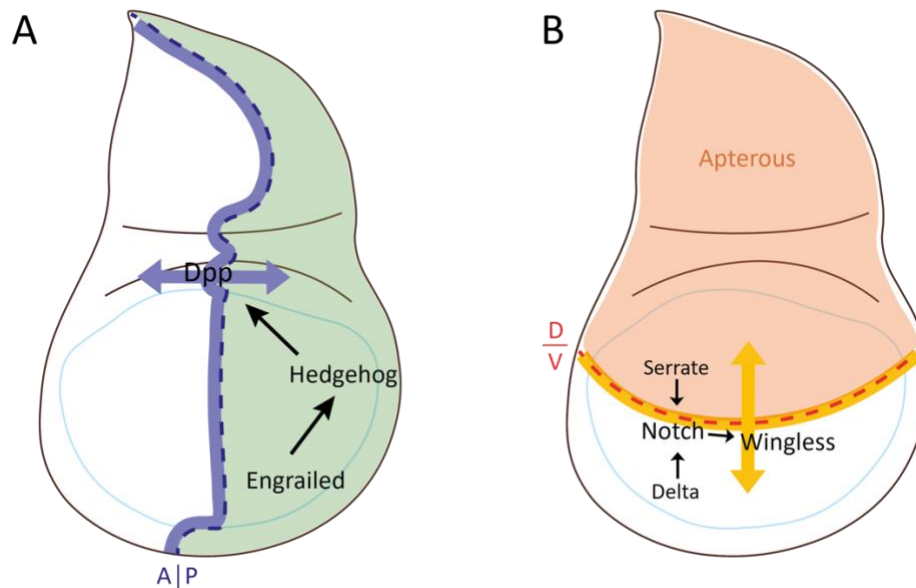


Figure 1.5. Compartmentalization of the wing disc.

(A) The anterior-posterior (A/P) compartment boundary of the wing disc (black dashed line) is established by the posterior-specific expression of Engrailed (green field). Engrailed induces the expression of Hedgehog, which diffuses anteriorly to activate the expression of Dpp in a small stripe of cells just anterior to the A/P boundary (blue). Dpp then diffuses in both directions setting up a signaling gradient that controls wing disc growth and patterning along the A/P axis. (B) The dorsal-ventral (D/V) boundary (red dashed line) is defined by the dorsal-specific expression of Apterous (orange field). At the boundary, Serrate and Delta induce Notch signaling, which in turn promotes the expression of Wingless (yellow). Wg then diffuses in both directions setting up a signaling gradient that controls wing disc growth and patterning along the D/V axis.

The subdivision of the wing into A/P compartments begins during embryogenesis, even before the formation of the disc primordia (Garcia-Bellido et al., 1973, 1976; Wieschaus & Gehring, 1976). This compartmentalization relies on the specific expression of the transcription

factor Engrailed (En) in the posterior region (Fig. 1.5A), a pattern established early in embryonic development and maintained through the rest of wing development via stable chromatin states (Breen et al., 1995; DeVido et al., 2008; T. Kornberg, 1981; Moazed & O'Farrell, 1992; Morata & Lawrence, 1975). On the other hand, D/V compartmentalization starts around the onset of the second larval instar stage and is defined by the dorsal-specific expression of the transcription factor Apterous (Ap) (B. Cohen et al., 1992; F. Diaz-Benjumea, 1993; Blair et al., 1994), driven by EGFR signaling (S.-H. Wang et al., 2000; Zecca & Struhl, 2002).

In the posterior compartment, Engrailed indirectly stimulates the expression of Hedgehog (Hh), a ligand in its own signaling pathway, while simultaneously suppressing the expression of Cubitus interruptus (Ci), a critical transcription factor in the Hh pathway (Eaton & Kornberg, 1990; Zecca et al., 1995; Méthot & Basler, 1999; Apidianakis et al., 2001; Bejarano et al., 2007; Bejarano & Milán, 2009). The complementary expression of Hh and Ci leads to activation of the Hh pathway in a stripe of cells immediately anterior to the A/P compartment boundary, with the width of this stripe reflecting the effective range over which Hh can spread. Downstream of Hh activation, expression of Dpp at the A/P boundary plays crucial roles in wing growth and patterning (Basler & Struhl, 1994; Tabata & Kornberg, 1994). In addition, and as discussed later, both Hh and Dpp signaling are major regulators in the establishment of the veins (Blair, 2007).

Signaling at the D/V boundary is orchestrated by the Notch pathway, which is activated along both sides of this boundary (Fig. 1.5B). In dorsal cells, Notch is triggered by the Delta (Dl) ligand, while in ventral cells, it responds to the Serrate (Ser) ligand (F. J. Diaz-Benjumea & Cohen, 1995; de Celis et al., 1996; Doherty et al., 1996). Notch at the D/V boundary regulates key genes such as Wingless (Wg) and Vestigial (Vg) (Kim et al., 1995, 1996; Rulifson & Blair, 1995), which are crucial for wing margin specification, growth and patterning. In fact, the D/V

boundary transforms into the margin of the adult wing, via a mechanism that involves a feedback loop between Wg and Notch (J. F. De Celis & Bray, 1997; Micchelli, et al., 1997). Additionally, during later stages of wing development, both the Notch and the Wnt signaling pathways are critical for establishing and refining veins (De Celis & Bray, 1997; Micchelli, et al., 1997).

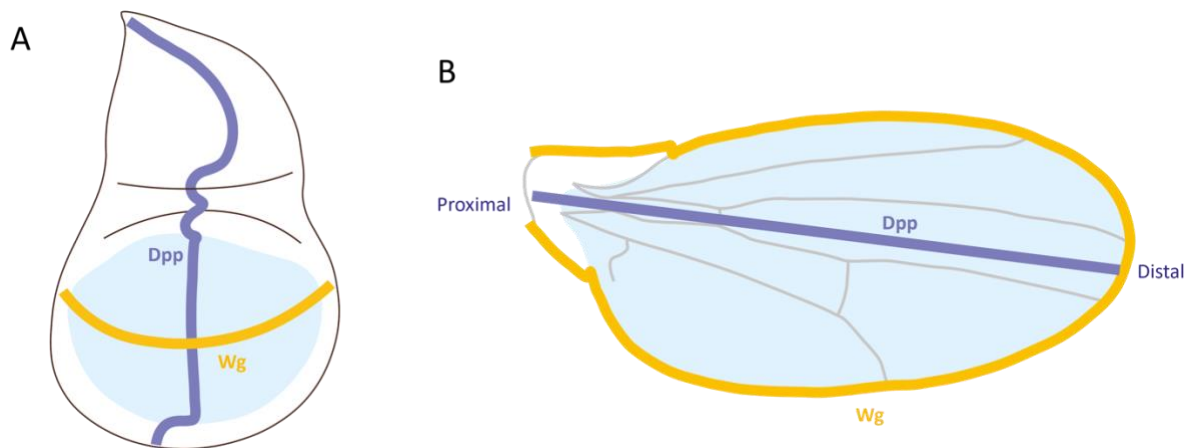


Figure 1.6. Proximal-distal patterning in the wing.

Schematics of wing structures showing the expression of Wg, and Dpp. The left diagram depicts these expressions in the wing disc, and the right diagram in the mature wing. The Wg and Dpp boundary stripes intersect in the middle of the wing pouch, which will correspond to the distal tip of the adult wing.

In addition to the A/P and D/V axes, appendages like the wing also feature a proximal-distal (P/D) axis, where cell fates are determined by their distance from the body. P/D patterning in the wing is governed by the coordinated action of Wg and Dpp, whose expression domains at the A/P and D/V boundaries, respectively, converge at the center of the wing disc pouch and extend to the distal tip of the adult wing (Fig. 1.6). Thus, Wg and Dpp collectively promote the expression of distal genes while suppressing proximal gene expression (Williams et al., 1993; Ng

et al., 1995; Kim et al., 1996; T. Klein & Arias, 1998; Azpiazu & Morata, 2000; J. Wu & Cohen, 2002; Weihe et al., 2004; Zirin & Mann, 2004). Although cells within different proximal-distal domains do not adhere strictly to lineage boundaries, they generally maintain distinct identities without intermixing (X. Liu et al., 2000; Villa-Cuesta et al., 2007).

An overview of pupal wing morphogenesis

In contrast to larval stages when the wing disc undergoes significant growth, expanding from approximately 25-30 to between 30,000 and 40,000 cells (McClure & Schubiger, 2005; Martín et al., 2009; Tripathi & Irvine, 2022), cell proliferation during pupal stages is comparatively limited, with the disc estimated to reach around 52,000 cells during this period (Garcia-Bellido & Merriam, 1971; Milán et al., 1996a, 1996b). Pupal wing development is instead best characterized by substantial morphological changes that reshape the larval wing disc into a structure resembling the adult wing (reviewed in Diaz De La Loza & Thompson, 2017) (Fig. 1.7). In this sense, while the initial formation of the veins observed in adult wings begins during third instar larval stages, the majority of vein differentiation, spatial patterning, and boundary refinement occurs during pupal development (de Celis, 2003; Blair, 2007; Antson et al., 2022).

Shortly after puparium formation, in the initial hours of pupal development, the (proper) wing disc epithelium undergoes eversion, folding over itself with the apical side of the newly juxtaposed epithelial layers oriented towards the outside (Fig. 1.7). This process also entails rupture of the overlying peripodial epithelium/membrane, resulting in only two epithelial layers whose basal sides converge in the lumen or inside (Fristrom & Fristrom, 1975; Fristrom, 1976;

Pastor-Pareja et al., 2004; Aldaz et al., 2010, 2013). Subsequently, the wing pouch expands and elongates into a large, flat wing blade by 7-8 hours after puparium formation (APF) (Fristrom & Fristrom, 1975; Fristrom, 1976; Aldaz et al., 2010, 2013). During the elongation phase, wing disc epithelial cells transition from columnar to cuboidal shapes, leading to an increase in their apical surface area (Fristrom, 1976). These cell shape changes, combined with cell intercalation, facilitate tissue elongation in a proximal-distal manner (Diaz De La Loza & Thompson, 2017).

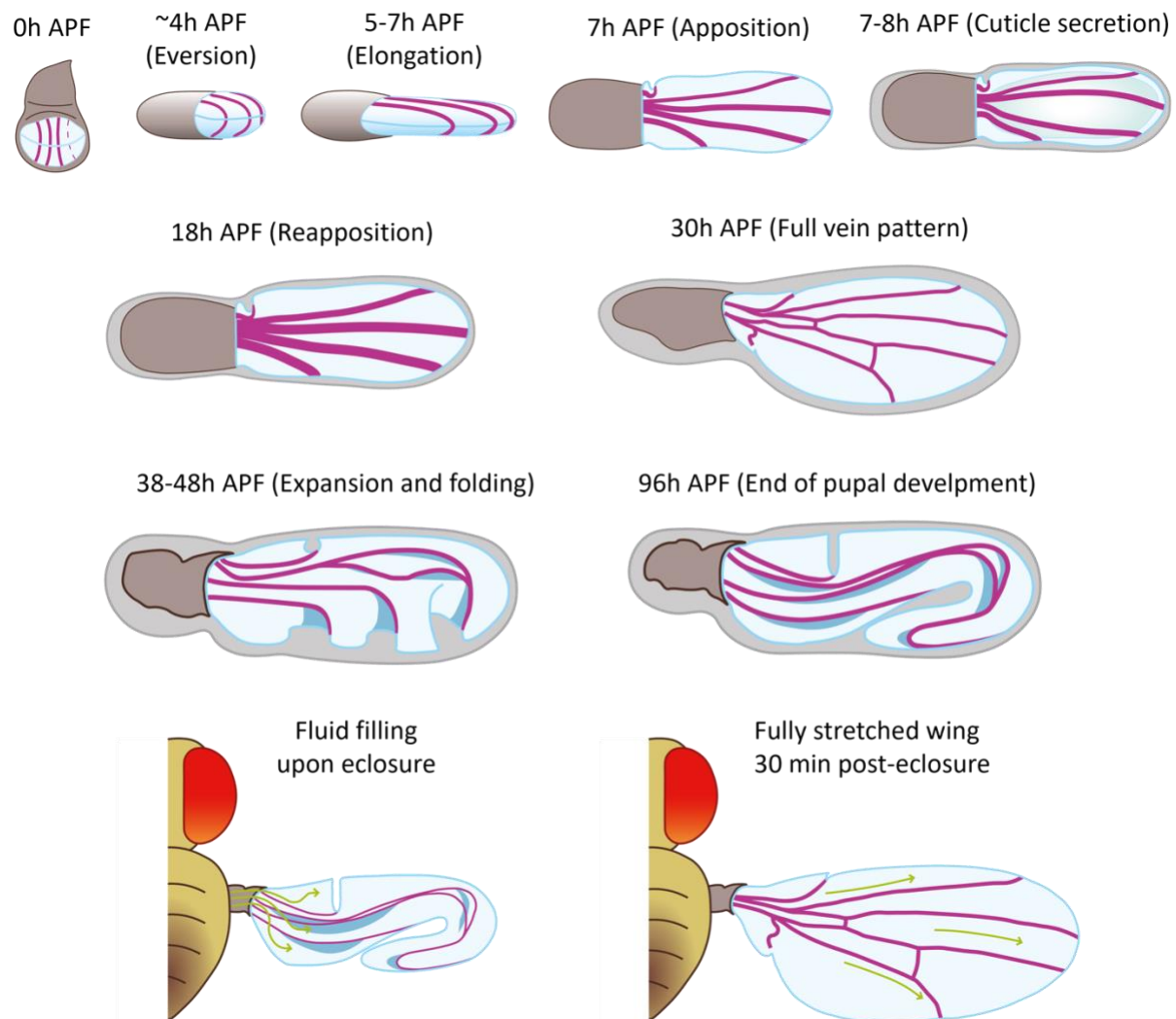


Figure 1.7. *Drosophila*'s pupal wing development.

Summary of the main events involved in pupal wing morphogenesis and the approximate timing of their occurrence. Early pupal wings undergo eversion and elongation, with its dorsal and ventral surfaces coming together by 7-8h APF. Following a period of cuticle secretion, the wing epithelial layers rejoin around 18h APF. Also this stage, the immature vein regions begin their full deployment and refinement, with the complete vein pattern being visible approximately at 30h APF. Mid and late pupal stages involve further tissue expansion and folding. Finally, shortly after adult eclosion, fluid filling stretches the wing to its final mature form. Adapted from Diaz de la Loza and Thompson, 2017.

Following eversion and elongation, the pupal wing disc (also known as the pupal wing) experiences swelling caused by fluid accumulation between its dorsal and ventral epithelial layers, accompanied by the deposition of the pupal cuticle (Waddington, 1939). The wing then returns to a flat shape as the dorsal and ventral compartments come back together along their basal surfaces around 18h APF (Fig. 1.7) (Waddington, 1939; Fristrom et al., 1993; Brown, 1993; Brown et al., 2002), although not completely in the veins, where narrow channels are maintained for fluid flow (Blair, 2007; O'Keefe et al., 2007, 2009). Indeed, besides providing structural support to the wing, the veins serve as conducts for trachea, nerves, hemocytes and hemolymph (de Celis, 2003; Blair, 2007).

Later stages of pupal wing morphogenesis feature the main vein development events (reviewed in Blair, 2007), and hinge contraction coupled to further wing blade elongation in the proximal-distal axis (Turner & Adler, 1995; Etournay et al., 2015; Ray et al., 2015), thus shaping the wing to its adult stage form by 40h APF (Waddington, 1939, 1940) (Fig. 1.7). Subsequently, the tissue folds over its posterior margin beneath a newly-secreted adult cuticle, maintaining this structure until the emergence of the adult fly (Fristrom et al., 1993). Shortly after eclosion, fluid filling through the veins facilitates wing spreading (Bilousov et al., 2012; Honegger et al., 2008;

Johnson & Milner, 1987; Loveall & Deitcher, 2010), while the death of the epithelial cells leaves behind mostly cuticle material in the adult wing (Garcia-Hughes et al., 2015; Kiger et al., 2007).

Remarks

The first section of this introductory chapter has covered the embryonic origin of the wing, the fundamental cellular and regional organization of the wing disc, the signaling interactions that establish specialized wing compartments, and the main events of pupal wing morphogenesis. This foundational knowledge outlines significant wing development milestones, establishes essential wing terminology, and introduces key molecular factors that recurrently appear throughout this dissertation. In that regard, several signaling pathways that specify wing primordia in the embryo and pattern larval wing discs are reused in subsequent processes of vein development during late third instar and pupal stages. The following section provides a detailed exploration of those events.

1.2 Signaling integration during *Drosophila* wing vein patterning

The *Drosophila* wing veins

The veins are the most conspicuous features of the *Drosophila* wing, meticulously positioned and reliably formed during its development. This biological paradigm has captivated researchers for decades, leading to the identification of numerous mutations that disrupt vein formation by deleting, adding, or altering their locations (reviewed in Blair, 2007). These studies

highlight the involvement of at least the five major signaling pathways (EGFR, BMP, Wnt, Hh, and Notch) in specifying and maintaining the precise positioning of veins and intervein regions in the wing. The study of venation not only exemplifies how these pathways integrate with each other to orchestrate patterning but also serves as a robust model for uncovering novel components within these regulatory networks.

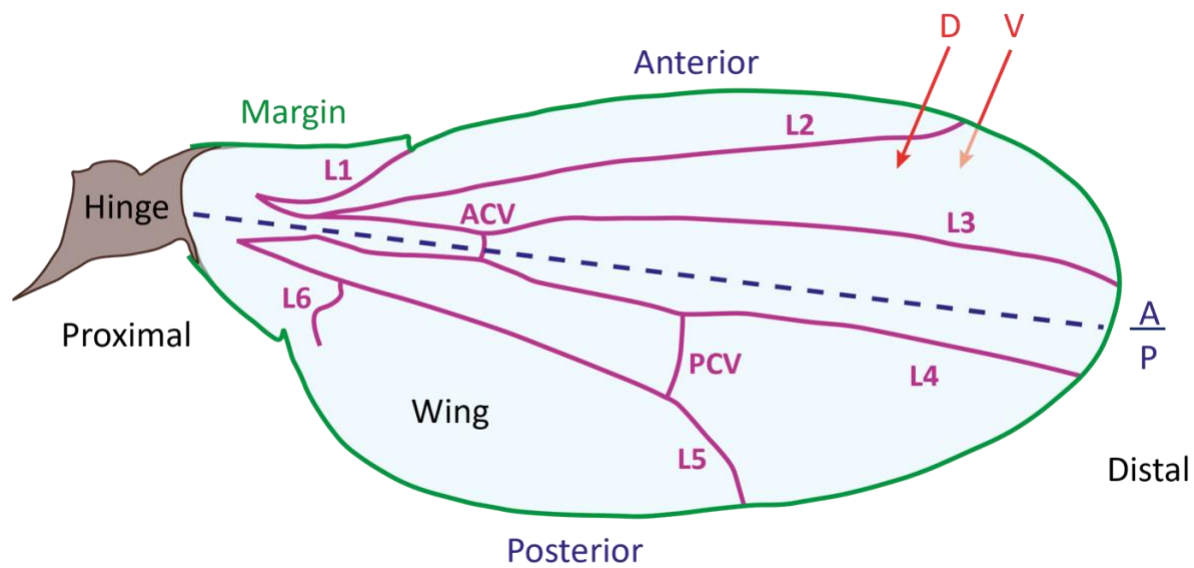


Figure 1.8. The *Drosophila* wing veins.

Schematic of an adult wing showing the approximate locations of longitudinal veins L1–L6, as well as the anterior crossvein (ACV) and posterior crossvein (PCV), all in purple. The anterior and posterior domains are highlighted in blue font, the dorsal and ventral domains are shown in orange font, and the proximal and distal regions are indicated in black font. The anterior-posterior (A/P) boundary is indicated by a blue dashed line, the hinge is marked in brown, and the margin is shown in green. While L2–L5 span the proximal-distal (P/D) axis, L1 integrates into the margin and L6 remains proximal. The ACV connects L3 and L4, while the PCV bridges L4 and L5.

The veins are linear cuticular structures that can be recognized on both surfaces on the adult wing. They are characterized by thicker, more pigmented cuticle and a denser packing of cells compared to the intervein regions located between them. Despite some discrepancies in vein nomenclature (Blair, 2007), there is a solid consensus regarding names of the main veins present in the wing blade: six longitudinal veins (L1-L6), the anterior crossvein (ACV) and the posterior crossvein (PCV) (Fig. 1.8). L1-L5 veins run proximodistally in the wing blade, with L2-L5 spanning the full length, while L1 integrates into the anterior margin at the proximal side of the wing. Vein L6 is very short, branching from the proximal region of L5 towards its posterior side. In the case of the crossveins (CVs), the ACV bridges L3 and L4 at the proximal blade, while the PCV connects L4 and L5 in the middle portion of the blade. This dissertation focuses on the most prominent veins, L2-5, ACV and PCV.

Based on classical morphological examination, the longitudinal veins (LVs) manifest in two distinct phases of wing development (Waddington, 1940; J. D. Mohler & Swedberg, 1964; Murray et al., 1984; Fristrom et al., 1993; Fristrom & Fristrom, 1993; Murray et al., 1995; Conley et al., 2000). At the beginning of pupariation, as the wing disc everts, the basal sides of the dorsal and ventral wing epithelia converge, except in the broad gaps where the LV proveins are situated. These proveins exhibit less defined thickness compared to mature LVs and their pattern is not yet complete. Around 7-8h APF, the pupal wing starts inflating, causing the dorsal and ventral surfaces to separate, while remaining connected only along their edges. The veins are not observable during this swelling period. However, between 18-30h APF, as the wing surfaces rejoin, the LV pattern re-emerges, now resembling that of the adult wing. Additionally, at this stage, the CVs are visible for the first time, with all vein structures persisting throughout the remainder of wing development.

Cell signaling in the early proveins

The formation of wing veins does not involve invasion or significant cell migration (Bryant, 1970; Garcia-Bellido et al., 1976). Instead, adjacent epithelial cells within the wing disc differentiate into either vein or intervein, a dynamic process influenced by signaling interactions established as early as larval stages. Starting from the third instar stage, EGFR signaling, likely mediated by the combined action of the ligands Spitz, Keren, and Vein (Guichard et al., 1999; Simcox et al., 1996; Reich & Shilo, 2002; Blair, 2007), becomes apparent in early proveins. This is evidenced by rolled (rl) (*Drosophila* MAPK/Erk) phosphorylation and the expression of target genes such as *rhomboid* (*rho*), *Star*, and *argos* (*aos*) (Heberlein et al., 1993; Sturtevant et al., 1993; Sawamoto et al., 1994; Sturtevant & Bier, 1995; Schweitzer et al., 1995; Golembo et al., 1996; Gabay et al., 1997; Guichard et al., 1999) (Fig . 1.9). Concurrently, provein cells downregulate the expression of the *Drosophila* Serum Response Factor (DSRF, also called Blistered (Bs)), whereas intervein cells maintain its expression (Fristrom et al., 1994; Montagne et al., 1996). EGFR signaling is crucial for suppressing DSRF expression within the wing disc, and it is both necessary and sufficient for promoting vein differentiation (Roch et al., 1998; Ralston & Blair, 2005).

The EGFR targets Rhomboid and Star are proteins involved in cleavage-processing of EGF ligands, which facilitates pathway activation (Urban et al., 2001; Tsruya et al., 2002; Reich & Shilo, 2002). This complexity complicates our understanding of how EGFR signaling localizes along the veins. Given that Rhomboid and Star expression correlates with active EGFR signaling, their specific expression in veins may both trigger and result from EGFR activation

(Blair, 2007). Further investigation is needed to elucidate the precise mechanisms of EGFR activation in provein cells and to explore potential contributions from additional pathway inputs.

In addition to the EGFR pathway, BMP signaling also governs the specification of early proveins. While predominantly observed in early L3 and L4 proveins (Fig. 1.9), gradients of BMP signaling are believed to extend to L2 and L5, directing the expression of genes involved in pre-patterning veins (J. F. De Celis, 1997; Tanimoto et al., 2000; Blair, 2007). These genes typically stimulate the expression of Rhomboid and Star, although they may also provide information independently of EGFR signaling. Importantly, during the formation of crossveins (CVs) in later pupal stages, localized BMP signaling acts instructively before the requirement for localized EGFR signaling emerges (discussed later in this section).

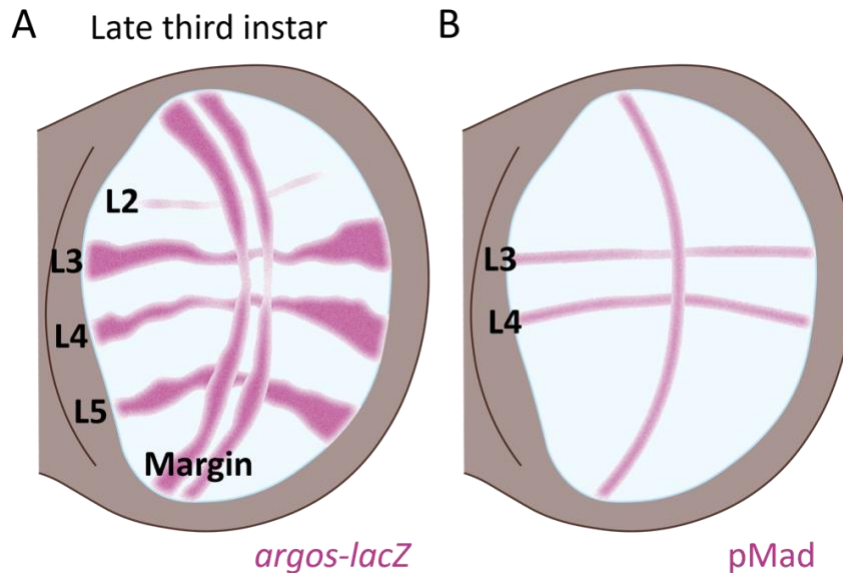


Figure 1.9. Early provein signaling in the wing. Representations of provein signals in third instar wing discs. (A) *argos-LacZ* and (B) *pMad* serve as reporters for EGFR and BMP signaling, respectively. Adapted from Blair, 2007.

Developing the longitudinal veins

While the development of LVs begins at the third instar larval stage, the majority of their growth, spatial patterning and boundary refinement takes place during the initial third of pupal development. As the early pupal wing elongates proximo-distally, and its dorsal and ventral epithelia come into apposition, LVs fully emerge and are accurately placed. These processes are controlled by the coordinated action of key signaling pathways.

The specification and positioning of longitudinal veins L3 and L4 are heavily influenced by the Hedgehog and BMP signaling pathways. As previously noted, Hh signaling is exclusively received by anterior cells, regulated by the anterior-specific expression of Ci. Consequently, anterior cells respond by expressing *Drosophila* BMP ligands such as Dpp and Glass bottom boat (Gbb) (Padgett et al., 1987; Doctor et al., 1992; Y. Chen et al., 1998; Khalsa et al., 1998). While Gbb is uniformly expressed across the wing, Dpp exhibits enriched expression along the anterior-posterior (A/P) boundary. Interestingly, their combined activity appears notably stronger where they overlap, potentially due to the formation of a Dpp-Gbb heterodimer (Ray & Wharton, 2001; Bangi & Wharton, 2006a, 2006b). This creates a gradient of BMP signaling that is particularly pronounced just anterior to the A/P boundary (Tanimoto et al., 2000).

Experimental evidence indicates that heightened Hh signaling directs the specification of intervein cells (Luis Mullor et al., 1997; Nestoras et al., 1997; Strigini & Cohen, 1997; Biehs et al., 1998). These intervein cells then emit a secondary signal to neighboring cells that receive minimal or no Hh signal, prompting them to differentiate into proveins (Phillips et al., 1990; Y. Chen & Struhl, 1996; Blair & Ralston, 1997) (Fig 1.10). This process is thought to establish the positioning of L3, the vein closest to the A/P boundary, which appears to be situated just outside

the effective range of Hh signaling. Similarly, this mechanism sets the stage for L4, which develops just posterior to the A/P boundary where cells do not detect the Hh signal. Downstream of Hh signaling, the EGF ligand Vein promotes vein identity in L3 and L4 (Simcox et al., 1996; Wessells et al., 1999), while the transcription factor Knot (also known as Collier) inhibits vein formation in the L3-L4 intervein (J. Mohler et al., 2000; Nestoras et al., 1997; Vervoort et al., 1999).

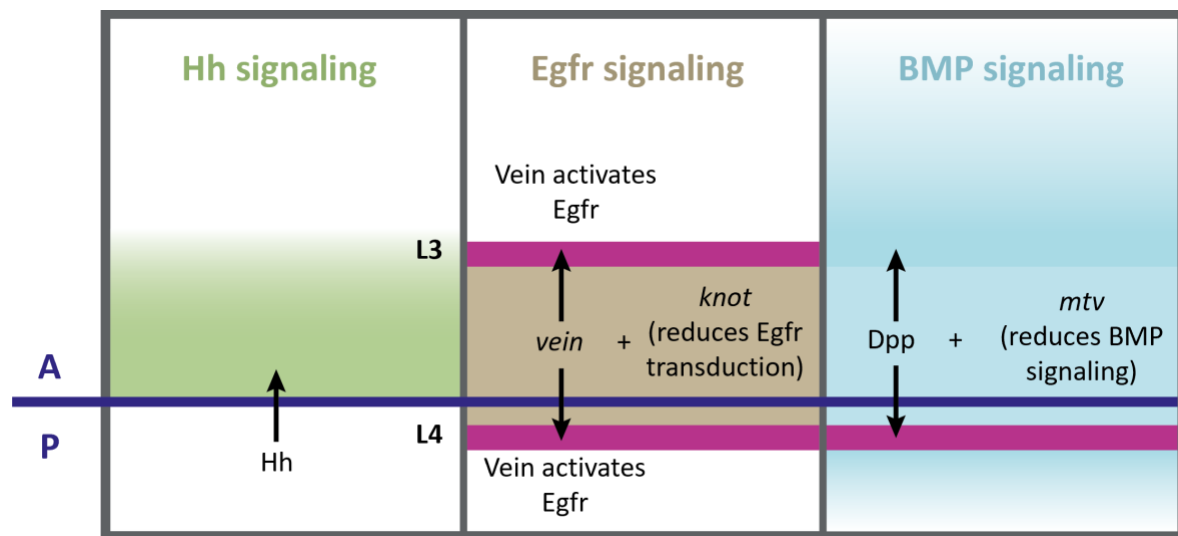


Figure 1.10. Signaling-dependent patterning of L3 and L4.

(Left Panel) Hh from the posterior side of the wing A/P boundary signals to cells on the anterior side, influencing the positioning of L3 and L4. (Center Panel) Hh signaling induces the EGFR ligand encoded by *vein* anterior to the A-P boundary but also activates the transcription factor encoded by *knot*, which inhibits EGFR reception. Consequently, Vein signals to cells with minimal Hh reception, positioning L3 anteriorly and L4 posteriorly. (Right Panel) Hh signaling induces the BMP ligand encoded by *Decapentaplegic* (*Dpp*) anterior to the A/P boundary, but also activates *master of thickveins* (*mtv*), which reduces BMP transduction. Thus, Dpp more effectively signals to cells with minimal Hh reception, promoting L3 formation anteriorly and L4 formation posteriorly. Adapted from Blair, 2007.

There is also evidence suggesting that BMP signaling, downstream of Hh and driven by Dpp and Gbb, acts redundantly with Vein (Tanimoto et al., 2000; Blair, 2007) (Fig 1.10). The effective range of BMP signaling is constrained by the Hh-dependent expression of Master of thickveins (Mtv) between L3 and L4 (Funakoshi et al., 2001). Additionally, Hh signaling may also play a direct role in determining the identity of L3 over L4 (Mullor & Guerrero, 2000). Specifically, the L3 provein receives a small amount of Hh signal, whereas L4 receives none. When Hh signaling is reduced in anterior cells, there is a loss of heightened expression of Iroquois Complex (Iro-C) transcription factors normally seen in the L3 provein during late third instar, resulting in these cells acquiring some features reminiscent of L4 in mature wings (Gómez-Skarmeta et al., 1996).

In the case of L2 and L5, they are positioned along the A/P boundary through the established gradient of Dpp signaling (Blair, 2007). Moderate levels of Dpp induce the expression of the transcription factors Spalt major (Salm) and Spalt-related (Salr) around the A/P boundary, extending just posterior to the L2 provein and just anterior to the L5 provein. This spatial expression pattern is crucial for specifying both proveins. Additionally, Dpp signaling drives the expression of Optomotor blind (Omb), which is also essential for specifying L5.

Clonal analysis experiments offer two interpretations for the mechanism for L2 specification (Fig. 1.11). One perspective suggests that cells expressing Salm do not directly differentiate into L2 but instead emit a signal that triggers L2 formation in adjacent anterior cells lacking Salm expression (Sturtevant et al., 1997; Lunde et al., 1998). Another viewpoint proposes that cells with high levels of Salm and Salr cannot form L2, whereas lower levels of expression at the anterior edge of the Salm/Salr domain specify L2 formation (J. F. De Celis & Barrio, 2000). Regardless of the specific mechanism, this leads to the localized expression of

transcription factors Knirps (Kni) and Knirps-like (Knrl) along L2, subsequently activating EGFR signaling in this vein (Lunde et al., 1998, 2003).

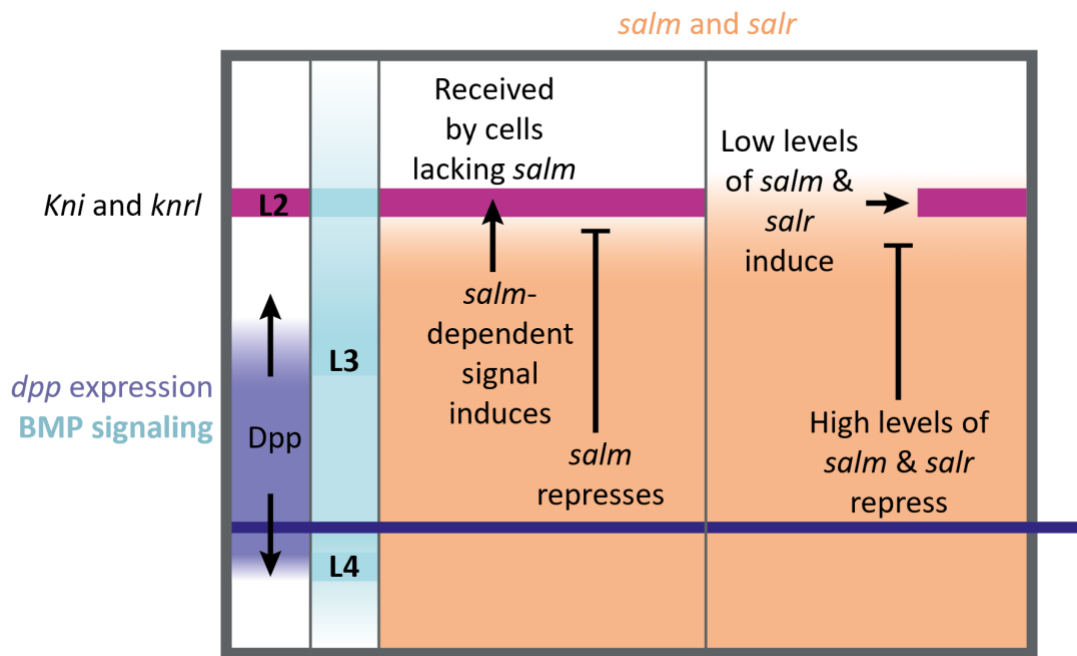


Figure 1.11. Signaling-dependent positioning of L2.

The BMP ligand Decapentaplegic (Dpp) is expressed just anterior to the wing A/P boundary, creating a gradient of BMP signaling and inducing the expression of the transcription factors encoded by *spalt major* (*salm*) and *spalt-related* (*salr*). Two models for the action of Salm and Salr are depicted: (Left Model) Cells expressing *salm* do not form L2 but produce an unknown signal that induces L2 formation in adjacent *salm*-negative cells. (Right Model) Cells with high *salm* and *salr* levels do not form L2, while cells at the anterior boundary with lower levels of these factors do. Once specified, L2 expresses *knirps* (*kni*) and *knirps-like* (*knrl*). Adapted from Blair, 2007.

At least two distinct inputs govern the specification of L5 (Fig 1.12). In the simplest case, Salm and Salr act to suppress L5 formation and define its anterior boundary (J. F. De Celis, Barrio, et al., 1996, p. 96; J. F. De Celis & Barrio, 2000). Potentially in combination, Omb

requires lower levels of Dpp signaling compared to *Salm* and *Salr*, thereby promoting L5 fate beyond the domain of *Salm* and *Salr* expression (Cook et al., 2004). In this context, the posterior boundary is determined by the absence of *Omb* expression. Additionally, akin to L3, L5 is characterized by the expression of Iroquois Complex (*Iro-C*), which is partially required for this specific vein fate (Gómez-Skarmeta et al., 1996).

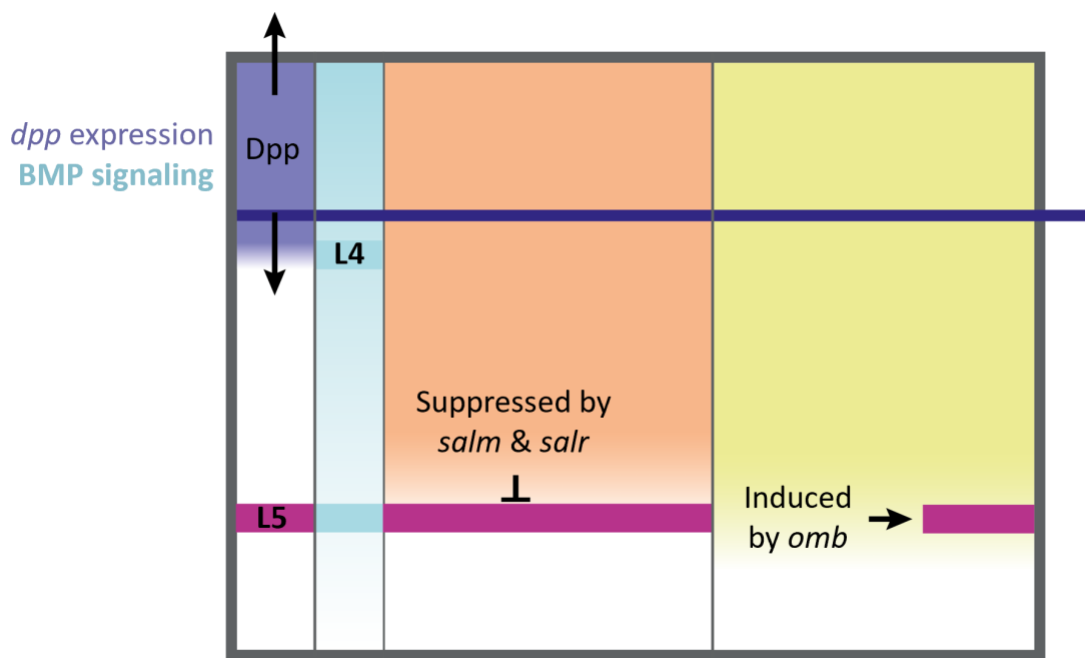


Figure 1.12. Signaling-dependent positioning of L5.

The BMP ligand Decapentaplegic (*Dpp*) is expressed just anterior to the wing A/P boundary, establishing a gradient of BMP signaling. This induces a region expressing the transcription factors encoded by *spalt major* (*salm*) and *spalt-related* (*salr*), and a slightly broader region expressing *optomotor blind* (*omb*). (Left Panel) *salm* and *salr* suppress L5 formation. (Right Panel) *omb* stimulates L5 formation. Once specified, L5 expresses high levels of transcription factors encoded by the *Iroquois complex* (*iro-C*) and *abrupt* (*ab*). Adapted from Blair, 2007.

In summary, the development of LVs involves distinct yet overlapping signaling pathways and transcriptional regulators, reflecting a nuanced interplay between localized signals and spatial gradients. Hh and BMP gradients primarily influence L3 and L4, whereas Dpp and associated factors drive the patterning of L2 and L5. The accurate arrangement of LVs is further refined by integrating vein-promoting signals with vein-repressing signals, which together define the vein-intervein boundaries (see “vein refinement” subsection).

Developing the crossveins

The positioning and development of crossveins in the *Drosophila* wing represent a distinct patterning challenge compared to longitudinal veins (Antson et al., 2022). Unlike LVs, CVs must be precisely placed along both the A/P and P/D axes of the wing. Intriguingly, CV positioning appears to be independent of marginal (distal)-promoting signals like Wingless (Wg), highlighting our limited understanding of the precise mechanisms governing CV development (unpublished data mentioned in Blair, 2007).

CVs form after the initial specification of LVs, during the second apposition of wing surfaces (Waddington, 1940). Markers characteristic of LVs are not evident in CVs until approximately 18-30h APF, with locally heightened BMP signaling observed as the earliest discernible marker between 18-22h APF (Conley et al., 2000; Yang et al., 2022). In fact, the initial development of the PCV relies on BMP signaling rather than EGFR signaling (Ralston & Blair, 2005). This exclusive reliance on BMP signals during the early stages underscores the particular sensitivity of CV development to changes or reductions in BMP pathway activity (O'Connor et al., 2006; Antson et al., 2022).

Initially absent in CVs, the key BMP ligand Dpp necessitates long-range BMP signaling for CV formation until Dpp itself becomes expressed within the CVs (reviewed in Antson et al., 2022). In the case of the PCV, BMP signaling requires both Dpp and Gbb (Conley et al., 2000; Ray & Wharton, 2001; Ralston & Blair, 2005). The transport of both Dpp and Gbb likely involves a complex containing two extracellular BMP-binding molecules: Short gastrulation (Sog) and Crossveinless (Serpe et al., 2005; Shimmi et al., 2005; Vilmos et al., 2005; Matsuda & Shimmi, 2012) (Fig 1.13). Downstream of BMP signaling, EGFR signaling induces the expression of Rhomboid and Star, thereby aligning CV signaling with that of LVs (Ralston & Blair, 2005).

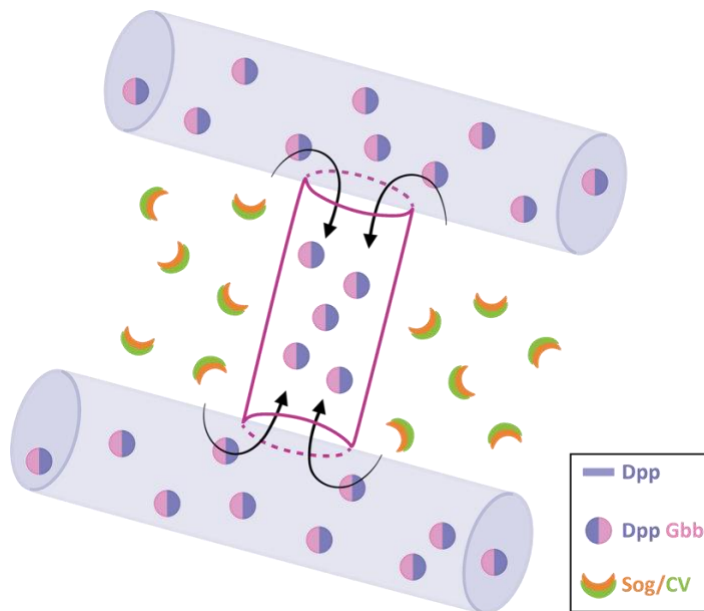


Figure 1.13. Development of the PCV.

A schematic illustrating the long-range BMP signaling going into the posterior crossvein (PCV) region to promote PCV specification. The Sog/Cv complex facilitates the trafficking of Dpp/Gbb heterodimers from longitudinal veins (LVs) into the PCV region.

Thus, while both EGFR and BMP are critical vein-promoting signals in the development of CVs, BMP signaling, particularly through long-range effects, serves as the initial key cue for CV specification, in contrast to the pioneering role of EGFR in LVs. This distinction highlights the different mechanisms governing vein formation. The following two subsections explore the processes of vein maintenance and refinement, which are crucial for both LVs and CVs, focusing on the shared mechanisms that fine-tune vein patterning and vein-intervein boundary definition.

Vein maintenance

Once proveins are specified and positioned, they must be maintained throughout wing development. In this context, EGFR and BMP signaling are integral to maintaining vein stability during this process. Mechanisms mediated by these pathways are potentially established as early as the mid-third instar larval stage, when EGFR signaling first appears in proveins, and are believed to persist into at least early pupal development.

As previously mentioned, expression of EGFR pathway components such as Rhomboid and Star occurs downstream of EGFR signaling in longitudinal veins (Heberlein et al., 1993; Sturtevant et al., 1993; Sturtevant & Bier, 1995; Guichard et al., 1999). These proteins, in turn, facilitate EGFR signaling, potentially establishing a positive feedback loop that ensures the persistence of vein-inducing signals (Blair, 2007). Moreover, while EGFR signaling precedes BMP signaling in longitudinal veins, BMP signaling is crucial for maintaining the development of at least the distal vein cells via both Dpp and Gbb (Segal & Gelbart, 1985; Posakony et al., 1990; J. F. De Celis, 1997; Ray & Wharton, 2001; Sotillos & De Celis, 2006; Bangi & Wharton,

2006a, 2006b). Therefore, early maintenance of LVs might be initially mediated by EGFR signaling, with late maintenance involving both EGFR and BMP signaling.

In contrast to the longitudinal veins, crossveins initially rely exclusively on BMP pathway activity but eventually exhibit EGFR signaling activity (Blair, 2007). Loss of EGFR activity can lead to the loss of CVs, underscoring the pathway's necessity for their maintenance (Ralston & Blair, 2005). It is believed that similar maintenance mechanisms observed in LVs may eventually be established in CVs, including both EGFR self-regulation and the EGFR-BMP feedback loop.

Vein refinement

The initial proveins start as broad and somewhat undefined regions that undergo refinement into the narrower veins observed in adults. It has been speculated that this refinement is necessary to coordinate vein development on both the dorsal and ventral surfaces of the wing. Proveins initially form independently on these surfaces of the wing imaginal disc but must eventually align to form a coherent vein pattern. Therefore, vein refinement, primarily occurring during the second and final reapposition of wing surfaces (Herszterg et al., 2023 and this study), likely facilitates the vein alignment process (Blair, 2007). Moreover, more recent suggestions propose that variability in vein placement across wings and individuals converges into a reproducible pattern through the refinement process (Alba et al., 2021; Herszterg et al., 2023).

One mechanism that contributes to refining veins involves self-regulation of the EGFR pathway. As previously discussed, Argos is induced in veins in response to EGFR signaling (Sawamoto et al., 1994; Golembo et al., 1996; Martín-Blanco et al., 1999). Argos, a secreted

protein, sequesters the Spitz ligand, preventing it from binding to the EGF receptor (EGFR) and thereby reducing EGFR pathway activity (D. E. Klein et al., 2004; Schweitzer et al., 1995).

Argos has a longer range of action compared to Spitz, allowing it to inhibit cells more distally from being activated by low levels of Spitz, a dynamic interaction termed "remote inhibition" (Freeman, 1997). Consistently, the absence of Argos leads to the formation of additional veins in adult wings (Sawamoto et al., 1994). This suggests that Argos acts as a diffusible inhibitor that, although not potent enough to completely block EGFR signaling in veins, can inhibit the lower levels of EGFR activity typically present in adjacent intervein cells (Blair, 2007).

Another vein refining mechanism involves self-regulation of the BMP pathway. Experimental evidence has revealed that reducing the activity of the BMP receptor Thickveins (Tkv) leads to excessive vein formation, including both ectopic veins and vein thickening (J. F. De Celis, 1997). Since BMP signaling promotes vein formation, two potential explanations have been proposed to reconcile this seemingly paradoxical outcome. One hypothesis suggests that Tkv-mediated signaling in cells at the vein-intervein boundary suppresses the expression of Dpp (J. F. De Celis, 1997). Therefore, reducing Tkv activity would expand the stripes of Dpp, thereby enlarging the provein domains. Another possibility is that Tkv restricts the range of Dpp signaling by binding and sequestering Dpp within the veins, similar to what has been observed in other biological contexts (Lecuit & Cohen, 1998; Tanimoto et al., 2000). In this scenario, reduced Tkv activity would allow Dpp to spread further away from the veins, resulting in the formation of broader veins.

Last but not least, the most prominent mechanism of vein refinement (at least to my knowledge) involves regulation via the Notch pathway, the main protagonist of this dissertation. Vein patterning is highly sensitive to alterations in Notch activity, with insufficient signaling

leading to excessive vein formation and too much signaling leading to vein loss (Huppert et al., 1997). At the molecular level, following EGFR signaling, provein cells express Delta, which acts as a ligand in the Notch pathway (de Celis et al., 1997). As the Notch receptor is expressed ubiquitously in the pupal wing epithelium, Delta activates Notch signaling in cells immediately flanking the proveins (de Celis et al., 1997; Huppert et al., 1997) (Fig 1.14). As detailed by de Celis et al. (1997), Notch signaling leads to the activation of Enhancer of split $m\beta$ ($E(spl)m\beta$) expression, which in turn represses the expression of Veinlet (Ve), best known as Rhomboid. Ve/rho facilitates EGFR signaling via Torpedo (Top), best known as the EGF receptor (Price et al., 1989; Sturtevant et al., 1993). Therefore, Notch signaling indirectly represses EGFR signaling, consequently inhibiting provein fate. This sets up a negative feedback loop that confines high EGFR signaling to provein cells, which in turn promotes high Notch signaling in the adjacent cells (Fig. 1.15).

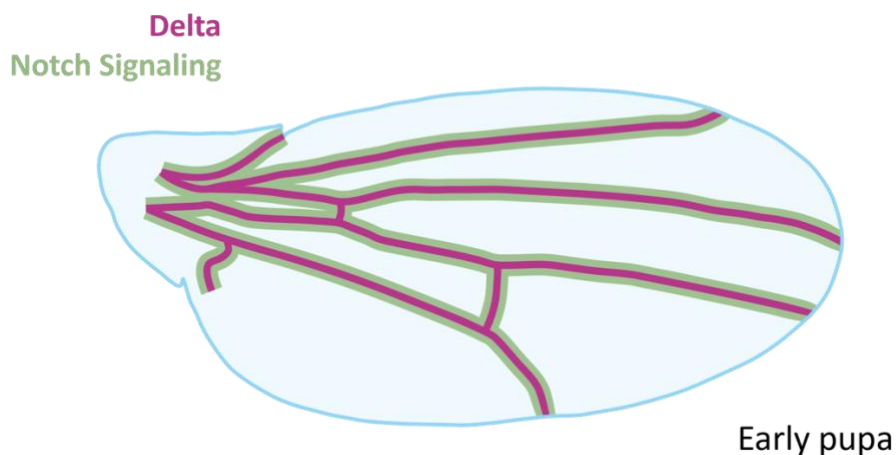


Figure 1.14. Delta-Notch signaling in the developing pupal wing. Delta (magenta), expressed in the provein cells, activates Notch signaling (green) in flanking intervein cells to regulate and prevent excessive vein formation.

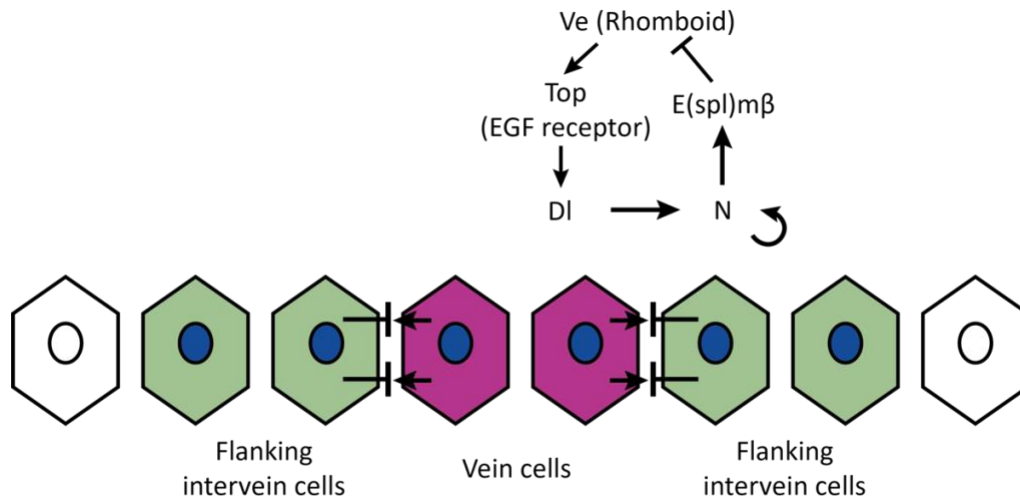


Figure 1.15. An EGFR-Notch negative feedback circuit establishes vein/intervein boundaries in the *Drosophila* wing.

Activation of Torpedo (Top, also known as the EGF receptor (EGFR)) mediated by Veinlet (Ve, also known as Rhomboid (rho)) drives expression of the Delta ligand in provein cells. This Delta activates Notch signaling in flanking intervein cells, which induces E(spl)m β expression. E(spl)m β represses Ve in these cells, thereby suppressing both vein fate and, in turn, Delta production. This feedback mechanism prevents the flanking cells from using the Delta ligand to activate Notch signaling in the provein cells. Adapted from De Celis et al, 1997.

Notably, because Delta expression is downstream of EGFR activation, the repression of EGFR signaling by Notch results in a distinct and mutually exclusive pattern of ligand expression and pathway activation. Specifically, Delta is prominently expressed within provein cells, while Notch signaling is heightened in the immediately adjacent cells. This negative feedback is further reinforced by Notch signaling's ability to regulate its own receptor expression (Huppert et al., 1997), solidifying the spatial segregation of Delta and Notch/Notch signaling. This introductory chapter later features a subsection dedicated to Delta-Notch feedback interactions, which establishes foundational knowledge require to navigate Chapter 3 of this dissertation.

1.3 History of Notch biology

Brief overview of the Notch signaling pathway

The highly evolutionarily conserved Notch pathway is a core communication mechanism used by cells to coordinate their behaviors, essential for constructing and maintaining living organisms (Bray, 2016; Gozlan & Sprinzak, 2023). Notch-mediated cell signaling orchestrates a wide array of cellular, developmental and homeostatic processes, spanning from neurogenesis to oncogenesis (Siebel & Lendahl, 2017; Engler et al., 2018; Alfred & Vaccari, 2018; Giaimo & Borggrefe, 2018; Gozlan & Sprinzak, 2023; Sachan et al., 2024). While discovered through *Drosophila* mutations over a century ago, ongoing research continues to unveil the broad impact of the Notch pathway across metazoans, highlighting its pivotal role in biological systems.

The Notch signaling pathway is named after the eponymous cell surface receptor. Notch is an integral membrane protein with a ligand-binding extracellular domain (NECD) and a signal transducing intracellular domain (NICD) (Wharton et al., 1985; Rebay et al., 1993; Struhl et al., 1993; Lieber et al., 1993) (Fig. 1.15). Canonical activation of Notch signaling involves cell surface interactions with DSL (Delta/Serrate/Lag2)-family ligands (Fig 1.16) (De Strooper et al., 1999; Brou et al., 2000; Kopan & Ilagan, 2009; Kopan, 2012; Hori et al., 2013; Kovall et al., 2017; Zhou et al., 2022). After binding, transendocytosis of the NECD-ligand complex is initiated in the signal-sending cell (covered in Yamamoto et al., 2010). This process pulls the receptor and exposes the S2 cleavage site on the NECD (Nichols et al., 2007; Meloty-Kapella et al., 2012). Subsequent cleavage at the S2 site by the proteinase ADAM in the signal-receiving cell (Brou et al., 2000) might allow transendocytosis to complete.

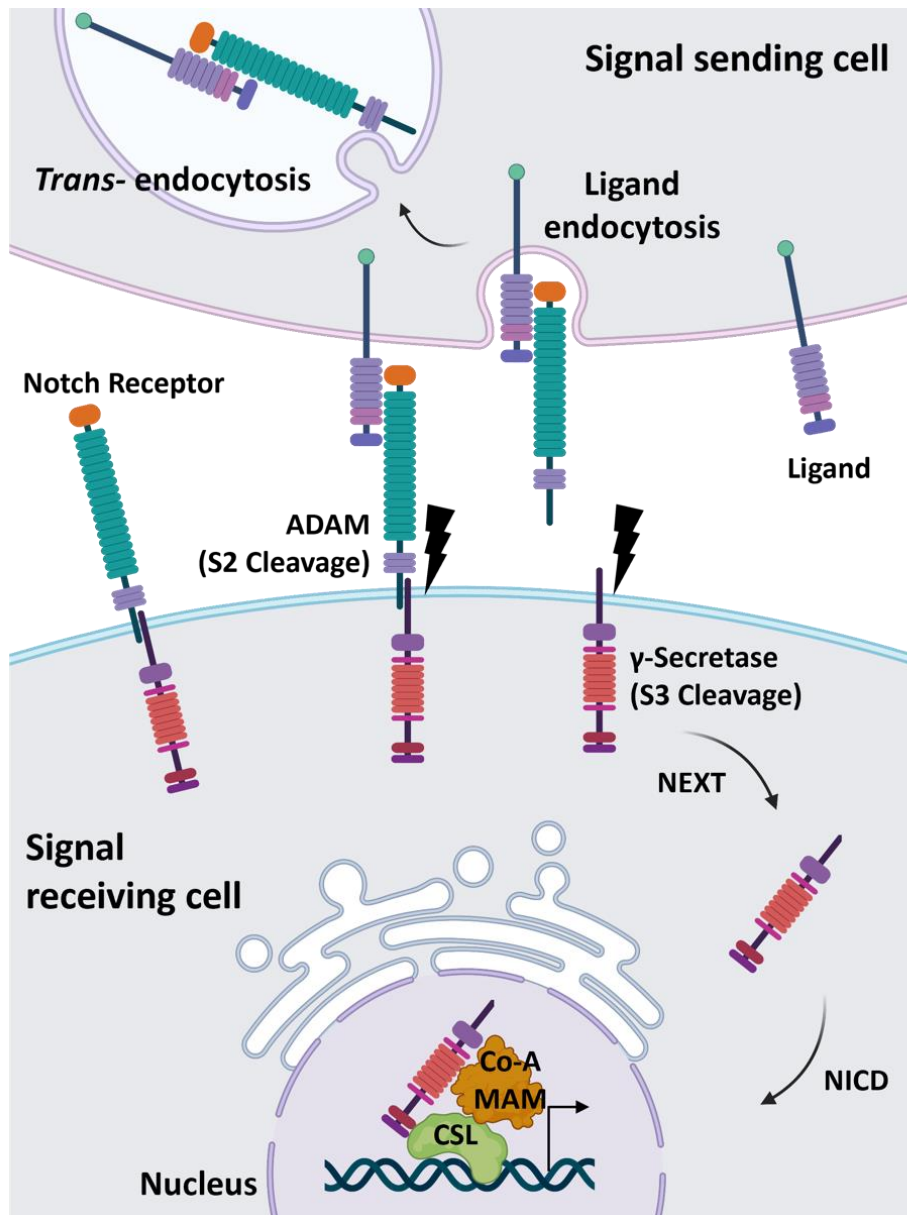


Figure 1.16. Overview of the canonical Notch signaling pathway. Receptor-ligand interaction triggers S2 cleavage within the extracellular domain (NECD), mediated by the metalloprotease ADAM. The cleaved extracellular subunit of the Notch receptor is then trans-endocytosed by the neighboring ligand-expressing cell. Subsequently, S3 cleavage occurs within the transmembrane domain of the remaining receptor structure, known as NEXT, and is mediated by the γ -secretase complex. This process releases the intracellular domain of Notch (NICD), which then translocates to the nucleus and binds to the transcription factor CSL. This binding results in transcriptional activation through the displacement of co-repressors (CoR) and the recruitment of co-activators (CoA), such as Mastermind. Adapted from Sachan et al, 2024, and made with BioRender.

Following S2 cleavage, another cleavage site is exposed which triggers cleavage and release of the NICD by γ -secretase and presenilin, deemed S3 cleavage (De Strooper et al., 1999). This process is thought to happen both at the cell surface and in endocytic vesicles (discussed in Baron, 2012). Once cleaved, activated Notch (free NICD) enters the nucleus and forms a complex with the CSL protein (CBF1/RBPJ, Su(H), Lag-1), Mastermind, and potentially other co-transcriptional partners (Bailey & Posakony, 1995; J. Wu & Cohen, 2002; Wilson & Kovall, 2006; Kopan & Ilagan, 2009). When assembled, this complex drives activation of target genes such as the *Enhancer of split* family of genes, which are downstream of Notch in many developmental contexts (Kopan & Ilagan, 2009; Kopan, 2012; Hori et al., 2013; Housden et al., 2014; Dearden, 2015; Zhou et al., 2022).

Whereas *Drosophila* expresses one Notch receptor and two ligands, termed Serrate and Delta, mammals including mice and humans express four Notch receptors, along with two Serrate homologues known as Jagged (JAG) 1 and 2, and three Delta homologues named Delta-like (DLL) 1, 3, and 4 (Fig 1.17) (reviewed in Giaimo & Borggreffe, 2018, and Zanotti & Canalis, 2020). Notch receptors are heterodimers composed of two polypeptides encoded by the same gene, which produces a protein that is cleaved during processing (Blaumueller et al., 1997). Their ECD consists of a signal peptide, a tandem array of epidermal growth factor-like (EGF-like) repeats, and three cysteine-rich repeats known as Lin-12-Notch repeats (LNR). The EGF repeats facilitate ligand binding and subsequent pathway activation, while the LNR repeats prevent premature receptor activation by metalloprotease cleavage (Bray, 2006; Fiúza & Arias, 2007; Siebel & Lendahl, 2017). The NICD comprises an RBPjk association module (RAM) domain, a nuclear localization signal (NLS), followed by seven ankyrin repeats. Additionally, the C-terminal Notch-ICD contains a proline/glutamic acid/serine/threonine-rich (PEST) sequence,

which is involved in regulating NICD turnover (Fortini, 2002; Andersson et al., 2011; Siebel & Lendahl, 2017) .

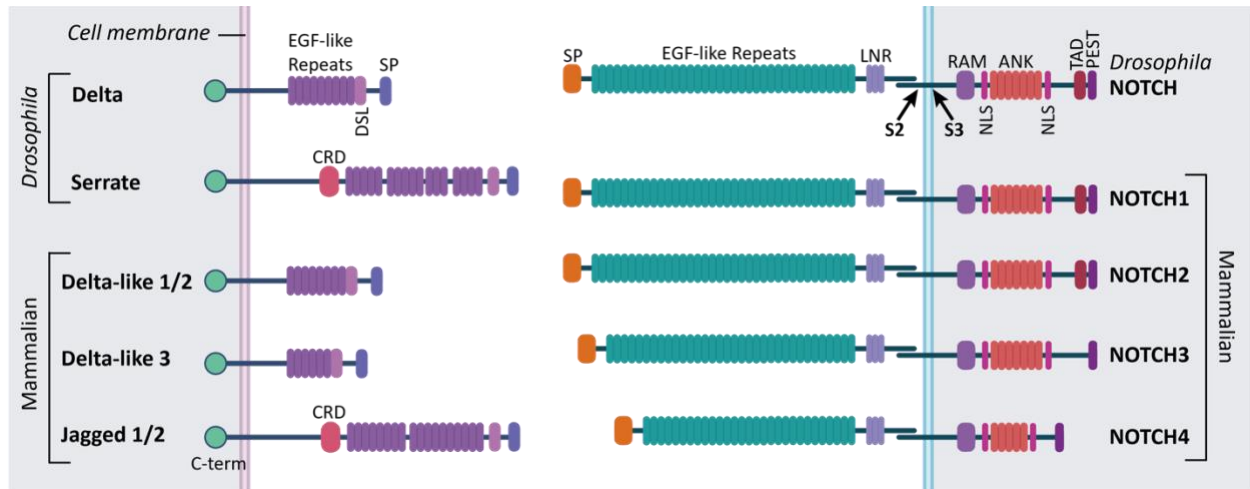


Figure 1.17. Domain organization of Notch receptors and DSL-family Notch ligands. (Left side) Schematics depict the various domains of *Drosophila* and human Notch ligands, including DSL (Delta/Serrate/Lag2), EGF-like repeats, the cysteine-rich domain (CRD), and a conserved C-terminal domain (C-term). (Right side) Schematics illustrate the domains of *Drosophila* and human Notch receptors. The organization of key domains is shown: signal peptide (SP), EGF-like repeats, Lin12/Notch (LNR) domains, RAM (Recombination signal binding protein for immunoglobulin kappa J region/CBF1 Associated Motif) domain, ankyrin (ANK) repeats, transcriptional activation domain (TAD), nuclear localization signal (NLS), and the PEST (Pro-Glu-Ser-Thr) sequence. Based on Hori, 2013; Zanotti & Canalis, 2020; Seib & Klein, 2021; and Sachan et al, 2024. Made with BioRender.

The ligands are monomers that span the cell membrane, with most of their domains located in the extracellular space. They typically include a signal peptide, a DSL domain, and an array of EGF-like repeats (Siebel & Lendahl, 2017; Zanotti & Canalis, 2020). The DSL domain of ligands binds specific EGF-like repeats on receptors, a crucial interaction for both Notch trans-activation and cis-inhibition (Nichols et al., 2007; Cordle et al., 2008; Sprinzak et al., 2010;

Del Álamo et al., 2011; Luca et al., 2015). Additionally, Serrate and its homologs feature a cysteine-rich region (CR) (Siebel & Lendahl, 2017; Zanotti & Canalis, 2020). The C-terminal intracellular portion of the ligands is required for both routine ligand endocytosis and transendocytosis of the ligand-receptor complex (Meloty-Kapella et al., 2012; Nandagopal et al., 2018; Zanotti & Canalis, 2020; Seib & Klein, 2021). For a more detailed review on the biochemistry of Notch pathway receptors and ligands, see Zanotti & Canalis, 2020; Zhou et al., 2022; and Sachan et al., 2024.

Notch in the early years: from inheritance marker to developmental regulator

In the 1910s, pioneering work established Notch as one of the earliest studied developmental signaling pathways. The oldest known "Notch phenotype," characterized by notching of the *Drosophila* wing blade, emerged as spontaneous dominant sex-linked mutations in fly stocks from multiple laboratories, drawing the attention of researchers such as Thomas Hunt Morgan, John Dexter and Calvin Bridges (T. H. Morgan, 1911; Dexter, 1914; T. H. Morgan & Bridges, 1916; Mohr, 1919). Initially, this phenotype was described as "beaded" wings (T. H. Morgan, 1911), later renamed to "notched" wings over the course of the decade, which eventually led to naming the gene responsible for these mutations as "Notch". I suppose "Beaded" did not get as many votes.

The initial work on Notch focused on using it as a paradigm to study the nature of genes, sex-linked inheritance, haploinsufficiency (hence the dominant phenotype), and recessive-lethal heredity (T. H. Morgan & Bridges, 1916; T. Morgan, 1917; Mohr, 1919). Considering the rudimentary tools at the time, the development of an understanding of genetic laws by these

scientists is remarkable. However, these early limitations might also explain why it took several decades for the next breakthroughs in the Notch research field.

It was not until the 1940s when Donald Poulson, regarded as the founding father of the Notch field in *Drosophila*, wrote the next major chapter in the history of this pathway. Poulson was the first one to describe the hallmark phenotype of homozygous *Notch* null mutant embryos, which involves an excessive production of nervous system cells at the expense of epidermal tissue (D. Poulson, 1939; D. F. Poulson, 1940) (Fig. 1.18A,B). This finding, later referred to as the “neurogenic” phenotype (Lehmann et al., 1983), is thought to have marked the start of developmental genetics as a field.

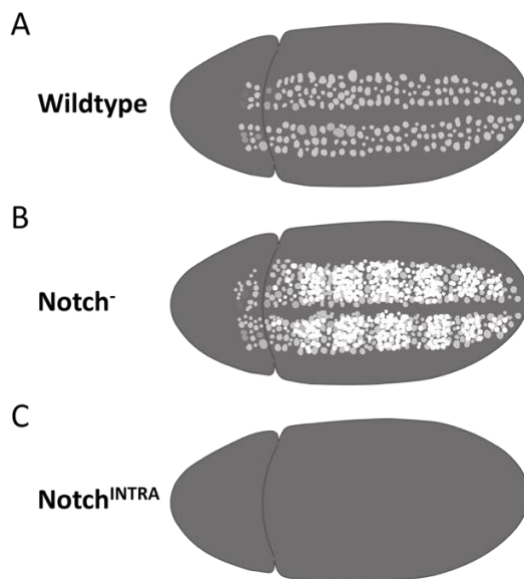


Figure 1.18. The role of Notch in *Drosophila*'s embryonic neurogenesis.

(A) A wildtype embryo displaying the normal pattern of neuroblast segregation. (B) A *Notch*^{-/-} embryo, where all ventral ectodermal cells adopt a neuroblast fate, the classic “neurogenic phenotype”. (C) An embryo expressing *Notch*^{INTRA} protein ubiquitously under heat-shock control, showing that all ventral ectodermal cells retain an ectodermal identity. Adapted from Greenwald, 2012.

Over the next few decades, researchers prioritized the genetic characteristics of Notch alleles rather than their behaviors in development. It was not until Cristiane Nüsslein-Volhard and Eric Wieschaus conducted the Heidelberg screen that the field of developmental genetics saw its major breakthrough (Nüsslein-Volhard & Wieschaus, 1980; Nüsslein-Volhard et al., 1984; Jürgens et al., 1984; Wieschaus et al., 1984; Wieschaus & Nüsslein-Volhard, 2016). Their groundbreaking work was inspired by Edward Lewis's earlier research (Lewis, 1978), for which they later shared the Nobel Prize in Physiology or Medicine, awarded for their discoveries concerning the genetic control of embryonic development. From this effort, they identified approximately 200 genes essential for fly development, including many of the genes that we now know comprise the Notch pathway.

Cell fate determination by the neurogenic signaling pathway

Shortly after the Heidelberg screen, the *C. elegans* nematode community began delving into the study of developmental genes, focusing particularly on vulval cell lineages, another classic context for studying Notch. *lin-12*, a *C. elegans* Notch, emerged from a genetic screen performed by Chip Ferguson in Bob Horvitz's lab (Ferguson & Horvitz, 1985; Ferguson et al., 1987). Subsequently, Iva Greenwald identified the key role of LIN-12 in mediating binary cell fate decisions in vulval development (I. S. Greenwald et al., 1983; I. Greenwald, 1985). In wildtype *C. elegans*, there are two equipotent cells in the hermaphrodite gonad. LIN-12 mediates their specification in anchor cell (AC) or ventral uterine precursor cell (VU) by repressing AC fate in one of the two precursors. Consistent with this, while the *lin-12(0)* null allele results in

both cells becoming ACs, the hypermorphic *lin-12(d)* allele leads to the development of two VUs.

Concurrently with the investigations in *C. elegans*, Ruth Lehmann and José Campos-Ortega, while screening the Heidelberg collection, uncovered a set of genes recapitulating the neurogenic phenotype in *Drosophila* embryos (Lehmann et al., 1983). This study identified seven allelic groups now known as the “neurogenic genes”, which included key Notch pathway members such as: *Notch* (*N*, the receptor), *Delta* (*Dl*, a key ligand), *enhancer of split* (*e(spl)*, a major Notch pathway target), *mastermind* (*mam*, a member of the transcriptional complex downstream Notch activation) and *neuralized* (*neu*, an E3 ubiquitin ligase involved in ligand processing). Additionally, two other genes whose involvement in the pathway is much less clear were also identified: *big brain* (*bib*, an aquaporin channel protein) and *almondex* (*amx*, a transmembrane protein).

While the neurogenic genes were being identified, different research groups had begun the molecular characterization of Notch, during a period when molecular biology tools were becoming available for the first time. Independently and collaboratively, scientists from both the fruit fly and nematode research communities undertook the molecular cloning of the *Notch* locus (Artavanis-Tsakonas et al., 1983; Kidd et al., 1983; I. Greenwald, 1985; Wharton et al., 1985; Yochem et al., 1988; Yochem & Greenwald, 1989). These studies identified that the *Notch* gene, now known to be conserved from flies to humans, encodes a transmembrane protein that features EGF-like repeats in its extracellular domain (Fig. 1.15). This discovery was particularly significant as it represented only the second known instance where gene sequence similarity was identified between regulators of invertebrate and vertebrate development (Notch vs. EGF) (I. Greenwald, 2012).

Since the EGF protein had been identified as a signal between cells that is generated from a transmembrane protein precursor (Gray et al., 1983; Scott et al., 1983), subsequent efforts concentrated on elucidating whether Notch could similarly function as an intercellular signal. This inquiry, in turn, raised questions regarding the autonomy of the role of Notch in determining cell fates. In this context, *C. elegans* research once again made key contributions (Seydoux & Greenwald, 1989; Wilkinson et al., 1994). Iva Greenwald's group determined that signaling between the two gonad cells, mediated by LAG-2, a LIN-12 ligand (Henderson et al., 1994), leads to the signal-sending cell becoming an AC and the signal-receiving cell adopting a VU fate. They further showed that this also involves a transcriptional feedback loop where *lin-12* expression is activated and *lag-2* expression is repressed in the signal-receiving cell. The process by which a cell that has adopted a specific fate instructs its neighbor not to adopt the same fate was termed "lateral inhibition", and is characteristic of many Notch-controlled intercellular interactions.

At around the same time, researchers achieved the molecular cloning of the fly neurogenic locus *Delta* (Vässin et al., 1987; Kopczynski et al., 1988). This gene was found to also encode a transmembrane protein with EGF-like repeats in its extracellular domain. Given that EGF-like repeats binding each other had been reported to mediate protein-protein interactions, this raised the possibility that Delta and Notch bind each other via these protein domains. This proposition gained support from experiments demonstrating that Delta-expressing cells aggregate with Notch-expressing cells in *Drosophila* S2 cell culture (Fehon et al., 1990). Furthermore, in that study, Rick Fehon and collaborators observed that Notch and Delta concentrate at contact points between these cells and that these proteins can coimmunoprecipitate, suggesting that they can directly interact *in vivo*.

While the Delta-Notch interaction was initially thought to have a passive role in mediating cell-cell adhesion, it was soon shown that Delta-Notch signaling interactions regulate binary cell fate decisions (Heitzler & Simpson, 1991). “The Simpson experiments”, as I know them, featured somatic mosaics of *Notch* and *Delta* in the *Drosophila* thoracic epidermis. During development, this tissue consists of proneural cells that face a binary fate decision: they must choose between becoming Sensory Organ Precursors (SOPs), which develop into bristles on the adult fly notum, or remaining epidermal cells. Heitzler and Simpson first found that *Notch*⁻ cells consistently adopt the SOP fate, implying an autonomous role of Notch in promoting the epidermal fate. Subsequently, they showed that *Delta*⁻ cells become SOPs only in the center of clones, away from clonal borders, suggesting this phenotype is suppressed at clone borders where *Delta*⁻ cells contact wildtype (*Delta*⁺) cells. This indicates that mutant cells that lack *Delta* but have *Notch* are kept from becoming SOPs by neighboring *Delta*-expressing cells. Therefore, they concluded that Delta acts non-autonomously to prevent the SOP fate in neighboring cells via the activity of Notch, in alignment with Delta being a ligand and Notch a receptor.

A few years later, Pascal Heitzler elucidated the existence of a transcriptional feedback loop between Notch and Delta that orchestrates SOP patterning in the fly notum (Heitzler et al., 1996). This mechanism involves transcriptional activation of *Notch* and repression of *Delta* in epidermal cells downstream of Notch activation (Fig. 1.19). Notably, this setup mirrors the previously described Delta-Notch genetic circuit that governs vein patterning in the *Drosophila* wing and vulval development in *C. elegans*. Thus, lateral inhibition involving a negative feedback loop seems to be a hallmark of several biological processes regulated by the Notch pathway. Personally, I find this genetic circuitry to be one of the most elegant in all biology.

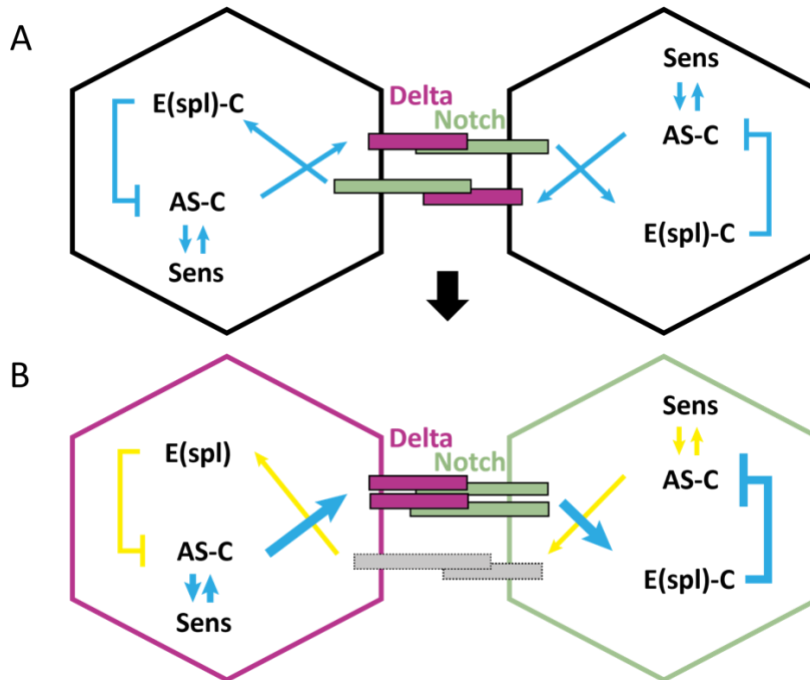


Figure 1.19. The Delta-Notch negative feedback that orchestrates SOP development. Schematics illustrating lateral inhibition during the selection of a sensory organ precursor (SOP) cell in the fly notum. (A) Initially, two neighboring cells have the potential to become an SOP. As development progresses, small initial differences between the cells are amplified through a Delta-Notch transcriptional feedback loop. (B) The cell that becomes the dominant signal sender develops into the SOP (labeled in magenta), while the cells receiving the predominant signal adopt the epithelial cell fate (labeled in green). Adapted from Salazar & Yamamoto, 2018.

Transducing the Notch signal

Notch research in the 1990s began with the identification of Notch as an oncogene in mammals (Ellisen et al., 1991). The discovery that human NOTCH1 is frequently rearranged in T cell lymphoblastic leukemias (T-ALL) marked a major advance in the study of Notch signal transduction. The genomic rearrangement results in the expression of an incomplete Notch variant that lacks the extracellular domain, which was interpreted to “activate” the oncogenic

potential of the receptor. This interpretation by no means implied what we now know as “activating mutations” (gain of function mutations); however, it spurred the investigation of engineered truncated forms of Notch to gain insights into Notch signal transduction.

It was indeed a major breakthrough when Struhl *et al.* (1993) employed the hs-Flp technology to overexpress the Notch intracellular domain (Notch^{INTRA} or NICD) in both flies and worms, ultimately finding phenotypes suggestive of Notch gain-of-function. Specifically, they found that NICD overexpression in the fly embryo promoted epidermal fate at the expense of neurons, the opposite of the neurogenic phenotype (Fig 1.18C). In the case of the worm, it recapitulated the effect of the *lin-12(d)* hypermorphic allele. Strikingly, using an NICD antibody, they observed nuclear localization of overexpressed NICD, contrasting with the normal Notch distribution. Gary Struhl's ability to interpret these findings was particularly impressive. He speculated that “signal transduction mediated by wildtype Notch involves cleavage and transport of its ICD to the nucleus, with the possibility that the NICD may be directly involved in transcriptional regulation”. In support of this, Toby Lieber published similar results a few months after (Lieber *et al.*, 1993).

Coincidentally, in the same issue of *Cell* as the Struhl *et al.* publication, another seminal study reported the effect of overexpressing various Notch truncations. This work was conducted by my own mentor, Ilaria (Rebay *et al.*, 1993). Among a set of dominant activated and dominant negative forms of the Notch receptor, this study identified the Notch Δ E variant, which lacked much of the extracellular domain but remained membrane-tethered. This version of Notch exhibited constitutive activity and resembled the receptor state following S2 cleavage by the ADAM protease. The Notch^{INTRA} form mimics the receptor state after both S2 and S3 cleavage (Fortini *et al.*, 1993). Thus, this pivotal knowledge facilitated further delineation of the pathway

during the 1990s, which involved elucidation of the mechanisms involved in cleavage processing of the receptor (reviewed in Greenwald, 2012)).

Notch in development, homeostasis and disease

While Notch research in the 20th century delineated the fundamental cellular and molecular events of what is now recognized as canonical Notch signaling, the 21st century has emphasized regulatory strategies that precisely adjust pathway activity, including “non-canonical” mechanisms of Notch modulation. Importantly, what are presently regarded as unconventional modes of Notch regulation may eventually constitute integral aspects of the pathway. Before delving into those mechanisms, it is important to first understand the significance of achieving and maintaining appropriate levels of Notch signaling. To illustrate this, I will provide some examples of its critical roles in development, homeostasis and disease.

Thus far in this introductory chapter, I have discussed four classic developmental contexts regulated by the Notch pathway: Notch limits neuronal specification in the neuron-epidermis cell fate decision during both *Drosophila* embryogenesis and thoracic bristle development (D. Poulson, 1939; D. F. Poulson, 1940; Heitzler & Simpson, 1991; Heitzler et al., 1996), Notch restricts vein formation during fruit fly wing development (de Celis et al., 1997; Huppert et al., 1997), and Notch represses AC differentiation in the AC-VU fate decision during *C. elegans* vulval development (Seydoux & Greenwald, 1989; Wilkinson et al., 1994). While more discrete changes in Notch signaling levels influence the AC-VU decision in *C. elegans*, nuanced variations in pathway activity dictate the extent of neural or vein tissue formation in *Drosophila*. The prior introduction of these Notch-controlled systems is unsurprising, given the

longstanding intimate relationship between Notch and both fly and worm models. However, Notch's impact transcends these model organisms.

A major role that Notch plays in vertebrate development is during somitogenesis (reviewed in Carrieri & Dale, 2017, and Liao & Oates, 2017). Somitogenesis is a rhythmic process occurring in the presomitic mesoderm (PSM) to segment the undifferentiated tissue of the vertebrate embryo into body axis segments called somites. These somites give rise to segmented structures in the adult anatomy, including vertebrae, neural and hemal arches, ribs, muscles, and skin. The cyclic and tightly controlled production of somites depends on precise pulses of Notch signaling. Consequently, inadequate Notch activity disrupts somite boundaries, while excessive signaling can accelerate oscillations and lead to the production of extra somites. Therefore, both the strength and precise temporal dynamics of Notch signaling are critical for proper somitogenesis.

Notch signaling also plays crucial roles in vertebrate hematopoietic development. Specifically, Notch1 activity is upregulated in emerging hematopoietic cells (Del Monte et al., 2007; Richard et al., 2013), and *Notch1*⁻ cells do not contribute to hematopoiesis (Hadland et al., 2004). The hematopoietic system branches into two main lineages: the lymphoid lineage, which gives rise to T cells, B cells, and natural killer (NK) cells; and the myeloid lineage, which generates cells such as macrophages, monocytes, megakaryocytes, mast cells, and erythrocytes. Notch activity is required in many fate decisions along these two lineages, but the most well characterized is likely its role in T-cell development (reviewed in Siebel & Lendahl, 2017, and Vijayaraghavan & Osborne, 2018). Notch1 signaling has been shown to be both necessary and sufficient to promote T-cell differentiation in the lymphoid lineage (Pui et al., 1999; Radtke et al., 1999; Han et al., 2002). Subsequently, Notch plays a crucial role in subdividing early T-cell

progenitors. In particular, Notch1 favors CD8 over CD4 and $\alpha\beta$ and $\gamma\delta$ T-cell lineage (Robey et al., 1996; Washburn et al., 1997; Fowlkes & Robey, 2002). In lymphoid development's B-cell lineage, Notch2 is essential for the differentiation of marginal zone B cells and follicular zone B cells within the spleen (Tanigaki et al., 2002; Saito et al., 2003; Hozumi et al., 2004). In hematopoietic system development, precise regulation of Notch activity governs the production of specific cell fates in accurate numbers.

Turning to homeostasis, Notch plays significant roles in the vertebrate cutaneous and intestinal systems. In the former, Notch regulates differentiation within both the skin and hair follicles, with distinct roles in preserving stem cell fate in hair follicles and promoting differentiation in skin tissue (reviewed in Nowell & Radtke, 2013). In the intestine, Notch signaling is critical for maintaining the stem cell pool within the intestinal crypts and driving absorptive cell (enterocyte) differentiation while suppressing the secretory cell fate (for a review, see Dempsey et al., 2018). Studies in *Drosophila* have actually contributed significantly to our understanding of Notch's role in intestinal tissue development and maintenance (Z. Guo & Ohlstein, 2015). Notably, across both skin and intestine, finely tuned Notch signaling is essential for ensuring the correct balance of cell types.

For a comprehensive review of the roles of Notch in development and homeostasis, Siebel & Lendahl, 2017 provide valuable insights. Notch's involvement in development spans multiple organs and systems including somite-derived organs, vasculature, heart, hematopoietic system, nervous system, the eye, the pancreas, etc. Regarding its role in homeostasis, Notch function is key in the preservation of liver, skin, lung, vasculature, hematopoietic system, intestine, skeletal muscle, etc.

The significance of model system genes in understanding human disease has been a landmark achievement in developmental genetics, and Notch exemplifies this relationship. Mutations in human Notch genes contribute to a wide spectrum of biological disorders with diverse clinical manifestations. From a historical perspective, 1991 marked the start of the relationship between Notch and human diseases, when aberrant Notch activity was linked to T-cell acute lymphoblastic leukemia (T-ALL) (Ellisen et al., 1991). It was not until the next decade, however, that the biological basis underlying this and an immense range of cancer diseases was uncovered.

Mutations in Notch present diverse patterns in cancer and, among them, three significant types are well-documented (see Aster et al., 2017; Anusewicz et al., 2021; and Shi et al., 2024). Firstly, gain-of-function mutations, seen most commonly in T-ALL, enhance Notch signaling and often involve the oncogene *Myc*, contributing to oncogenesis. Secondly, mutations that affect the Notch intracellular PEST domain lead to increased ligand-dependent signaling, particularly observed in B-cell leukemias. Thirdly, loss-of-function mutations, found in squamous cell cancers, diminish Notch's tumor-suppressive functions. The diverse impact of Notch on cancer spans crucial processes such as cell survival, metabolism, immune response evasion, inflammation, and metastasis.

Notch's involvement extends beyond oncogenic development to encompass several classic diseases, including Alagille syndrome, Adams-Oliver syndrome (AOS), CADASIL syndrome, and Spondylocostal dysostosis, among others (reviewed in Hori et al., 2013; Siebel & Lendahl, 2017; Giaimo & Borggreffe, 2018; Zhou et al., 2022; and Sachan et al., 2024). Alagille syndrome is characterized by developmental abnormalities affecting the heart, liver, kidney, eye, and skeleton, attributed to insufficient Notch signaling dependent on JAG1/2 (L. Li et al., 1997;

McDaniell et al., 2006; Turnpenny & Ellard, 2012). In Adams-Oliver syndrome, a rare disorder marked by limb malformations, abnormal skin development, and thin skull bones, loss of Notch1 function is typically implicated (Meester et al., 2015; Stittrich et al., 2014). CADASIL syndrome, caused by mutations in the Notch3 receptor, manifests as a hereditary vascular disorder associated with ischemic strokes, dementia, and premature mortality (Karlström et al., 2002). Spondylocostal dysostosis results from Dll3-dependent downregulated Notch signaling during somitogenesis, leading to bone malformations such as vertebrae and rib malsegmentation (Bonafé et al., 2003).

In summary, achieving and maintaining precise levels and spatiotemporal dynamics of Notch signaling is critical for normal development, homeostasis, and disease prevention. Understanding the mechanisms that regulate Notch activity will shed light on how subtle adjustments in signaling impact health and pathology. The final section of this introductory chapter delves into these fine-tuning mechanisms and their significance.

1.4 Fine tuning of Notch signaling

The on/off state of Notch signaling plays fundamental roles in cell proliferation, differentiation, and apoptosis across species. However, the pleiotropic functions of Notch appear contradictory to that seemingly simple binary nature. Critical studies in the 21st century have highlighted that nuanced changes in Notch activity levels underlie various developmental disorders and diseases, indicating sensitivity to Notch signaling dosage (reviewed in Shen et al., 2021). More specifically, subtle alterations in pathway activity influence binary cell fate determinations, as well as cell proliferation and differentiation processes during many cellular

and developmental transitions. This suggests that changes in Notch signaling strength can be as crucial as the on/off switch of the signaling pathway. Further emphasizing the importance of precise Notch activity tuning, Michael Elowitz's group has reported that variations in the dynamics of NICD production can lead to qualitative differences in pathway outcomes (Nandagopal et al., 2018).

In contrast to numerous other signaling pathways, Notch features a distinct architecture characterized by the release of a single NICD upon ligand-receptor binding (Lecourtois & Schweisguth, 1998; Schroeter et al., 1998; Struhl & Adachi, 1998; Bray, 2006), without involving intermediate signals or kinase cascades (Kopan & Ilagan, 2009; Kopan, 2012). Consequently, the intensity and duration of Notch signaling are determined by the amount of free NICD (activated Notch) produced, as CSL and other transcriptional co-factors remain consistently present in the cell nucleus. After the NICD activates transcription, it is degraded via the proteasome (Fryer et al., 2004; Antfolk et al., 2019), ensuring that signaling remains tightly regulated and transient.

What mechanisms are employed to finely adjust Notch signaling activity? Several factors can impact the generation of activated Notch, thereby adjusting pathway activity. Traditionally, in ligand-triggered signaling, the abundance of ligands and receptors on cell surfaces (regulated by endocytosis) (Hounjet & Vooijs, 2021; Sala et al., 2012; Seib & Klein, 2021), the specific ligand-receptor complex formed (Benedito et al., 2009; Gama-Norton et al., 2015; Groot et al., 2014; Nandagopal et al., 2018), glycosylation of the receptor's EGF repeats (Kakuda & Haltiwanger, 2017; Stanley & Okajima, 2010; Takeuchi & Haltiwanger, 2010) and lipid-ligand interactions (Suckling et al., 2017) can all affect Notch activation and NICD release dynamics. Moreover, regardless of ligand presence, the localization of Notch in distinct

endocytic/endosomal compartments can influence receptor activation and modify signaling levels, complementing the role of the endolysosomal pathway in regulating protein abundance (Vaccari et al., 2008; Windler & Bilder, 2010; Palmer & Deng, 2015; Alfred & Vaccari, 2018). Detailed discussion of how endolysosomal trafficking modulates Notch pathway activity is explored further below and serves as the main focus of Chapter 2.

Further enhancing the diversity and specificity of the Notch signaling, Falo-Sanjuan and Bray (2020) recently reviewed the dynamics of NICD-CSL binding to various target genes, noting variations in binding strength. This strength can be influenced not only by the specific sequence and distribution of CSL binding sites but also by interactions with context-specific co-transcriptional partners, such as Twist and Dorsal (Falo-Sanjuan et al., 2019). Additionally, contributing to the regulation of Notch activity at the transcriptional level, the context-specific control of NICD turnover through phosphorylation and ubiquitination can modulate the duration of pathway activity (Fryer et al., 2004; Antfolk et al., 2019).

Additional mechanisms for adjusting Notch pathway activity include feedback mechanisms at the transcriptional level. Several Notch target genes, such as Hes/Hey, participate in feedback regulation of the pathway. This underlies their ability to negatively regulate their own expression, contributing to an oscillatory control that, for example, directs the rhythmic production of body segments during somitogenesis (reviewed in Liao & Oates, 2017). Another possibility is the co-regulation of Notch and other signaling pathway, such as the positive feedback loop established between the Wnt and Notch signaling pathways during the establishment the dorsoventral boundary at the larval wing disc, and consequent margin fate specification (J. F. De Celis & Bray, 1997). Furthermore, a prominent feature of Notch signaling includes transcriptional loops between Notch and Delta leading to a mutually exclusive

expression pattern between neighboring cells. This configuration enhances initial disparities in protein abundance and pathway activation among adjacent cells, thereby influencing cell fate decisions (see "Cell fate determination by the neurogenic pathway"). The Delta-Notch feedback mechanism is further elaborated on in a subsequent subsection and serves as a major topic in Chapter 3 of this dissertation.

Regulation of pathway activity by Notch endosomal trafficking

Integral membrane proteins, such as the Notch receptor, are continuously endocytosed and transported through endocytic compartments, a process essential for protein recycling and turnover (Schmid et al., 2014). The endosomal pathway is integral to multiple facets of Notch signaling, encompassing both ligand-dependent and independent activation, as well as the degradation of the Notch receptor (Schnute et al., 2018). Work mostly done in *Drosophila* during this century shows that full-length Notch undergoes constitutive trafficking through the pathway in a ligand-independent manner for eventual degradation in the lysosome (Fig. 1.20) (Vaccari et al., 2008; Schnute et al., 2022). This research is predominantly conducted in the epithelial cells of imaginal tissue, and particularly in the context of the wing disc.

Membrane protein endocytosis involves the interaction of coat proteins, adaptors, and cargo at precise locations on the cell's plasma membrane (Huotari & Helenius, 2011). Cargo proteins can bind to the adaptor protein AP-2 directly through sorting signals like the di-leucine motif, or they may require ubiquitination for recognition by specific adaptors such as Epsin (Mellman, 1996; Simpson et al., 1996; Sen et al., 2012). Ubiquitination involves a series of enzymes including E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and

substrate-specific E3 ubiquitin ligases. These ligases attach ubiquitin molecules to lysine residues within the intracellular domain (ICD) of transmembrane proteins (TMPs) (Moretti & Brou, 2013). AP-2 starts the process of creating Clathrin-coated pits on the cell's plasma membrane, which then bend inward and detach to become Clathrin-coated vesicles (CCVs) (Pearse & Robinson, 1990).

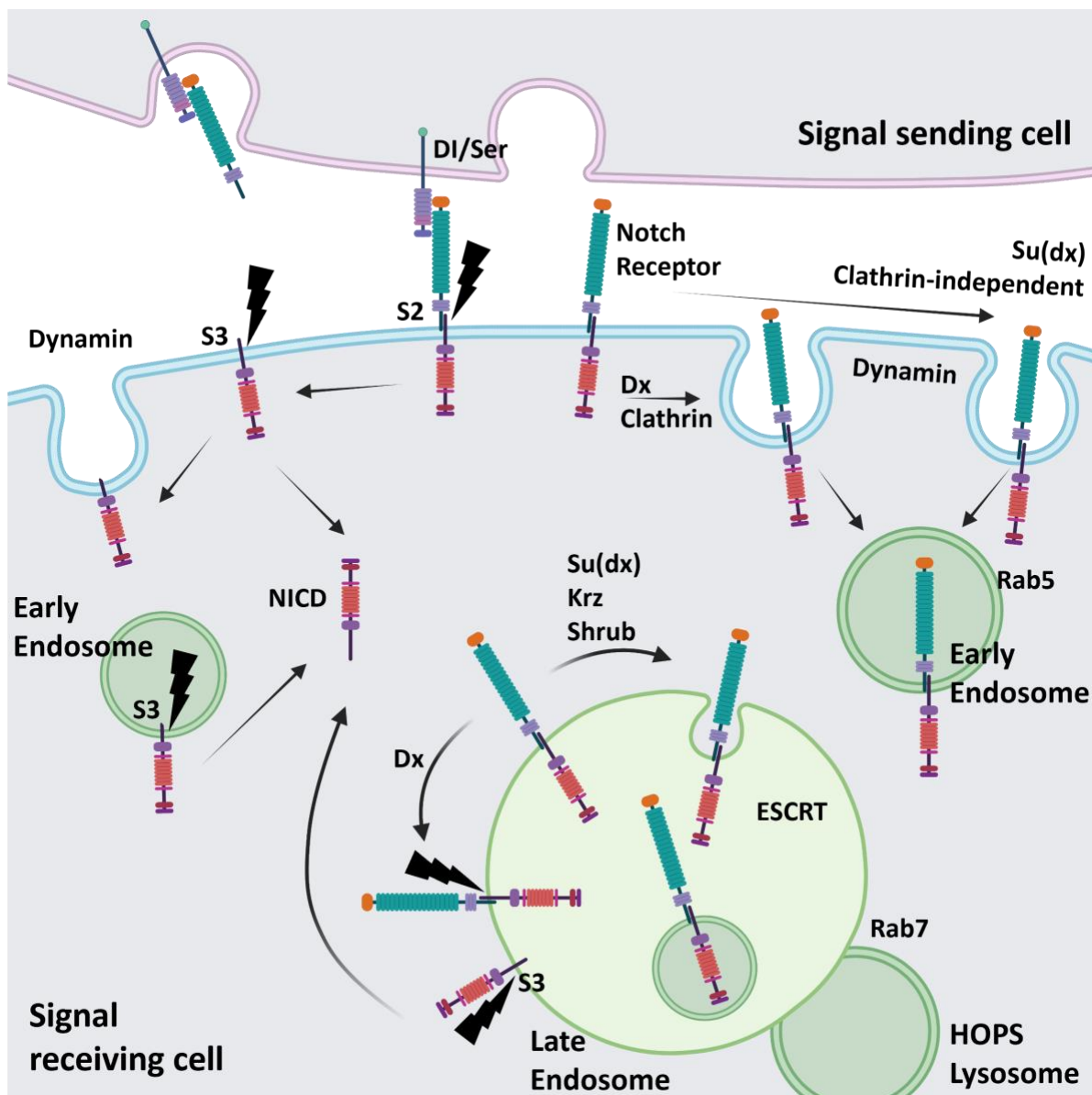


Figure 1.20. Endocytic trafficking in the Notch pathway.

Mechanisms of ligand-dependent (canonical, left) and ligand-independent, endosomal trafficking-mediated (non-canonical, right) Notch activation are depicted. In canonical activation, DSL ligand-induced S2 cleavage is followed by S3 cleavage, which can occur either at the cell surface or within endocytic vesicles, leading to the release of the Notch intracellular domain (NICD). In the non-canonical pathway, the E3 ubiquitin ligases Deltex (Dx) and Suppressor of Deltex (Su(dx)) mediate clathrin-dependent and clathrin-independent endocytosis of Notch, respectively. After transport through upstream endocytic vesicles and Rab5⁺ early endosomes, the positioning of Notch at Rab7⁺ late endosomes (LEs) impacts receptor activation. Dx stabilizes Notch at the LE membrane, promoting receptor cleavage and NICD release. This effect can be counteracted by Su(dx), Kurtz (Kz), and Shrub, which promote Notch internalization into multivesicular bodies (MVBs) via Endosomal Sorting Complex Required for Transport (ESCRT) complexes. This process targets Notch for lysosomal degradation, with HOPS facilitating endosomal-lysosomal fusion. Based on Shimizu et al, 2014; Shimizu et al, 2024; and Sachan et al, 2024. Made with BioRender.

Research in *Drosophila* has demonstrated that Notch endocytosis can proceed through a clathrin-dependent pathway involving AP-2, although it can also occur via a clathrin-independent route (Windler & Bilder, 2010). Clathrin-independent endocytosis of Notch takes place through GPI-enriched membrane domains that are also rich in cholesterol (Shimizu et al., 2014), a mechanism observed for other transmembrane proteins as well (Huotari & Helenius, 2011). Notably, endocytosis of Notch via different routes is promoted by the activity of two E3 ubiquitin ligases: Deltex (Dx) in the clathrin-dependent pathway and Suppressor of Deltex (Su(dx)) in the clathrin-independent pathway (Shimizu et al., 2014).

Following its initial endocytosis, Notch progresses through upstream/early endocytic vesicles (EEVs) towards the early endosome (EE). This transportation process relies on the functions of the GTPase Rab5 and Avalanche (Avl), a member of the *Drosophila* Syntaxin family (Lu & Bilder, 2005; Vaccari et al., 2009). Disruptions in Notch endosomal trafficking that hinder its transfer to EEs result in abnormal accumulation of the receptor at or below the cell

surface, leading to reduced Notch signaling (Vaccari et al., 2008). Upon reaching EEs, Notch faces a decision: it can either enter the recycling pathway to return to the plasma membrane or remain in the EE to proceed towards the lysosome for degradation. The majority of Notch appears to remain in the EE. However, recent research indicates that a small fraction of Notch undergoes recycling, facilitated by the DNAJ protein Rme-8 in *Drosophila* via a Rab4⁺ recycling pathway (Gomez-Lamarca et al., 2015). Of note, perturbations in Notch endosomal trafficking that cause receptor accumulation in Avl⁺ EEs do not seem to affect Notch pathway activity (Alfred & Vaccari, 2018).

As Notch travels towards the lysosome, it must be encapsulated into intraluminal vesicles (ILVs) (Schnute et al., 2018). This step is crucial for the complete degradation because it ensures that Notch receptors (and other transmembrane receptors) are fully enclosed within the lumen of the maturing endosome (ME), also referred to as the late endosome (LE), or multivesicular body (MBV) once the ILVs have formed (reviewed in Fortini & Bilder, 2009, and Huotari & Helenius, 2011). ILV formation is a continuous process during endosomal maturation and is facilitated by a sequence of four ESCRT (endosomal sorting complex required for transport) complexes: ESCRT-0, -I, -II, and -III, along with the Vps4 complex (reviewed in Hurley, 2015). These complexes shuttle between the cytosol and the endosomal membrane, either as pre-formed complexes (ESCRT-0, -I, -II) or as monomers (ESCRT-III).

ILV formation begins with the recruitment of ESCRT-0, comprising Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and STAM (signal-transducing adaptor molecule), to specific sites on the endosomal membrane. This recruitment process relies on Rab5, Vps34, and PI(3)P. Subsequently, ESCRT-0 recruits ESCRT-I, followed by ESCRT-II. This sequential recruitment leads to the formation of a large supercomplex that concentrates

cargo (reviewed in Schmidt & Teis, 2012). Cargo incorporation into this complex is marked by ubiquitin, often attached to cargo at membranes by specific E3 ligases. Consistent with its pivotal role in endosomal maturation, dysfunction in the ESCRT machinery leads to a significant reduction or complete failure in ILV formation (Hurley, 2010). Impaired incorporation of Notch into ILVs, whether due to dysfunctional ESCRT activity or other mechanisms, results in the accumulation of the receptor at the membrane of Hrs⁺ EEs or Rab7⁺ LEs, leading to increased Notch signaling (Vaccari et al., 2008; M. Wilkin et al., 2008; Shimizu et al., 2014; Schnute et al., 2022; Shimizu et al., 2024).

The E3 ligases Dx and Su(dx) also regulate Notch at LEs where they interact to coordinate the amount of Notch that is incorporated into ILVs (Fig 1.19) (Hori et al., 2011; Shimizu et al., 2014; Schnute et al., 2022; Shimizu et al., 2024). This function is temperature-dependent and serves as a buffering system to ensure appropriate levels of Notch activation across a wide temperature range (Shimizu et al., 2014). In the LE regulation of Notch, Dx plays a critical role by monoubiquitinating the NICD, which appears to prevent its incorporation into ILVs (Hori et al., 2004; M. Wilkin et al., 2008; Hori et al., 2011; Yamada et al., 2011; Shimizu et al., 2014; Schnute et al., 2022; Shimizu et al., 2024), possibly because this modification is not effectively recognized by the ESCRT machinery (Ren & Hurley, 2010). Strikingly, Notch that remains at the limiting membrane becomes activated during endosomal maturation (Vaccari et al., 2008; M. Wilkin et al., 2008; Shimizu et al., 2014; Schnute et al., 2022; Shimizu et al., 2024). Therefore, another essential role of ILV incorporation is to regulate the pathway activation by preventing uncontrolled activation of Notch situated in the LE limiting membrane (Baron, 2012).

Interestingly, when the *Drosophila* non-visual arrestin Kurtz binds to Dx, it seems to alter the function of the E3 ligase, resulting in polyubiquitination of Notch. As the ESCRT machinery

shows a preference for the polyubiquitinated cargo (Ren and Hurley, 2010), polyubiquitination by Dx/Kurtz directs Notch into the lumen of LEs (Hori et al., 2011). Su(dx) also counteracts the activity of Dx, which occurs by inducing polyubiquitination of Notch in a manner influenced by temperature (M. B. Wilkin et al., 2004; Sakata et al., 2004; Jennings et al., 2007; M. Wilkin et al., 2008; Shimizu et al., 2014; Schnute et al., 2022; Shimizu et al., 2024). Thus, these proteins establish a regulatory module that dictates whether Notch is incorporated into the lumen or remains at the limiting membrane of LEs/MVBs.

In overexpression experiments, it has been demonstrated that Su(dx) exerts dominant activity over Dx, leading to the incorporation of Notch into the LE lumen (Schnute et al., 2022; Shimizu et al., 2014). The different types of ubiquitination might provide an explanation for the outcome of this interaction (Schnute et al., 2018). Su(dx) may attach additional ubiquitin molecules to sites where Dx has already monoubiquitinated Notch, or to new sites, prompting the ESCRT machinery to internalize Notch into the LEs. Importantly, while activation of Notch at the LE membrane has long been thought to depend on LE-lysosomal fusion, very recent work by Martin Baron suggests that alternative, yet still poorly understood, mechanisms may also be involved (Shimizu et al., 2024). The intricate interplay between Dx and Su(dx) regulates LE-mediated processes in various cellular and developmental contexts (Shimizu et al., 2014; Schnute et al., 2022).

Notch is transported to the lysosome through fusion of the LE with this organelle. This fusion process relies on Rab7, which recruits and activates the HOPS tethering complex (Hori et al., 2011). Loss of Rab7 function or its guanine nucleotide exchange factor (GEF), crucial for Rab7 recruitment, results in similar phenotypes characterized by markedly enlarged endosomes containing supernumerary ILVs (Yousefian et al., 2013). This suggests that ILV formation

remains unaffected despite the loss of Rab7 activity. Furthermore, studies in *Drosophila* indicate that while endosome maturation progresses normally, the fusion of these endosomes with the lysosome is impaired in the absence of Rab7 (Yousefian et al., 2013). This underscores Rab7's essential role in orchestrating the fusion of the maturing endosome with the lysosome.

The observation that Notch can become activated within the endosome raises the question about whether this mode of activation, which does not require ligands, is utilized during development (Schnute et al., 2018). In the cases reported thus far, this form of Notch activation arises from disruptions in genes that control Notch ubiquitination and transport through endosomal compartments. However, the Dx/Krz/Su(dx) module might represent a specific sorting mechanism evolved to regulate the amount of Notch present at the limiting membrane of the LE in a manner influenced by temperature. According to this model, the endosomal trafficking-mediated ligand-independent activity of Notch is essential in addition to ligand-dependent activation for certain developmental processes (Shimizu et al., 2014).

To date, only one developmental process has been identified that necessitates ligand-independent activation of Notch (T. Mukherjee et al., 2011). Crystal cells, which are *Drosophila* platelet-like immune cells, rely on Notch signaling for their survival even in the absence of direct ligand stimulation. Notch activity in these cells has been observed to occur independently of ligands, via retention of the receptor at Hrs⁺ EEs. However, it is important to interpret this finding cautiously, considering that crystal cells are mobile within the body and may encounter Notch ligands during circulation. In Chapter 4 of this dissertation, I discuss what I believe to be the first identified developmental function of ligand-independent Notch signaling in non-circulating cells.

Lateral inhibition and Delta-Notch feedback

The concept of lateral inhibition was first introduced by Wigglesworth in 1940 (Wigglesworth, 1940), but it was not until the 1990s that these ideas were formalized into a precise mathematical model (Collier et al., 1996). In essence, lateral inhibition patterning is a process where initially identical cells differentiate into alternating patterns of cell fates by symmetry breaking interactions. This involves local competition among neighboring cells, where each cell strives to adopt a specific fate while inhibiting its neighbors from doing the same. Within each group of cells, one cell emerges as dominant and suppresses its neighboring cells, typically through Notch signaling (Binshtok & Sprinzak, 2018; Sjöqvist & Andersson, 2019; Bocci et al., 2020).

A fundamental mechanism underlying lateral inhibition involves an intercellular feedback loop between the Notch receptor, which mediates Notch signaling, and its ligand, typically Delta (Collier et al., 1996). Initially, higher Delta expression or activity in one cell induces Notch signaling in neighboring cells. Subsequently, Notch activity in these receiving cells inhibits *Delta* expression and often induces expression of *Notch* itself. This downregulation of *Delta* in the responding cells prevents them from signaling back to the original inducing cell, establishing a mutually exclusive pattern of Notch and Delta domains.

As this feedback loop progresses, it has the capacity to amplify initial differences between cells, leading to one cell expressing high levels of Delta ligand while its neighbor exhibits high levels of Notch receptor and pathway activation. This process is thought to underlie the characteristic "checkerboard" pattern observed in lateral inhibition (Fig. 1.21A) (Collier et al., 1996; Binshtok & Sprinzak, 2018; Sjöqvist & Andersson, 2019; Bocci et al., 2020). As

discussed earlier, lateral inhibition mediated by Delta-Notch negative feedback has been implicated in various developmental processes, including bristle patterning in the *Drosophila* notum (Heitzler et al., 1996; Heitzler & Simpson, 1991), vein patterning in the *Drosophila* wing (de Celis et al., 1997; Huppert et al., 1997) and AC-VU development in the *C. elegans* vulva (Seydoux & Greenwald, 1989; Wilkinson et al., 1994).

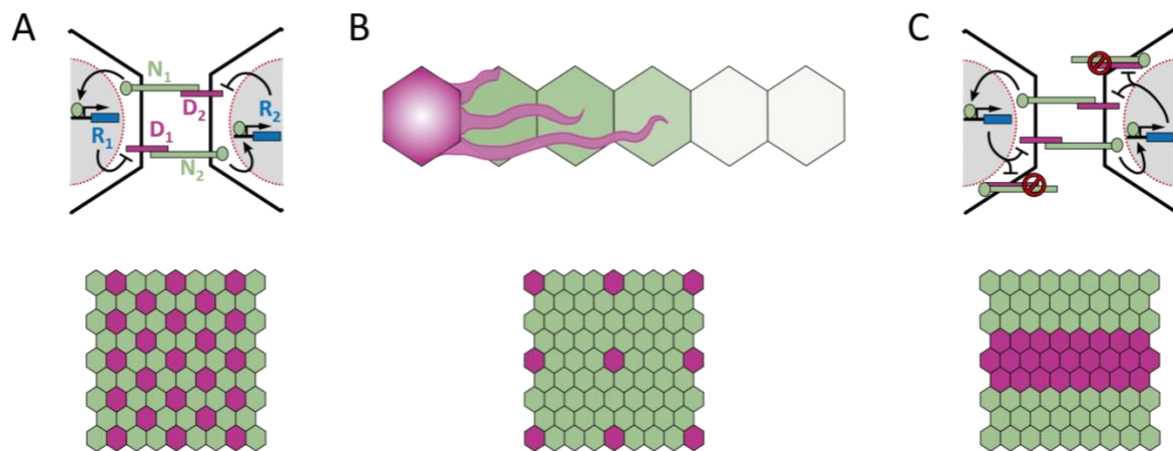


Figure 1.21. Fundamental aspects of lateral inhibition mediated by Delta-Notch signaling. (A) A Delta-Notch transcriptional feedback loop generates the characteristic "checkerboard" pattern seen in various lateral inhibition contexts. (B) Cellular projections enable the propagation of the Delta signal, often activating Notch signaling effectively in three rows of neighboring cells. (C) Cis-inhibition permits the coexistence of adjacent cells with high Delta expression. Adapted from Binshtok & Sprinzak, 2018.

In the context of lateral inhibition, a novel concept that has emerged recently suggests that cells can communicate through extended cellular projections such as filopodia or cytonemes, allowing direct signaling over significant distances (T. B. Kornberg, 2014) (Fig. 1.21B). These protrusions can be extremely narrow, with diameters as small as 0.1 μm , and can extend over

100 μ m length. Recent studies have provided experimental evidence supporting instances where Notch signaling occurs through such protrusions in developmental patterns. Examples include the patterning of bristles on the *Drosophila* notum (M. Cohen et al., 2010; G. L. Hunter et al., 2016), the spotted skin patterns in pearl danio fish (Eom et al., 2015), and striped skin patterns in zebrafish (Hamada et al., 2014; Eom et al., 2015). The possibility for long-range signaling through these protrusions provides a viable explanation for various feedback-driven lateral inhibition scenarios, where cells expressing elevated Delta levels are spaced further apart than a single cell diameter. Additionally, it could account for instances where Notch signaling extends beyond the immediate neighbors of Delta-expressing cells such as the *Drosophila*'s bristle and patterning processes (see Corson et al., 2017, and Chapter 3 of this dissertation).

Another important aspect of feedback-dependent lateral inhibition systems concerns ligand-receptor cis-inhibition interactions (Fig. 1.21C). Briefly, it has been recognized for a considerable period that Notch receptors and their ligands can engage in interactions not just between neighboring cells (in-trans) but also within the same cell (in-cis) (J. F. De Celis & Bray, 1997; T. Klein et al., 1997; Micchelli, et al., 1997; Del Álamo et al., 2011; Palmer et al., 2014; Nandagopal et al., 2019). In contrast to trans-interactions that activate Notch receptors, cis-interactions serve to inhibit Notch signaling (Del Álamo et al., 2011).

At the most essential level, cis-inhibition provides a plausible explanation for the stripes of contiguous Delta-expressing cells observed transiently during bristle patterning in the fruit fly notum (Corson et al., 2017) and throughout *Drosophila* wing vein patterning (Chapter 3). Additionally, mathematical modeling suggest that ligand-receptor cis-inhibition serves as an error suppression mechanism (Barad et al., 2010; Sprinzak et al., 2010). These studies propose that lateral inhibition involving a transcriptional feedback circuit can exhibit delayed response

times, potentially leading to higher error rates than observed in nature. Thus, modeling predicts that faster dynamics mediated by cis-inhibition are expected to mitigate errors associated with such delays. Experimental work *in vivo* will be required to validate these predictions.

Remarks and thesis overview

The final section of this introductory chapter highlighted the critical importance of precisely regulating Notch pathway activity to achieve proper biological outcomes, a central theme in both contemporary Notch research and this dissertation. Using wing venation as an experimental system, my thesis focuses on two key aspects of Notch regulation: endosomal trafficking mechanisms and lateral inhibition via Delta-Notch feedback. While the role of ubiquitination in Notch trafficking is well-established, the phosphorylation-based regulation of this process remains a key gap in the field, which my work begins to address (Chapter 2). Additionally, although the basic principles of Delta-Notch negative feedback are known, its spatiotemporal deployment across biological contexts is still poorly understood. By examining vein-intervein binary decisions during wing development, my work provides new insights into these dynamics (Chapter 3). My thesis also briefly explores a ligand-independent mode of Notch activation that contributes to wing vein development, although the exact mechanism remains unidentified (Chapter 4). This novel area of Notch regulation represents an exciting frontier that has yet to be thoroughly studied and is now the focus of ongoing research by new trainees in our lab.

Taken together, my studies illustrate how Notch signaling integrates various regulatory inputs to achieve accurate pathway activity, mirroring how developmental processes coordinate

multiple signaling pathways to produce proper biological outcomes. This intricate regulatory choreography ensures that biological systems achieve both precision and robustness, underscoring the reliability and sophistication of development.

CHAPTER 2

THE ABELSON KINASE COOPERATES WITH THE NEDD4-FAMILY UBIQUITIN LIGASES TO REGULATE THE LATE ENDOSOMAL PASSAGE OF NOTCH AND LIMIT SIGNALING

This chapter is based on the manuscript titled “*The Abelson kinase and the Nedd4-family E3 ligases co-regulate Notch trafficking to limit signaling,*” which was submitted for publication on July 12, 2024. It is currently under positive review at a peer-reviewed journal and is available as a preprint at: <https://www.biorxiv.org/content/10.1101/2024.07.12.603272v1>.

Statement of contributions:

While the majority of the work presented in this chapter is my own, it was significantly enhanced by the contributions of some exceptional collaborators. Through his study of Abl function in the eye disc, Nicelio Sanchez-Luege made seminal early observations on Abl’s regulation of Notch, which largely inspired this work. He also performed the molecular cloning of pGEX-NICD, pGEX-NICD^{Y2328F}, and pMT-Notch^{Y2328F}, as well as the *in vitro* kinase assay. Under my supervision, Fernando Valbuena established the Notch/Rab7 colocalization assay in S2 cultured cells and validated the impact of known Notch trafficking regulators (Dx and Su(dx)) on the LE localization of Notch in this system. Additionally, Chyan Rangel, under my mentorship, performed part of the *abl* clonal analysis experiments and contributed to assessing the role of Nedd4 isoforms in Notch signaling in both *Drosophila* wings and S2 cells. The remaining work was completed by me with guidance from Dr. Ilaria Rebay.

2.1 Introduction

Metazoan development relies on spatiotemporal coordination of cellular behaviors. Cells communicate with each other through precisely regulated biochemical signals, with either too little or too much signaling linked to developmental disorders and pathologies (Berridge, 2014). Manipulations of the highly conserved Notch signaling pathway exemplify the sensitivity of developmental events to signal transmission levels (Gozlan & Sprinzak, 2023; Siebel & Lendahl, 2017). Notch orchestrates a diverse array of cellular and developmental processes, including cell fate determination and establishment of tissue boundaries. In some contexts, the *on* versus *off* Notch signaling state induces distinct outcomes, whereas in other cases more modest differences in signaling levels drive specific transitions (Ohlstein & Spradling, 2007; Van de Walle et al., 2009; Ninov et al., 2012; Gama-Norton et al., 2015; Basch et al., 2016; Contreras et al., 2018). Because both Notch and its ligands are broadly expressed, this versatility in function requires meticulous spatiotemporal control of the initiation, duration and strength of signaling (Hori et al., 2013).

Notch is an integral membrane protein with a ligand-binding extracellular domain (NECD) and a signal transducing intracellular domain (NICD) (Lieber et al., 1993; Rebay et al., 1993; Wharton et al., 1985). Canonical activation of Notch signaling (Kovall et al., 2017) involves cell surface interactions with Delta/Serrate/Lag2-family ligands that trigger endocytosis and proteolytic cleavage of the Notch receptor to release the NICD (De Strooper et al., 1999; Brou et al., 2000). Notch activation can also occur independent of ligand binding. Unbound Notch receptor is continuously endocytosed (Maitra et al., 2006) and either recycled back to the cell surface or delivered to the lysosome for degradation (Yamamoto et al., 2010). Ligand-

independent release of the NICD from specific endosomal compartments is possible during this transport (Fortini & Bilder, 2009; Palmer & Deng, 2015; Hounjet & Vooijs, 2021). Once cleaved, activated Notch (free NICD) enters the nucleus and forms a protein complex that drives target gene transcription (Bailey & Posakony, 1995; Wilson & Kovall, 2006; L. Wu et al., 2000). The amount of free NICD quantitatively correlates with the overall transcriptional outcome of signaling (Shen et al., 2021) and different dynamics of NICD production can elicit distinct downstream responses (Nandagopal et al., 2018).

Multiple factors can influence the production of activated Notch and thereby fine-tune pathway activity. In ligand-induced signaling, the ligand/receptor abundance at cell surfaces (regulated by endocytosis) (Hounjet & Vooijs, 2021; Sala et al., 2012; Seib & Klein, 2021), the specific ligand-receptor complex formed (Benedito et al., 2009; Gama-Norton et al., 2015; Groot et al., 2014; Nandagopal et al., 2018), glycosylation of the receptor's EGF repeats (Kakuda & Haltiwanger, 2017; Stanley & Okajima, 2010; Takeuchi & Haltiwanger, 2010) and lipid-ligand interactions (Suckling et al., 2017) can all affect Notch activation and NICD release dynamics (Shen et al., 2021). In ligand-independent signaling, residence of Notch at distinct endocytic/endosomal compartments can affect receptor activation and modify signaling levels (Alfred & Vaccari, 2018; Palmer & Deng, 2015; Vaccari et al., 2008; Windler & Bilder, 2010). Therefore, in both ligand-dependent and ligand-independent contexts, precise regulation of Notch endosomal transit must be ensured to modulate NICD release and pathway signaling output.

Endosomal trafficking of integral membrane receptors is controlled by post-translational regulatory mechanisms, most frequently involving ubiquitination and phosphorylation (Foot et al., 2017; Offringa & Huang, 2013). Most insights into the roles of ubiquitination in modulating

Notch endocytic flux and signaling stem from studying three specific E3 ubiquitin ligases: Deltex (Dx) and the Nedd4-family members Suppressor of dx (Su(dx)) and Nedd4 (Sakata et al., 2004; M. B. Wilkin et al., 2004; M. B. Wilkin & Baron, 2005; Yamada et al., 2011; Baron, 2012; Hori et al., 2012; Moretti & Brou, 2013; Schnute et al., 2018; Dutta et al., 2022; Revici et al., 2022). Genetic, molecular and cell biological approaches have characterized Dx as a positive regulator and Su(dx) and Nedd4 as negative regulators of Notch (Sakata et al., 2004; M. B. Wilkin et al., 2004; Shimizu et al., 2014; Schnute et al., 2022; Xu & Artavanis-Tsakonas, 1990; Matsuno et al., 1995; Fostier et al., 1998; Cornell et al., 1999; Matsuno et al., 2002; Hori et al., 2004; M. Wilkin et al., 2008; Hori et al., 2011). Monoubiquitination of the NICD by Dx stabilizes Notch at the limiting membrane of late endosomes/multivesicular bodies (LEs/MVBs) (Yamada et al., 2011; Shimizu et al., 2014; Schnute et al., 2022; Hori et al., 2004; M. Wilkin et al., 2008; Hori et al., 2011; Shimizu et al., 2024), a topology permissive to NICD cleavage and pathway activation (Vaccari et al., 2008; Shimizu et al., 2014; Schnute et al., 2022; M. Wilkin et al., 2008; Shimizu et al., 2024). In contrast, recognition of the proline-rich PPxY motif in the NICD by the WW domains of Su(dx) or Nedd4 leads to polyubiquitination and downstream degradation of Notch (Sakata et al., 2004; Shimizu et al., 2014; Schnute et al., 2022; Jennings et al., 2007; Shimizu et al., 2024). Although unclear for Nedd4 (Sakata et al., 2004; M. B. Wilkin et al., 2004), the Su(dx)-mediated downregulation of Notch involves internalization of the receptor into the intraluminal vesicles (ILVs) of the LEs/MVBs (Shimizu et al., 2014; Schnute et al., 2022; M. B. Wilkin et al., 2004; M. Wilkin et al., 2008; Sakata et al., 2004; Jennings et al., 2007; Shimizu et al., 2024). This topologically prevents release of cleaved NICD to the cytoplasm and targets Notch for lysosomal degradation (Vaccari et al., 2008; Shimizu et al., 2014; Schnute et al., 2022; M. Wilkin et al., 2008; Shimizu et al., 2024).

Although receptor trafficking is commonly regulated by both ubiquitination and phosphorylation, phosphorylation-based regulation of Notch endocytic trafficking remains largely unexplored (Sjöqvist et al., 2014). The Abelson (Abl) cytoplasmic tyrosine kinase offered an opportunity to address this gap. Abl is best studied as a regulator of actin cytoskeleton and adherens junction dynamics and Abl-Notch genetic interactions have been demonstrated during axon guidance and planar cell polarity establishment (Crowner et al., 2003; Giniger, 1998; Kannan et al., 2017; Koca et al., 2022; Kuzina et al., 2011; Le Gall et al., 2008; Song & Giniger, 2011). However, in these contexts, the underlying mechanism involves Notch-mediated organization of cytoplasmic protein complexes that modulate Abl function, rather than Abl-mediated regulation of Notch signaling. An additional and different role for Abl-Notch interactions was uncovered in the pupal eye disc, where we found that Abl loss impairs clearance of endocytosed Notch (Xiong et al., 2013). Because reducing the genetic dose of Notch or its transcriptional effector Su(H) suppressed other *abl* mutant phenotypes in the eye, we proposed that Abl-mediated regulation of Notch trafficking might limit signaling. However, the complexity of the *abl* mutant phenotypes in the pupal eye disc (Sun et al., 2024; Xiong et al., 2013) made this hypothesis difficult to test.

In this study we used the *Drosophila* wing vein pattern and S2 cultured cells to investigate the mechanisms by which Abl regulates Notch endocytic trafficking and signaling. We show that loss of Abl results in truncated veins, suggesting excessive Notch activity, and that increasing Abl leads to ectopic vein formation, consistent with insufficient Notch signaling. Subcellularly, loss of Abl results in aberrant accumulation of Notch at the LE membrane, a topology that increases Notch signaling output. Conversely, increased Abl activity drives Notch internalization into the LE lumen and reduces signaling. The LE regulation of Notch by Abl is

kinase activity-dependent, and may involve phosphorylation of the NICD's PPxY, a motif used by Nedd4-family ubiquitin ligases to recognize Notch. Mutation of this motif renders Notch insensitive to regulation by Abl and Su(dx), and partially resistant to a different Nedd4 family member, Nedd4Lo, that can also promote Notch internalization into the LE/MBV lumen. Genetic interaction experiments suggest that the Abl-mediated LE regulation of Notch requires Su(dx), with Nedd4Lo providing parallel PPxY-dependent and -independent inputs. We propose that LEs and the PPxY motif, respectively, provide subcellular and molecular integration points for multiple Notch regulatory inputs. Together or separately, they offer fine-tuning routes applicable to both ligand-dependent and ligand-independent signaling contexts.

2.2 Results

Loss of *abl* increases Notch signaling and disrupts wing vein pattern

Wing vein patterning is highly sensitive to Notch signaling levels, with too little resulting in excessive vein formation, and too much leading to vein loss (de Celis et al., 1993; de Celis & Garcia-Bellido, 1994; Johannes & Preiss, 2002). The pattern arises during early pupal development when provein cells expressing Delta activate Notch signaling in flanking intervein cells to repress vein fate, ultimately producing the stereotypical vein-intervein pattern by 30-32h after puparium formation (APF) (de Celis et al., 1997; Herszterg et al., 2023; Huppert et al., 1997; Sotillos & De Celis, 2005). If Abl modulates Notch signaling during wing vein patterning, Abl loss should produce phenotypes characteristic of Notch misregulation.

To test this, we first examined adult wings. Inconveniently, although *abl* null mutants

complete pupal development, they die as pharate adults (Henkemeyer et al., 1987) and, in accordance with our earlier observations (Xiong et al., 2009), RNAi-mediated Abl knockdown did not produce wing phenotypes, presumably because of Abl protein perdurance. Therefore, we used the deGradFP system (Caussinus & Affolter, 2016) to knockdown Abl protein levels in animals carrying an endogenously GFP-tagged allele (Nagarkar-Jaiswal et al., 2015) over a null allele (*abl^{GFP}/abl²*). Adding spatiotemporal control (McGuire et al., 2004) allowed us to target the knockdown specifically to the posterior compartment of developing pupal wings, thereby bypassing any earlier requirements for Abl function in proliferation or patterning of the larval wing disc (Singh et al., 2010). The vein truncations found in 29% of otherwise wildtype-looking Abl knockdown adult wings (Fig. 2.1A,B; Table 1) suggested a disruption in the vein patterning process, and resembled Notch *gain of function* phenotypes. Genetic interactions further implicated Abl as a potential negative regulator within the Notch pathway: reducing *abl* dose, which on its own did not perturb pattern, suppressed the induction of excessive vein material associated with Notch heterozygosity or Su(dx) overexpression and enhanced Dx overexpression-induced vein loss (Table 1).

Multiple signaling pathways regulate wing vein patterning (Blair, 2007; Sotillos & De Celis, 2005). Therefore, to confirm that the Abl loss-of-function phenotypes resulted from misregulation of Notch, we evaluated the expression of a GFP reporter driven by a Notch Responsive Element (NRE) (Furriols & Bray, 2001; Housden et al., 2012; Zacharioudaki & Bray, 2014). As previously shown (Herszterg et al., 2023; Huppert et al., 1997; Lyman & Yedvobnick, 1995), in wildtype wings Notch signaling was detected as early as 16h APF in cells flanking the presumptive L3/L4 veins (Fig. 2.1C) and increased in intensity over time as the pattern refined into two stripes flanking each vein (Figs. 2.1E, 2.1G, S2.1). At 18h APF,

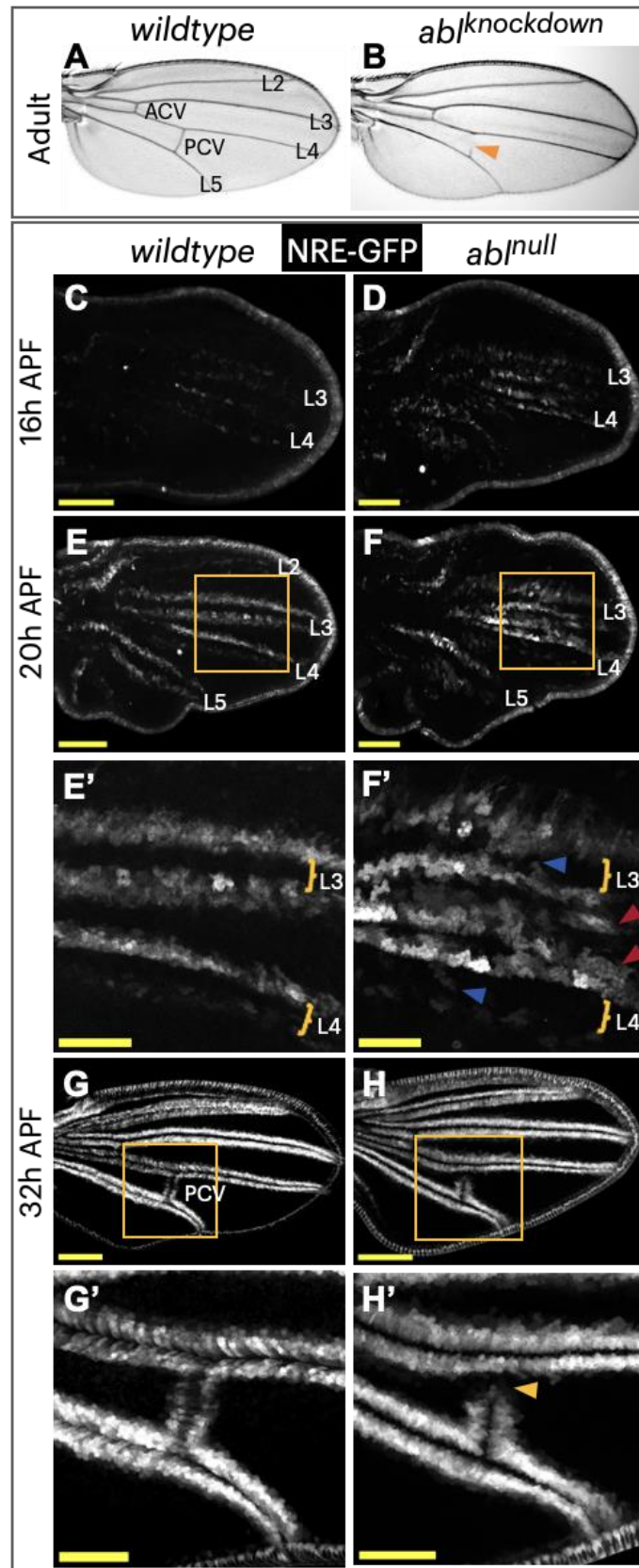


Figure 2.1. Abl is required to limit Notch signaling during wing vein patterning. (A,B) Adult wings. (A) Wildtype, with L2, L3, L4, L5, ACV and PCV veins indicated. (B) *en-Gal4>UAS-deGradFP; abl^{GFP}/abl²* (*abl^{knockdown}*) adult wings. Orange arrowhead points to a gap in the PCV. (C-H) Maximal projections comparing NRE-GFP reporter expression in wildtype and *abl* null (*abl¹/abl²*) pupal wings at 16h (C,D), 20h (E-F) and 32h (G,H) APF. Zoomed insets in (E',F') highlight the “noisy” signaling events in L3 and L4 vein (blue arrowheads) and intervein (red arrowheads) cells associated with *abl* knockdown. Zoomed insets in (G',H') highlight the loss of NRE-GFP expression in a presumptive PCV gap (yellow arrowhead) that occurs upon loss of *abl*. Scale bars = 100µm (C-H) or 50µm (E'-H').

GENETIC PERTURBATION	GAIN/LOSS OF VEINS?	PENETRANCE (% OF WINGS)	REGIONS AFFECTED
Wildtype	-	0% (N=50)	-
<i>Abl^{DegradFP}</i> (knockdown)	Loss	29% (N=70)	PCV
<i>Notch⁵⁴¹⁹/+</i>	Gain	94% (N=54)	L3,L4,L5
<i>Notch⁵⁴¹⁹/+ ; abl²/+</i>	Gain	80% (N=76)	L3,L4,L5
<i>Su(dx)</i>	Gain	62% (N=40)	PCV, occasionally ACV, L4, L5, interveins.
<i>Su(dx) + abl²/+</i>	Gain	43% (N=63)	PCV
<i>Dx</i>	Loss	60% (N=80)	L4, occasionally L5, PCV
<i>Dx + abl²/+</i>	Loss	71% (N=44)	L4, occasionally L5, PCV
<i>Abl</i>	Gain	52% (N=65)	PCV
<i>Abl^{K417N}</i> (kinase dead)	Gain	4% (N=48)	PCV
<i>Abl + Su(dx)</i>	Gain	85% (N=83)	PCV, ACV, L4, L5, interveins
<i>Su(dx)^{RNAi}</i>	-	0% (N=30)	-
<i>Abl + Su(dx)^{RNAi}</i>	Gain	18% (N=60)	PCV
<i>Nedd4^{RNAi}</i>	-	0% (N=40)	-
<i>Abl + Nedd4^{RNAi}</i>	Gain	24% (N=54)	PCV
<i>Nedd4S</i>	Gain	9% (N=34)	PCV
<i>Nedd4Lo</i>	Gain	90% (N=42)	L4, L5, ACV, PCV

Table 2.1. Penetrance of vein pattern phenotypes upon manipulation of Abl and other Notch endosomal trafficking regulators. All genetic perturbations, except the *Notch⁵⁴¹⁹/+;abl²/+* interaction, used *engrailed-Gal4* to drive expression of UAS-transgenes in the posterior compartment of developing pupal wings (see methods). Abl knockdown (*Abl^{DegradFP}*) was performed in an *abl^{GFP}/abl²* background. ACV/PCV, anterior/posterior cross vein.

zooming into the L3/L4 region (Fig. S2.1A,A'), small patches of faint reporter expression were

detected in the presumptive vein cells (blue arrowheads) and in intervein territory (red arrowheads). This “noisy” signaling activity was resolved by 20hr APF (Fig. 2.1E,E’). The reporter was also detected at the wing margin at all stages examined, with no obvious change upon *abl* loss (Figs. 2.1 and S2.1).

In comparison to wildtype, wings from *abl¹/abl²* null mutants (*abl^{null}*) exhibited stronger NRE-GFP signal in the presumptive L3/L4 regions at 16h and 18hr APF (Figs. 2.1D and S2.1A-B’). At 18h and 20h APF, the pattern of NRE-GFP appeared “noisier” than in wildtype, with broader-ranged signaling activity in flanking intervein cells and more frequent ectopic signaling events in vein and intervein regions (Figs. 2.1F,F’, S2.1A-C). This signaling “noise” was resolved by 24hr APF, after which the pattern and intensity of NRE-GFP reporter expression matched that of comparably staged wildtype wings (Fig. S2.1D-G). Together these results argue that Abl limits Notch signaling activity during early stages of pupal wing vein patterning.

Starting at 32hr APF, gaps in the NRE-GFP pattern indicative of truncated vein structures were occasionally found in *abl^{null}* wings (5%, N=40; Fig. 2.1G-H’). The frequency of these gaps increased over time (21% at 38h APF, N=78; Fig. S2.1H-J), consistent with the 29% observed in Abl knockdown adult wings (Table 1). Therefore, endpoint-looking vein pattern defects associated with loss of Abl can be observed as early as the developmental stage when the mature pattern first appears in wildtype (~32h APF).

While the increased and ectopic NRE-GFP signals detected in early stage *abl^{null}* wings and the final wing vein gaps both supported a Notch gain-of-function interpretation, the loss of reporter expression in flanking intervein cells between 32h-38h APF (Figs. 2.1H’, S2.1I,J) at first seemed contradictory. However, consideration of the Delta-Notch negative feedback loop that

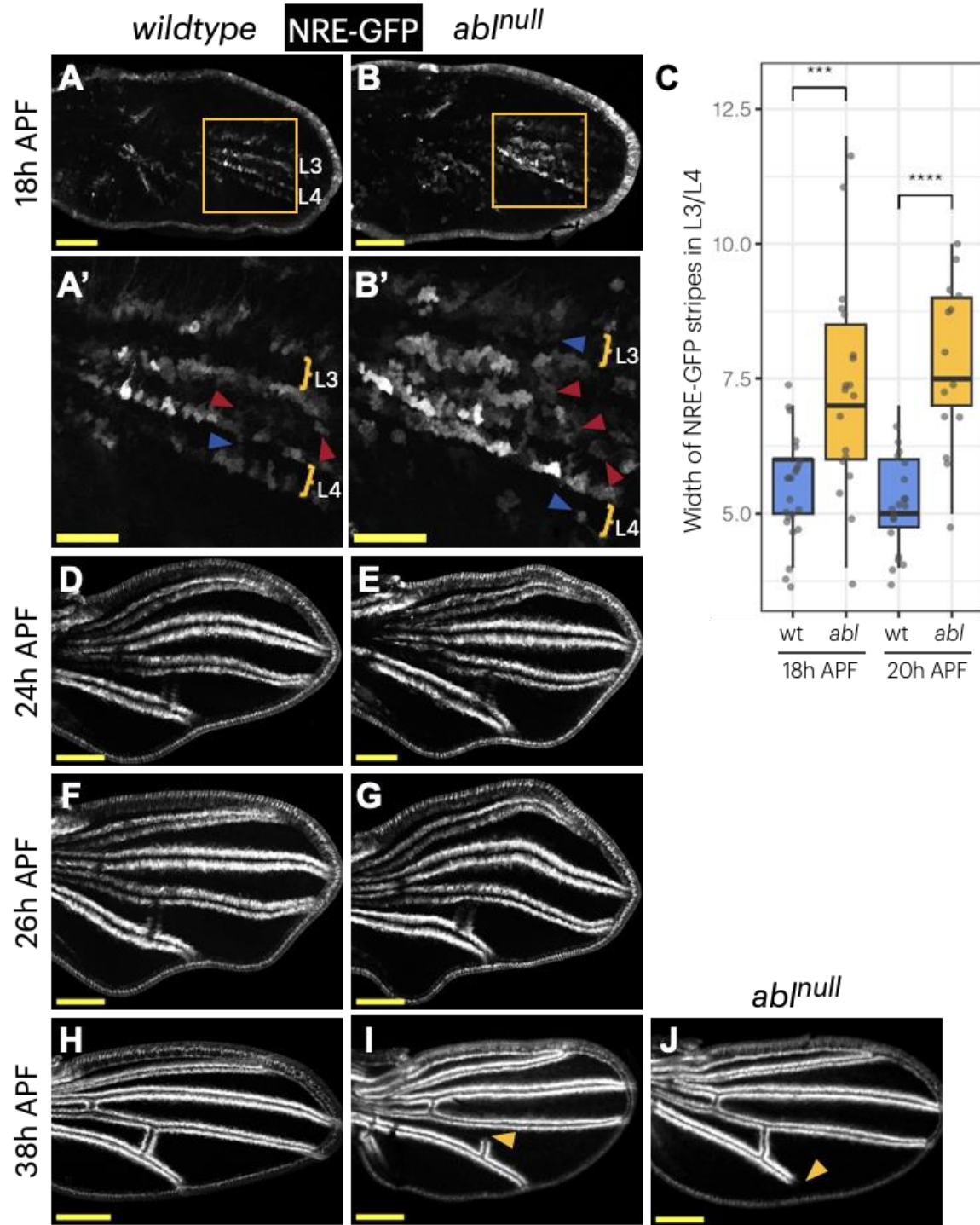


Figure S2.1. Abl is required to limit Notch signaling during wing vein patterning. (A,B,D-J) Maximal projections of wildtype and *abl*^{null} pupal wings. (A,B) 18h APF, with zoomed insets in (A',B'). (D-E) 24h APF. (F-G) 26h APF. (H-K) 38h APF. Blue and red arrowheads mark ectopic signaling events at intervein and vein cells, respectively. Yellow

arrowheads mark a loss of signaling event. Scale bars = 100µm. (C) Plot showing the maximum number of cell rows expressing NRE-GFP reporter in L3/L4 veins at 18h and 20h APF. Each dot represents the measurement from an individual wing. Sample size (# of wings evaluated): wt 18h (N=23), *abl* 18h (N=19), wt 20h (N=20) and *abl* 20h (N=14). A t-student means comparison test was performed and p-values <0.05 (*), <0.01 (**), <0.001(***) and <0.0001 (****) are indicated.

patterns the vein-intervein boundary (de Celis et al., 1997; Huppert et al., 1997) offers a logical explanation. According to the model, excessive Notch signaling in presumptive vein cells should abrogate Delta ligand expression and vein fate in those cells, which in turn should reduce ligand-induced Notch signaling in the adjacent cells. If sustained, this should manifest as gaps in NRE-GFP expression and ultimately vein loss. Although in-depth study of Notch-Delta feedback temporal dynamics will be required to confirm this explanation, as predicted by the model, *abl^{null}* vein gaps at times had ectopic NRE-GFP reporter expression within the provein region (Fig. 2.1H', yellow arrowhead). Altogether, our analysis of Abl loss-of-function phenotypes argues that Abl represses Notch signaling during wing vein patterning.

Loss of Abl results in endocytic accumulation of Notch

To elucidate the cellular-level consequences of Abl loss that might increase Notch signaling, we compared the subcellular distribution of Notch protein in wildtype versus *abl^{null}* pupal wings. As previously described (Huppert et al., 1997), in wildtype 32h APF wings, Notch is expressed ubiquitously, with the highest levels of expression (Fig. 2.2A) and activation (Fig. 1G) in cells immediately flanking the presumptive veins. Subcellularly, Notch appeared enriched at the plasma membrane and in the cytoplasm (Fig. 2.2A'-A''), with a punctate distribution that

was particularly obvious in the vein regions (arrowheads in Fig. 2.2A').

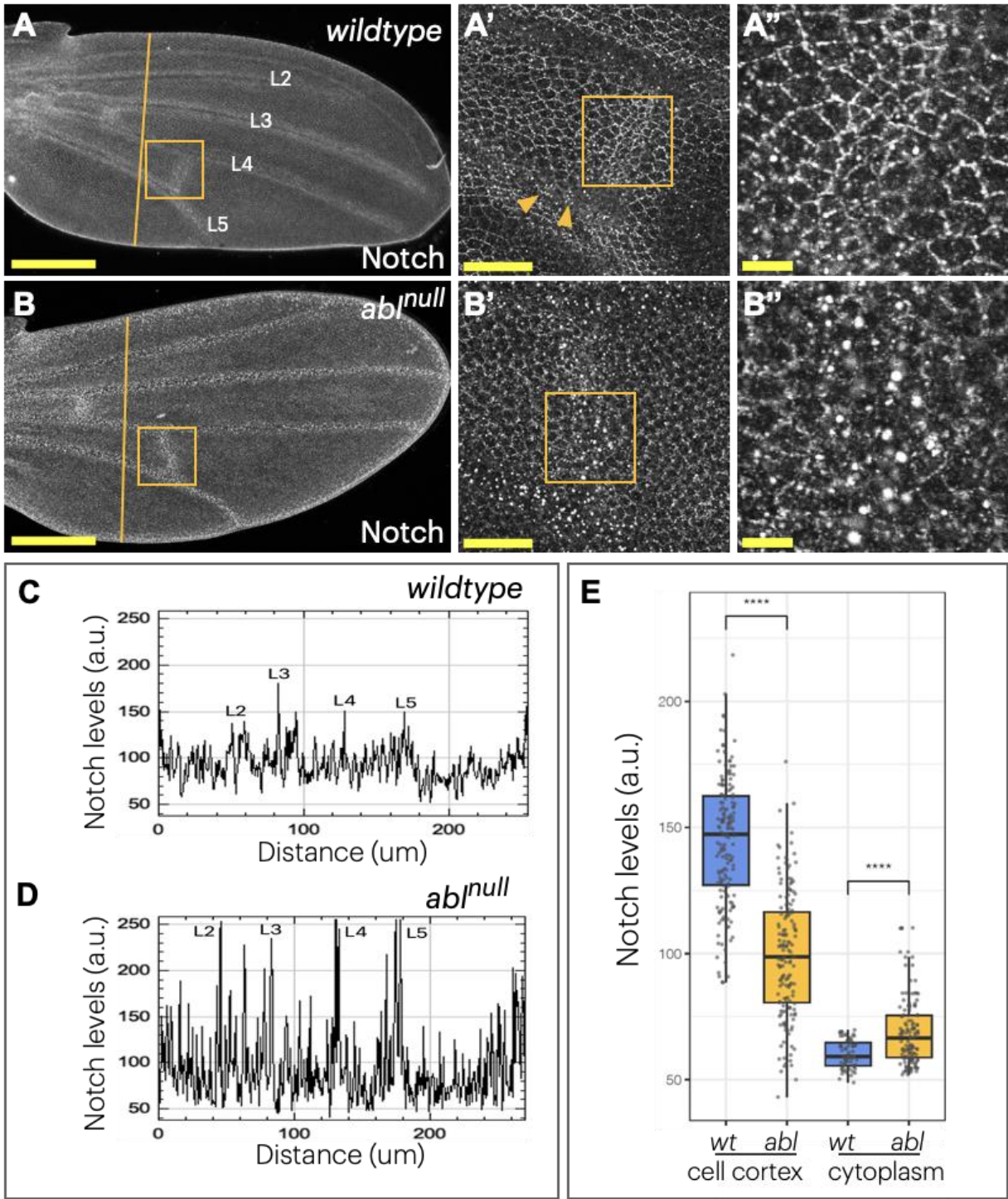


Figure 2.2. Loss of *abl* increases Notch levels and endocytic accumulation.

(A-B'') Maximal projections showing Notch protein expression detected by indirect immunofluorescence with anti-Notch in 32h APF wildtype and *abl^{null}* pupal wings, with zoomed views of the PCV region in (A',A'',B',B''). Scale bars = 100μm (A, B), 20μm (A', B') or 5μm (A'', B''). (C,D) Plots of Notch fluorescent intensity values across the anterior-posterior axis of the wing blade (yellow lines in (A,B)). (E) Quantification of Notch levels at the cell cortex and cytoplasm of cells shown in (A'') and (B'') are plotted in (E). Each dot represents the individual measurement of a cell border (N=158 in wt and N=156 in *abl*) or a cell cytoplasm (N=70 in wt and N=80 in *abl*). A t-student means comparison test was performed and p-values <0.05 (*), <0.01 (**), <0.001(***) and <0.0001 (****) are indicated.

In agreement with our prior work (Xiong et al., 2013), Notch levels appeared elevated in *abl^{null}* wings, with the most pronounced increase in the vein regions (Figs. 2.2B-D). Higher magnification revealed a reduction in membrane-localized Notch and an increase in cytoplasmic Notch (Figs. 2.2B'', 2.2E, S2.2A-C). The enrichment of Notch in cytoplasmic puncta was particularly striking in the vein regions (Figs. 2.2B',B''). Side by side comparison of *abl^{null}* and wildtype tissue in somatic mosaics confirmed these differences (Fig S2.2D,D').

The localization and turnover of membrane proteins is largely regulated by endocytosis (Cullen & Steinberg, 2018; MacGurn et al., 2012), and previous studies have shown that perturbing Notch flux through the endocytic pathway can impact signaling (Alfred & Vaccari, 2018; Palmer & Deng, 2015; Vaccari et al., 2008). Therefore, to assess whether altered patterns of residence in specific endocytic compartments could account for the increased Notch activity observed in *abl^{null}* wings, we examined the localization of Notch relative to three endomembrane compartments: upstream endocytic vesicles (Eps15); early endosomes (Hrs); and late endosomes (Rab7) (Dunst et al., 2015; Raiborg et al., 2002; Shearer & Petersen, 2019; Tebar et al., 1996). In comparison to wildtype, in *abl^{null}* wing cells, Notch enrichment was significantly increased at Eps15⁺ and Rab7⁺ structures, and significantly decreased at Hrs⁺ structures (Fig. 2.3A-G). We

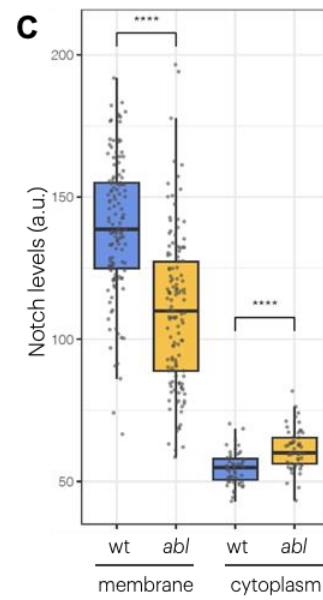
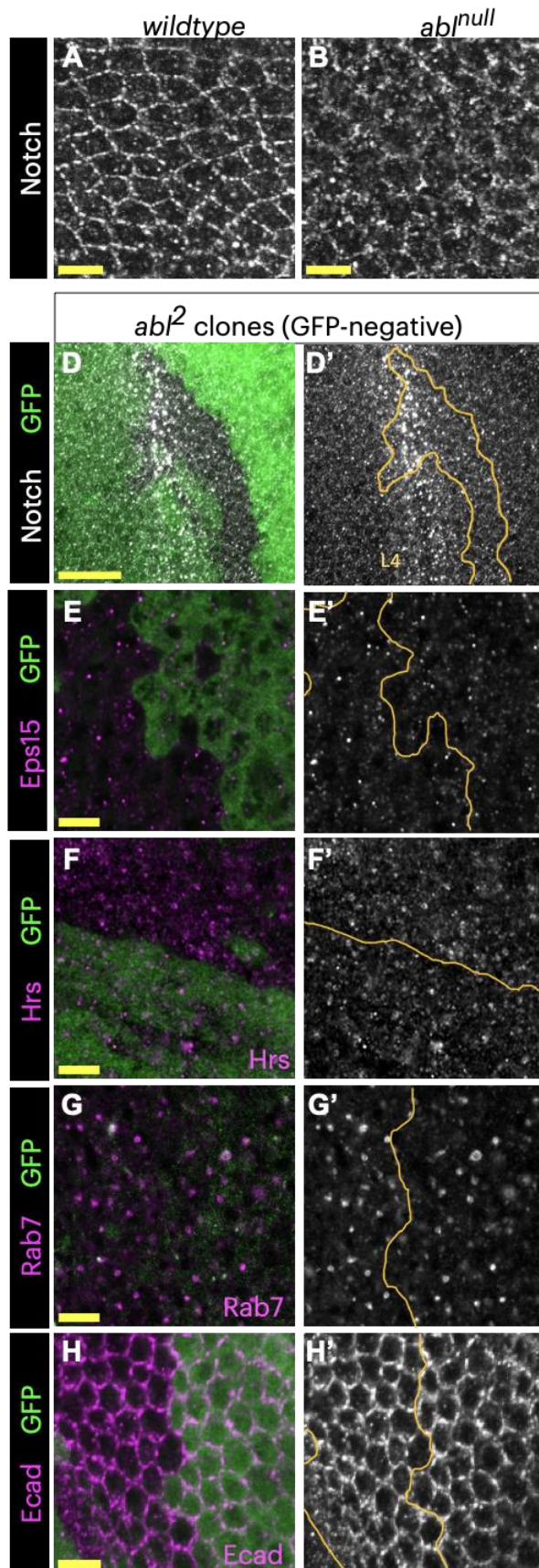


Figure S2.2. Loss of *abl* selectively perturbs Notch endocytic trafficking

(A-B) Maximal projections of wildtype and *abl^{null}* pupal wing tissue at 32h APF. Panels shown anti-Notch immunostaining in intervein cells between L3 and L4, proximal to the PCV. Scale bars = 5µm. (C) Quantification of Notch fluorescent intensity at the cortex and cytoplasm of cells shown in (A) and (B). Each dot represents the individual measurement of a cell border (N=130 in wt and N=129 in *abl*) or a cell cytoplasm (N=52 in wt and N=53 in *abl*). A t-student means comparison test was performed and p-values <0.05 (*), <0.01 (**), <0.001(***) and <0.0001 (****) are indicated. Results are similar to those measured at vein areas (Fig. 2E). (D-H) 32hr APF pupal wing tissue with *abl²* null clones marked by lack of GFP (green) and stained with the indicated marker (magenta or white). Yellow lines in D'-H' mark the clonal boundaries. (D) Notch, (E), Eps15, (F) Hrs, (G) Rab7, (H) E-cad. A maximal projection is shown in (D) and representative single confocal sections are shown in (E-H). Scale bars = 20µm (D) or 5µm (E-H).

did not detect obvious differences in overall intensity or pattern of these three markers in wildtype versus *abl* null clones (Figs. S2.2E-G'), nor in the subcellular distribution of E-cadherin (Fig. S2.2H,H'), a junctional adhesion receptor subject to endocytic regulation in many epithelial tissues (Brüser & Bogdan, 2017; De Beco et al., 2009, 2012; Kowalczyk & Nanes, 2012). This suggests that Abl is not a general endocytic pathway modulator, and that Abl-mediated regulation specifically impacts Notch trafficking.

Prior studies of Notch endocytic regulation offer insight into which of the altered patterns of Notch endocytic residence might underly the gain-of-function wing vein phenotypes associated with Abl loss. While accumulation of Notch in upstream endocytic vesicles has been linked to decreased signaling (Vaccari et al., 2008), accumulation in Hrs⁺ compartments has been linked to increased signaling (T. Mukherjee et al., 2011; Vaccari et al., 2008; Vaccari & Bilder, 2005). Therefore, although the respectively increased and decreased localization to Eps15⁺ and Hrs⁺ compartments reflect aspects of the Notch trafficking/turnover defects caused by Abl loss, neither would be predicted to promote Notch activity. In contrast, Rab7-marked late endosomes

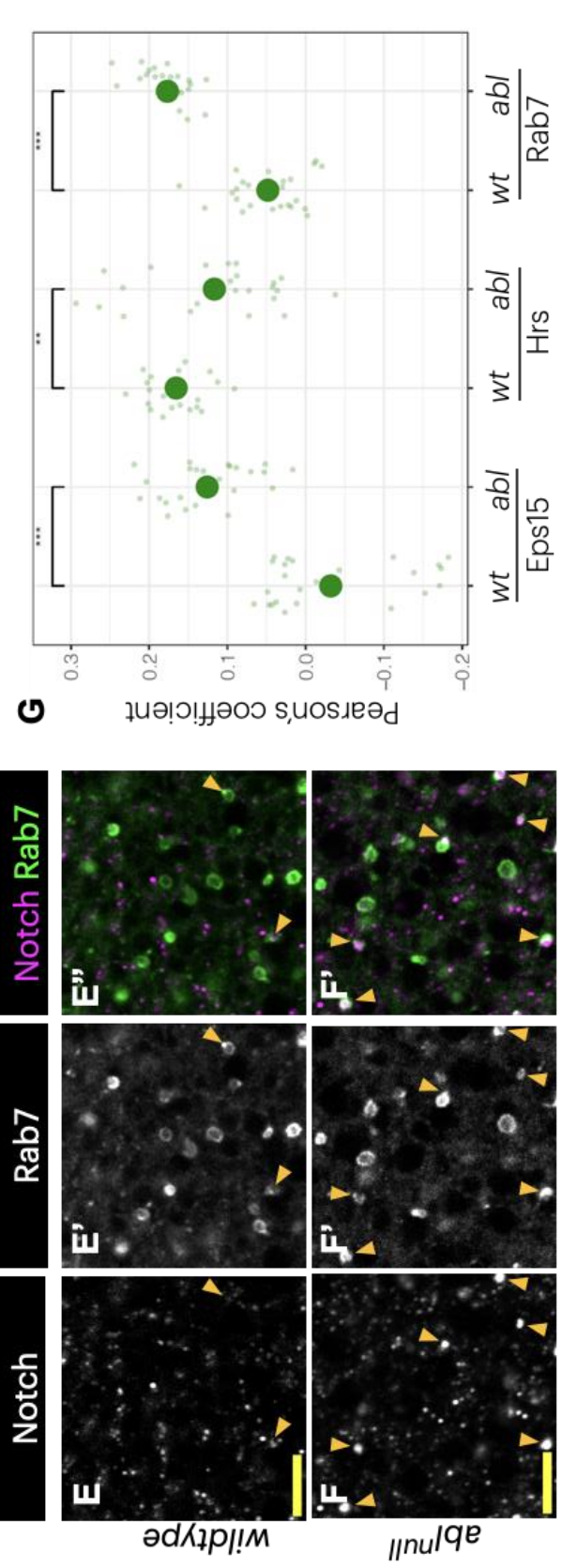
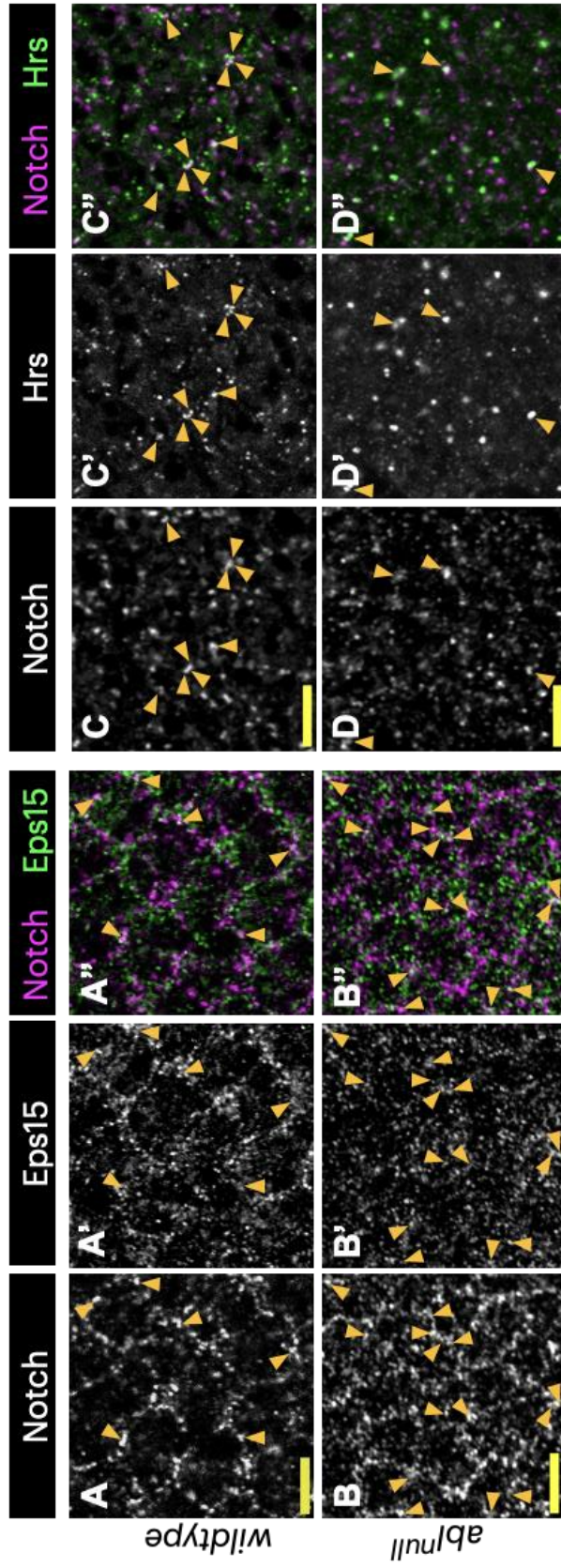


Figure 2.3. Loss of Abl alters Notch colocalization with endosomal markers.

(A-F) Single confocal sections showing Notch and endosomal markers in 32h APF wildtype and *abl^{null}* wings. (A,B) anti-Notch and anti-Eps15. (C,D) anti-Notch and anti-Hrs. (E,F) anti-Notch and endogenous Rab7-EYFP. Yellow arrowheads point to examples of Notch-endosomal marker colocalization. Scale bars = 5µm. (G) Pearson's coefficient-based co-localization analysis. Each dot represents the Pearson's coefficient from a group of approximately 100 cells in an individual wing. 24 different wings were evaluated per condition. A t-student means comparison test was performed and p-values <0.05 (*), <0.01 (**), <0.001(***) and <0.0001 (****) are indicated.

(LEs) and multivesicular bodies (MVBs) are competent compartments for Notch activation (Alfred & Vaccari, 2018; Palmer & Deng, 2015; Schnute et al., 2022; Shimizu et al., 2014, 2024; Vaccari et al., 2008; M. Wilkin et al., 2008). Therefore, the increased association with Rab7⁺ structures suggested a potential mechanism for Abl-mediated regulation of Notch activity.

Abl promotes Notch internalization into the late endosome lumen to limit signaling

The specific topology of Notch within LEs determines signaling competence (Schnute et al., 2022; Shimizu et al., 2014, 2024; Vaccari et al., 2008; M. Wilkin et al., 2008). When Notch localizes to the limiting membrane of the organelle, ICD cleavage and cytoplasmic release can activate nuclear signaling. In contrast, internalization of Notch into the LE lumen sequesters the NICD from the cytoplasm and prevents signaling. Therefore, if the increase in Notch localization to Rab7⁺ compartments in *abl^{null}* cells activates signaling, Notch should be preferentially enriched at the LE limiting membrane.

Because the small size of wing cells did not offer the resolution needed to distinguish clearly between membrane and luminal domains of Rab7⁺ compartments, we turned to *Drosophila* S2 cells, a cultured cell system the field has used to connect Notch cell biology with

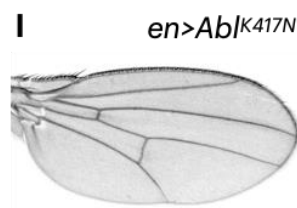
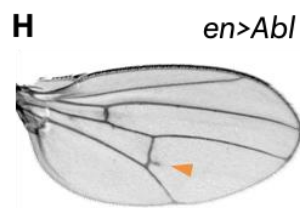
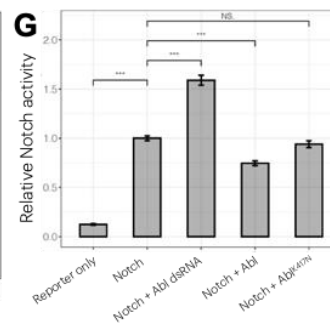
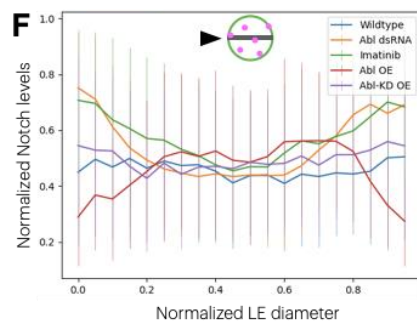
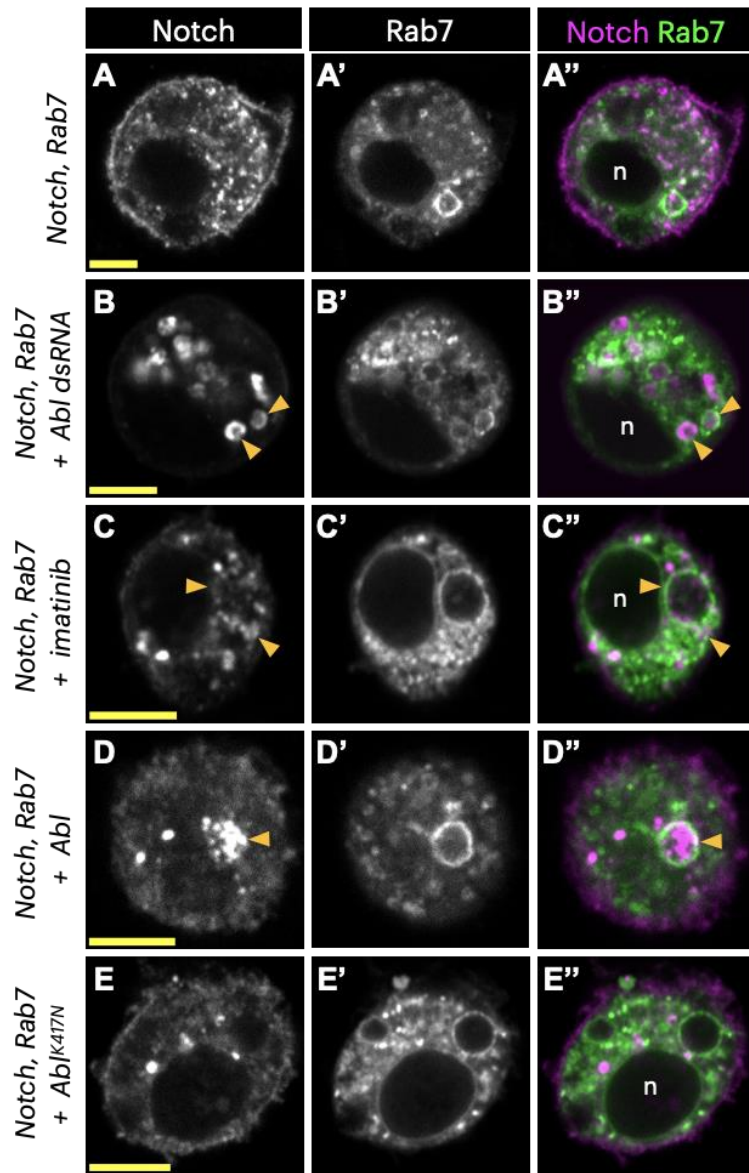


Figure 2.4. Abl promotes Notch internalization into the late endosome lumen to limit signaling. (A-E) Single confocal sections of representative S2 cells showing Notch (anti-Notch indirect immunofluorescence, magenta) and Rab7-EYFP (green). “n” marks the cell nucleus. Transfections and treatments shown: (A) Notch, (B) Notch + Abl dsRNA, (C) Notch + imatinib mesylate (Gleevec), (D) Notch + Abl, and (E) Notch + Abl^{K417N} (kinase dead). Scale bars = 5µm. (F) Plot showing profiles of Notch levels across late endosome diameters in conditions in (A-E), as depicted by the black arrowhead in cartoon schematic. A 0° cross-sectional line was used to measure Notch fluorescent intensity across LEs. Both Notch levels and LE diameters were normalized. Sample size (# of LEs evaluated): Notch (N=38), Notch + Abl dsRNA (N=32), Notch + imatinib (N=50), Notch + Abl (N=27), Notch + Abl^{K417N} (N=52). (G) Relative Notch activity in manipulations shown in (A-E). A t-student means comparison test was performed and p-values <0.05 (*), <0.01 (**), and <0.001 (***) are indicated. (H-I) en>Abl and en>Abl^{K417N} adult wings, respectively. Orange arrowhead in (H) points to an ectopic vein at the PCV.

pathway activity (Fehon et al., 1990; Shimizu et al., 2014; Zacharioudaki & Bray, 2014). To validate the system, we first confirmed that Abl limits Notch signaling output. Since S2 cells express Abl endogenously (Y. Zhang et al., 2010) but do not express Notch (Fehon et al., 1990), we transiently transfected Notch cDNA with or without Abl dsRNA, and then assessed signaling output using the well-validated NRE>luciferase transcriptional reporter (J. Li et al., 2014; Zacharioudaki & Bray, 2014). Abl dsRNA led to significantly higher levels of Notch signaling in comparison to transfection with a control dsRNA (Figs. 2.4G, S2.3F), indicating that Abl negatively regulates Notch activity in S2 cells.

We next asked if the cellular response to Abl knockdown was comparable to that observed in the wing by examining Notch subcellular distribution in cells expressing Rab7-YFP (Dunst et al., 2015). Consistent with previous descriptions (Fehon et al., 1990; Lake et al., 2009; A. Mukherjee et al., 2005; Schnute et al., 2022; Shimizu et al., 2014, 2017), in cells transfected with Notch alone, and therefore in the presence of endogenous Abl, Notch protein accumulated at the cell cortex and in the cytoplasm, often in small punctate structures (Fig. 2.4A), some of

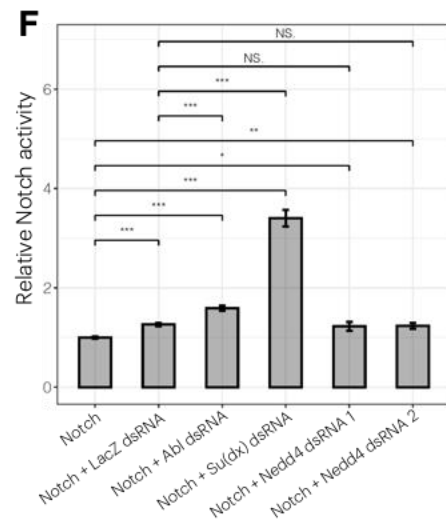
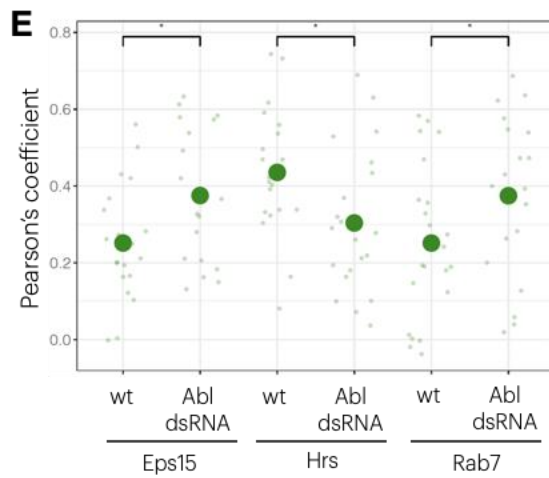
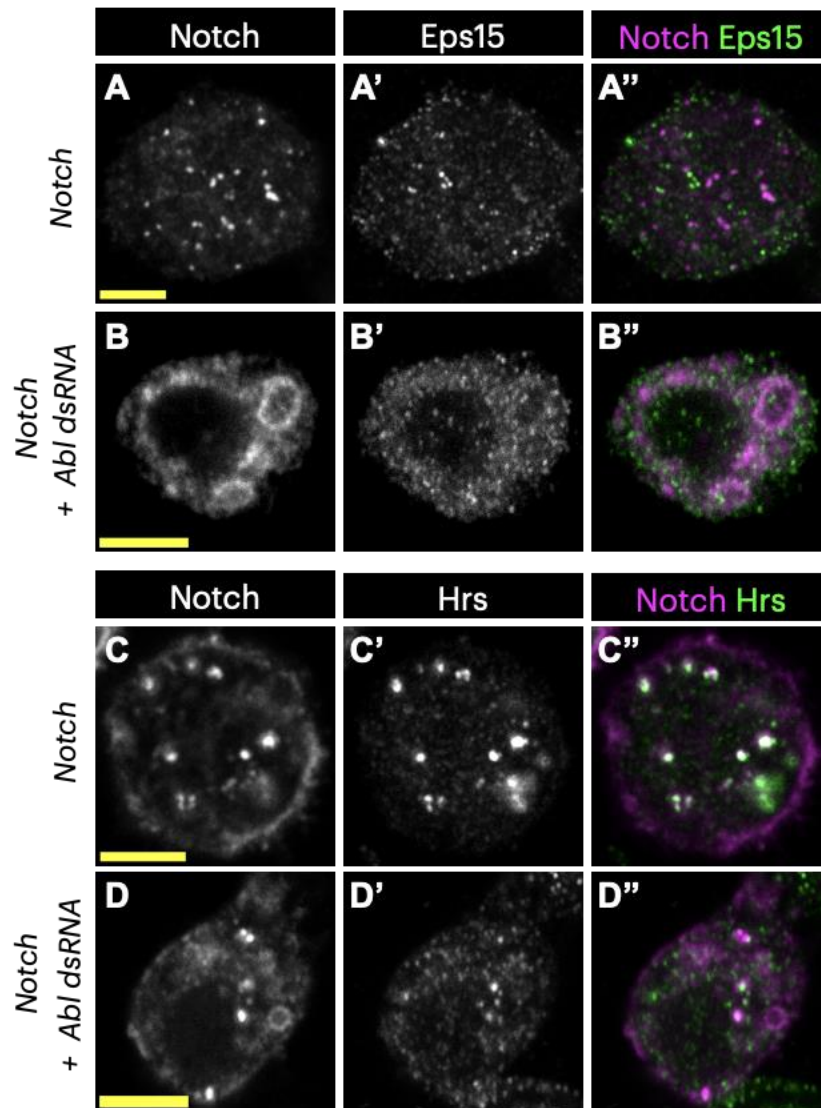


Figure S2.3. Abl knockdown alters Notch endocytic distribution in S2 cells similarly to *abl* loss in wings.

(A-D) Co-imaging of Notch and endosomal markers in wildtype and Abl dsRNA-treated S2 cells. Representative single confocal sections are shown. Transfections: Notch in (A) and (C), and Notch + Abl dsRNA in (B) and (D). Stainings: Notch and Eps15 in (A-B), and Notch and Hrs in (C-D). Scale bars = 5µm. (E) Pearson's coefficient-based co-localization analysis of Notch and endosomal markers shown in (A-D). Each dot represents the Pearson's coefficient from an individual S2 cell, with an average of 20 evaluated per condition. A t-student means comparison test was performed and p-values <0.05 (*), <0.01 (**), <0.001 (***) and <0.0001 (****) are indicated. (F) Relative Notch activity (NRE>luciferase reporter) produced by co-transfection of dsRNAs targeting Notch trafficking regulators, in comparison to co-transfection of LacZ dsRNA or transfection of Notch alone. A t-student means comparison test was performed and p-values <0.05 (*), <0.01 (**) and <0.001 (***) are indicated.

which were Rab7⁺ (Fig. 2.4A',A''). Within Rab7⁺ compartments, Notch could be found both at the membrane and in the lumen, with no preferential enrichment in either domain (Fig. 2.4F). Abl knockdown produced a visually striking shift, with reduced cortical Notch and pronounced enrichment in ring-shaped Rab7⁺ cytoplasmic foci (Figs. 2.4B, S2.3E). Notch residence at other endosomal compartments was also impacted, with trends similar to those in the wing (Fig. S2.3A-E, compare to Fig. 2.3G). Treatment with imatinib mesylate, a specific inhibitor of Abl kinase activity (T. Hunter, 2007), similarly shifted Notch subcellular localization from the cell cortex into cytoplasmic foci (Fig. 2.4C-C''). The predominant enrichment of Notch at the outer membrane of Rab7⁺ LEs was significant for both Abl loss-of-function manipulations (Figs. 2.4B'',C'',F).

Since internalization of Notch into the luminal space of LEs/MVBs targets the receptor for lysosomal degradation (Vaccari et al., 2008; Shimizu et al., 2014; Schnute et al., 2022; M. Wilkin et al., 2008; Shimizu et al., 2024; Cornell et al., 1999), Abl might limit Notch signaling by promoting Notch transit into the lumen of these compartments. To test this, we overexpressed

Abl in S2 cells and then assessed Notch topology within Rab7⁺ LEs and NRE signaling output. Abl overexpression significantly shifted Notch localization to a predominantly luminal distribution in these compartments (Figs. 4D-D'',F) and reduced signaling output (Fig. 2.4G). In contrast, overexpression of a well-validated kinase-dead Abl mutant (Abl^{K417N}) (O'Donnell & Bashaw, 2013; Wills et al., 1999; Xiong et al., 2009) did not promote Notch internalization into the LE lumen or attenuate signaling activity (Figs. 2.4E-G), further confirming the necessity of Abl's kinase activity with respect to Notch regulation (Fig. 2.4C-C''),. We corroborated these findings in the wing, using the same spatiotemporal control strategy described for the Abl knockdown analysis (Fig 2.1) to show that overexpression of Abl, but not of kinase-dead Abl^{K417N}, induced ectopic veins (Fig. 2.4H,I and Table 1). Altogether, our results suggest that, in a kinase activity-dependent fashion, Abl promotes Notch relocation from the membrane into the lumen of LEs, thereby negatively regulating Notch signaling by limiting its activation at the LE membrane.

Abl requires Su(dx) to regulate Notch trafficking and signaling

Targeting of Notch to different LE domains is orchestrated by E3 ubiquitin ligases (Sakata et al., 2004; M. B. Wilkin et al., 2004; Yamada et al., 2011; Shimizu et al., 2014; Schnute et al., 2022; Cornell et al., 1999; Hori et al., 2004; M. Wilkin et al., 2008; Hori et al., 2011; Shimizu et al., 2024). For example, while Notch is stabilized at the LE membrane by the activity of Dx (Fig. S2.4A,D), Su(dx) counters this by promoting Notch luminal internalization (Fig. S2.4B,D). These opposing inputs respectively promote and restrict Notch signaling during

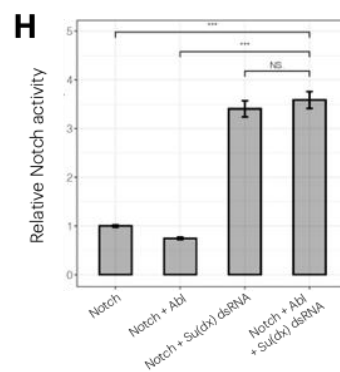
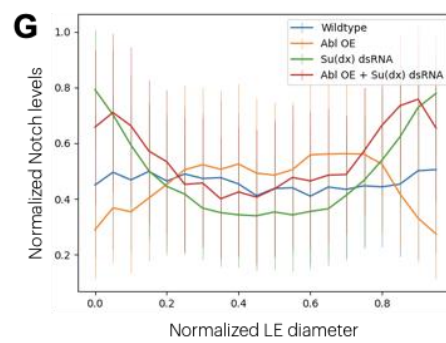
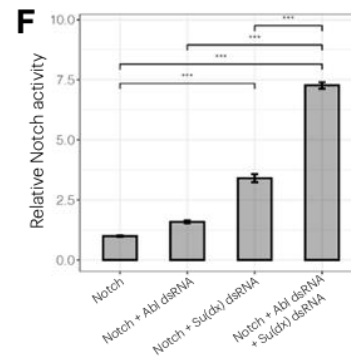
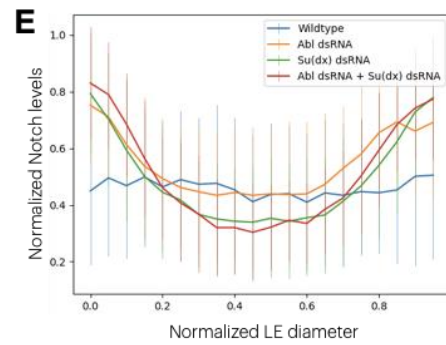
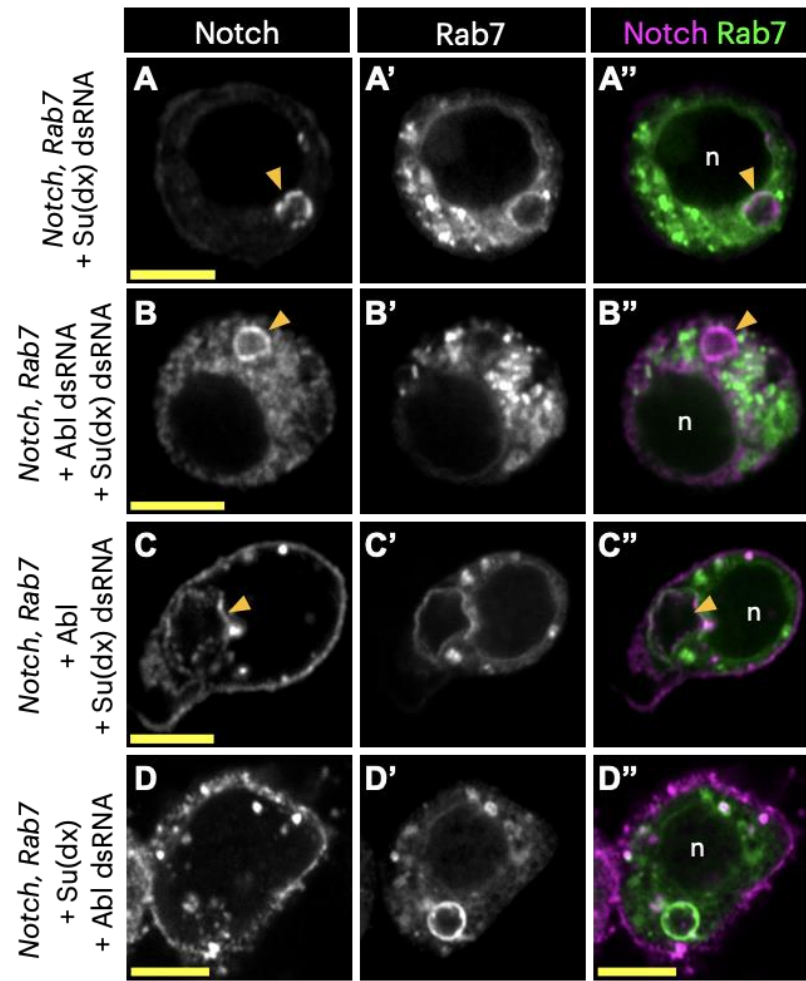


Figure 2.5. Abl requires Su(dx) to internalize Notch into late endosomes and attenuate signaling. (A-D) Single confocal sections of representative S2 cells showing Notch (indirect immunofluorescence) and Rab7-EYFP. “n” marks the cell nucleus. Transfections shown: (A) Notch + Su(dx) dsRNA, (B) Notch + Abl dsRNA + Su(dx) dsRNA, (C) Notch + Abl + Su(dx) dsRNA, and (D) Notch + Su(dx) + Abl dsRNA. Scale bars = 5µm. (E,G) Plot showing profiles of Notch levels across late endosome diameters in the indicated manipulations. Sample size (# of LEs evaluated): Notch (N=38), Notch + Abl dsRNA (N=32), Notch + Su(dx) dsRNA (N=68), Notch + Abl dsRNA + Su(dx) dsRNA (N=53), Notch + Abl (N=27), Notch + Abl + Su(dx) dsRNA (N=27). (F,H) Relative Notch activity in the indicated manipulations. A t-student means comparison test was performed and p-values <0.05 (*), <0.01 (**) and <0.001 (***) are indicated.

wing development (Cornell et al., 1999; Fostier et al., 1998; Matsuno et al., 1995, 2002; Schnute et al., 2022; Shimizu et al., 2014; Xu & Artavanis-Tsakonas, 1990) (Figs. S2.4H,I and Table 1).

Given the similarities between Abl and Su(dx) with respect to Notch, we examined genetic interactions between them to probe potential pathway relationships, using signaling output in S2 cells as a quantitative metric. As expected, dsRNA-mediated depletion of Su(dx) increased Notch localization to the membrane of LEs (Figs. 2.5A,E) and upregulated signaling activity (Fig. 2.5F). Co-transfection of Abl dsRNA further increased signaling (Figs. 2.5B,E,F), suggesting a cooperative relationship between Abl and Su(dx) in the LE regulation of Notch. Supporting this, co-overexpression of Abl and Su(dx) led to a greater reduction in signaling than with either Abl or Su(dx) alone, and to the expected Notch LE luminal enrichment (Figs. S2.4C-E). Such functional synergy could result from Abl and Su(dx) either working together in the same pathway or providing parallel inputs that converge on Notch.

To assess the epistatic relationship, we tested whether Su(dx) is required for Abl-mediated regulation of Notch, or vice versa. Depletion of Su(dx) completely masked the effects of Abl overexpression, producing an enrichment of Notch at LE membranes and an increased

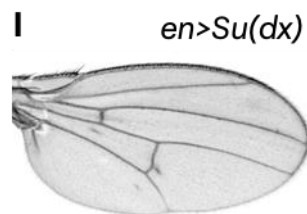
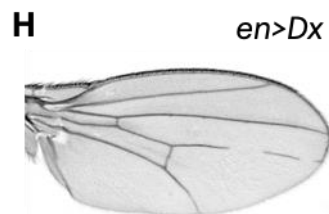
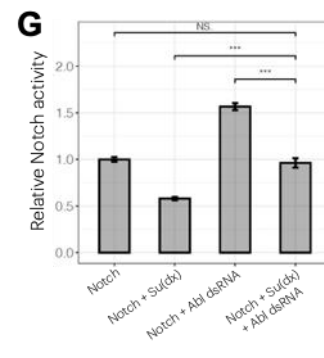
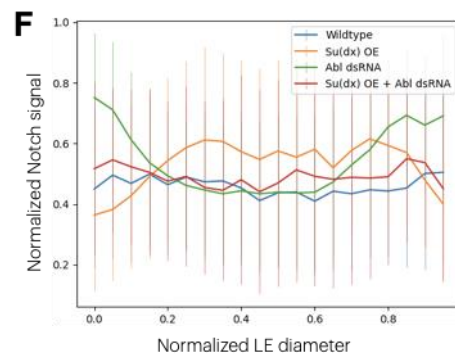
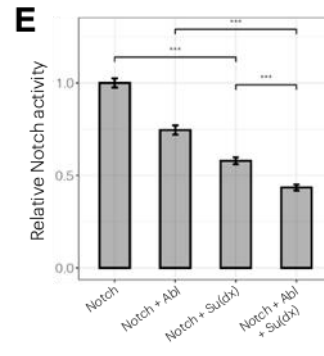
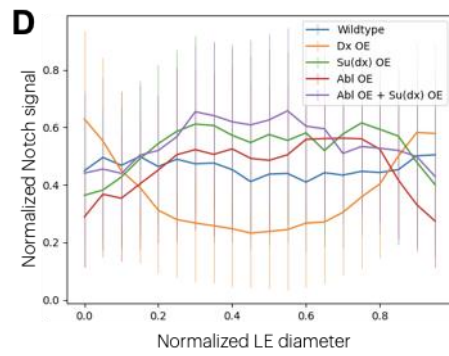
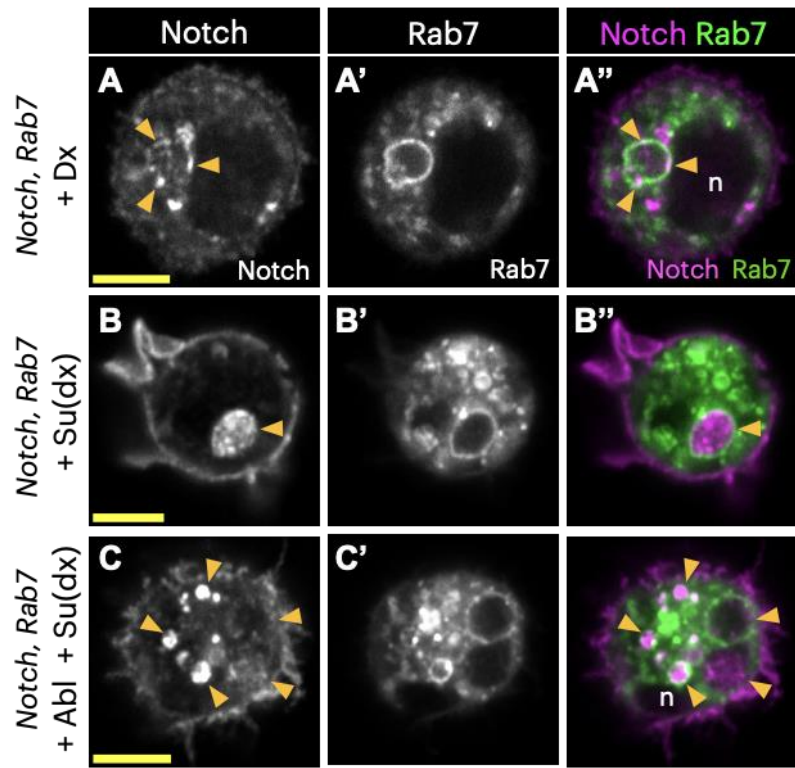


Figure S2.4. Abl and Su(dx) cooperate to promote Notch internalization into the late endosomal lumen.

(A-C) Single confocal sections of representative S2 cells showing Notch (indirect immunofluorescence) and Rab7-EYFP. “n” marks the cell nucleus. Transfections shown: (A) Notch + Dx, (B) Notch + Su(dx), and (C) Notch + Abl + Su(dx). Scale bars = 5µm. (D,F) Plot showing profiles of Notch levels across late endosome diameters in the indicated manipulations. Sample size (# of LEs evaluated): Notch (N=38), Notch + Dx (N=68), Notch + Su(dx) (N=51), Notch + Abl (N=27), Notch + Abl + Su(dx) (N=25), Notch + Su(dx) + Abl dsRNA (N=47). (E,G) Relative Notch activity in the indicated manipulations. A t-student means comparison test was performed and p-values <0.05 (*), <0.01 (**), and <0.001(***) are indicated. (H-I) en>Dx and en>Su(dx) adult wings, respectively.

signaling output comparable to that produced by Su(dx) dsRNA alone (Figs. 2.5C,G,H). The epistatic relationship suggested that Abl-mediated regulation of Notch is dependent on Su(dx). Performing the experiment in the other direction, depletion of Abl in Su(dx)-overexpressing cells restored the balance of Notch LE luminal versus membrane localization and signaling output to that typical of cells transfected with Notch alone (Figs. 2.5D, S2.4F,G). The simplest interpretation of this mutual suppression is that the activity of Su(dx) on Notch is not mediated by Abl, consistent with Abl working upstream of Su(dx).

Suggesting a comparable Abl-Su(dx) synergy and epistatic relationship *in vivo*, the penetrance of ectopic veins resulting from co-overexpression of Abl and Su(dx) exceeded that of each individual manipulation (Table 1). Moreover, while Su(dx) RNAi did not modify the vein pattern in an otherwise wildtype background (Table 1), it substantially suppressed the ectopic vein formation induced by Abl overexpression (Table 1). Altogether, our results suggest that Abl and Su(dx) cooperatively regulate Notch trafficking and signaling, with Abl function dependent on Su(dx).

The NICD's PPxY motif integrates Abl, Su(dx) and Nedd4Lo-mediated regulation of Notch trafficking and signaling

Given the necessity of Abl's kinase activity in the regulation of Notch trafficking and signaling (Figs. 2.4C,E,F) and the presence of a tyrosine residue, Y2328, in the PPxY motif within the NICD that is recognized by Nedd4-family ligases (Jennings et al., 2007; Sakata et al., 2004; M. B. Wilkin et al., 2004), we hypothesized that this motif might integrate Abl and Nedd4-family regulatory inputs. Using an established *in vitro* kinase assay (Xiong et al., 2009), we found that Abl can phosphorylate GST-NICD but not GST alone (Fig. 2.6A). Mutation of the Y2328 tyrosine reduced phosphorylation of the GST-NICD^{Y2328F} substrate (Fig. 2.6A), indicating that the Notch PPxY tyrosine can be a direct target of Abl activity. The residual phosphorylation signal in GST-NICD^{Y2328F} was expected, as previous work has shown that Abl can phosphorylate other NICD tyrosine residues (Kannan et al., 2017).

To test the functional significance of the PPxY tyrosine, we introduced a tyrosine to phenylalanine substitution to generate Notch^{Y2328F}. When transiently transfected into S2 cells, the enrichment of Notch^{Y2328F} at the limiting membrane of LEs (Fig. 2.6B) was enhanced relative to wildtype Notch (Fig. 2.6F). Consistent with its enrichment at the LE membrane, signaling output from Notch^{Y2328F} was increased relative to wildtype Notch (Fig. 2.6G). If the PPxY tyrosine provides a molecular target for Abl-mediated antagonism of Notch signaling, then Notch^{Y2328F} localization and activity should be insensitive to Abl overexpression. As predicted, neither the increased localization of Notch^{Y2328F} to LE membranes nor its elevated signaling output were modified by co-transfection of Abl (Figs. 2.6C,F,G). Notch^{Y2328F} localization and activity was also insensitive to Su(dx) overexpression (Figs. 2.6D,F,G), arguing that the Notch Y2328 is

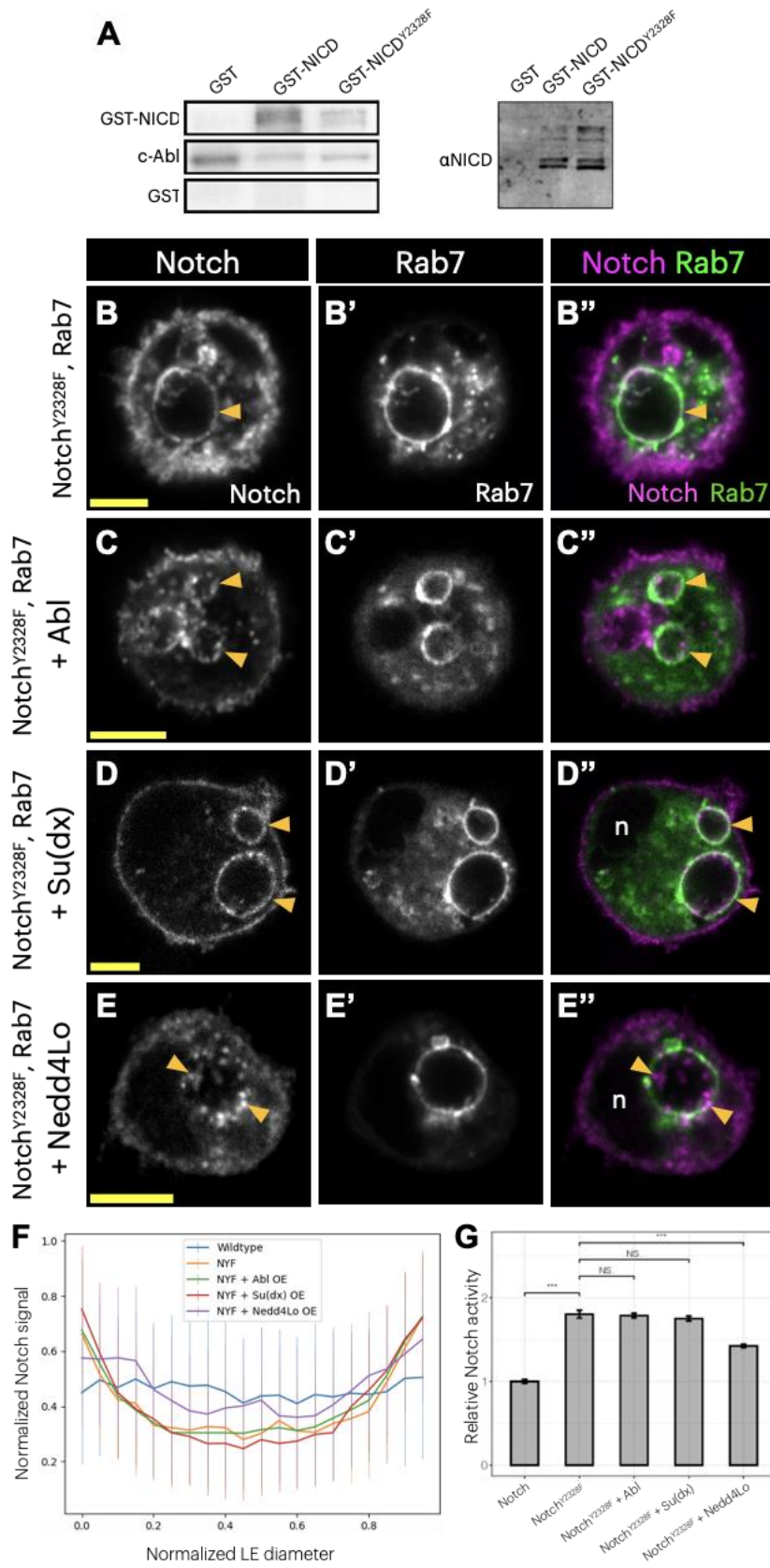


Figure 2.6. The NICD's PPxY tyrosine integrates Abl, Su(dx) and Nedd4Lo-mediated regulation of Notch trafficking and signaling at LEs.

(A) Left panel: *in vitro* kinase assay showing that recombinant c-Abl can phosphorylate the NICD, with some activity targeted to the Y2328 residue. Right panel: Western blot showing GST-NICD loading in samples evaluated on left panel. (B-E) Single confocal sections of representative S2 cells showing Notch (indirect immunofluorescence) and Rab7-EYFP. "n" marks the cell nucleus. Transfections shown: (B) Notch^{Y2328F}, (C) Notch^{Y2328F} + Abl, (D) Notch^{Y2328F} + Su(dx), and (E) Notch^{Y2328F} + Nedd4Lo. Scale bars = 5µm. (F) Notch signal profiles across late endosome diameters in manipulations in (B-E). Sample size (# of LEs evaluated): Notch (N=38), Notch^{Y2328F} (N=43), Notch^{Y2328F} + Abl (N=73), Notch^{Y2328F} + Su(dx) (N=38), Notch^{Y2328F} + Nedd4Lo (N=52). (G) Relative Notch activity in manipulations in (B-E). A t-student means comparison test was performed and p-values <0.05 (*), <0.01 (**) and <0.001 (***) are indicated.

required for both the Abl- and Su(dx)-mediated LE internalization and signaling modulation.

In addition to mediating regulation by Abl and Su(dx), the NICD PPxY motif has also been implicated in Nedd4-mediated ubiquitination and degradation of Notch (Sakata et al., 2004; M. B. Wilkin et al., 2004). Nedd4 encodes two major isoforms, Nedd4short (Nedd4S) and Nedd4long (Nedd4Lo), which have been suggested to have distinct genetic requirements and cell biological functions (Safi et al., 2016; Wasserman et al., 2019; Zhong et al., 2011). We were therefore curious whether Nedd4 might also participate in Notch LE regulation, perhaps with different isoform specificity. To assess this, we examined the effects of Nedd4 depletion or overexpression on Notch LE localization and signaling in S2 cells. Although neither Nedd4 dsRNA nor expression of the short isoform (Nedd4S) altered Notch signaling (Fig S2.3F), expression of the long Nedd4 isoform (Nedd4Lo) significantly decreased signaling (Fig. S2.5D) and enriched Notch localization to LE lumens (Figs. S2.5A-C). Consistent with the isoform-specificity uncovered in S2 cells, overexpression of Nedd4Lo strongly induced excessive vein formation in 90% of wings, a phenotype consistent with reduced Notch activity, while Nedd4S

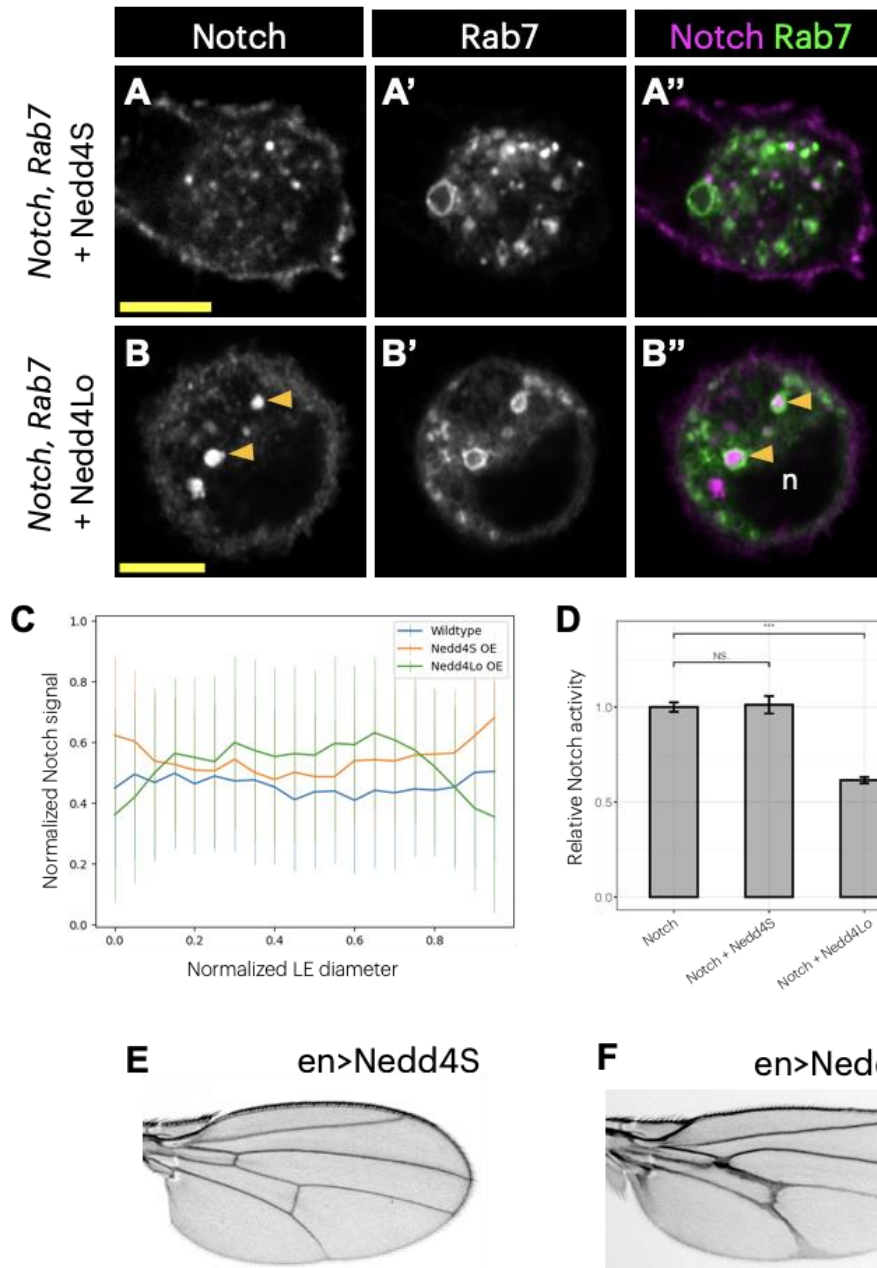


Figure S2.5. Nedd4Lo promotes Notch internalization into the late endosome lumen to attenuate signaling.

(A-B) Single confocal sections of representative S2 cells showing Notch (indirect immunofluorescence) and Rab7-EYFP. “n” marks the cell nucleus. Transfections shown: (A) Notch + Nedd4S, (B) Notch + Nedd4Lo. Scale bars = 5 μ m. (C) Plot showing profiles of Notch levels across late endosome diameters in (A-B). Sample size (# of LEs evaluated): Notch (N=38), Notch + Nedd4S (N=27), Notch + Nedd4Lo (N=36). (D). Relative Notch activity in manipulations in (A-B). A t-student means comparison test was performed and p-values <0.05

(*), <0.01 (**) and <0.001 (***) are indicated. (E-F) en>Nedd4S and en>Nedd4Lo adult wings, respectively.

only rarely perturbed pattern (Figs. S2.5E,F; Table 1). Nedd4 knockdown also suppressed the ectopic vein formation induced by Abl overexpression (Table 1), suggesting that, like Su(dx), it may act downstream of Abl.

Returning to the S2 cells, we asked if the PPxY motif was necessary for Nedd4Lo-mediated regulation of Notch LE localization and signaling. In contrast to our finding that Notch^{Y2328F} was resistant to regulation by both Su(dx) and Abl, Nedd4Lo transfection led to a significant (~20%) reduction in Notch^{Y2328F} signaling (Fig. 2.6G). Consistent with the reduced signaling, LE luminal Notch^{Y2328F} subcellular localization was readily detected upon Nedd4Lo transfection. Given that Nedd4Lo reduced wildtype Notch signaling by ~40% (Fig. S2.5D), the ~20% reduction in Notch^{Y2328F} signaling (Fig. 2.6G) suggests that Nedd4Lo regulates Notch via both PPxY-dependent and independent mechanisms. Together with the previous report of a partial requirement for the PPxY in the Nedd4-mediated ubiquitination of Notch (Sakata et al., 2004), our result offers a logical mechanistic connection between Notch ubiquitination, subcellular localization and signaling. Altogether, our results highlight LEs and the PPxY motif as versatile regulatory hubs that can integrate multiple inputs to finely modulate Notch signaling output.

2.3 Discussion

Endocytic internalization and trafficking of transmembrane signaling receptors play key roles in modulating pathway output (Barbieri et al., 2016; Di Fiore & von Zastrow, 2014;

Schmid, 2017; Sigismund et al., 2012). Given the Notch pathway's distinctive lack of a cytoplasmic signaling cascade, regulation of receptor passage through endosomal compartments provides each cell, whether ligand-stimulated or not, with autonomous control over Notch levels and pathway activity (Alfred & Vaccari, 2018; Baron, 2012; Fortini & Bilder, 2009; Pagliaro et al., 2020; Palmer & Deng, 2015; Revici et al., 2022; Schnute et al., 2018). In this study we identify the cytoplasmic tyrosine kinase Abelson as a novel regulator of Notch trafficking and signaling. Our findings reveal a kinase-dependent role for Abl in promoting the clearance of endocytosed Notch and establish an unanticipated connection between Abl and a regulatory network of ubiquitin ligases that controls the topology, accessibility and enrichment of Notch at LEs to modulate signaling output. We propose that LEs provide dynamic subcellular hubs, capable of integrating multiple regulatory inputs to finely tune Notch activity to levels optimal for specific cellular and developmental contexts.

Previous work has demonstrated the key role of E3 ubiquitin ligases in controlling Notch dynamics at LEs (Sakata et al., 2004; Schnute et al., 2022; Shimizu et al., 2014, 2024; M. Wilkin et al., 2008; M. B. Wilkin et al., 2004; Yamada et al., 2011), but the molecular events that control their activity have remained poorly understood (Conner, 2016). At the molecular level, Nedd4-family proteins use their WW domains to recognize and bind PPxY motifs within their substrates (Fedoroff et al., 2004; Ingham et al., 2004; Jennings et al., 2007; Jung et al., 2014; Otte, 2003; Sakata et al., 2004), an interaction involved in forming complexes such as Su(dx)/Nedd4-Notch in *Drosophila* and the analogous human WWP2-NOTCH3 (Jennings et al., 2007; Jung et al., 2014; Sakata et al., 2004). The connections between the ubiquitin ligases network and the Abl kinase uncovered in our study mark the Notch PPxY motif as an important molecular integration point for WW domain-based and tyrosine phosphorylation inputs that

modulate Notch subcellular localization and signaling. Although we tried to generate an endogenous *Notch*^{Y2328F} allele via CRISPR/Cas9, the recovered heterozygous females were too sick and infertile to establish a genetic line. However, those flies exhibited a marked reduction in small thoracic bristle density (Sanchez-Luege, 2018), a phenotype suggestive of excessive Notch activity (M. Guo et al., 1996; Hartenstein & Posakony, 1990). Alternative genetic strategies will therefore be needed to explore how the Notch PPxY motif integrates kinase and ubiquitin ligase inputs to regulate trafficking and signaling in developing tissues.

While protein structure disruptions could underlie the insensitivity of Notch^{Y2328F} to LE regulators, we favor a model in which the phosphorylation state of the Notch PPxY motif influences its interaction with and regulation by Nedd4-family members. Prior work has shown that PPxY phosphorylation can either enhance or reduce a particular interaction (Gao et al., 2006; Y. Liu et al., 2016; Otte, 2003). For example, phosphorylation of the c-Jun PPxY motif in T-cells prevents Nedd4-family ligase binding, thereby protecting the transcription factor from degradation (Gao et al., 2006). c-Abl was implicated as the kinase, which combined with our findings, hints at a role that might extend to diverse target proteins and biological contexts.

In contrast to the c-Abl/c-Jun relationship, our results suggest Abl-mediated phosphorylation of the Notch PPxY motif potentiates regulation by Nedd4-family ligases. By creating a more favorable interaction platform for Su(dx) or Nedd4Lo recruitment, phosphorylation could increase the probability of ubiquitination patterns that drive Notch into signaling-incompetent endosomal domains. Given that Su(dx) recognizes Notch using a unique WW4 domain absent in other Nedd4-family members (Fedoroff et al., 2004; Ingham et al., 2004), Su(dx) and Nedd4Lo might also have distinct PPxY motif phosphorylation preferences or affinities, enabling them to compete and collaborate in different ways. Therefore, in some

situations, the activity of Abl on Notch might rely primarily on Su(dx) or on Nedd4Lo. In others, for example the pupal wing epithelium, where RNAi knockdown of either Su(dx) or Nedd4 suppressed the ectopic vein phenotype associated with Abl overexpression (Table 1), contributions from multiple Nedd4-family ligases may be required.

Beyond mediating Abl-Nedd4 ligases interactions, the Notch PPxY motif could provide a molecular hub whose phosphorylated versus unphosphorylated states are read and acted upon by additional regulators. These could include tyrosine phosphatases, phosphotyrosine binding proteins, WW domain containing proteins, among others. By organizing diverse molecular complexes with different regulatory and signaling capabilities, these interactions could provide highly dynamic control of Notch activity. In addition, because Notch-Abl interactions have also been shown to influence Abl activity (Kannan et al., 2017; Koca et al., 2022), the possibility of multiple layers of feedback regulation offers essentially limitless opportunities for each cell to fine-tune its Notch levels, subcellular localization and signaling output.

The NICD PPxY motif need not be the only target of Abl. For example, tyrosine phosphorylation of Nedd4-family proteins has been shown to relieve autoinhibitory mechanisms (Dalton et al., 2011; Mund & Pelham, 2009; Persaud et al., 2014). Therefore, Abl could facilitate the LE internalization of Notch by unlocking or enhancing the regulatory potential of Nedd4 ligases in more than one way. While the molecular events that mediate recognition of Nedd4 ligases by tyrosine kinases have not been elucidated, the latter typically use their Src Homology (SH) domains to bind other proteins (Nash, 2013). Such interactions could facilitate targeting and tyrosine phosphorylation of NEDD4-1 by c-Src (Persaud et al., 2014), for example. Akin to Src, Abl possesses SH2 and SH3 domains (Colicelli, 2010) and, in particular, SH3 domains have been shown to bind proline-rich motifs in Nedd4-family ligases (Baumann et al., 2010; Grabs et

al., 1997; Safi et al., 2016). In *Drosophila*, Nedd4Lo differs from Nedd4S in the presence of extra proline-rich regions in both its N-terminal and Mid domains (Safi et al., 2016). In the case of the Mid domain, the proline-rich region separates the WW1 from the WW2 and WW3 domains (Safi et al., 2016; Zhong et al., 2011), an organization similar to the separation of the Su(dx) WW3 and WW4 domains by a proline-rich region (Fedoroff et al., 2004). Hence, the unique molecular structure of certain Nedd4 family members, even at the isoform level, could nucleate specific complexes that interact with Notch in both PPxY-dependent and independent manners.

In conclusion, we propose that the spatiotemporal patterning of Notch post-translational modifications offers a versatile regulatory strategy to modulate endosomal trafficking and thereby tune Notch signaling levels to the particular cellular or developmental transition. In the developing wing, the enrichment of Abl and Su(dx) in cells flanking proveins (Bennett & Hoffmann, 1992; Cornell et al., 1999) suggests that tight regulation of LE trafficking may be critical to attenuating Notch signaling in cells that are exposed to high concentration of ligand. On the other hand, the ubiquitous Notch trafficking defects observed in *abl^{null}* mutant wings, even outside of the vein areas (Fig. S2.2A,B), imply that cells can use the endocytic pathway to modulate Notch activation even in the absence of ligand stimulus. LE regulation can thus provide a versatile system to adjust Notch signaling output in both ligand induced and independent contexts. As dysregulated Notch activity is a key feature in oncogenic disorders linked to dysfunction of Abl, Dx, Su(dx) and Nedd4 (Aljedai et al., 2015; X. Liu et al., 2021; X. Wang et al., 2017; Wen et al., 2017; Z. Zhang et al., 2023), our study highlights cellular processes and molecular interactions that could be potential targets for therapeutic interventions.

2.4 Methods

Drosophila genetics

Flies were raised on standard *Drosophila* food at 26°C unless otherwise noted. The following lines were used: *Notch*⁵⁴¹⁹ (a gift from Rick Fehon), *abl*¹ (Henkemeyer et al., 1987), *abl*² (Henkemeyer et al., 1987), *abl-GFP* (Nagarkar-Jaiswal et al., 2015), NRE-EGFP (BDSC 30727), *rab7-myc-EYFP* (Dunst et al., 2015), *en>Gal4, tub>Gal80ts*, UAS-deGradFp (Caussinus & Affolter, 2016), UAS-Abl-GFP (O'Donnell & Bashaw, 2013), UAS-Abl^{K417N}-GFP (O'Donnell & Bashaw, 2013), UAS-Flag-Dx (a gift from Spyros Artavanis-Tsakonas), UAS-Su(dx) (BDSC 15664), UAS-Nedd4Short-Flag (BDSC 81605) (Zhong et al., 2011), UAS-Nedd4Long-Flag (BDSC 81604) (Zhong et al., 2011), UAS-Su(dx) RNAi (BDSC 67012), and UAS-Nedd4 RNAi (BDSC 34741). For UAS-driven genetic manipulations, crosses were established at 18°C and individual animals were transferred to 26°C at 0h APF. They were either dissected during early pupal development or allowed to grow to adulthood. To generate heatshock-driven *abl* clones in wing tissue, *y, w, hs>Flp;; ubi-GFP, FRT80B/TM6, Tb* females crossed to *abl*², *FRT80B/TM6, Tb* males. Parents laid eggs overnight, offspring were allowed to develop for 32h before heatshock treatment at 37°C for 45min, and heatshocked animals were transferred back to 26°C until dissection.

Immunostaining of fly tissue, antibodies and co-localization analysis

16-38h APF wing dissections: pupae were decapitated, fixed in 4% paraformaldehyde

(PFA) in PBS for 12-24h at 4°C, washed 3x and then dissected in PBS. Dissected wings were immediately blocked in 1% normal goat serum in PBT for 30min at room temperature or overnight at 4°C, incubated overnight at 4°C with primary antibodies in PBT, washed 3x in PBT, incubated overnight with secondary antibodies at 4°C, washed 3x in PBT, mounted in n-propyl gallate mounting medium and imaged on a Zeiss LSM 880 confocal microscope. The Airyscan function was used for Notch-Rab7 colocalization experiments. GFP-tagged proteins were imaged using the endogenous fluorescent signal. Antibodies: mouse anti-NECD (1:100, Developmental Studies Hybridoma Bank (DHSB) C458.2H), mouse anti-Delta (1:500, DHSB C594.9C), guinea pig anti-Eps15 and anti-Hrs (1:500, gifts from Hugo Bellen), goat anti-mouse Cy3 and Alexa 488 (1:2000, Jackson ImmunoResearch), goat anti-guinea pig Cy3 and Alexa488 (1:2000, Jackson ImmunoResearch). Co-localization analysis between Notch and endosomal markers was performed by calculating the Pearson's correlation coefficient between Notch and endosome channels using the COLOC2 plug-in of Image J/Fiji. Regions of approximately 100 cells were evaluated per wing, and 24 different wings were evaluated per genotype and marker. Profiles of Notch distribution across LE diameters were obtained from all LEs > 0.8µm (N=30-70) for each condition, and normalized to allow comparisons between experiments.

S2 cell transfection and dsRNA/imatinib treatments

S2 cells were cultured in Schneider's insect medium (Sigma catalog S9895-10X1L) supplemented with Penicillin and Streptomycin at 25°C. For all assays, 2.25×10^6 cells were transfected with dimethyldioctadecylammonium bromide (DDAB, Sigma D2779-10G), pMT expression plasmids, dsRNA, and pMT vector to equalize the total amount of nucleic acid

transfected, up to 1 μ g, Transfection was conducted for 30min, followed by incubation for 24h at 25°C, before induction with 0.7mM CuSO₄ for 24h at 25°C.

Plasmid and dsRNA transfection amounts: pMT-Notch (150 ng), pMT-Notch^{Y2328F} (150 ng), pMT-Abl-Myc (250ng), pMT-Su(dx)-Flag (150 ng), pMT-Dx-Flag (150ng), pMT-Flag-Nedd4S (150ng), pMT-Flag-Nedd4Lo (150ng), Abl dsRNA (350ng), Su(dx) dsRNA (250ng), Nedd4 dsRNA (250ng), LacZ dsRNA (250-350ng), pMT-EYFP-Rab7 (250ng, a gift from Martin Baron) and pMT vector. Transcription assays also included 2 ng of NRE-Luciferase (Firefly) reporter (a gift from Sarah Bray), and 20 ng of actin-Renilla normalizing control. For imatinib mesylate treatment, imatinib mesylate solution in water was added to transfected cells at a final concentration of 50-100 μ M at the time of induction.

For dsRNA preparation, target sequences from pMT-Abl-Myc(Xiong et al., 2009), pMT-Su(dx)-Flag (a gift from Martin Baron) and pcDNA3-Flag-Nedd4Lo (a gift from Daniela Rotin) were PCR-amplified using the following primers: 5'-

TAATACGACTCACTATAGGGAGAAAAATTTGTTTGGCCTTTTCAA-3' and 5'-

TAATACGACTCACTATAGGGAGAACGGTCATCCTATATCTTTT-3' for Abl

dsRNA(Rohn et al., 2011), 5'-

TAATACGACTCACTATAGGGAGATACATCACCTCATGACG-3' and 5'-

TAATACGACTCACTATAGGGAGATCATTCTGGCAGAAGCC-3' for Su(dx) dsRNA (C. Wang et al., 2015), 5'-

TAATACGACTCACTATAGGGAGAGTCACAATAGTATAGAGGACAA-3' and 5'-

TAATACGACTCACTATAGGGAGAGGTCGTTCTGCAAACCTGGTC-3' for Nedd4 dsRNA 1(Farny et al., 2008), and 5'-

TAATACGACTCACTATAGGGAGACGACTTGAAGAGCAGCAACA-3' and 5'-

TAATACGACTCACTATAGGGAGATAGGTTTCGTGTGGTTCACCA-3' for Nedd4 dsRNA

2. Transcription of amplified sequences was done using the Ambion MEGAscript™ kit (ThermoFisher Scientific) and subsequent purification was done via standard ethanol precipitation.

S2 cell-based subcellular localization and transcription/luciferase assays

For subcellular localization assays, cells were settled on poly-L lysine coated slides for 1h at 25°C, fixed for 15 minutes in 4% PFA in PBS, washed 3x in PBS, incubated for 1h at room temperature with primary antibodies in PBT+1% normal goat serum, washed 3x in PBS, incubated with secondary antibodies in PBT for 2h at room temperature, washed 3x in PBS, mounted in n-propyl gallate mounting medium, and imaged on a Zeiss LSM 880 confocal microscope. The same antibodies used for fly tissue were used for S2 cell staining and a minimum of 20 cells were imaged and considered for further examination. Co-localization analysis between Notch and endosomal markers was done as described above. Profiles of Notch distribution across LE diameters were obtained from all LEs > 0.8 μm (N=25-75) for each condition. Both the Notch signal and the LE diameter were normalized to a 0-1 scale to allow comparisons between experiments. Custom python script available upon request.

For transcription assays, each biological replicate in our plots represents a separate transfection. Cells were lysed in 100mM Potassium Phosphate, 0.5% NP-40, 1 mM DTT, pH 7.8 for 1h on ice. Lysates were loaded in triplicate into an Autolumat Plus LB 953 luminometer. Firefly and Renilla luciferases were activated in separate reactions using Firefly buffer (10mM Mg Acetate, 100mM Tris Acetate, 1mM EDTA, 4.5mM ATP, and 77uM D-luciferin, pH 7.8)

and Renilla buffer (25mM sodium pyrophosphate, 10mM Na Acetate, 15mM EDTA, 500mM Na₂SO₄ 500mM NaCl, 4mM coelenterazine, pH 5.0), respectively. The ratio of Firefly RLU to Renilla RLU was averaged across technical replicates, and activation values were normalized to Notch activity.

Molecular cloning of pGEX-NICD, pGEX-NICD^{Y2328F}, and pMT-Notch^{Y2328F}

pMT-NotchY2328F was made by subcloning the XhoI-XbaI NICD fragment from pMT-Notch into pBluescript. The Y2328F point mutation was introduced using Quickchange PCR using the following primers: 5'-AAGCAGCCGCCGAGCTTTGAGGATTGCATCAAG-3'; 5'-CTTGATGCAATCCTCAAAGCTCGGCGGCTGCTT-3'. The mutated XhoI-XbaI fragment, confirmed by sequencing, was cloned back into pMT-Notch to create pMT-Notch^{Y2328F}. Wildtype and Y2328F XhoI-NotI NICD fragments were subcloned into SalI-NotI digested pGEX-4T-2 plasmid to create the bacterial expression constructs.

In vitro kinase assay

GST-fusion proteins were purified from BL21 *E. coli* cells as described in Rebay and Fehon, 2009. Kinase assays were performed as described in Xiong et al., 2009 (Xiong et al., 2009). The relative intensity of full-length wildtype and Y2328F GST-NICD bands, as detected on western blots with mouse anti-NICD (1:1000) antibody, was used to estimate comparable amounts of substrate for the reactions. Samples were run on a 10% polyacrylamide gel, transferred to a PVDF membrane using standard methods and exposed on a Storm

phosphoimager prior to immunoblot analysis to confirm protein loading.

2.5 Acknowledgements

We would like to thank Spyros Artavanis-Tsakonas, Rick Fehon, Wei Du and Hitoshi Matakatsu for fly strains, Sarah Bray, Martin Baron and Daniela Rotin for S2 cell expression plasmids, Hugo Bellen and Rick Fehon for antibodies, and Lucy Godley for imatinib mesylate. We also thank Hideyoshi Shimizu and Hitoshi Matakatsu for advice on pupal wing dissection, Allison Zajac, Jacob Decker and Jose Velarde for advice on co-localization analysis, Xiao Sun and Christine Cao for experimental assistance, and Rick Fehon for helpful comments on the manuscript. In addition, we thank all members of the Rebay Lab, Rick Fehon, Chip Ferguson, Sally Horne-Badovinac, Aaron Turkewitz and Paschalis Kratsios for helpful discussions.

CHAPTER 3

PRECISE DEPLOYMENT OF NOTCH SIGNALING VIA DELTA-NOTCH FEEDBACK DYNAMICS REGULATES WING VEIN PATTERNING IN *DROSOPHILA*

This chapter constitutes the basis for a manuscript that will be submitted for publication after the completion of this dissertation.

Statement of contributions:

While the work presented in this chapter predominantly reflects my own ideas and scientific curiosity, it was significantly advanced by the experimental contributions of three exceptional trainees and collaborators: Chyan Rangel, Zach Baker, and Carol Dilts. Chyan, a summer student in the Research Experience for Undergraduates (REU) program at the University of Chicago, distinguished herself through her outstanding performance and dedication. She was the first one to characterize how Delta-Notch signaling dynamics respond to perturbations in pathway activity, uncovering new insights into how lateral inhibition via Delta-Notch feedback is spatially and temporally deployed in biological systems. Chyan's experimental dexterity and keen intellect laid the groundwork for this summer project to develop into a full research paper. Later, Carol and Zach joined the team, making invaluable contributions as we explored how the Delta-Notch feedback interacts with various vein development signals, including EGFR and BMP.

3.1 Introduction

Precise patterning is a fundamental aspect of metazoan development. Acquiring specific cell fates at the appropriate locations and times ensures the reliable assembly of functional structures, which is essential for both the construction and maintenance of living tissues and organs (Wolpert et al., 2019; Barresi & Gilbert, 2023). The formation of well-ordered multicellular structures relies on meticulous intercellular coordination, where cells interact to influence their neighbors' behaviors, impacting processes such as division, differentiation, migration, and more (Barresi & Gilbert, 2023). Disruptions in achieving the correct number of cells, appropriate cell types or proper cell placement can lead to developmental disorders and pathologies (Veraksa et al., 2000; Derry, 2023), underscoring the critical need for tightly regulated cell-cell communication.

A classic paradigm of such intercellular coordination is the process of lateral inhibition (Waddington, 1940; Collier et al., 1996). In this mechanism, a group of initially equivalent cells engages in dynamic local competition, generating a feedback loop that amplifies differences between adjacent cells. As one cell transitions to a distinct fate, it simultaneously inhibits its neighbors from doing the same. This competition creates spatial patterns of alternating cell types, with differentiated cells interspersed among those that did not achieve the desired fate (Binshtok & Sprinzak, 2018; Sjöqvist & Andersson, 2019). Notable examples of lateral inhibition shaping developmental patterns include the bristle arrangements on the *Drosophila notum* (Heitzler & Simpson, 1991; Heitzler et al., 1996; M. Cohen et al., 2010; G. L. Hunter et al., 2016), the spotted skin patterns on pearl danio fish (Eom et al., 2015), the stripe distribution on the zebrafish body (Hamada et al., 2014; Eom et al., 2015), and the organization of secretory cells in

the mammalian intestine (Tóth et al., 2017). In each case, lateral inhibition resolves binary fate decisions through precise regulation of cell behaviors.

Intercellular interactions during developmental patterning are mediated by cell-cell signaling mechanisms (Perrimon et al., 2012), with the Notch pathway serving as the primary conduit in lateral inhibition events (Perrimon et al., 2012; Binshtok & Sprinzak, 2018; Sjöqvist & Andersson, 2019). Lateral inhibition mediated by Notch signaling involves Delta-driven activation of the Notch receptor coupled with a transcriptional negative feedback loop (Collier et al., 1996; Binshtok & Sprinzak, 2018; Sjöqvist & Andersson, 2019; Bocci et al., 2020). In this mechanism, cells with initially elevated Delta expression, whether due to stochastic variation or upstream induction, activate Notch signaling in adjacent cells. Activation of the Notch pathway in signal-receiving cells inhibits *Delta* expression and often promotes the expression of *Notch* itself, thereby preventing these cells from signaling back to the original inducers while also enhancing their responsiveness to inductive inputs. As this feedback loop progresses, it amplifies initial differences among cells, resulting in a mutually-exclusive spatial arrangement where some cells exhibit high levels of Delta ligand and low pathway activity while their neighbors display elevated Notch receptor and strong pathway activation.

Lateral inhibition via Delta-Notch feedback is integral to various developmental processes including the AC/VU fate decision in the *C. elegans* vulva (Seydoux & Greenwald, 1989; Wilkinson et al., 1994), the arrangement of bristles on the *Drosophila* notum (Heitzler et al., 1996; Heitzler & Simpson, 1991), and vein patterning in the *Drosophila* wing (de Celis et al., 1997; Huppert et al., 1997). In *C. elegans*, stochastic variations establish the Delta-Notch circuit (Seydoux & Greenwald, 1989; Wilkinson et al., 1994), while self-organizing Delta-Notch signaling shapes bristle patterns in the *Drosophila* notum (Corson et al., 2017). In contrast, vein

patterning in the *Drosophila* wing is initially guided by tightly regulated upstream inductive cues (Yu et al., 1996; J. F. De Celis, 1997; Gabay et al., 1997; Martín-Blanco et al., 1999; Sotillos & De Celis, 2005; Blair, 2007; Antson et al., 2022), with the Delta-Notch feedback acting downstream to refine tissue domain boundaries. The sophisticated, signaling-driven orchestration of wing vein patterning makes this system an attractive model for investigating lateral inhibition-based regulatory networks.

Vein specification in the *Drosophila* wing begins at the third instar stage, with the majority of vein growth, spatial patterning, and boundary refinement occurring during the initial third of pupal development (Blair, 2007). As the early pupal wing elongates proximo-distally and its dorsal and ventral epithelia converge, vein formation is driven by spatiotemporally organized EGFR and BMP signaling (Yu et al., 1996; J. F. De Celis, 1997; Gabay et al., 1997; Martín-Blanco et al., 1999; Sotillos & De Celis, 2005; Blair, 2007; Antson et al., 2022). Broad vein regions initially lack clear boundaries but are refined by Delta-Notch signaling approximately between 16h-32h after puparium formation (APF) (Huppert et al., 1997; Herszterg et al., 2023). During this period, EGFR and BMP signaling promote Delta expression in developing vein cells (proveins) (Sturtevant & Bier, 1995; de Celis et al., 1997; Huppert et al., 1997; J. F. De Celis, 1997; Tanimoto et al., 2000; Blair, 2007). This Delta activates Notch signaling in adjacent intervein cells (referred to as “flanking cells” to distinguish them from the “proper intervein cells”), which in turn represses vein fate and *Delta* expression, creating a feedback loop that prevents Notch activation within the proveins (de Celis et al., 1997; Huppert et al., 1997). While the essential role of Notch signaling in wing vein patterning is well established, the detailed progression of the Delta-Notch feedback across a large cell field, involving different cell types and unfolding over time, remains poorly understood.

In this study, we investigated the spatiotemporal dynamics of Delta-Notch signaling during wing vein development. Our results indicate that continuous refinement and correction of unexpectedly noisy Notch signaling episodes, likely through Delta-Notch feedback, plays an essential role in shaping the vein pattern. This process drives the accurate establishment of vein and intervein domains, as well as the boundaries between them, from 16h APF and extending beyond 32h APF. Although the Delta-Notch feedback is typically active only within vein areas, it can be triggered elsewhere in the tissue in response to inappropriate Notch activity, revealing a tissue-wide surveillance mechanism that ensures proper patterning. Notably, our findings also show that, while this quality-control system can effectively respond to a range of perturbations, it can be overwhelmed and blocked by extreme disruptions in Notch pathway activation, shedding light on both its capabilities and limitations. Overall, our study offers key new insights into the precise deployment of Delta-Notch feedback to safeguard the integrity of tissue domains and boundaries in response to inadequate signaling activity.

3.2 Results

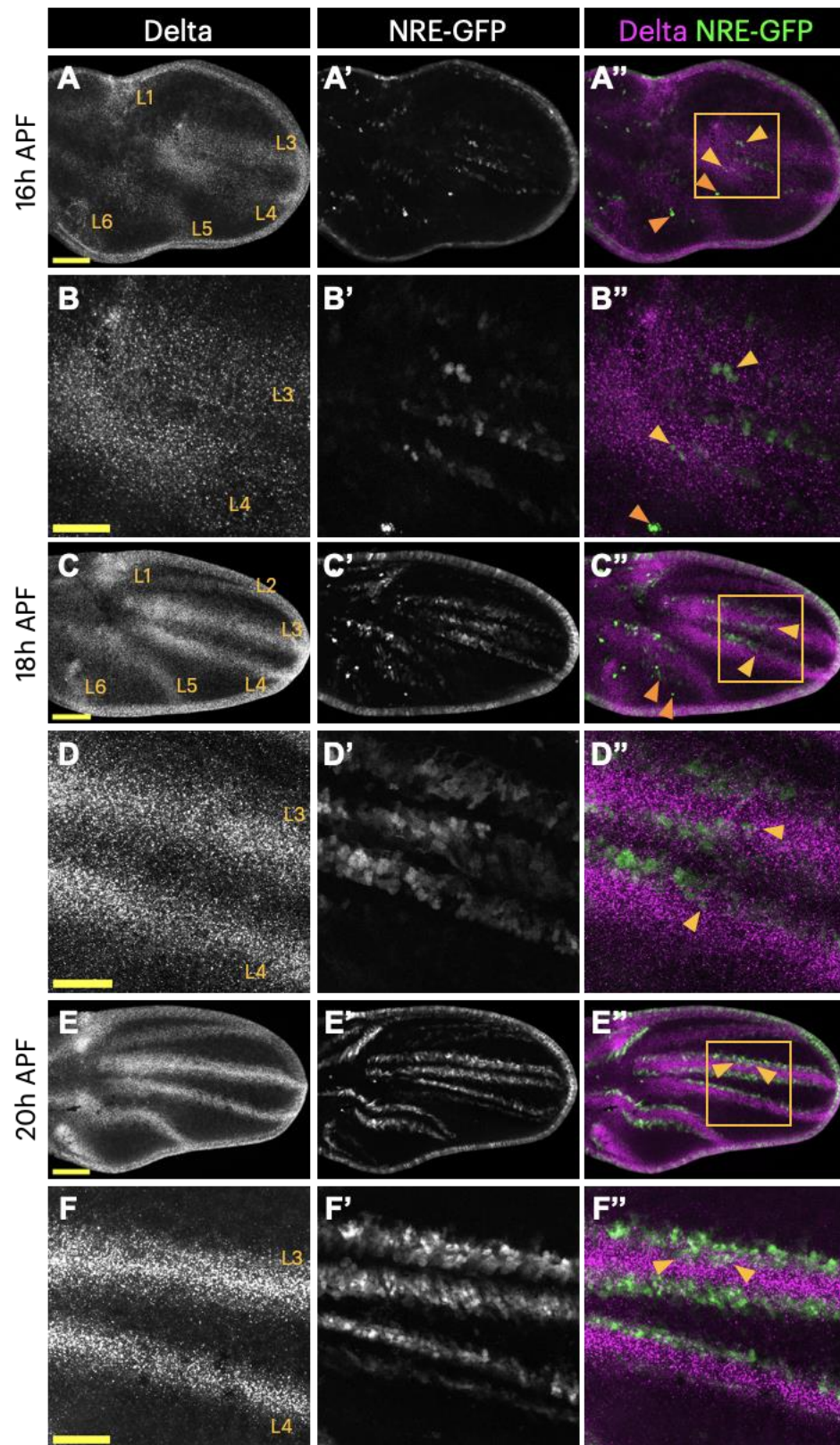
Refinement of noisy Delta-Notch signaling during vein-intervein boundary establishment

The stereotyped vein-intervein boundaries in the *Drosophila* wing reflect the culmination of a pattern refinement program that occurs primarily between 16h-32h APF, with Notch signaling playing a pivotal role (Lyman & Yedvobnick, 1995; J. F. De Celis & Bray, 1997; Huppert et al., 1997; Sotillos & De Celis, 2005; Blair, 2007; Herszterg et al., 2023). To gain a deeper understanding of how Delta-Notch signaling and feedback unfold in both space and time

during this process, we examined the expression patterns of Delta and a Notch signaling reporter (NRE-GFP, Notch Responsive Element), at 2-hour intervals throughout this critical period. Although not the focus of our analysis, both Delta and NRE-GFP were detected at the wing margin at all stages examined (Fig 3.1), consistent with the well-established role of Notch signaling in wing margin development (de Celis et al., 1996; J. F. De Celis & Bray, 1997). Qualitatively, Delta levels appeared to peak around 18h APF while NRE-GFP reached its maximum around 24h APF; both reached a steady-state lower level of wing margin expression by 28h APF.

Focusing on expression within the wing blade, we detected both Delta and NRE-GFP as early as 16h APF (Figs. 3.1A-B”). Although upstream protein signaling via the EGFR and BMP pathways is more distinctly organized into stripe patterns at this stage (Fristrom et al., 1994; J. F. De Celis, 1997; Conley et al., 2000; Sotillos & De Celis, 2005), Delta expression in the presumptive L1 and L3-L6 protein regions remains broad and somewhat discontinuous, with poorly defined borders between the emerging stripe-like structures along the proximal-distal axis. Concurrently, NRE-GFP expression is sparse, generally localized to cells at the edges of the Delta expression domains. Strikingly, the signaling reporter is also detected within Delta⁺ areas (yellow arrows in Figs. 3.1A”,B”), as well as in Delta⁻ cells distant from the presumptive vein structures (orange arrows in Figs. 3.1A”,B”). This suggested “noisy” Notch signaling activity outside of the expected vein-intervein boundary domains.

At 18h APF, Delta expression begins to consolidate into defined stripe-like regions that correspond to each of the six proveins (Fig. 3.1C). The NRE-GFP signal is noticeably stronger and more widespread, surrounding most of the provein structures with the exception of the newly emerged L2, whose Delta levels are lower than those in the other proveins (Figs. 3.1C’,C”). Faint



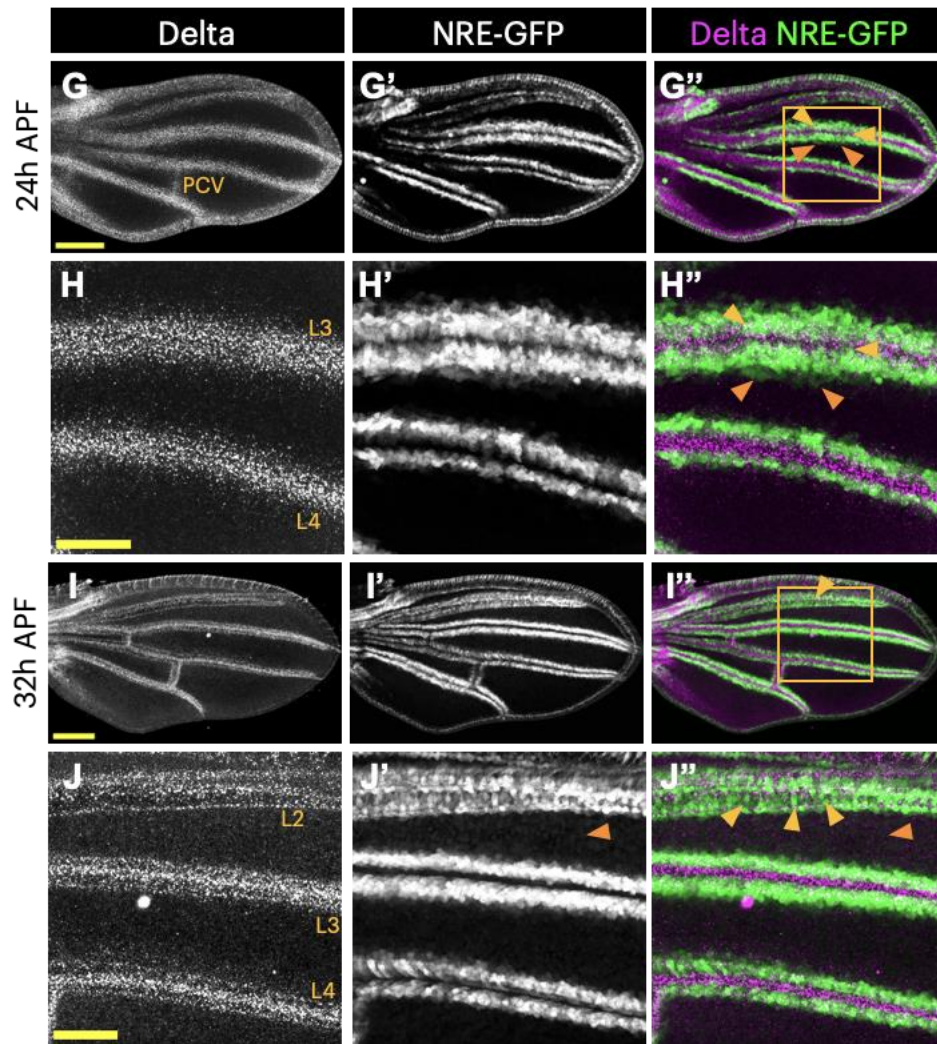


Figure 3.1. Spatiotemporal dynamics of Delta-Notch signaling during early pupal wing development I.

(A,J) Maximal projections of wildtype wings showing Delta expression and Notch signaling activity (NRE-GFP). Longitudinal veins L1-6, and crossveins ACV and PCV are indicated. (A,B) 16h APF. (C,D) 18h APF. (E,F) 20h APF. (G,H) 24h APF. (I,J) 32h APF. Insets in A, C, E, G, and I are shown in B, D, F, H, and J, respectively. Yellow arrows mark Notch signaling activity in provein domains. Orange arrows indicate Notch signaling activity in intervein domains. Scale bars = 100 μ m (A, C, E, G, I) or 50 μ m (B, D, F, H, J).

patches of reporter expression can also be detected in both presumptive vein cells (yellow arrows in Figs. 3.1C”,D”) and intervein territories (orange arrows in Fig. 3.1C”), once again indicating noisy Notch signaling.

At 20h APF, as the longitudinal proveins extend further proximally towards the hinge, the Delta expression stripes appear more consolidated, with the L2 stripe now more clearly visible and L5 fully formed (Fig. 3.1E). Simultaneously, the NRE-GFP reporter exhibits increased intensity and tissue coverage relative to 18h APF, and the signal around L2 is now discernible, though still sparse (Figs. 3.1E’,E”). While ectopic signaling activity has been largely resolved in the interveins, it remains unresolved in some provein areas (yellow arrows in Figs. 3.1E”,F”). Nevertheless, the pattern of NRE-GFP in cells flanking the edges of the L2-L5 Delta expression stripes now more clearly demarcates the proveins (Fig. 3.1E”).

At 22h and 24h APF, refinement of the Delta⁺ provein stripes continues as the longitudinal veins (LVs) extend further proximally (Figs. S3.1A, 3.1G). Meanwhile, the NRE-GFP signal continues to intensify, peaking around L3 by 24h APF. This peak in intensity is frequently accompanied by a resurgence of signaling noise in both presumptive vein and intervein cells (yellow and orange arrows in Figs. 1.3G”,H”, respectively). Additionally, following the upstream BMP signaling that initiates crossvein development around 18h APF (Yu et al., 1996; Conley et al., 2000; Ralston & Blair, 2005; J. Chen et al., 2012; Gui et al., 2018; Yang et al., 2022), and the subsequent activation of EGFR signaling in those regions around 20h APF (Martín-Blanco et al., 1999), Notch pathway markers are now detected in the posterior crossvein (PCV). Delta appears first at 22h APF, and NRE-GFP starts to follow around 24hr APF (Figs. S3.1A-A”, 3.1G-G”).

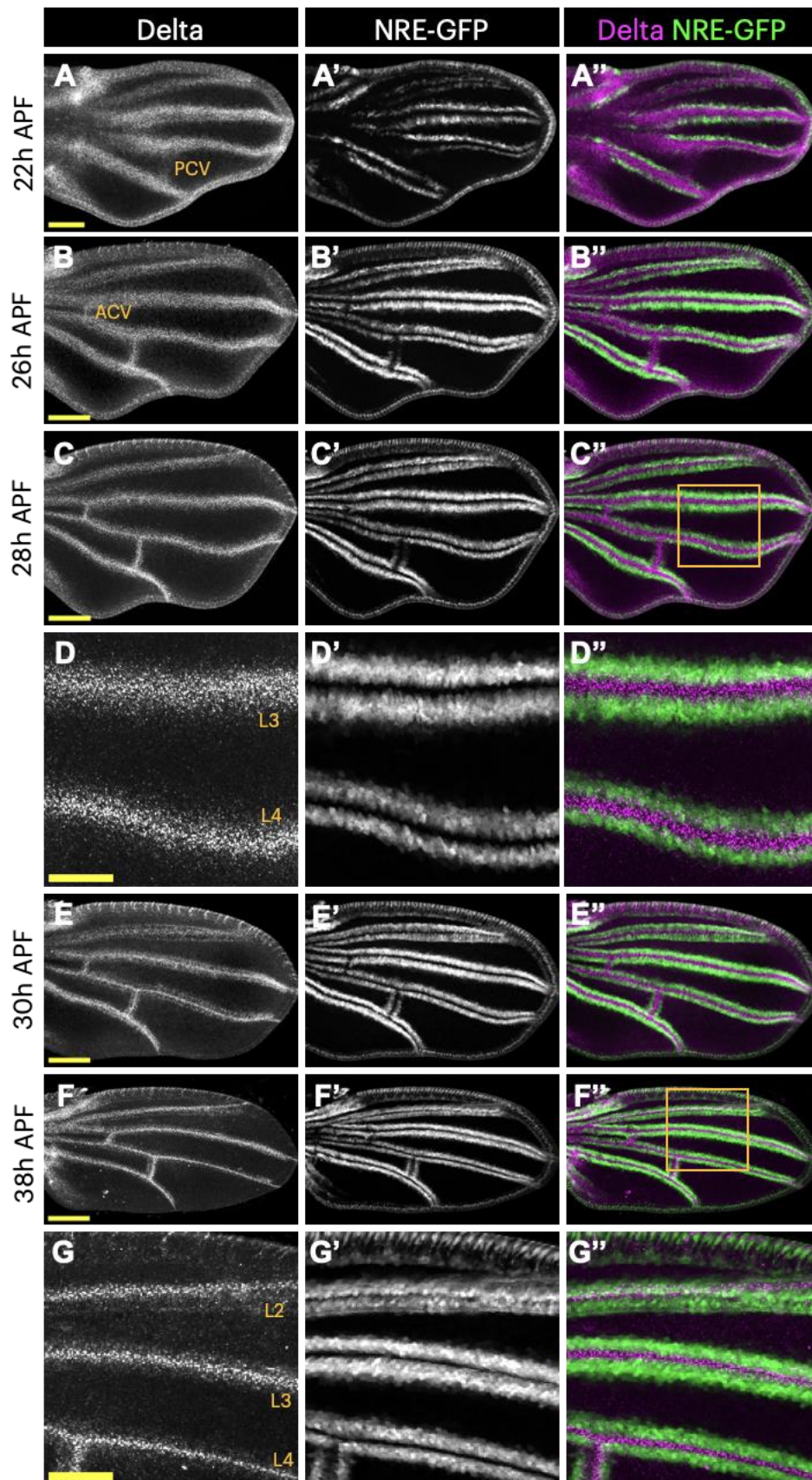


Figure S3.1. Spatiotemporal dynamics of Delta-Notch signaling during early pupal wing development II.

(A,G) Maximal projections of wildtype wings showing Delta expression and Notch signaling activity (NRE-GFP). Longitudinal veins L1-6, and crossveins ACV and PCV are indicated. (A) 22h APF. (B) 26h APF. (C,D) 28h APF. (E) 30hAPF. (F,G) 38h APF. Insets in C and F are shown in D and G, respectively. Yellow arrows mark Notch signaling activity in provein domains. Orange arrows indicate Notch signaling activity in intervein domains. Scale bars = 100µm (A-C, E-F) or 50µm (D,G).

At 26h and 28h APF, as the LVs complete their extension to the proximal end of the wing blade, the Delta⁺ provein stripes refine further (Figs. S3.1B,C). By 28h APF, the NRE-GFP signal further surrounds the posterior crossvein (PCV) and fully delineates L2 (Fig. S3.1C-C’). The intensity of the NRE-GFP signal in the middle portion of the wing blade, particularly around L3, has moderated, with much of the earlier signaling noise resolved (Figs. S3.1D-D’). Delta also marks the newly apparent anterior crossvein (ACV) at 26h APF, with NRE-GFP surrounding it by 28h APF (Fig S3.1B-B’, C-C’).

At 30h-32h APF, the Delta⁺ provein stripes approach their final, refined thickness (Figs. S3.1E, 3.1I). During these stages, there is also noticeable broadening of the NRE-GFP stripes around L2 compared to earlier timepoints, along with some apparent signaling noise in and around this provein (yellow arrows in Figs. 3.1I’-J’), which is partially resolved by 38h APF (Figs. S3.1F-G’). Notably, further vein refinement of vein thickness is observed by 38h APF in comparison to 32h APF (compare Figs. 3.1I,J and S3.1F,G). While tissue folding prevented further analysis beyond 38h APF, these results suggest that both vein thickness and aberrant Notch signaling events continue to be refined beyond 32h APF.

Overall, our results reveal distinct temporal trajectories for different vein structures and uncover previously overlooked aspects of Notch signaling during vein patterning, particularly

noisy pathway activity. Delta marking of the proveins is consistently observed first, with NRE-GFP⁺ active Notch signaling typically emerging about 2 hours later. Delta⁺ vein structures form in a distal-to-proximal direction in LVs and from the anterior-posterior edges towards the center in the PCV. As the broad, diffuse Delta⁺ provein domains become more sharply delineated and narrow over time, noisy Notch signaling is detected, likely reflecting the process of refining initially imprecise vein-intervein boundaries. In addition, although Delta-Notch signaling is fully deployed across all wing veins by approximately 32h APF, our observations suggest ongoing refinement of both the vein pattern and signaling activity beyond this point.

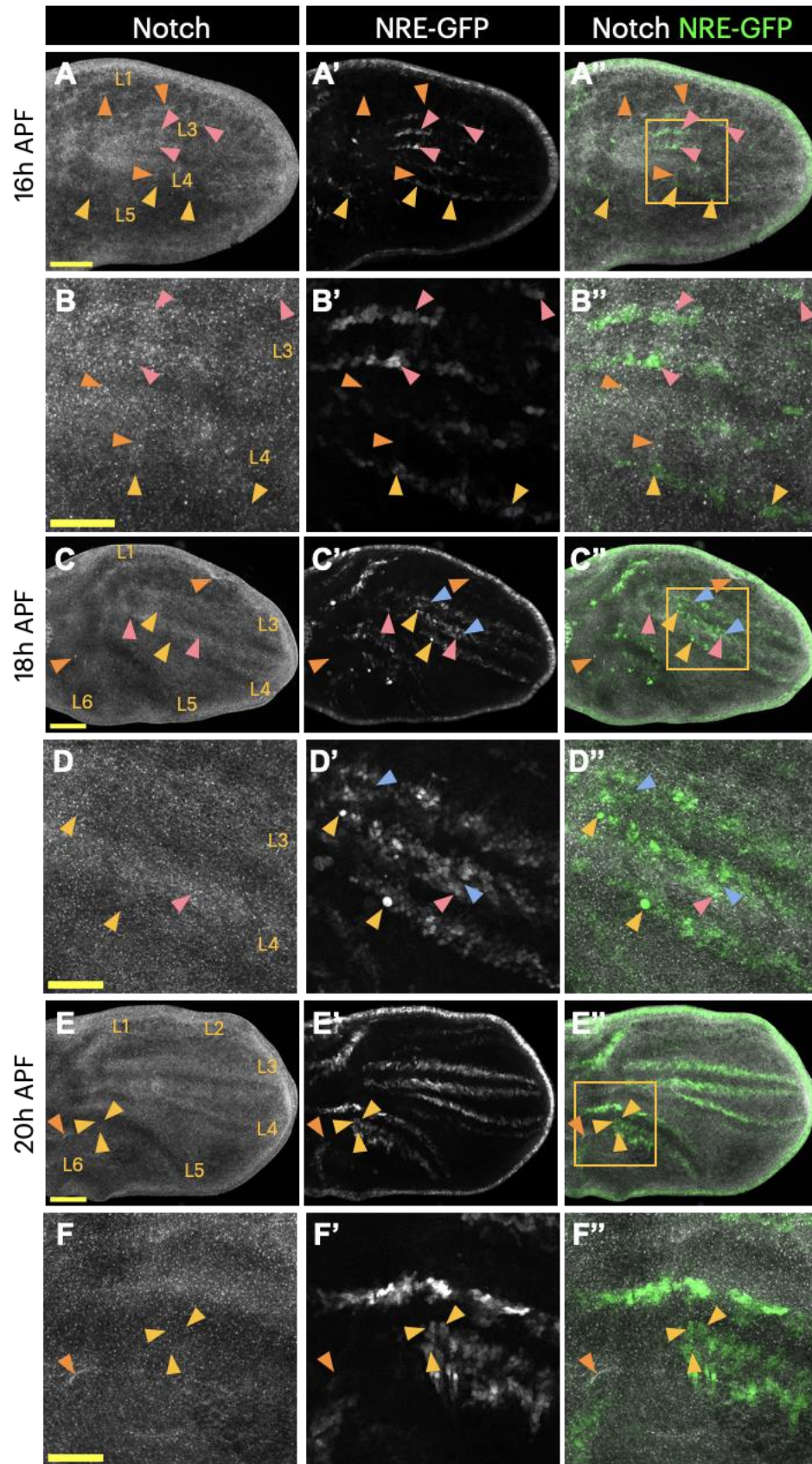
Mutual induction of Notch expression and signaling activity during wing vein patterning

The concurrent induction of *Notch* and repression of *Delta* gene expression in signal receiving cells is a central feature of Delta-Notch lateral inhibition feedback circuits (Collier et al., 1996; Heitzler et al., 1996; Binshtok & Sprinzak, 2018; Sjöqvist & Andersson, 2019; Bocci et al., 2020). Given that Delta expression is pre-patterned in the wing cell field by upstream provein signaling, we wondered whether Notch receptor expression is similarly pre-patterned to favor pathway activation around vein areas, or whether it is primarily driven by Delta-dependent Notch signaling. Prior studies have shown complementary patterns of Delta and Notch expression as early as 6h APF (Fehon et al., 1991; Kooh et al., 1993), a time when Notch signaling might not yet be active, based on our detection of NRE-GFP starting at 16h APF. While at later time points (24h–28h APF) Notch expression and Notch signaling appear to overlap (J. F. De Celis & Bray, 1997), how their spatial distribution is dynamically coordinated

during wing vein patterning remains unclear. To address this gap, we investigated the expression pattern of the Notch receptor and NRE-GFP at 2-hour intervals between 16h and 32h APF.

Examination of 16h APF wings revealed ubiquitous Notch receptor expression, with notable enrichment both in the central portion of the wing blade and at the wing margin (Fig. 3.2A). The central enrichment, which corresponds to L3-L4 structures, mirrors the Delta pattern, while the margin enrichment is more pronounced than that of Delta. Presumptive L1 and L5-6 structures are less clearly detected than with Delta marking, with L5-6 often being undetectable. Meanwhile, NRE-GFP expression follows a similar pattern to what we described above, appearing around the presumptive L1 and L3-L6 proveins, with some noisy signaling activity near L3-L4 (Fig 3.2A'-A'', B'-B''). While there is general overlap between regions of Notch enrichment and visible NRE-GFP signal at this stage (pink arrows in 3.2A-A'',B-B''), we also identified tissue areas where NRE-GFP is present but Notch enrichment is less clear (yellow arrows in 3.2A-A, B-B''), as well as regions where Notch receptor expression is elevated but NRE-GFP is absent (orange arrows in 3.2A-A''). Consistent with previous literature, these findings suggest that Notch signaling upregulates expression of the Notch receptor. However, they also suggest that the relationship may work in reverse, with Notch receptor expression influencing the activation of Notch signaling.

By 18h APF, Notch enrichment in the central wing blade has significantly dispersed (Fig. 3.2C), more so than the reduction in Delta concentration observed between 16h and 18h APF. This dispersion is accompanied by a clearer delineation of stripe-like structures extending from the central Notch concentration to the distal margin, particularly around the presumptive L3-4 proveins (Figs. 3C-D). Presumptive L1 and L5-6 structures also become identifiable at this stage.



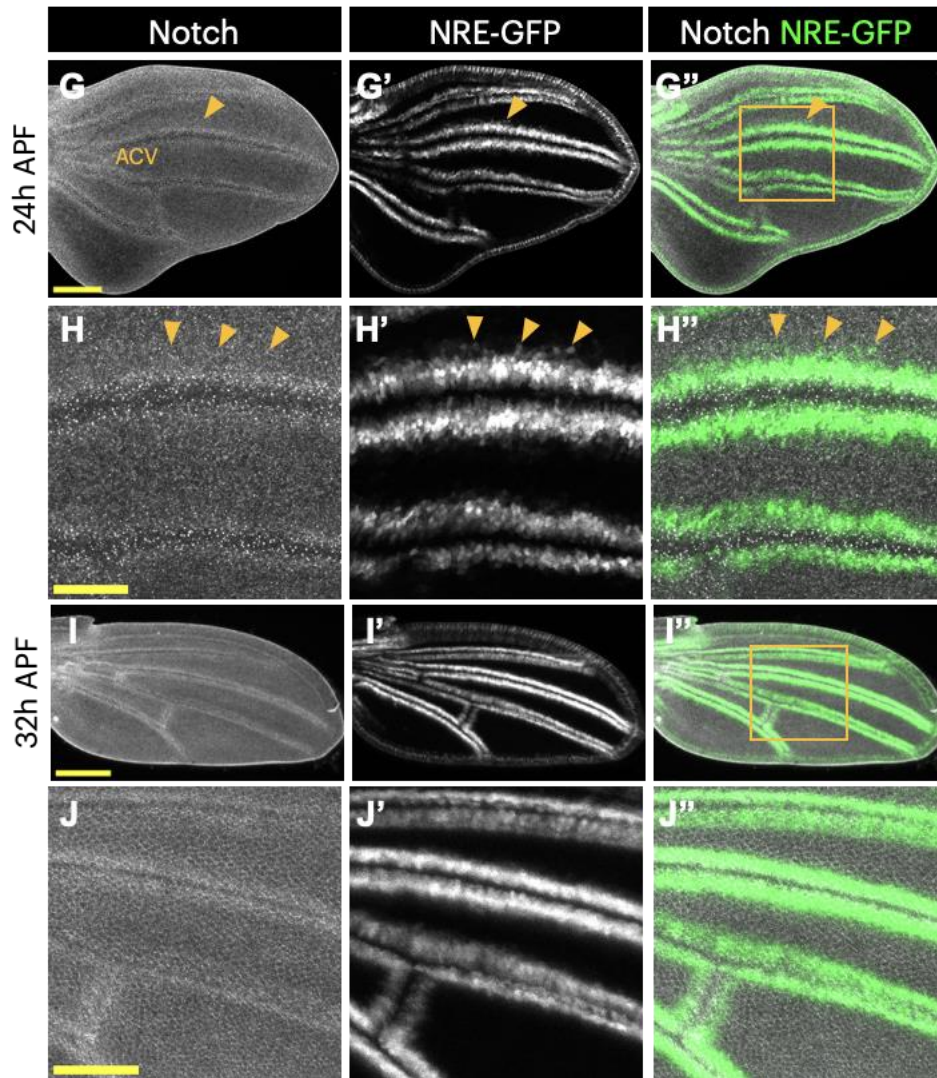


Figure 3.2. The Notch receptor-Notch signaling interplay during early pupal development I. (A,J) Maximal projections of wildtype wings showing Notch expression and Notch signaling activity (NRE-GFP). Longitudinal veins L1-6, and crossveins ACV and PCV are indicated. (A,B) 16h APF. (C,D) 18h APF. (E,F) 20h APF. (G,H) 24hAPF. (I,J) 32h APF. Insets in A, C, E, G, and I are shown in B, D, F, H, and J, respectively. Pink arrows highlight areas displaying enrichment of both Noth receptor and NRE-GFP. Yellow arrows mark presence of signaling reporter and lack of Notch enrichment. Orange arrows show Notch enrichment and absence of signaling reporter. Blue arrows highlight the presence of multiple NRE-GFP⁺ stripes around a provein. Scale bars = 100µm (A, C, E, G, I) or 50µm (B, D, F, H, J).

Notably, elevated Notch expression is detected both in and around presumptive proveins, rather than predominantly around them, suggesting that the complementary spatial distribution of Delta and Notch remains unresolved at this stage (Figs. 3C-D''). Concurrently, the NRE-GFP reporter persists in localizing to areas of Notch receptor enrichment (pink arrows in Figs. 3.2C-C'', D-D''), though regions with only Notch or only NRE-GFP are still observed (orange and yellow arrows in Figs. 3.2C-C'', D-D'', respectively). In addition, noisy Notch signaling is observed as multiple NRE-GFP⁺ stripes on one side of a provein (blue arrows in Figs. 3.2C'-C'', D'-D'').

By 20h APF, Notch expression in the central wing blade has further dissipated, resembling the changes seen with Delta (Figs. 3.1E, 3.2E). The shift in Notch expression from central to proximal regions of the wing blade coincides with the continued extension of the LV-associated stripes towards the proximal wing. By 20h APF, Notch expression in the central wing blade has further dissipated, resembling the changes seen with Delta (Figs. 3.1E, 3.2E). The shift in Notch expression from central to proximal regions of the wing blade accompanies the further extension of the LV-associated stripes towards the proximal wing blade. Notably, marking of L2 by Notch is first detected at this timepoint, albeit it appears as a single stripe (Fig. 3.2E). This contrasts with the now evident Notch-marking of two distinct stripes flanking the other primordial LVs, consistent with the expected upregulation of Notch specifically in cells adjacent to the proveins. Meanwhile, although minimal NRE-GFP signal is detected around the L2 provein, the reporter more clearly delineates the other LVs (Figs. 3.2E'-E''). Furthermore, the incomplete overlap between Notch and NRE-GFP at this stage seems to result mostly from noisy Notch signaling, with NRE-GFP present in areas where Notch protein enrichment is not evident (yellow arrows in Figs. 3.2E-E'', F-F''), although some Notch enrichment in the absence of NRE-GFP was still seen (orange arrow in Figs. 3.2E-E'', F-F'').

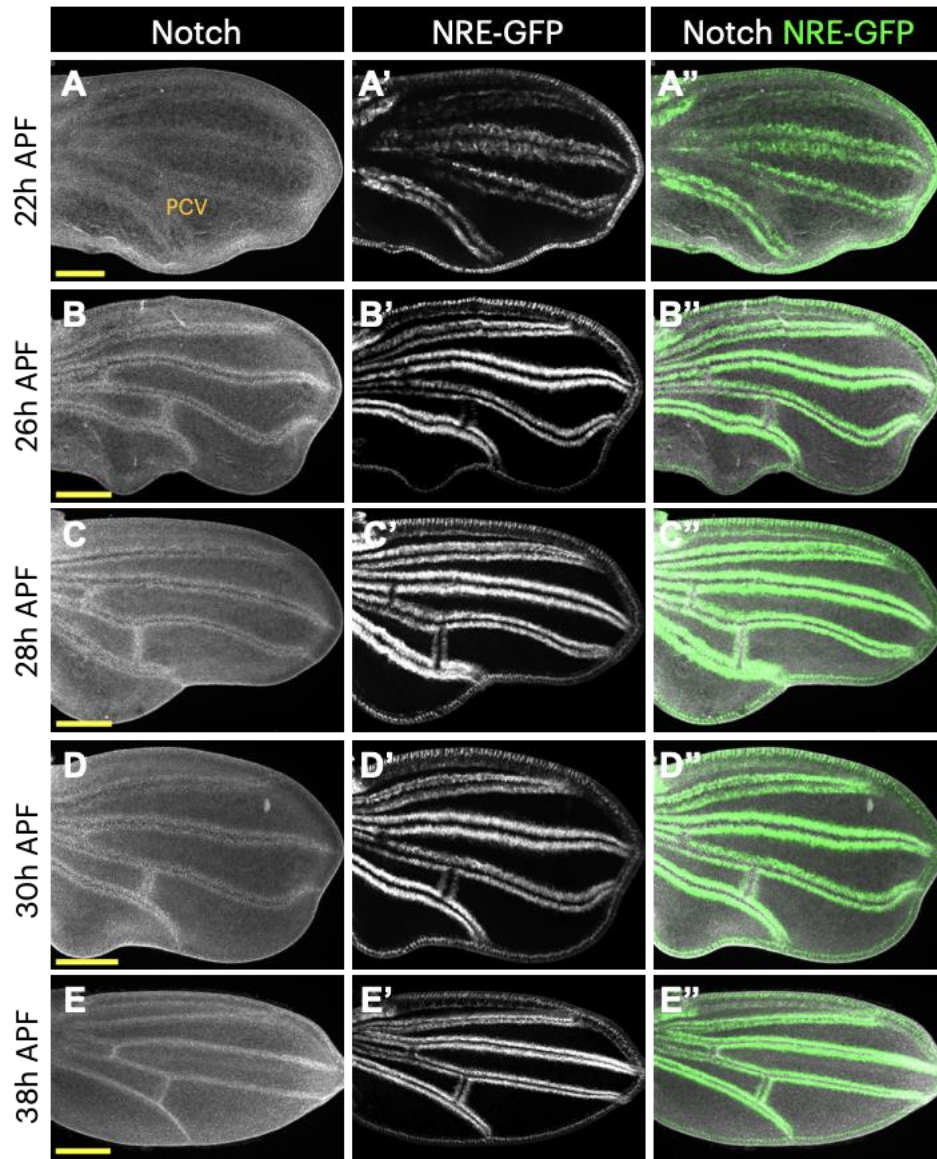


Figure S3.2. The Notch receptor-Notch signaling interplay during early pupal development II. (A,J) Maximal projections of wildtype wings showing Notch expression and Notch signaling activity (NRE-GFP). Longitudinal veins L1-6, and crossveins ACV and PCV are indicated. (A) 22h APF. (B) 26h APF. (C) 28h APF. (D) 32APF. (E) 38h APF. Scale bars = 100 μ m.

At 22h and 24h APF, the thickness of both presumptive proveins and the Notch⁺ stripe-like structures around them decreases (Figs. S3.2A, 3.2G). The central clump of Notch signal in the wing blade has completely dissipated, and Notch⁺ stripes have extended further towards the proximal side of the wing. At these stages, Notch marks crossveins structures for the first time. Notably, while there is a mild increase in Notch expression around the PCV area starting at 22h APF (Fig. S3.2A), more clearly defined by 24h APF (Fig. 3.2G), NRE-GFP does not begin to match the PCV-flanking stripes until around 24h APF (Figs S3.2A-A'', 3.2G-G''). In the case of the ACV, an initially moderate elevation in Notch signal at 22h APF becomes more defined by 24h APF, although without accompanying NRE-GFP signal (Figs S3.2A-A'', 3.2G-G''). In LVs, the alignment between Notch expression and NRE-GFP continues to improve, nearing fully accurate correspondence by 24h APF, although some signaling noise remains around L3, with active NRE-GFP expression observed in the absence of elevated Notch (yellow arrows in Figs. 3.2G-H'').

At 26h and 28h APF, the Notch⁺ stripes and the intervening provein regions undergo further refinement, seemingly reaching their final thickness by 28h APF (Figs. S3.2B,C). During these stages, the LV-associated Notch⁺ stripes extend fully to the proximal end of the wing blade, and the ACV-associated Notch⁺ stripes are closely matched by the NRE-GFP signal (Figs. S3.2B-B'', C'-C''). Furthermore, by 28h APF, the expression patterns of Notch and NRE-GFP align perfectly in the LVs. At 30h and 32h APF, the final stages of vein-intervein boundary refinement are observed, with NRE-GFP perfectly matching the Notch signal in both LVs and CVs by 32h APF (Figs. S3.2D-D'', 3.2I-J'').

By 38h APF, no significant changes in the synchrony between Notch and NRE-GFP are observed (Fig. S3.2E-E''), indicating that these patterns remain stable beyond 32h APF. Overall,

our results demonstrate how the spatial organization of Notch protein and Notch signaling can mutually influence each other throughout the wing vein patterning process. The positive feedback loop established between them helps amplifies initial molecular differences among wing cells, contributing to the establishment of distinct cell fates and tissue boundaries.

A multi-step Delta-Notch feedback responds to aberrant signaling events in the pupal wing

The persistent occurrence of “noisy” Notch signaling during wildtype vein patterning suggested the need for mechanisms to correct errors and establish accurate vein-intervein boundaries. Given that a primary feature of this “noise” is pathway activity in Delta⁺ areas, the Delta-Notch intercellular feedback, known for resolving Delta and Notch expression domains (Collier et al., 1996; Heitzler et al., 1996; de Celis et al., 1997; Huppert et al., 1997; Binshtok & Sprinzak, 2018; Sjöqvist & Andersson, 2019; Bocci et al., 2020), presented an attractive candidate for a quality-control mechanism that ensures proper signaling deployment. Thus, to investigate the Delta-Notch circuit’s potential to respond to noisy signaling, we examined the expression of Delta and the NRE reporter across Notch gain-of-function conditions.

The Notch pathway ligand Delta (Dl) (Fehon et al., 1990; Heitzler & Simpson, 1991), Enhancer of split mβ (E(spl)mβ), a known target and downstream effector of Notch signaling (Heitzler et al., 1996; J. F. De Celis, Celis, et al., 1996; de Celis et al., 1997)), and Deltex (Dx), a well-established positive regulator of Notch pathway activity (Shimizu et al., 2014; Schnute et al., 2022), are all known to induce significant vein loss in adult wings when overexpressed (Matsuno et al., 1995; de Celis et al., 1997; Huppert et al., 1997) (Figs. S3.3A-C). Consistent with Notch pathway upregulation, at 32h APF, en>gal4-mediated overexpression of each of these pathway components leads to an overall increase in NRE reporter activity throughout the

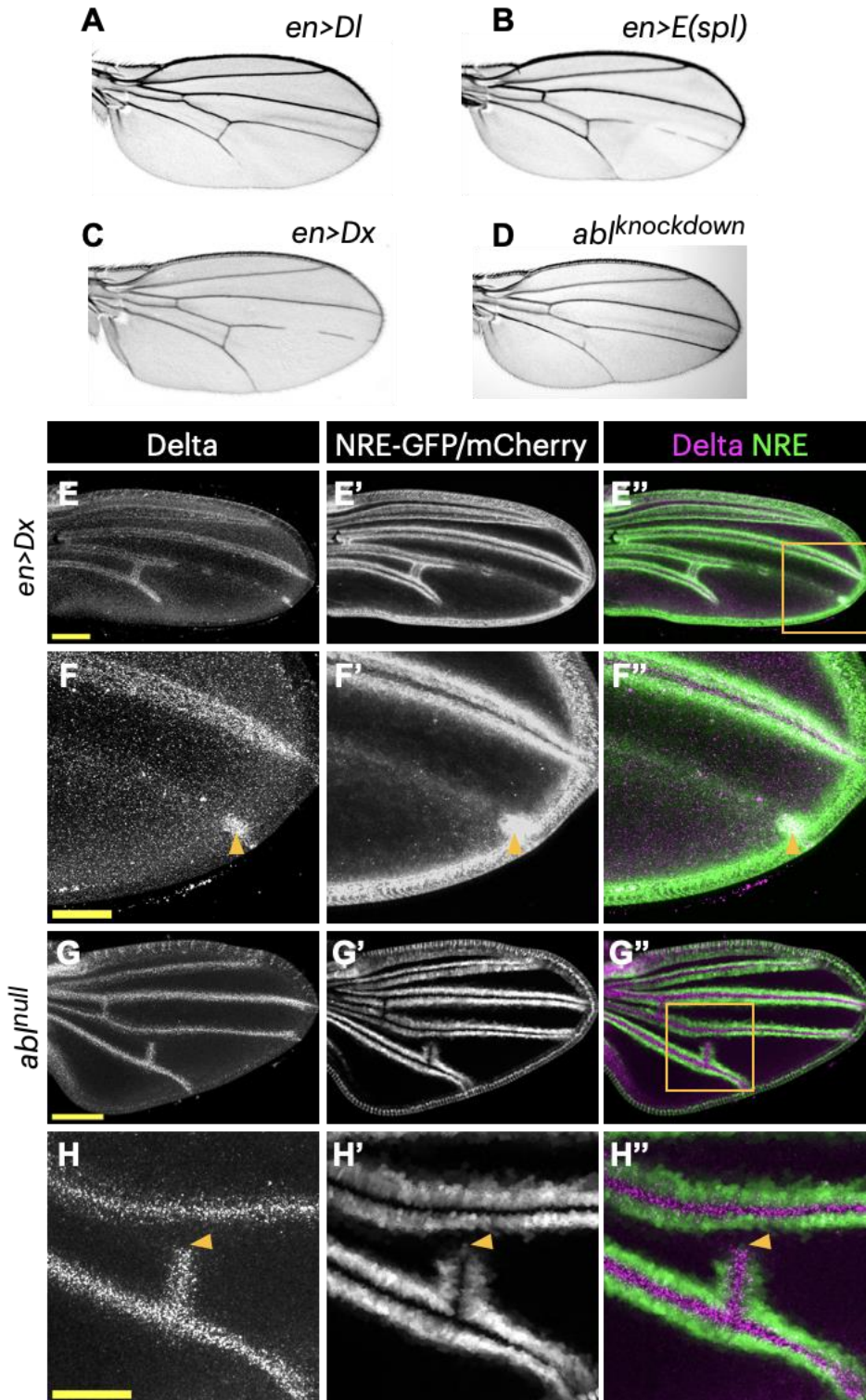


Figure S3.3. Notch gain-of-function conditions induce gaps in the vein pattern.
 (A-D) Adult wing phenotypes of the following manipulations: (A) *en>Dl*, (B) *en>E(spl)*, (C)

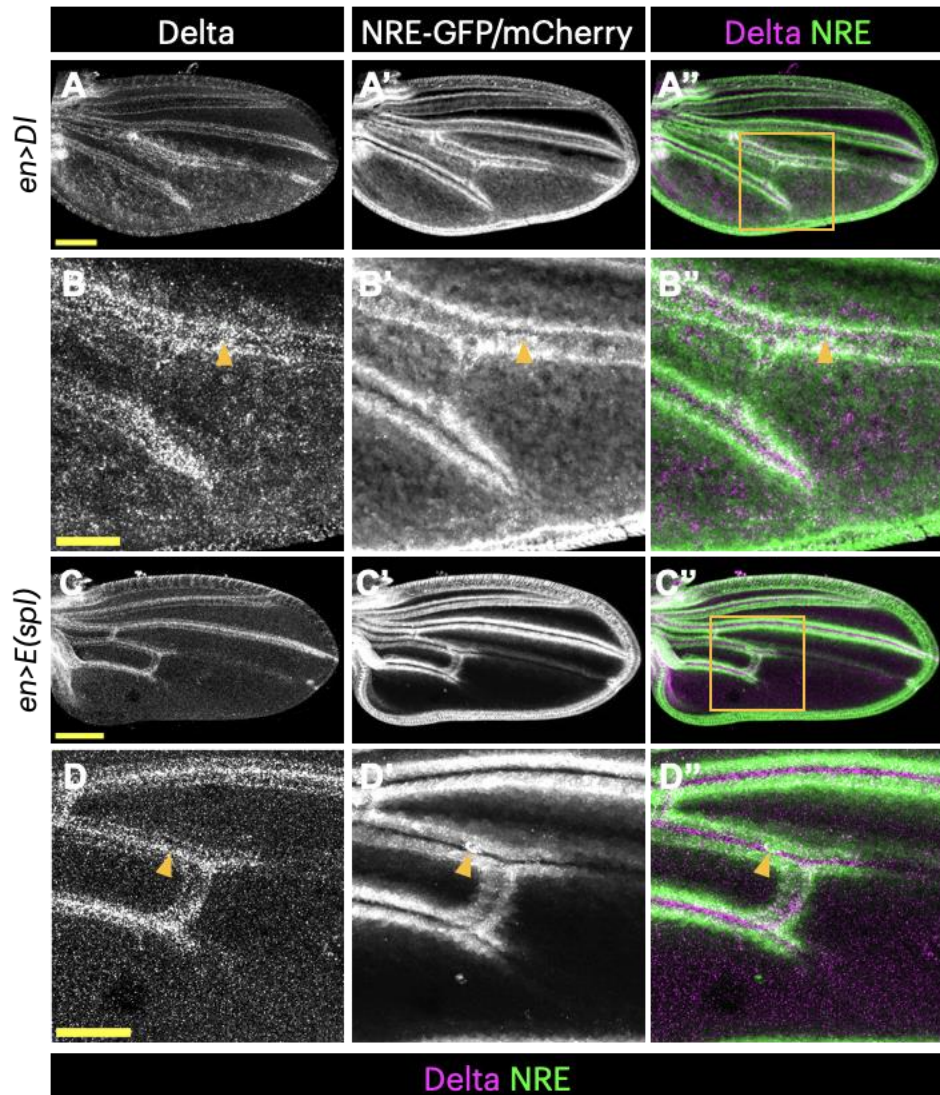
en>Dx, and (D) *en-Gal4>UAS-deGradFp; abl^{GFP}/abl² (abl^{knockdown})*. (E-H) Maximal projections of wings showing Delta expression and/or Notch signaling activity (NRE-GFP/mCherry) at 32h APF. (E,F) *en>Dx*. (G,H) *abl¹/abl²*. Insets in E and G are shown in F and H, respectively. Yellow arrows mark ectopic signaling events in provein domains. Scale bars = 100µm (E,G) or 50µm (F,H).

posterior compartment of wings, with different degrees of induction (Figs. 3.3A',C'; S3.3E').

Closer inspection revealed occasional ectopic Notch signaling in the provein cells of primordial vein structures (yellow arrows in Figs. 3.3B-B'', D-D''; S3.3F-F''), resembling the signaling noise observed in wildtype vein patterning.

Under Delta-Notch feedback conditions, (1) excessive Notch signaling in presumptive vein cells is expected to (2) suppress Delta ligand expression and vein fate in those cells, thereby (3) reducing ligand-induced Notch signaling in the adjacent cells. If sustained, this should manifest as gaps in the NRE pattern and, ultimately, vein loss. Consistent with this, pupal wings overexpressing *Dl*, *Dx*, or *E(spl)* exhibited truncated provein structures, which are characterized by a loss of Delta expression and reduced NRE reporter in the surrounding cells (Fig. 3.3E). These truncated patterns, which mirror the vein gaps observed in adult wings following these manipulations, connect the last two steps of the propose Delta-Notch intercellular circuit model.

To determine whether truncated vein structures arise from prior aberrant signaling in their provein cells, we employed a transcriptional timer system to track the progression of Notch activity in wing tissue displaying vein gaps. This system utilizes a fast-folding, fast-decay NRE reporter (NRE-sfGFP) to indicate current signaling events, and a slow-folding, slow-decay NRE reporter (NRE-RFP) to reflect past signaling activity (He et al., 2019). Since both Transtimer and transgene overexpression tools rely on the UAS/Gal4 system, we assessed the temporal dynamics of Notch signaling in wings null for a previously validated negative regulator of



E. Delta-Notch feedback in Notch gain-of-function conditions

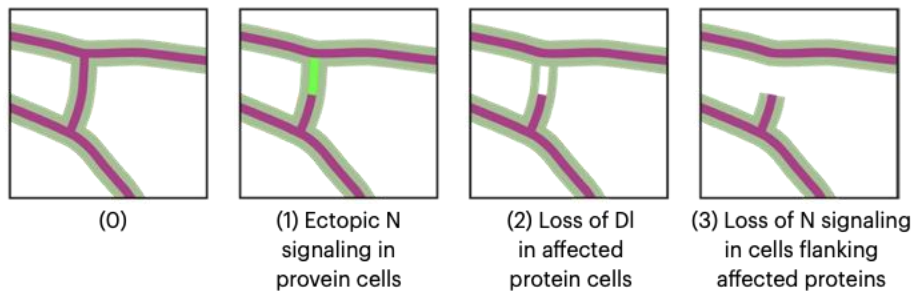


Figure 3.3. The Delta-Notch feedback mechanism involves a complex sequence of intercellular interactions.

(A-E) Maximal projections of wings showing Delta expression and Notch signaling activity (NRE-GFP/mCherry) at 32h APF. (A,B) *en>Dl*. (C,D) *en>E(spl)*. Insets in A and C are shown

in B and D, respectively. Yellow arrows mark ectopic signaling events in provein domains. Scale bars = 100µm (A,C) or 50µm (B,D). (E) Proposed sequence of events in the Delta-Notch feedback mechanism that responds to Notch gain-of-function conditions.

Notch, the Abelson kinase (Abl) (Miranda-Alban et al., 2024; Chapter 2). Loss of Abl leads to gaps in adult vein patterns (Fig. S3.3D), and *abl^{null}* pupal wings display both occasional aberrant signaling events (yellow arrows in Figs. S3.3D-D'') and truncated primordial veins, marked by the absence of both Delta and NRE reporter expression (Fig S3.3D-D'').

NRE>Transtimer was evaluated at 38h APF, the timepoint at which we observed the highest frequency of NRE loss in the flanking cells of *abl^{null}* wings. Examination of wildtype wings revealed broader flanking cell stripes with the NRE-sfGFP reporter compared to those marked by NRE driving a non-superfolding protein (compare Figs. S3.1F'' and 3.4A''), indicating that NRE-sfGFP has higher sensitivity for detecting Notch activity. NRE-RFP showed a pattern generally consistent with NRE-sfGFP, but with thicker signaling regions around the proveins and wing margin (Figs. 3.4A'-A''), possibly reflecting the refinement of signaling stripes during vein patterning. In the PCV, a region typically associated with vein loss upon Notch hyperactivation, NRE-sfGFP and NRE-RFP displayed similar patterns with a clear lack of signal between NRE stripes, delineating the provein cells (Figs. 3.4B-B''). In comparison, while *abl^{null}* wings also showed a good correlation between NRE-sfGFP and NRE-RFP (Figs. 3.4C-C''), key differences were observed in truncated PCV structures. Specifically, the loss of high signaling activity in PCV-associated flanking cells, as shown by both current and past signaling reporters, was accompanied by a more intense past signaling marker within the PCV provein domain compared to the current signaling marker (Figs. 3.4D-D''). These results indicate that the

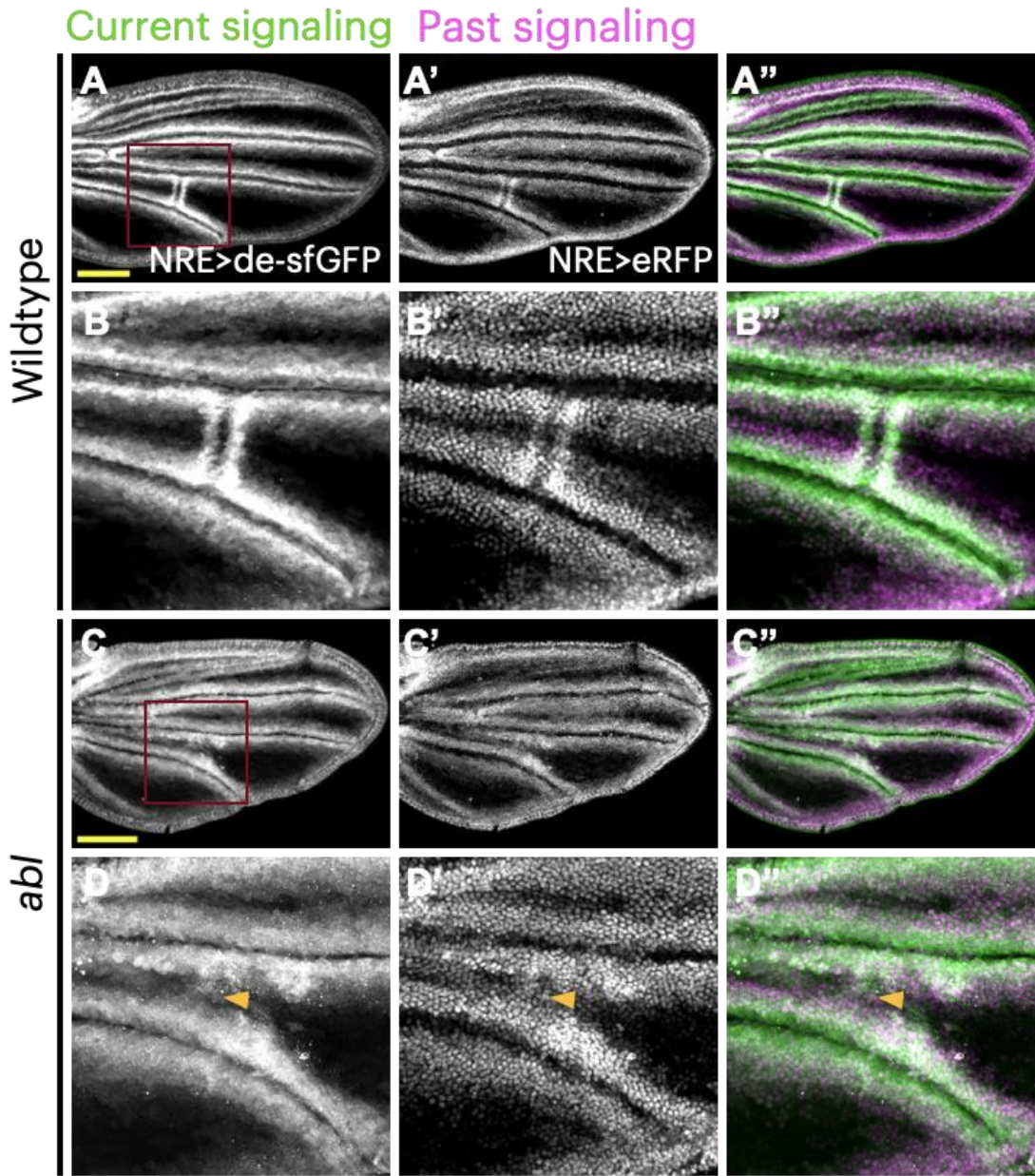


Figure 3.4. Ectopic Notch signaling precedes the depletion of provein structures. (A-D) Maximal projections of wings showing NRE>de-sfGFP or NRE-eRFP, which mark current and past Notch signaling events, respectively. (A,B) Wildtype wings at 38h APF. (C,D) *abl¹/abl²* wings at 38h APF. Insets in A and C are shown in B and D, respectively. Yellow arrows in (D) mark the presence of past signaling reporter in the provein region of a truncated vein structure. Scale bars = 100µm.

loss of Notch signaling in the flanking cells of truncated primordial veins represents a later stage in a process that earlier involved ectopic signaling in the provein cells of these vein structures.

Altogether, our findings suggest that inappropriate Notch signaling triggers dynamic intercellular interactions mediated by Delta-Notch feedback. In Notch gain-of-function conditions, which cause vein loss in adult wings, we observed a range of phenotypes at the pupal stage that likely represent sequential events in a multi-step process. Specifically, we identified evidence for: (1) upregulation of Notch signaling in provein cells, followed by (2) loss of Delta in these cells, which leads to (3) a reduction of Notch activity in adjacent flanking cells (Fig. 3.3E). Our work highlights the complexity of the intercellular coordination deployed to maintain the integrity of the *Drosophila* wing vein pattern and positions the Delta-Notch feedback circuit as a quality-control mechanism that can help manage naturally occurring pathway “noise”.

The Delta-Notch feedback can be activated anywhere in the pupal wing blade

While primarily associated with vein refinement, noisy Notch signaling is observed throughout much of the pupal wing epithelium during wildtype development. This prompted us to consider whether the wing tissue might employ a similarly widespread quality-control mechanisms to correct inappropriate pathway deployment. Specifically, we investigated whether the Delta-Notch feedback circuit could be activated to counteract aberrant venation outside of the expected provein areas.

Excessive vein formation was induced through two main approaches. First, we upregulated provein signals by manipulating the EGFR and BMP pathways. This involved overexpressing key EGFR pathway components, including Rhomboid (rho) (Sturtevant et al.,

1993; de Celis et al., 1997; Urban et al., 2001) (Fig. S3.4A), and constitutively active versions of the EGFR receptor (EGFR^{CA}) (Fig. S3.4B) (Livneh et al., 1985; Price et al., 1989; F. J. Diaz-Benjumea & Garcia-Bellido, 1990; Lesokhin et al., 1999) and the Ras GTPase (Ras^{V12}) Fig. S3.4C) (Seeburg et al., 1984; Barbacid, 1987; Lee et al., 1996; Doroquez & Rebay, 2006). Similarly, we enhanced BMP signaling by overexpressing the BMP-binding protein Crossveinless (Cv) (Shimmi et al., 2005; Vilmos et al., 2005) (Fig. S3.4D) or a constitutively active form of the BMP receptor TkV (TkV^{CA}) or (Brummel et al., 1994; Xie et al., 1994; J. F. De Celis, 1997; Adachi-Yamada et al., 1999) (Fig. S3.4E). Second, we impaired vein-repressive signaling by downregulating Notch pathway activity. This was achieved by overexpressing negative regulators of Notch, such as Abelson (Abl) and Suppressor of Deltex (Su(dx)) (Figs. S3.4F,G) (Miranda-Alban et al., 2024; Schnute et al., 2022; Shimizu et al., 2014).

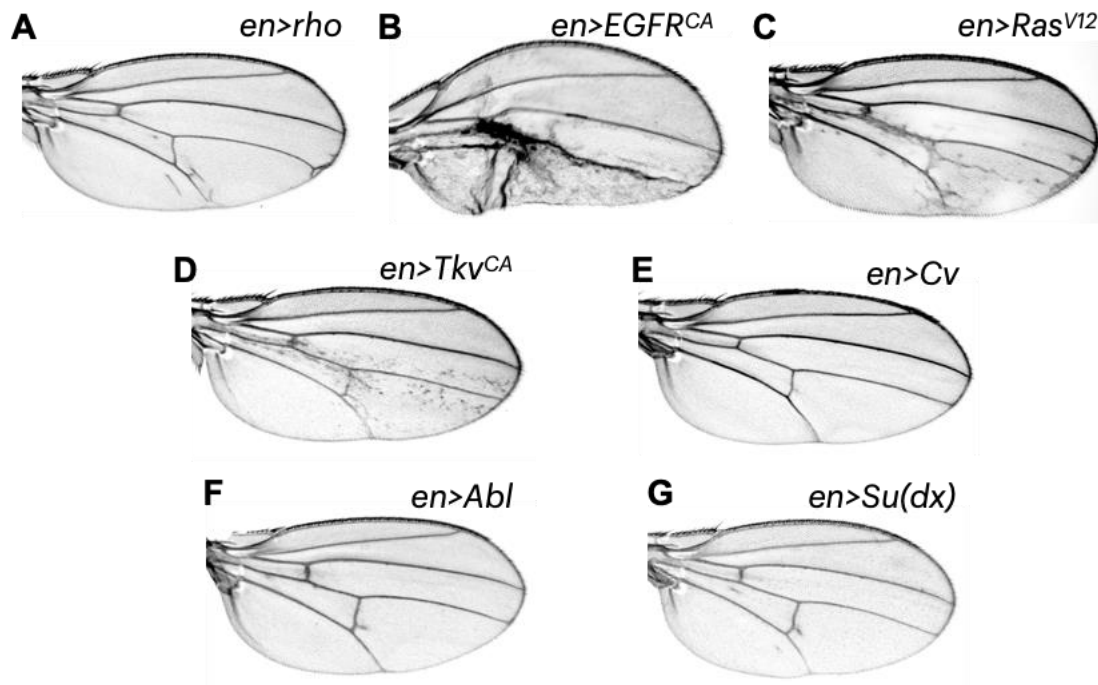


Figure S3.4. Vein expansion can be induced by either upregulation of EGFR or BMP signaling, or by downregulation of Notch activity.

(A-E) Adult wing phenotypes of the following manipulations: (A) *en>rho*, (B) *en>EGFR^{CA}*, (C) *en>Ras^{V12}*, (D) *en>Tkv^{CA}*, (E) *en>Cv*, (F) *en>Abl*, and (G) *en>Su(dx)*.

At 32h APF, using Delta as a marker, we observed extensive vein expansion in wings overexpressing EGFR or BMP pathway components (Figs. S3.5A-E”). This expansion manifested as ectopic veins, vein branching, vein thickening, and the appearance of deltas at the distal end of L4-L5. Notably, the most pronounced effects were detected upon EGFR^{CA} overexpression, where vein growth extended throughout nearly the entire posterior compartment (Fig. S3.5B). In wings with elevated Abl or Su(dx) expression, aberrant vein formation was observed primarily in the form of ectopic veins and deltas at L4-L5 (Figs. 3.5A,B,C,D). In all conditions, the extra vein material was consistently surrounded by NRE reporter expression, indicating that aberrantly induced veins are capable of activating Notch signaling in adjacent intervein cells (Figs. S3.5A-E”; 3.5A-D”). These findings suggest that, although the Delta-Notch quality-control mechanism is normally active only at endogenous veins, it can be triggered throughout the tissue in response to vein expansion, thereby preventing further growth of aberrant provein structures.

While the presence of excessive provein structures marked by Delta and delineated by NRE aligned with the phenotypes observed in adult wings following the manipulations described above, the detection of “ectopic” NRE reporter in Notch loss-of-function conditions presented a slight conundrum. However, the Delta-Notch feedback circuit once again provides a logical mechanistic explanation. In this model: (1) loss of Notch signaling in intervein cells flanking a provein leads to (2) de-repression of Delta in those cells, which can result in expansion of the Delta domain into intervein territory. As the Delta-Notch quality-control mechanism attempts to address this issue,

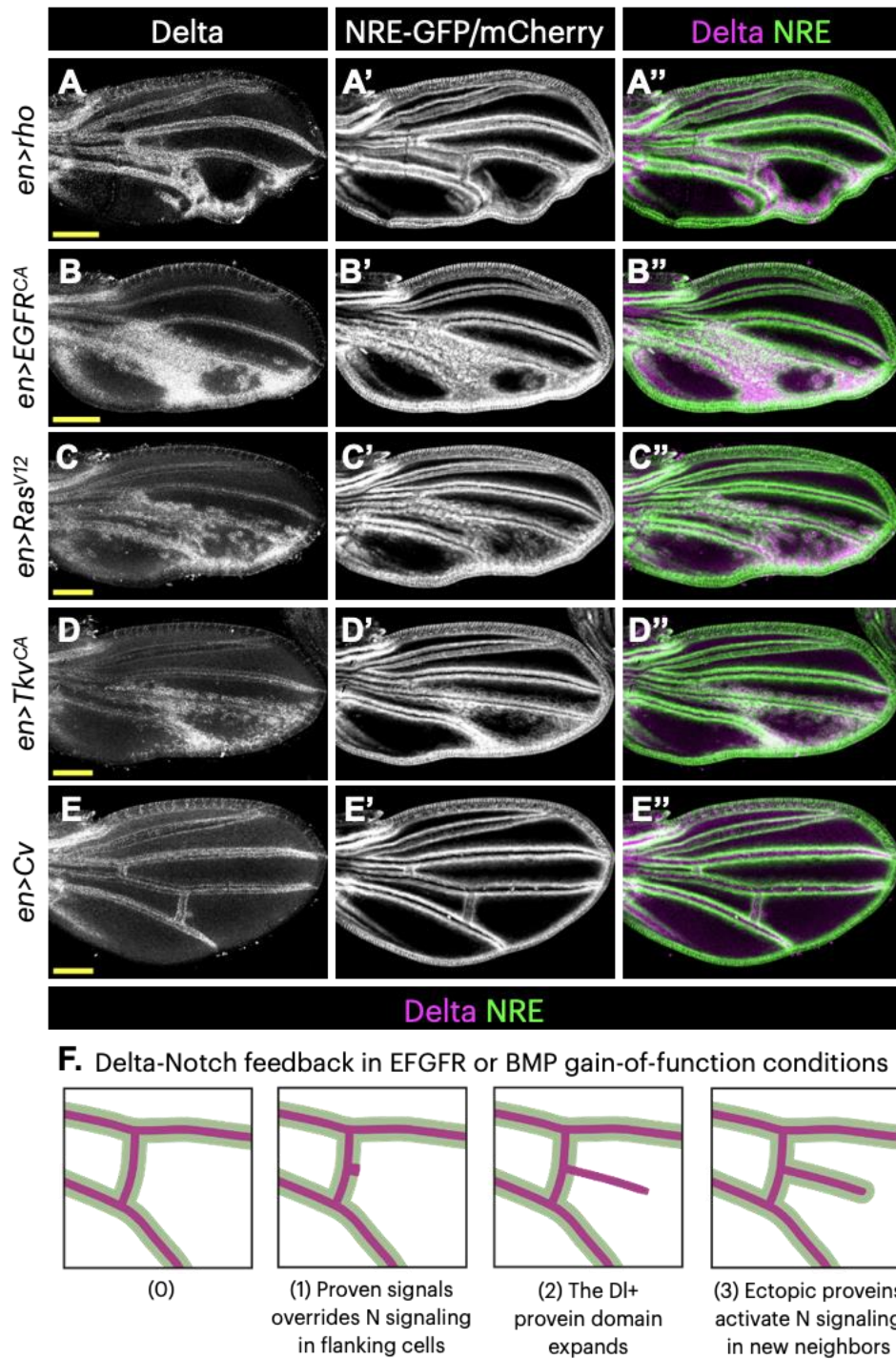
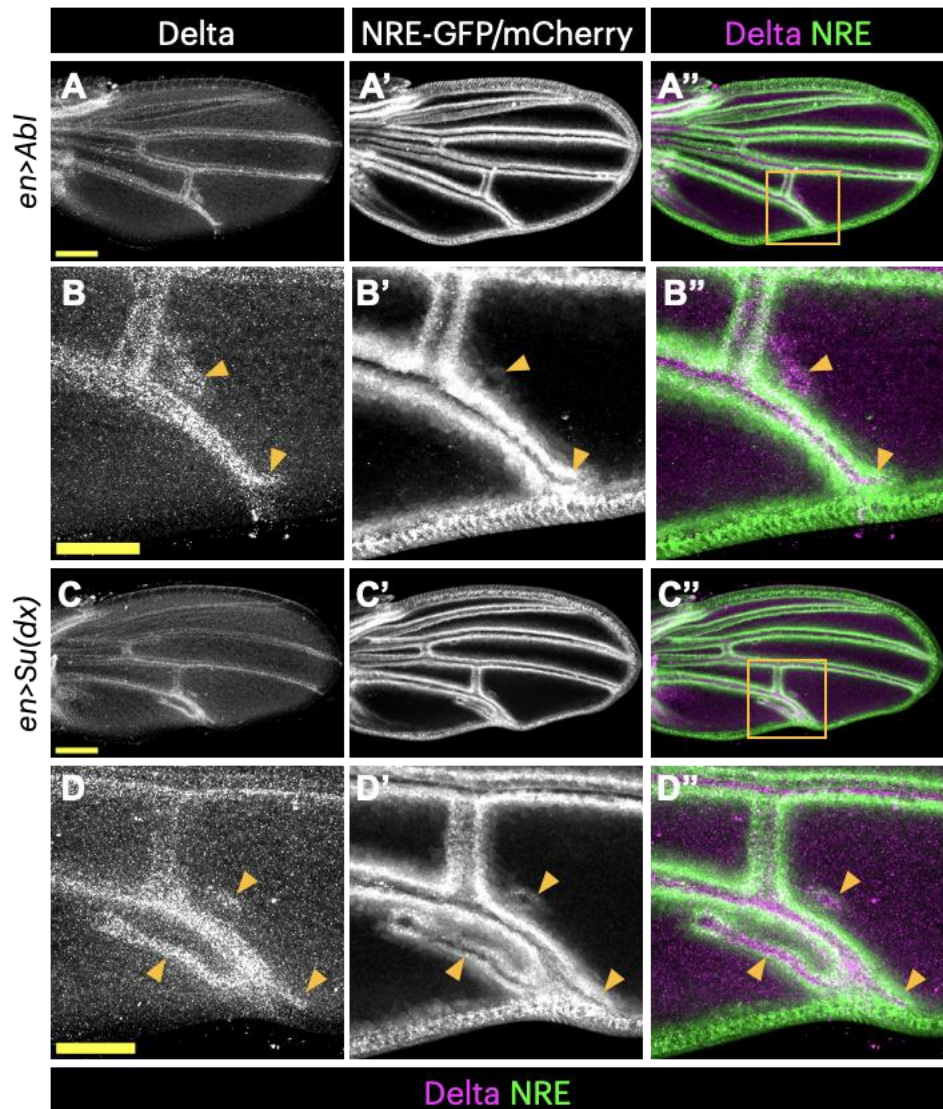


Figure S3.5. The Delta-Notch feedback responds to aberrant vein expansion throughout the pupal wing.

(A,E) Maximal projections of wings showing Delta expression and/or Notch signaling activity (NRE-GFP/mCherry) at 32h APF. (A) *en>rho*. (B) *en>EGFR^{CA}*. (C) *en>Ras^{V12}*. (D) *en>Tkv^{CA}*, *en>Cv*.

(E) *en>Cv*. Scale bars = 100 μ m. (F) Proposed sequence of events in the Delta-Notch feedback mechanism that responds to EGFR or BMP gain-of-function.



E. Delta-Notch feedback in Notch loss-of-function conditions

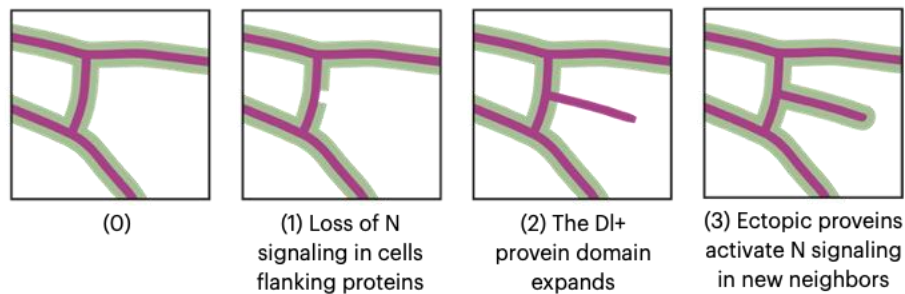


Figure 3.5. The Delta-Notch feedback responds to inappropriate Notch signaling activity throughout the pupal wing.

(A,D) Maximal projections of wings showing Delta expression and Notch signaling activity (NRE-GFP/mCherry) at 32h APF. (A,B) *en>Abl*. (C,D) *en>Su(dx)*. Insets in A and C are shown in B and D, respectively. Scale bars = 100µm (A,C) or 50µm (B,D). (E) Proposed sequence of events in the Delta-Notch feedback mechanism that responds to Notch loss-of-function conditions.

(3) ectopic veins eventually activate Notch signaling in neighboring cells, which prevents further expansion of these ectopic structures (Fig. 3.5E). In the case of EGFR or BMP gain-of-function conditions, loss of Notch signaling does not occur in step (1). Instead, potentiation of provein signals may overpower Notch-mediated vein repression, allowing the veins to expand. Later in the process, Delta-Notch signaling may intervene to counteract the aberrant vein growth (S3.5F).

Although further study of the temporal dynamics of Delta-Notch feedback under vein expansion conditions is needed to confirm the proposed models, we observed that, as predicted by our logic, parts of the growing Delta⁺ ectopic veins occasionally lack significant NRE expression in the surrounding cells (Figs. 3.5A-B”; S3.5E-E”). This observation supports the idea that the growth of a Delta⁺ expanding vein precedes the appearance of “ectopic” Notch signaling, which then limits further growth. Taken together, our results suggest that Delta-Notch feedback signaling can be activated throughout the pupal wing blade to counteract inappropriate vein development.

Strong disruption of Notch signaling reveals the limits of the Delta-Notch feedback mechanism

Our analysis of Delta-Notch signaling in disrupted wing vein development revealed a range of phenotypes within the same tissue following each manipulation. While these

phenotypes are thought to reflect distinct sequential steps in an intercellular coordination mechanism, they raised the question of whether a given phenotype represents an intermediate step in an ongoing process or the endpoint that particular event can reach. This prompted us to investigate whether any inappropriate signaling event can complete the full Delta-Notch feedback process, or if the feedback cannot effectively respond in certain cases.

Under conditions of excessive vein repression, specifically Notch gain-of-function, ectopic NRE reporter activity is expected to be the primary outcome. However, we observed that loss of NRE reporter activity in cells surrounding missing proveins was also a prominent phenotype. Since ectopic Notch signaling marks the initial step of the proposed Delta-Notch feedback sequence in excessive vein repression scenarios (Fig. 3.3E), the prominent presence of the final step suggests that the feedback system may be robust enough to counteract the effects of Notch hyperactivation under the manipulations evaluated above. This led us to ask whether there are conditions that could exceed the capacity of the feedback mechanism and, if so, what the ultimate outcome would be. To address this, we overexpressed the Notch intracellular domain (Notch-ICD), one of the most potent Notch gain-of-function manipulations known in the context of wing vein development (Fig. S3.6A) (Huppert et al., 1997).

En>NICD pupal wings exhibited a pronounced hyperactivation of the NRE reporter across all cell types, which was accompanied by a large loss of Delta⁺ provein structures (Figs. 3.6A-A”). In comparison to milder Notch gain-of-function conditions, NICD overexpression led to more frequent NRE activity in provein cells (yellow arrows in Fig 3.6B-B”). Thus, stronger upregulation of Notch activity increases the likelihood of detecting ectopic signaling within provein regions. Since ectopic signaling represents the initial step in the Delta-Notch feedback sequence leading to truncated vein structures in gain-of-function conditions, these results suggest

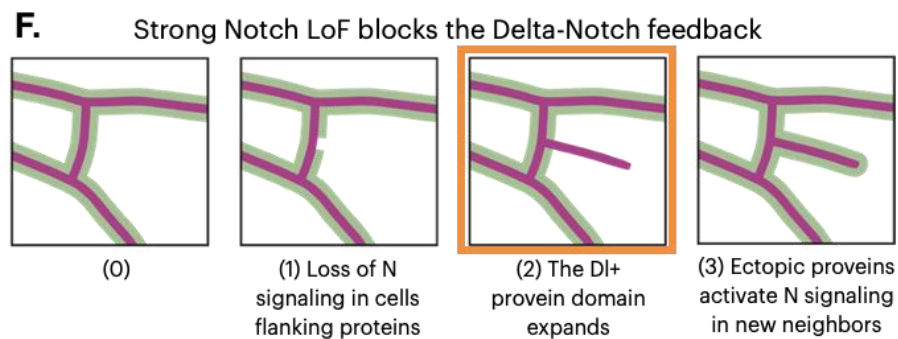
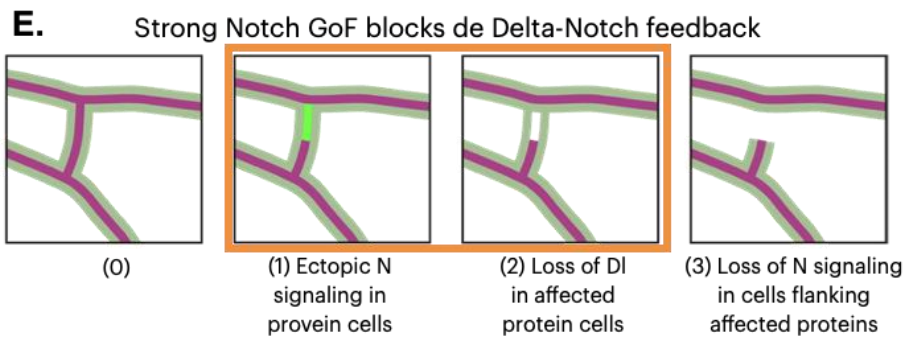
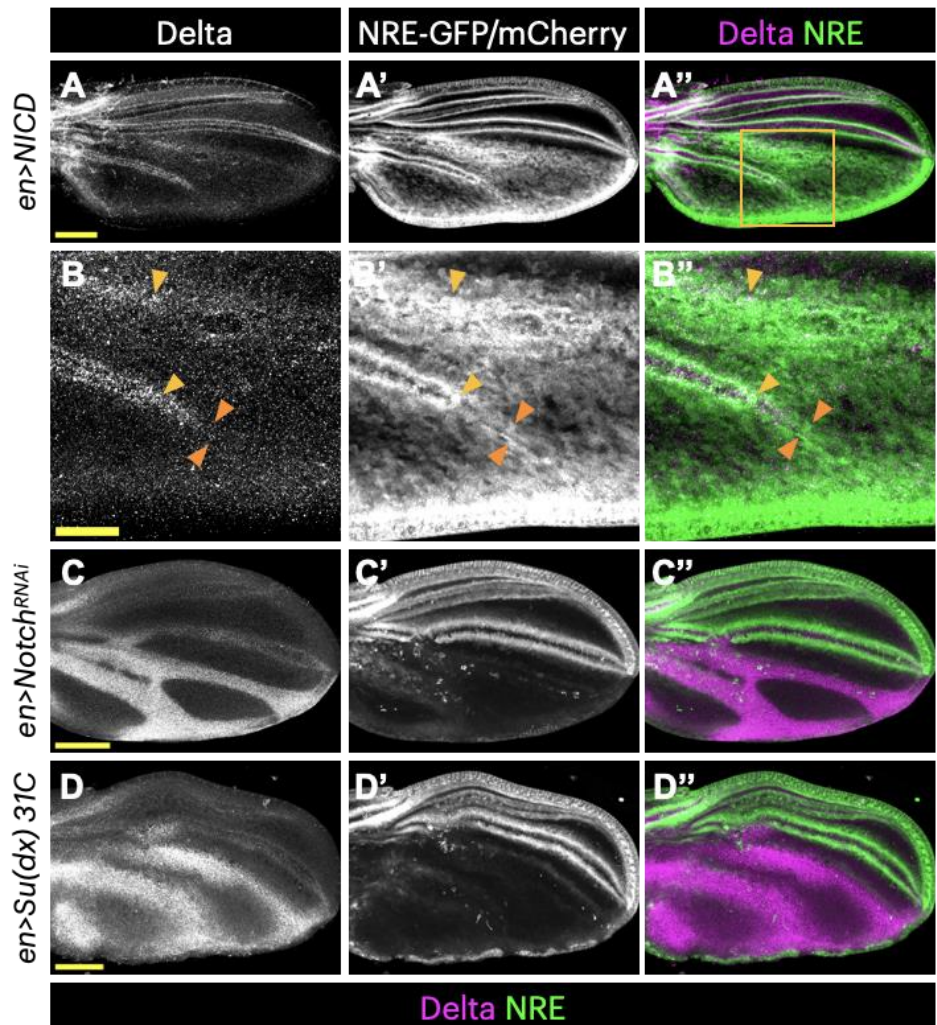


Figure 3.6. Strong manipulations of Notch signaling block the Delta-Notch feedback mechanism I.

(A-D) Maximal projections of wings showing Delta expression and Notch signaling activity (NRE-GFP/mCherry) at 32h APF. (A,B) *en>NICD*. (C) *en>Notch^{RNAi}*. (D) *en>Su(dx)* at 31°C. Inset in A is shown in B. Yellow arrows mark ectopic signaling in provein cells. Orange arrows indicate signaling activity in flanking cells despite the absence of a Delta ligand source. Scale bars = 100µm (A,C,D) or 50µm (B). (E) Steps at which strong Notch gain-of-function conditions block the Delta-Notch intercellular feedback. (F) Step at which strong Notch loss-of-function conditions block the Delta-Notch intercellular feedback.

that NICD overexpression might prevent effective deployment of the quality-control system (Fig. 3.6E). Therefore, the capabilities and limits of Delta-Notch intercellular coordination under excessive Notch activity appear to be determined by the intensity of Notch hyperactivation.

We also observed that in regions of primordial vein structures where Delta was completely absent from provein cells, often there was a notable presence of NRE signal in the adjacent intervein cells (orange arrows in Figs. 3.6B-B''), a phenomenon not seen in milder Notch gain-of-function manipulations. This could reflect an intermediate stage leading to gaps in both Delta and NRE patterns, or it may suggest that strong Notch gain-of-function allows for the maintenance of NRE expression in flanking cells even in the absence of ligand, at least temporarily. To test this possibility, further investigation is needed to monitor the fate of these remaining NRE⁺ cells in the absence of a significant Delta source.

In cases of excessive vein formation, our analysis provided key insights into the deployment of the Delta-Notch feedback. When vein expansion was induced by upregulation of EGFR or BMP, Delta-Notch signaling was activated to counteract the vein overgrowth, resulting in NRE reporter activity around the aberrantly induced extra veins (Figs. S3.5A-E''). This response was observed even in extreme instances of vein expansion, such as EGFR^{CA}

overexpression (Fig. S3.5B-B’). Remarkably, similar outcomes were also seen when vein expansion followed downregulation of Notch activity, where loss of NRE reporter activity would typically be the hallmark phenotype. While NRE was indeed lost in a subset of flanking cells in these contexts, ectopic expression of NRE in intervein regions also emerged as a prominent phenotype (Fig. 3.5A-D’). Since loss of Notch signaling marks the initial step in the Delta-Notch feedback sequence under compromised vein repression contexts (Figs. S3.5F; 3.5E), the predominant observation of the final step suggests that the feedback system may be sufficiently robust to counteract the initial effects of vein expansion, even under reduced Notch activity. However, given the mild vein expansion produced by Abl or Su(dx) overexpression, and the apparent inability of the Delta-Notch feedback to be deployed under extreme Notch hyperactivation, we wondered whether the feedback circuit might also be overwhelmed by extreme downregulation of Notch activity.

To investigate conditions that might exceed the capacity of the Delta-Notch feedback, we evaluated several potent Notch loss-of-function manipulations. These included overexpression of Notch^{RNAi} (Fig. S3.6B) (Zacharioudaki & Bray, 2014), Delta^{RNAi} (Fig. S3.6C) (Perkins et al., 2015), a dominant-negative form of Delta (Delta^{DN}) (Fig. S3.6D) (Parks et al., 2006), and Nedd4Lo, a strong negative regulator of Notch signaling (Fig. S3.6E) (Miranda-Alban et al., 2024). In all cases, Delta expression revealed a dramatic expansion of the provein domains, characterized by severe vein thickening (Figs. 3.6C, S3.6F,G,H). This was accompanied by a substantial loss of Notch signaling across the entire compartment (Figs. 3.6C’, S3.6F’,G’,H’). Not only was the NRE reporter severely reduced in the stripes of cells surrounding the provein regions, but we also observed very little ectopic signaling compared to milder manipulations. These results suggest that stronger loss-of-function manipulations increase the likelihood of

detecting significant reductions in signaling, a hallmark of such manipulations. Furthermore, since reduced signaling represents an initial step in the Delta-Notch feedback sequence under Notch loss-of-function conditions, these findings imply that more severe disruptions in signaling impair the effectiveness of the intercellular quality-control mechanism (Fig. 3.6F). Therefore, the capabilities and limitations of Delta-Notch intercellular coordination in insufficient Notch activity conditions are determined by the extent of Notch downregulation.

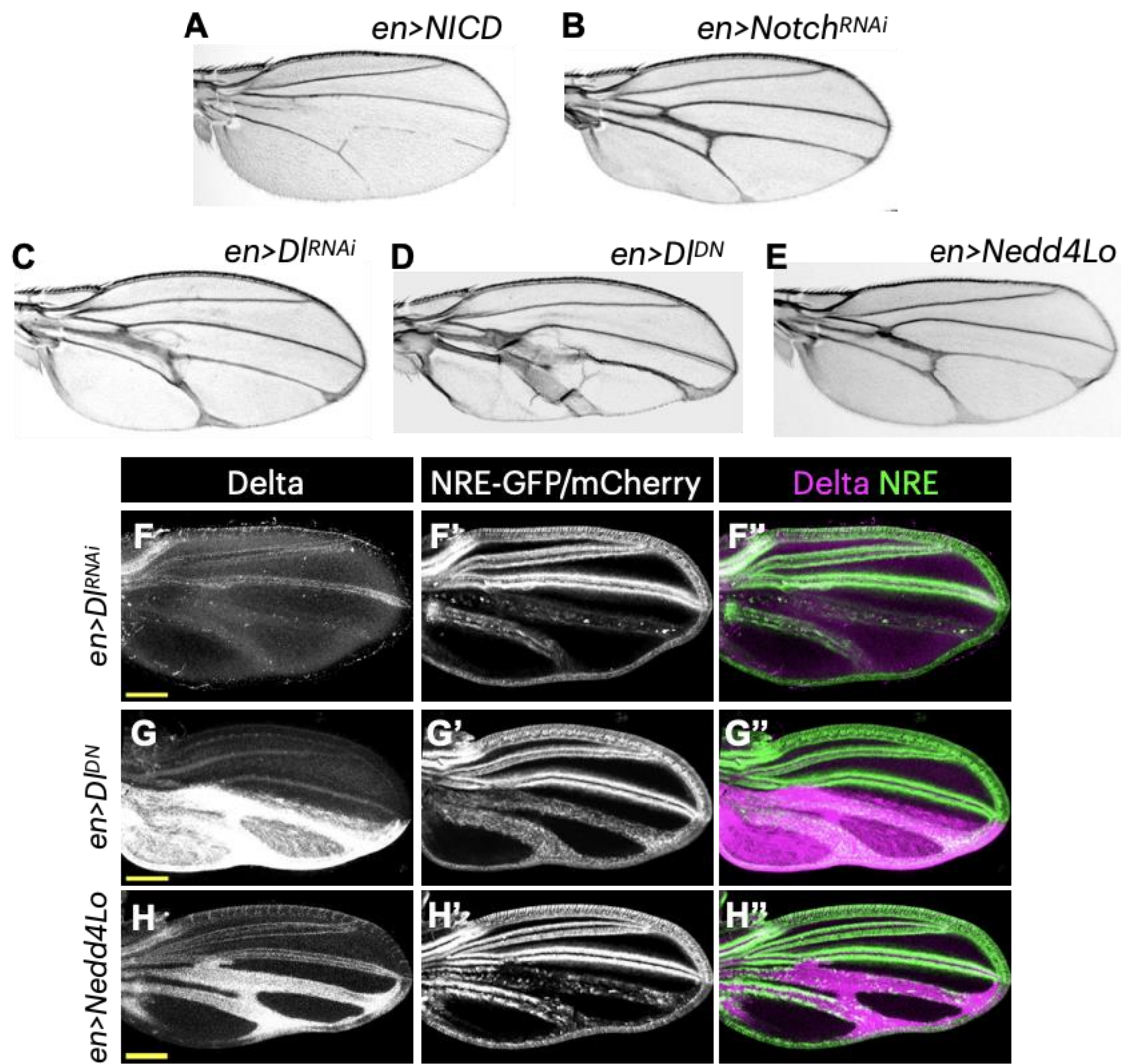


Figure S3.6. Strong manipulations of Notch signaling block the Delta-Notch feedback mechanism II.

(A-E) Adult wing phenotypes of the following manipulations: (A) *en>NICD*, (B) *en>Notch^{RNAi}*, (C) *en>Dl^{RNAi}*, (D) *en>Dl^{DN}*, and (E) *en>Nedd4Lo*. (F,H) Maximal projections of wings showing Delta expression and Notch signaling activity (NRE-GFP/mCherry) at 32h APF. (F) *en>Dl^{RNAi}*, (G) *en>Dl^{DN}*, and (H) *en>Nedd4Lo*. Scale bars = 100µm.

In contrast to tissue with increased Abl or Su(dx) activity, pupal wings overexpressing Notch^{RNAi}, Delta^{RNAi}, Delta^{DN} or Nedd4Lo exhibited a distinct absence of Notch signaling activity around the aberrantly expanding vein domains. This lack of signaling could either indicate an intermediate stage leading to eventual activation of significant Notch activity or suggest that, under strong Notch loss-of-function conditions, expanding veins fail to effectively induce Notch activity in neighboring cells. Consequently, this failure prevents the expanding structure from being constrained. To confirm this interpretation, further investigation, including time-lapse imaging, will be necessary to monitor the ultimate fate of cells involved in a vein expansion process.

The degree of loss of Notch function predicts whether vein expansion manifests as vein thickening or as ectopic veins.

A notable difference between wings overexpressing either Abl or Su(dx) and those with Notch^{RNAi}, Delta^{RNAi}, Delta^{DN} or Nedd4Lo is that the former typically have ectopic veins while the latter exhibit vein thickening. Although different genetic manipulations of Notch activity can affect vein patterning in diverse ways (Blair, 2007), this difference suggested that the severity of the Notch loss-of-function condition influences the type of vein expansion observed. If so, then

increasing the strength of a genetic manipulation that usually induces ectopic veins could shift in the phenotype to vein thickening.

To test this, we increased the temperature at which we induced Su(dx) overexpression from 26°C to 31°C. Remarkably, this resulted in a phenotype closely resembling that of pupal wings overexpressing Notch^{RNAi}, Delta^{RNAi}, Delta^{DN} or Ned4Lo (Figs. 3.6C; S3.6F-H”), with dramatically thickened Delta⁺ proveins and substantial loss of NRE throughout the posterior compartment (Fig. 3.6D-D”). These results address a long-standing question in the field: what determines whether Notch loss-of-function conditions lead to ectopic veins or vein thickening? Our data suggest that the extent of Notch activity loss, which largely influences the effectiveness of Delta-Notch feedback deployment, plays a critical role in shaping the final phenotype of insufficient Notch signaling during wing vein development.

3.3 Discussion

The reliable acquisition of cell fates in precisely organized spatiotemporal patterns is essential for establishing tissue domains and boundaries during organismal development. In this process, lateral inhibition signals between neighboring cells mediate intercellular coordination, resolving binary fate decisions and generating patterns with single-cell precision. The primary molecular toolkit for lateral inhibition during developmental patterning is the Notch signaling pathway, whose activity is finely tuned by a Delta-Notch feedback mechanism across various biological systems (Seydoux & Greenwald, 1989; Heitzler & Simpson, 1991; Wilkinson et al., 1994; Heitzler et al., 1996; de Celis et al., 1997; Huppert et al., 1997; Binshtok & Sprinzak, 2018; Sjöqvist & Andersson, 2019; Bocci et al., 2020). Using the *Drosophila* wing vein pattern

as a model system, this study provides detailed insights into the progressive spatiotemporal deployment of the Delta-Notch intercellular circuit as it shapes diverse cell types across a dynamic cell field. By genetically perturbing Notch and other vein development regulators, we reveal how naturally occurring noisy Notch signaling is refined to establish vein-intervein boundaries and how Delta-Notch feedback-mediated coordination counteracts inappropriate levels of Notch pathway activity to safeguard biological patterns.

Our investigation first demonstrates how the spatial distribution of Delta ligand expression, downstream of provein signaling, is progressively established during wildtype development. The Delta domain is gradually surrounded and constrained by areas of high Notch receptor and signaling activity, with each component of this scheme influencing the others. Notably, as the initially disorganized distribution of Delta is consolidated into properly spaced provein stripes, high signaling reporter expression is frequently observed not only in presumptive flanking intervein cells, but also in both Delta⁺ provein cells and within the Delta⁻ proper intervein region. This reveals that the process of establishing vein-intervein boundaries is regulated by noisy Notch signaling activity and, consequently, is error-prone, underscoring the need for quality-control mechanisms in Notch pathway deployment.

Through genetic manipulations of venation, we discovered that, although normally active only in the provein regions, dynamic Delta-Notch signaling can be activated throughout the pupal wing epithelium. Whether in conditions of excessive vein induction or suppression, a series of both cell-autonomous and non-cell-autonomous responses are triggered, coordinating a multi-step Delta-Notch intercellular mechanism that can potentially involve all cell types across the wing epithelium. In particular, under excessive vein production due to upregulation of EGFR or BMP signaling, or following downregulation of Notch activity, the Delta-Notch feedback

system appears to compensate by introducing new, ectopic vein-repressing cues in the extended vein regions. Conversely, excessive vein repression due to Notch hyperactivation in provein regions depletes vein identity in these cells, leading to a loss of ligand-sustained Notch activity in neighboring cells. Altogether, these data suggest that the Delta-Notch intercellular circuit both ensures deployment of vein-suppression mechanisms where vein-promoting signals need active regulation, and prevents vein-repressing activity in regions where vein-promoting signals are no longer present.

In the context of wildtype development, the frequent occurrence of aberrant Notch signaling events likely reflects ongoing tissue-level fluctuations in Notch activity, as cells compete to resolve their fates through lateral inhibition. Over time, proper organization emerges from this “chaotic” process as the Delta-Notch circuit catalyzes cell fate assignment dynamics. Thus, we propose that the Delta-Notch quality-control system, which can respond to disruptions in vein development, whether through the Notch pathway or independently of it, plays a crucial role in ensuring precise Notch signaling deployment to preserve the integrity of the vein pattern.

As we examined the capabilities and limitations of the Delta-Notch intercellular mechanism, we found that while it can effectively respond to a variety of perturbations in vein development signaling, it can be overwhelmed by extreme disruptions in the activity of Notch itself, revealing its constraints. Both strong downregulation and upregulation of the Notch pathway tend to block the Delta-Notch feedback circuit at early stages of its proposed temporal sequence, leading to outcomes that align more closely with the nature of the perturbation. For example, strong Notch gain-of-function conditions resulted in widespread ectopic signaling across all cell types, whereas strong Notch loss-of-function conditions caused a dramatic reduction in signaling activity. By comparing phenotypes at the pupal and adult stages, we have

learned that disruptions exceeding the Delta-Notch feedback system's capacity often lead to dramatic adult wing phenotypes.

Our exploration of how Delta-Notch signaling responds to various strengths of genetic manipulation has also addressed a long-standing question in the field. While both ectopic vein formation and vein thickening are associated with Notch loss-of-function conditions (de Celis et al., 1993; J. De Celis & García-Bellido, 1994; Blair, 2007), the molecular mechanisms driving these distinct outcomes have remained unclear for decades. Our study has pinpointed a crucial factor in this decision-making process: the extent of Notch activity loss directly influences the Delta-Notch feedback mechanism's ability to manage disruptions. Specifically, moderate loss of Notch activity allows the feedback mechanism to generate ectopic veins, while excessive Notch loss overwhelms the system, leading to vein thickening due to inadequate quality control. This discovery argues that final biological outcomes, in the form of adult vein patterns, are shaped by the inherent capabilities and constraints of the Delta-Notch regulatory circuit.

Further emphasizing the complexities of vein development regulation by Delta-Notch signaling, we note that similar or related phenotypes observed at the adult stage can appear quite distinct when examined during pupal development. For instance, while both overexpression of Dx and NICD produce vein gaps in adult wings, only NICD leads to ligand-independent NRE expression around missing primordial veins at the pupal stage. Similarly, while both overexpression of Su(dx) and Notch^{RNAi} both result in excessive vein formation in adult wings, pupal wing analysis reveals that Su(dx) overexpression primarily causes ectopic signaling reporter expression, while Notch^{RNAi} leads to a substantial loss of reporter activity. Thus, evaluating vein patterns solely in adult wings may oversimplify the underlying biology. A

detailed examination of both adult and pupal wing phenotypes can provide deeper insights into the molecular mechanisms shaping final vein patterns.

Altogether, our study shows how tightly regulated Notch signaling orchestrates vein-
intervein boundary formation in the developing *Drosophila* wing. Through a series of
manipulations, we illustrate how vein-suppressing cues driven by the Notch pathway are
precisely deployed where vein-promoting signals require active regulation. This process is
governed by a robust Delta-Notch feedback circuit that acts as a tissue-wide surveillance
mechanism, responding to inappropriate vein development events. While this system is capable
of adapting to a wide range of manipulations, including strong upregulation of vein-promoting
signals, extreme disruptions in Notch activity can overwhelm the feedback circuit, resulting in
significant patterning defects. Ultimately, our findings underscore how the interplay between
signaling and feedback dynamics contributes to the robustness of developmental patterning and
the maintenance of tissue domains and boundaries.

3.4 Methods

Drosophila genetics

Flies were raised on standard *Drosophila* food at 26°C unless otherwise noted. The
following lines were used: *abl*¹ (Henkemeyer et al., 1987), *abl*² (Henkemeyer et al., 1987), NRE-
EGFP (BDSC 30727), NRE-mCherry (a gift from Sarah Bray), NRE-Gal4 (a gift from Norbert
Perrimon), UAS-Transtimer (a gift from Norbert Perrimon), en>Gal4, tub>Gal80ts, UAS-Delta
(BDSC 26694), UAS-E(spl)mβ (BDSC 26675), UAS-Flag-Dx (a gift from Spyros Artavanis-

Tsakonas), UAS-rho (BDSC 8858), UAS-EGFR^{CA} (BDSC 9533), UAS-Ras^{V12} (BDSC 64196), UAS TkV^{CA} (BDSC 36537), UAS-Abl-GFP (O'Donnell & Bashaw, 2013), UAS-Su(dx) (BDSC 15664), UAS-NICD (Go et al., 1998), UAS-Notch^{RNAi} (Zacharioudaki & Bray, 2014), UAS-DI^{DN} (BDSC 26697), UAS-DI^{RNAi} (BDSC 34322), and UAS-Nedd4Lo (BDSC 81604) (Zhong et al., 2011). For UAS-driven genetic manipulations, crosses were established at 18°C and individual animals were transferred to 26°C at 0h APF. They were either dissected during early pupal development, generally at 32h APF, or allowed to grow to adulthood.

Immunostaining of fly tissue, antibodies and co-localization analysis

16-38h APF wing dissections: pupae were decapitated, fixed in 4% paraformaldehyde (PFA) in PBS for 12-24h at 4°C, washed 3x and then dissected in PBS. Dissected wings were immediately blocked in 1% normal goat serum in PBT for 30min at room temperature or overnight at 4°C, incubated overnight at 4°C with primary antibodies in PBT, washed 3x in PBT, incubated overnight with secondary antibodies at 4°C, washed 3x in PBT, mounted in n-propyl gallate mounting medium and imaged on a Zeiss LSM 880 confocal microscope. Endogenous fluorescent signals were imaged directly. Antibodies: mouse anti-NECD (1:100, Developmental Studies Hybridoma Bank (DHSB) C458.2H), mouse anti-Delta (1:500, DHSB C594.9C), goat anti-mouse Cy3, Alexa 488 and Cy5 (1:2000, Jackson ImmunoResearch).

3.5 Acknowledgements

We would like to thank Nortbert Perrimon, Sarah Bray, Spyros Artavanis-Tsakonas,

Daniela Rotin, and Hitoshi Matakatsu for providing fly strains, as well as Rick Fehon for generously donating antibodies. We also appreciate advice on pupal wing dissection from Hideyoshi Shimizu and Hitoshi Matakatsu, as well as experimental assistance from Carol Dilts and Zach Baker. Additionally, we are grateful to all members of the Rebay Lab, as well as Rick Fehon, Chip Ferguson, and Sally Horne-Badovinac for valuable discussions.

CHAPTER 4

LIGAND-INDEPENDENT NOTCH SIGNALING ACTIVELY MAINTAINS INTERVEIN FATE DURING WING VEIN PATTERNING IN *DROSOPHILA*

This short chapter constitutes the foundation for a letter-format manuscript that will be submitted for publication after the completion of this dissertation.

Statement of contributions:

While the work presented in this chapter predominantly reflects my own ideas and scientific curiosity, it was significantly enhanced by the contributions of two exceptional collaborators: Zach Baker and Carol Dilts. Under my mentorship, Zach evaluated how various manipulations of the Notch pathway impact vein development at distinct regions of the *Drosophila* wing. His impressive technical dexterity along with his scientific insight have been crucial in bringing this project to fruition. Similarly, Carol's extensive assistance in investigating Notch-mediated wing vein patterning—through experiments directly related to this chapter as well as those extending beyond it—not only catalyzed my dissertation work but also sparked new research directions in our lab. As Carol has since developed her own PhD project, our stimulating scientific discussions have been instrumental in advancing our understanding of how Notch signaling is deployed across the fly wing. The remaining work was completed by me under the guidance of Dr. Ilaria Rebay.

4.1 Introduction

Successful construction of a functional tissue requires the precise formation and maintenance of distinct multicellular domains along with well-defined borders between them (Batlle & Wilkinson, 2012; Wolpert et al., 2019; Barresi & Gilbert, 2023). Cells with specialized roles often need to be physically separated to ensure that each group can perform its functions efficiently (Dahmann et al., 2011). At the core of this process, cell-cell communication mechanisms typically orchestrate the specification of regional tissue identities, with initially imprecise patterns gradually refined as the borders between tissue subdivisions become sharper and more defined. Disruptions in the proper localization of cells can lead to a range of developmental defects or diseased states, ranging from tissue malformation to the metastatic spread of tumorigenic cells (Batlle & Wilkinson, 2012).

The precise formation of tissue domains and boundaries is well exemplified by the stereotypical vein pattern of the *Drosophila* wing. In this system, the accurate specification of veins and intervein regions, along with the establishment of clear boundaries between them, relies on the coordinated deployment of signaling pathway mechanisms (Blair, 2007; Sotillos & De Celis, 2005). As the early pupal wing elongates proximo-distally and its dorsal and ventral epithelia converge, vein formation is driven by spatiotemporally organized EGFR and BMP signaling (Yu et al., 1996; J. F. De Celis, 1997; Gabay et al., 1997; Martín-Blanco et al., 1999; Sotillos & De Celis, 2005; Blair, 2007; Antson et al., 2022). Broad vein regions, that initially lack clear borders, are refined by Delta-Notch signaling approximately between 16h-32h after puparium formation (APF) (Huppert et al., 1997; Herszterg et al., 2023). During this period, EGFR and BMP signaling promote Delta expression in developing vein cells (proveins)

(Sturtevant & Bier, 1995; de Celis et al., 1997; Huppert et al., 1997; J. F. De Celis, 1997; Tanimoto et al., 2000; Blair, 2007). This Delta activates Notch signaling in adjacent intervein cells (referred to as “flanking cells” to distinguish them from the “proper intervein cells”), which in turn represses vein fate and *Delta* expression, creating a feedback loop that prevents Notch activation within the proveins (de Celis et al., 1997; Huppert et al., 1997).

Notably, through our studies of Notch signaling regulation in Chapters 2 and 3, we observed that under several conditions of compromised Notch activity, ectopic vein material appeared in the center of the large intervein areas (Figs 4.1A,B). The presence of these isolated vein structures suggested that this phenotype might not simply result from a failure to refine the initially broad provein domains. Instead, it could reflect independent vein formation events driven by insufficient Notch activity. This led us to hypothesize that Notch-dependent repression of vein formation might not only be required in cells immediately adjacent to the endogenous veins, but also deeper within the intervein regions.

In this study we have combined a sensitive signaling reporter with genetic mosaic approaches to investigate the requirement for Notch signaling in intervein areas distant from vein-intervein borders. Examination of superfolder GFP driven by a Notch responsive element, NRE>sfGFP, suggests that low levels of Notch signaling are widespread across the pupal wing epithelium. Moreover, genetic mosaic analyses confirm that Notch-mediated vein repression is actively required within intervein regions, including cells located far from a major ligand source. Consistent with this, while Delta is the primary driver of Notch signaling near the veins, we find that Notch activity is sustained within intervein domains in a Delta/Serrate-independent manner. To our knowledge, this is the first report of a developmental role for ligand-independent Notch activation in non-circulating cells. More broadly, our findings suggest that these low levels of

ligand-independent Notch activity may not only maintain intervein fate, but may also prime these cells to engage Delta-Notch feedback mechanisms that counteract excessive vein formation, thereby thoroughly preserving the integrity of vein-intervein patterning.

4.2 Results

Low levels of Notch signaling repress vein fate in the interveins

As we had previously noted, multiple Notch loss-of-function conditions, including overexpression of Abl or Su(dx), can produce ectopic vein structures that are not directly in contact with endogenous proveins (Figs. 4.1A,B). Examination of these manipulations in pupal wings reveals that this phenotype can be detected as early 32h APF (Figs. 4.1C-D”). These results indicate that Notch signaling is required to prevent vein formation across the entire wing blade and raised the question of whether the Notch activity extends beyond vein-intervein boundaries.

To further investigate this, we utilized the enhanced sensitivity of the NRE-sfGFP reporter, which allowed us to detect lower levels of Notch signaling that were previously undetectable by conventional reporters. Specifically, examination of NRE-sfGFP at 32h APF showed stripes of reporter activity that were significantly broader than those detected by NRE-GFP (Figs. 4.1E-F’). More specifically, while 2-3 rows of provein cells are typically flanked by approximately 3 rows of high NRE-GFP signal in most veins around at this timepoint, the high NRE-sfGFP signal extends well beyond 3 cell rows, often reaching up to 5-6 rows (Figs. 4.1E’,F’). Additionally, although the NRE-GFP reporter is rarely observed in proper intervein territories, detailed examination of pupal wings around the completion of vein patterning (32h

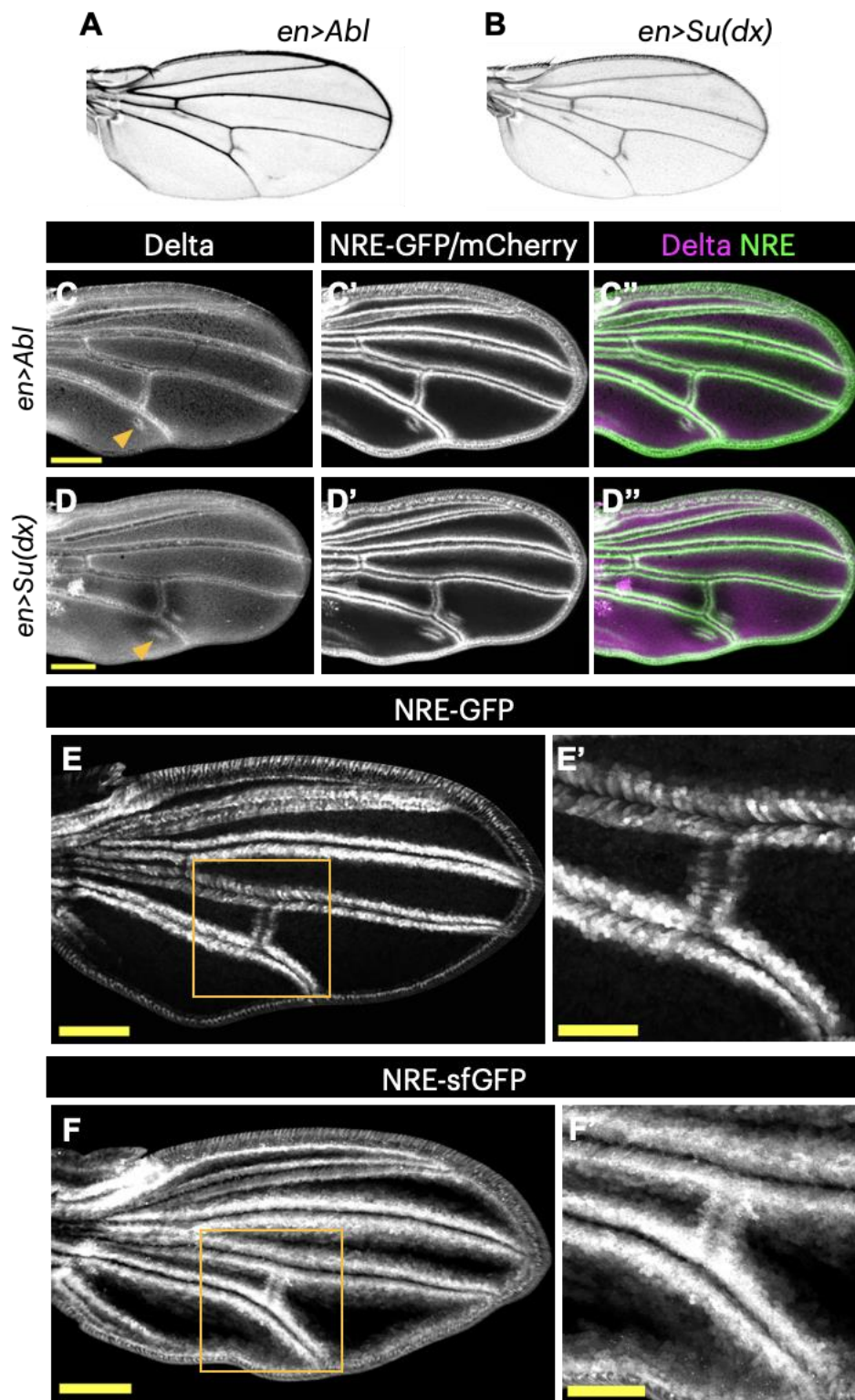


Figure 4.1. Low levels of Notch signaling repress vein fate in the interveins.

(A,B) *en>Abl* and *en>Su(dx)* adult wings, respectively. (C,D) Maximal projections of wings showing Delta expression and Notch signaling activity (NRE-GFP/mCherry) at 32h APF. (C) *en>Abl*. (D) *en>Su(dx)*. Yellow arrows in (C,D) highlight ectopic veins that are not associated with endogenous veins. (E,F) Maximal projections of wildtype wings showing Notch signaling activity, as indicated by reporters with varying sensitivity, at 32h APF. (E) NRE-GFP. (F) NRE-sfGFP. Scale bars = 100µm (C-D'', E,F) or 50µm (E',F').

APF) occasionally detected the NRE-GFP reporter in intervein regions, particularly near the L3 provein (orange arrow in Fig. 4.1E). These observations suggest that, to varying degrees, Notch signaling may be active throughout the wing blade, playing a crucial role in preventing ectopic vein formation outside of the expected areas.

Ligand-independent Notch signaling suppresses vein formation in the interveins

If low levels of Notch signaling actively repress vein fate within intervein domains, how is Notch activated in these regions? Delta and Serrate, the *Drosophila* Notch ligands, are both exclusively upregulated at provein structures (Figs. 4.2 A,B), with Delta being the key ligand driving Notch signaling in these regions, while Serrate is reported not to play a role in this specific context (Huppert et al., 1997). This led us to consider whether ligand-independent mechanisms might sustain Notch signaling in the interveins.

To test this hypothesis, we employed a genetic mosaic analysis approach. As previously reported, generation of *Notch*^{55e11} clones induced vein expansion in adult wings, manifesting as vein thickening or ectopic veins (Fig. 4.2C) (J. De Celis & García-Bellido, 1994). Notably, ectopic veins were observed both in direct contact with endogenous veins or isolated from them entirely (yellow arrows in Fig. 4.2C). In agreement with this, evaluation of Delta expression in

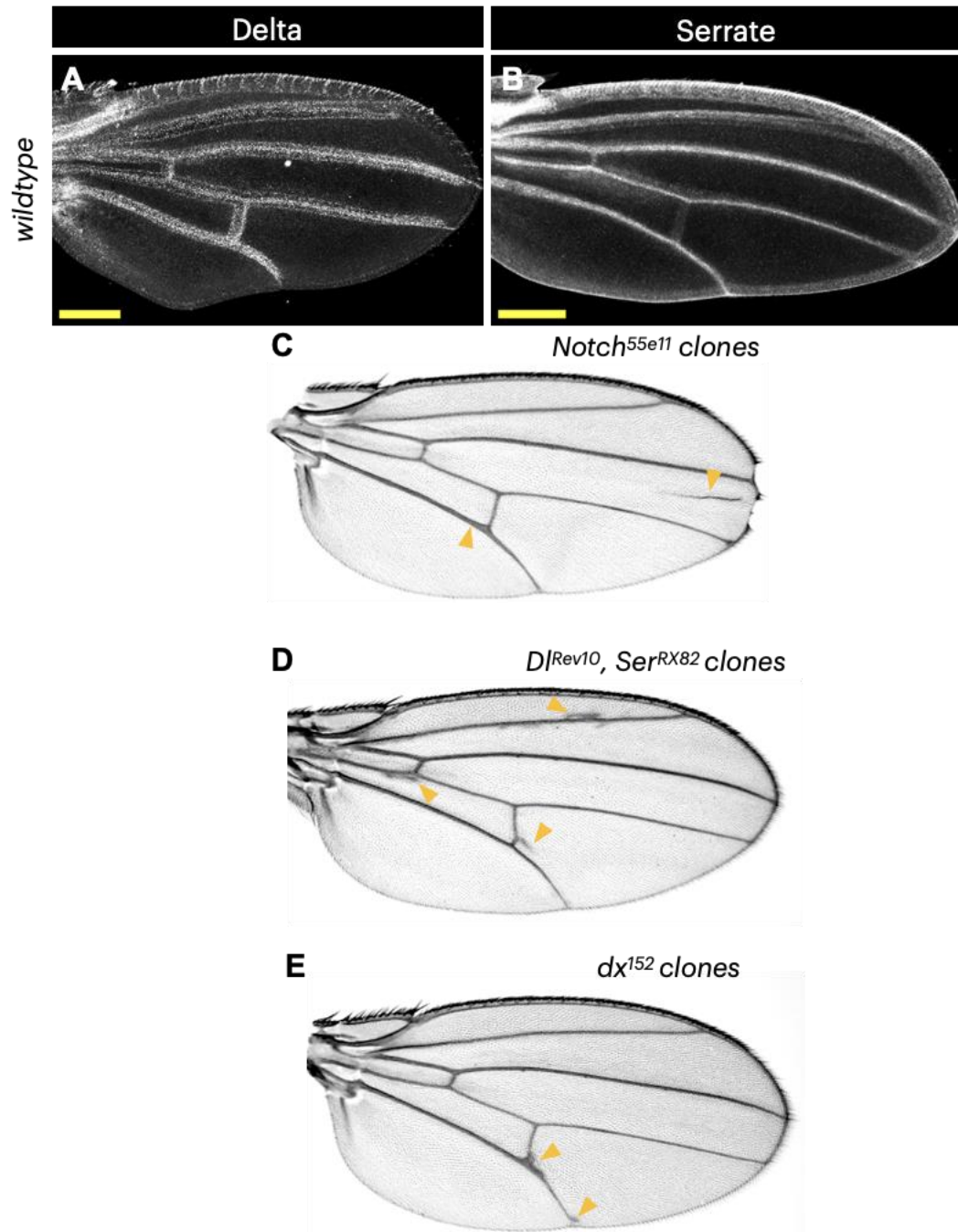


Figure 4.2. Delta, Serrate and Deltex are not required for Notch-mediated vein-suppression in the interveins.

(A,B) Maximal projections of wings showing expression of Delta (A) or (B) Serrate at 32h APF. While Delta expression was evaluated through immunofluorescence, Serrate expression was

detected via an endogenous Ser-GFP protein trap. Scale bars = 100µm. (C,E) Adult stage clonal wing tissue for the following manipulations: (C) *Notch*^{55e11}, (D) *Dl*^[RevF10], *Ser*^[RX82], and (E) *dx*¹⁵². Yellow arrows indicate aberrant vein expansion events.

pupal stage *Notch*^{55e11} clonal tissue showed the formation of Delta⁺ ectopic vein material both in *Notch*^{55e11} areas associated with or isolated from the endogenous primordial veins (Figs. 4.3A-A').

In contrast to *Notch*^{55e11} clones, evaluation of *Dl*^[RevF10], *Ser*^[RX82] mosaics in adult wings revealed excessive vein material only in direct contact with endogenous proveins (arrows in Figs. 4.2D). Surprisingly, in examining pupal stage *Dl*^[RevF10], *Ser*^[RX82] clonal tissue, we observed Delta protein marking vein structures within the “ligand-depleted” clones (Fig. 4.3B). This was unexpected, as the *Delta*^[RevF10] allele is thought to be amorphic due to the excision of the promoter region, transcription start site, and first exon (Heitzler & Simpson, 1991). Nevertheless, leveraging this marking, we found that Delta⁺ ectopic veins formed only when *Dl*^[RevF10], *Ser*^[RX82] tissue was neighboring endogenous proveins (Figs. 4.3B-B'). These findings suggest that *Delta*^[RevF10] produces an inactive Delta protein that fails to induce Notch signaling around vein primordia, leading to the formation of ectopic veins associated with these structures. Moreover, they argue that, while repression of vein fate through ligand-induced Notch signaling occurs specifically in the immediate vicinity of provein cells, ligand-independent Notch activity represses vein formation in the interveins. To our knowledge, this is the first report of a developmental role for ligand-independent Notch signaling in non-circulating cells.

Given that Notch ligands are not responsible for sustaining pathway activity in the intervein territory, we wondered how signaling is activated in these areas. To begin addressing this question, we investigated the potential of Deltex (Dx), a ubiquitin ligase that promotes Notch

activation through endocytic trafficking, to activate Notch in the interveins. Evaluation of both adult and pupal stage wings revealed that, similar to *Dl*^[RevF10], *Ser*^[RX82] somatic mosaics, *dx*¹⁵² clonal tissue produced extra vein material only when in physical contact with endogenous vein structures (Figs. 4.2E, 4.3C-C’'). These observations indicate that Dx might not be responsible for sustaining Notch signaling in intervein domains. Alternatively, multiple factors could contribute to this signaling, masking the role of Dx when abrogated alone.

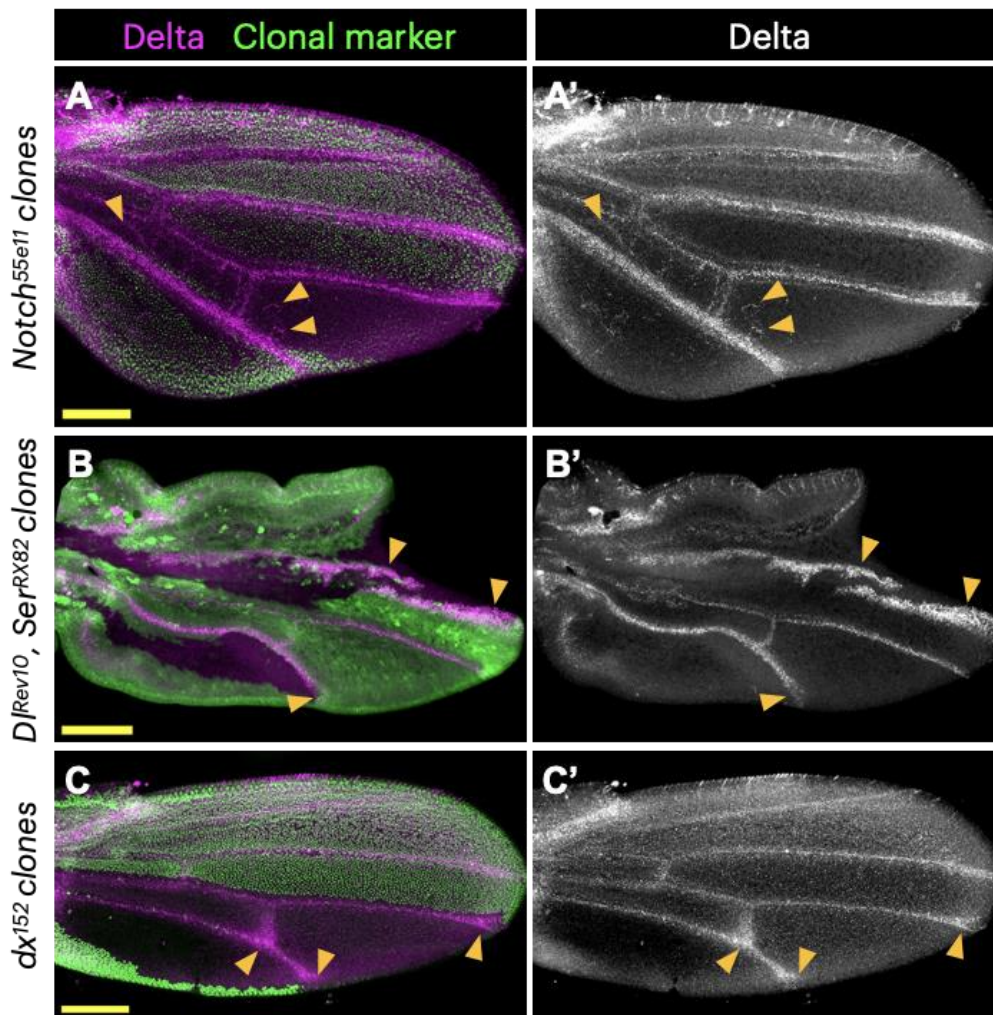


Figure 4.3. Delta/Serrate/Detlex-independent Notch signaling prevents vein formation in the interveins.

(A,B) Delta marking primordial veins in (A-A') *Notch*^{55e11}, (B-B') *Dl*^[RevF10], *Ser*^[RX82], and (C-C') *dx*¹⁵² somatic clones at 32h APF. Yellow arrows mark aberrant vein expansion events. Scale bars = 100µm.

4.4 Discussion

Tightly-regulated deployment of signaling mechanisms orchestrates wing vein development in the *Drosophila* wing. Specifically, pre-patterned EGFR and BMP signaling (Yu et al., 1996; J. F. De Celis, 1997; Gabay et al., 1997; Martín-Blanco et al., 1999; Sotillos & De Celis, 2005; Blair, 2007; Antson et al., 2022) drive vein formation, while the Notch pathway counteracts provein activity, ensuring that vein structures are confined to the appropriate areas observed in adult tissue (de Celis et al., 1997; Huppert et al., 1997). According to foundational studies (reviewed in Blair, 2007), Delta expression downstream of the vein fate program activates Notch signaling specifically in cells flanking the proveins. However, my work, along with that of others, has shown that Notch signaling is also crucial for repressing vein fate beyond vein-intervein boundaries, in regions distant from significant ligand sources. In this study, I provided multiple lines of evidence showing that Delta/Serrate-independent low levels of Notch signaling play a crucial role in maintaining intervein identity during wing vein patterning.

Although the formation of ectopic veins in *Notch* clones isolated in intervein territory was first observed decades ago (J. De Celis & García-Bellido, 1994), the significance of this phenotype in the study Notch signaling was not well appreciated at the time. In effect, careful characterization of the expression pattern of Notch pathway components in the context of wing

vein patterning was not achieved until the late 1990s (de Celis et al., 1997; Huppert et al., 1997). As a result, the concept that Notch-mediated vein-suppression is needed in the interveins, distant from ligand-expressing cells (i.e. ligand-independent signaling), was overlooked and likely faded from consideration over time. Our findings have now renewed attention to this earlier observation, arguing that Notch activity is required across the entire wing blade to actively repress vein fate. Supporting this, examination of a sensitive signaling reporter, NRE-sfGFP, indicates that active Notch signaling is potentially present in all cells of the pupal wing. This is consistent with the ubiquitous expression of Notch protein across this tissue and suggests that, in this system, the receptor can be activated regardless of ligand presence. Given the widespread expression of Notch in developing tissues across species, ligand-independent signaling may contribute to patterning mechanisms in many different contexts.

Interestingly, evaluation of the NRE-sfGFP reporter revealed a gradient of Notch signaling activation that is progressively diluted as it moves into intervein territory, which could suggest that ligand-induced Notch signaling extends into the interveins. Notwithstanding, the fact that *Notch*^{55e11} clones, unlike *Dl*^[RevF10], *Ser*^[RX82] clones, can form ectopic vein material away from endogenous veins argues that the Notch activity repressing vein formation within the interveins is likely Delta/Serrate-independent. While the exact mechanism driving Notch activation away from ligands remains to be identified, our findings show that Notch signaling can be deployed in the interveins even in the absence of Deltex. Ongoing work in our lab is investigating the roles of cytonemes, tissue mechanics, and other cellular mechanisms in establishing the Notch signaling gradient that refines vein-intervein boundaries in the fly wing.

Of importance, ligand-independent Notch activation had previously been linked to the development of *Drosophila*'s crystal cells, mobile blood cells that may encounter ligands during

circulation (T. Mukherjee et al., 2011). Since intervein cells are non-delaminating epithelial cells, our study is the first one to report a developmental role for ligand-independent Notch signaling in a non-circulating cell context. As NRE-sfGFP becomes more widely used in Notch signaling studies, we anticipate that additional developmental roles for ligand-independent Notch signaling will be uncovered, opening up an exciting and rapidly expanding area of research.

Among others, the discovery of ligand-independent Notch signaling in the interveins raises a critical question: why is vein repression actively required beyond vein-intervein boundaries? One possibility is that, as previously reported, EGFR and BMP signaling (the key drivers of vein formation) extend beyond the provein regions into the intervein areas, gradually diminishing as they spread further (Yan et al., 2009). This may explain why, even at very low levels, Notch signaling is necessary in intervein cells: to prevent them from adopting vein fate in response to active provein signaling. As a non-mutually exclusive possibility, ligand-independent Notch activation might complement and interact with other Notch regulatory mechanisms during wing vein patterning. For example, low levels of Notch induction in the interveins may provide basal pathway activity, enabling Delta-Notch signaling to be deployed more efficiently when the system needs to address vein patterning perturbations. Additionally, an interesting alternative model proposes that vein fate repression might be the default program in the pupal wing epithelium, with vein formation occurring only when active provein signaling is strong enough to override vein suppression.

In summary, our study uncovers a previously overlooked mode of Notch pathway activation in the developing *Drosophila* wing. Low levels of Notch signaling, independent of Delta or Serrate induction, help maintain intervein identity during the vein-intervein boundary establishment process. As Delta-Notch feedback signaling can be deployed throughout the tissue

in response to inappropriate vein formation, ligand-independent inductive cues might provide competency to engage this quality-control mechanism. Further increasing the diversity of mechanisms regulating Notch during wing vein patterning, our prior work shows how endosomal trafficking can contribute to both ligand-dependent and ligand-independent events in pupal wing epithelia (Miranda-Alban et al., 2024). In this regard, while our findings suggest that Dx might not be the primary driver of Notch signaling in the interveins, further exploration of endosomal trafficking mechanisms that could play this role is imperative. Ultimately, we propose that a complex interplay between ligand-dependent and ligand-independent Notch regulation ensures precise pathway deployment, which is crucial for the accurate establishment and maintenance of tissue domains and boundaries.

4.4 Methods

Drosophila genetics

Flies were raised on standard *Drosophila* food at 26°C unless otherwise noted. The following lines were used: *Notch*^{55e11} (BDSC 28813), *Dl*^[RevF10], *Ser*^[RX82] (BDSC 6300), *dx*¹⁵² (Fuwa et al., 2006), *Ser*-GFP (BDSC 59824), *NRE*-EGFP (BDSC 30727), *NRE*-Gal4 (a gift from Norbert Perrimon), *UAS*-Transtimer (a gift from Norbert Perrimon), *en*>Gal4, *tub*>Gal80ts, *UAS*-Abl-GFP (O'Donnell & Bashaw, 2013), and *UAS*-Su(dx) (BDSC 15664). For *UAS*-driven genetic manipulations, crosses were established at 18°C and individual animals were transferred to 26°C at 0h APF. They were either dissected during early pupal development, at 32h APF, or allowed to grow to adulthood.

To generate heatshock-driven *Notch*^{55e11} clones in wing tissue, *Notch*^{55e11}/*FM6* females were crossed to *y, w, hs>Flp, His2Av-GFP FRT19A/Y* males. To generate *Dl*^[RevF10], *Ser*^[RX82] clones, *y, w, hs>Flp;; ubi-RFP, FRT82B/TM6, Tb* females crossed to *Dl*^[RevF10], *Ser*^[RX82], *FRT82B/TM6, Tb* males. To generate *dx*¹⁵² clones, *y, w, hs>Flp, His2Av-GFP FRT19A* females were crossed to *dx*¹⁵² *FRT19A/Y* males. In all cases, parents laid eggs overnight, offspring were allowed to develop for 32h before heatshock treatment at 37°C for 45min, and heatshocked animals were transferred back to 26°C until dissection.

Immunostaining of fly tissue, antibodies and co-localization analysis

32h APF wing dissections: pupae were decapitated, fixed in 4% paraformaldehyde (PFA) in PBS for 12-24h at 4°C, washed 3x and then dissected in PBS. Dissected wings were immediately blocked in 1% normal goat serum in PBT for 30min at room temperature or overnight at 4°C, incubated overnight at 4°C with primary antibodies in PBT, washed 3x in PBT, incubated overnight with secondary antibodies at 4°C, washed 3x in PBT, mounted in n-propyl gallate mounting medium and imaged on a Zeiss LSM 880 confocal microscope. Endogenous fluorescent signals were imaged directly. Antibodies: mouse anti-Delta (1:500, DHSB C594.9C), goat anti-mouse Cy3, Alexa 488 and Cy5 (1:2000, Jackson ImmunoResearch).

4.5 Acknowledgements

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CHAPTER 5

CONCLUSIONS, IMPLICATIONS AND FUTURE DIRECTIONS

5.1 Introduction: Fine tuning of Notch signaling

The Notch signaling pathway, which orchestrates a broad spectrum of cellular behaviors and developmental processes, has captivated researchers for many decades. Despite its relatively simple design, lacking secondary messenger amplification and regulation, Notch exhibits remarkable versatility, guiding cells along highly diverse developmental paths in a context-dependent manner (Andersson et al., 2011; Sachan et al., 2024). This versatility relies on the accurate regulation of activated Notch (free NICD) production and its temporal dynamics (Nandagopal et al., 2018; Shen et al., 2021). Therefore, a variety of regulatory mechanisms must modulate NICD production to ensure that Notch signaling produces spatiotemporally appropriate outcomes (Shen et al., 2021).

While context-dependent integration of Notch signaling with other developmental cues or microenvironmental signals often underpins its multifaceted biological functions (LaFoya et al., 2016; Mishra et al., 2021; Sachan et al., 2024), research in the past two decades has uncovered intricate layers of regulation within the Notch pathway itself (see reviews by Schnute et al., 2018; Henrique & Schweisguth, 2019; Falo-Sanjuan & Bray, 2020; Shen et al., 2021). These mechanisms encompass optimization of Notch activity through post-translational modification and/or intracellular trafficking of both ligands and receptors, the geometry of ligand-receptor interactions, the nuclear turnover of the NICD, complex genetic circuits, and more. Although

several of these mechanisms are still emerging or consolidating within the Notch research field, I anticipate that some will ultimately become integral components of the pathway.

In this regard, using the establishment of the wing vein pattern in *Drosophila* as a model system, my doctoral dissertation investigated developmentally relevant approaches to precisely amplify or limit Notch inductive cues in space and time. In Chapter 2, I demonstrate how a network of post-translational modifiers converges at late endosomal vesicles to meticulously regulate Notch receptor activation from these compartments. This mechanism limits Notch activity in pupal wing epithelial cells irrespective of ligand exposure. In Chapter 3, I elucidate how a complex Delta-Notch feedback circuit, reliant on sequential intercellular interactions, is dynamically deployed to balance vein-promoting and vein-suppressing signals across the entire developing pupal wing. Furthermore, my work in Chapter 4 led to the discovery of a ligand-independent mode of Notch signaling that represses vein formation in intervein regions. Collectively, my findings highlight the rich variety of mechanisms that fine-tune Notch signaling during development, including intracellular, intercellular, ligand-dependent, and ligand-independent routes. I propose that this wide array of regulatory strategies is harmoniously integrated to assemble living systems with exceptional precision and robustness.

In this final chapter, I further explore the broad implications of my research on Notch pathway regulation and its significance in wing vein patterning and potentially other developmental contexts. I also discuss unresolved questions and propose potential avenues for future research. I am confident that the Notch pathway will continue to engage and inspire researchers for many years to come and hope that the questions arising from this work will offer stimulating challenges for future generations of developmental biologists.

5.2 Lessons from the late endosomal regulation of Notch and future work

The findings presented in this dissertation, along with previous research by others (M. B. Wilkin et al., 2004; M. Wilkin et al., 2008; Vaccari et al., 2008; Shimizu et al., 2014; Schnute et al., 2022; Shimizu et al., 2024), position late endosomes (LEs) as subcellular hubs capable of integrating multiple regulatory inputs to fine-tune Notch activity. While it was previously known that the ubiquitin ligases Deltex (Dx) and Suppressor of Deltex (Su(dx)) play crucial roles in regulating the precise topology of Notch at LEs, this dissertation identifies the Abelson kinase (Abl) and the long isoform of the Nedd4 ubiquitin ligase (Nedd4Lo) as additional key regulators of Notch at this compartment. This suggests the involvement of a broader network of post-translational modifiers in this regulatory process and raises the question of whether other LE regulators of Notch remain unidentified. Potential candidates for future investigation include: (1) Smurf, the third Nedd4-family member in *Drosophila*, which has reported functional overlap with Su(dx) (Ingham et al., 2004; M. B. Wilkin et al., 2004), (2) Ndfip, the *Drosophila* Nedd4-family interacting protein, known to modulate Notch signaling through mechanisms involving Rab7⁺ LEs (Dalton et al., 2011), and (3) the atypical protein kinase C (aPKC), which has been shown to regulate activation of a late endosomal fraction of Notch (Sjöqvist et al., 2014). Additionally, given that the Nedd4 ligases generally act as negative regulators of Notch activity, it remains an open question whether there are other positive regulators of Notch at LEs besides Dx.

Beyond the goal of comprehensively identifying all the LE regulators of Notch, a more fundamental question arises: Why would Notch require a broad array of regulators at this specific subcellular compartment? Could this indicate that LEs play a predominant role in the

endocytic trafficking-mediated activation of Notch? This question prompts further exploration into the contributions of other endocytic compartments to Notch pathway activity and whether the number of regulators is influenced by these contributions. In this regard, what determines the extent to which a compartment contributes to Notch signaling? Is it the compartment's abundance within the cytoplasm, or the variety of mechanisms through which Notch can be activated from these vesicles? Recent work from Martin Baron's lab indicates that Notch can be activated via multiple routes and mechanisms at LEs (Shimizu et al., 2024). These mechanisms may be dependent or independent of endolysosomal fusion and involve distinct sets of proteins, which could help explain the need for such precise regulation at these compartments.

Our investigation into the effects of Abl loss on Notch's subcellular localization revealed significant changes in the abundance of Notch at compartments other than LEs. Specifically, Abl loss led to increased Notch at Eps15⁺ upstream endocytic vesicles and decreased Notch at Hrs⁺ early endosomes (Figs. 2.3G, S2.3E). Notch accumulation in upstream endocytic vesicles is associated with reduced signaling (Vaccari et al., 2008), which could potentially lead to decreased Notch activity in *abl^{null}* tissue. Further, Notch enrichment at Hrs⁺ compartments is linked to increased signaling (T. Mukherjee et al., 2011; Vaccari et al., 2008; Vaccari & Bilder, 2005), raising the possibility that reduced Notch levels at these compartments in *abl^{null}* cells might compromise signaling. Despite these changes, however, we observed an overall increase in Notch activity in fly wing tissue. Does this suggest that LEs are indeed primary contributors to the Notch signaling pool regulated by endocytic trafficking?

Another intricate aspect of Abl loss is the significant reduction of Notch at the cell cortex observed in both wing and cultured cells (Figs. 2.2A",B",E; S2.2A-C; and 2.4A), with the underlying cause of this phenotype remaining unclear. Prior work has shown that the LE

regulators of Notch often play additional roles in the early stages of Notch endocytosis, typically promoting it (Sakata et al., 2004; Shimizu et al., 2014; Schnute et al., 2022). This raises the possibility that Abl may also influence other facets of Notch endocytic trafficking, given its effect on Notch distribution across multiple endocytic vesicles. To address this, a thorough investigation is needed to examine how Notch enrichment at different stages of the endocytic pathway is influenced by manipulating other LE regulators of the receptor. This would shed light on whether perturbations in Notch localization at a specific endocytic compartment indirectly affect its abundance at other stages of endosomal sorting. Key controls should include LE regulators known to impact other steps of Notch endocytosis as well as those not previously reported to do so.

Another possibility for the observed loss of Notch at the cell cortex in *abl^{null}* cells is that Abl loss may compromise the pool of Notch normally recycled back to the membrane. While the recycling of transmembrane proteins back to the cell cortex is known to primarily originate from early endosomes (Robinson, 2015; Cullen & Steinberg, 2018; Naslavsky & Caplan, 2018; O’Sullivan & Lindsay, 2020), a delicate balance between late endosomal and recycling fractions of Notch has been documented in a different fly tissue (Couturier et al., 2013; Reichardt & Knoblich, 2013; Johnson et al., 2016). Although the adaptor protein Numb has been implicated in this process (Reichardt & Knoblich, 2013), the specific post-translational modifiers that regulate this interplay remain to be identified.

The role of the endolysosomal pathway in developmental contexts extends beyond the regulation of Notch signaling. Recent research has demonstrated that targeted endocytosis and degradation of key components in major signaling pathways, such as Wingless, BMP, and Hippo, can either amplify or limit their activity (C. Wang et al., 2015; Tejeda-Muñoz & De

Robertis, 2022; Sharma et al., 2022, 2023). Notably, regulators of Notch at LEs are also involved in these mechanisms. For instance, Deltex has been shown to limit Wingless signaling in the *Drosophila* wing by promoting Armadillo degradation (Sharma et al., 2023). Similarly, Dx-mediated degradation of Dpp restricts the range of BMP signaling in the *Drosophila* wing (Sharma et al., 2022). Additionally, Su(dx) targets the tyrosine phosphatase Pez for degradation, which facilitates intestinal cell proliferation in *Drosophila* through Hippo signaling (C. Wang et al., 2015). These findings underscore the versatility of the endolysosomal pathway in fine-tuning various developmental signals and highlight the far-reaching implications of understanding Notch endosomal trafficking.

5.3 Lessons from the Delta-Notch feedback mechanism and future work

Our detailed examination of Notch activity in the developing pupal wing provided key insights into the spatial deployment of Delta-Notch signaling at single-cell resolution. Consistent with previous studies, we observed that Delta is highly expressed within the proveins, forming stripes approximately 2-3 cells wide by 30-32 hours APF (Fig. 3.7A) (Huppert et al., 1997; Herszterg et al., 2023). In addition, we confirmed previous findings that high levels of Notch activity are present in about 3 cell rows on each side of the proveins by the end of the critical vein refinement period (Huppert et al., 1997; Herszterg et al., 2023). This cellular arrangement raises several questions, including what determines the extent of the provein and its flanking cell regions, how this spatial organization is maintained, and what the biological significance of this pattern is.

While upstream provein cues, such as EGFR and BMP signaling, determine the localization of vein structures, these signals alone would lead to the formation of very thick and undefined vein structures (Blair, 2007). During the vein refinement process, Notch signaling activation causes provein structures, which initially encompass many cell diameters, to be reduced to about 2-3 cell rows. Strikingly, even optogenetically-induced ectopic veins, like their endogenous counterparts, narrow down to exactly 2-3 cell diameters (Herszterg et al., 2023). Why does Delta-Notch signaling refine the veins to precisely this width rather than a different one? One possibility is that this thickness represents a balance point where the high concentration of provein signals is no longer effectively countered by Notch-mediated suppression. Alternatively, having multiple provein cell rows instead of just one might enhance the structural integrity of the vein, making it more resilient to perturbations that could otherwise deplete the vein fate in some cells. If only a single cell row were present, ectopic Notch signaling could quickly truncate the vein; however, multiple provein cells might help restore balance through intercellular feedback using the remaining Delta⁺ cells. Nonetheless, the functional advantage of this specific width remains an area for further investigation.

Within the provein stripes, contiguous cells with high levels of Delta expression are present. Why don't these cells induce Notch signaling in each other to achieve an arrangement of isolated single cells, as seen in other contexts (Heitzler et al., 1996)? Research suggests that ligand cis-inhibition of Notch receptors is crucial for maintaining this spatial organization, playing a key role in various lateral inhibition processes (Fig. 1.20C) (Barad et al., 2010; Sprinzak et al., 2010; Del Álamo et al., 2011; Corson et al., 2017; Binshtok & Sprinzak, 2018; Herszterg et al., 2023). However, while these studies predominantly use mathematical modeling to highlight the importance of ligand cis-inhibition, to my knowledge, only one of them has

experimentally demonstrated this mechanism in the context of Delta-Notch signaling (Corson et al., 2017). In the developing *Drosophila* notum, during intermediate stages of bristle patterning, adjacent cells with high Delta expression (proneural/SOP/bristle cells) are maintained by ligand cis-inhibition before ultimately resolving into single proneural cells within the cell field. Thus, despite predictions from recent modeling work (Herszterg et al., 2023), the role of Delta-mediated cis-inhibition in wing vein patterning remains experimentally unverified and is currently a focus of research in our lab.

Delta from the proveins activates Notch signaling in flanking intervein cells. However, while this process typically involves direct cell surface contacts, expression of the *Notch-responsive element (NRE)* is observed in several rows of cells, with a decreasing gradient of high intensity spanning approximately 3 cell diameters, as indicated by GFP or mCherry reporters (Herszterg et al., 2023; and this study). As distance from the proveins increases, the NRE reporter signal weakens and eventually disappears. In this context, cellular projections are thought to assist in the propagation of Notch signaling across several cell diameters (Fig. 1.20B) (Binshtok & Sprinzak, 2018; Sjöqvist & Andersson, 2019; Bocci et al., 2020). Importantly, while cellular projections have been implicated in various Notch-mediated lateral inhibition contexts (M. Cohen et al., 2010; Hamada et al., 2014; Eom et al., 2015; G. L. Hunter et al., 2016), it remains unclear whether they play a role in establishing the Notch signaling gradient that surrounds the wing veins. An alternative hypothesis proposes that a spatial gradient of membrane tension may organize pathway activation. Given that Notch is responsive to mechanical tension (G. L. Hunter et al., 2019; Dullweber & Erzberger, 2023; Suarez Rodriguez et al., 2023), and that cells are significantly constricted at provein areas while becoming less constricted as distance from the proveins increases, this hypothesis is consistent with observed patterns. Investigating

the roles of cytonemes, tissue mechanics, and other cellular mechanisms in establishing the Notch signaling gradient that refines vein-intervein boundaries in the fly wing is thus an important focus of future research.

Regardless of the mechanism(s) involved in establishing the Notch signaling gradient around proveins, it is noteworthy that multiple studies have reported high levels of Notch pathway activation spanning about 3 cell diameters on each side of a provein (Huppert et al., 1997; Herszterg et al., 2023; this study). Similarly, in the context of bristle patterning in the *Drosophila* notum, high Notch activation is observed in exactly 3 rows of cells, reflecting a similar spatial organization (Corson et al., 2017). Initially, only two stripes of proneural (bristle) regions are partially pre-patterned on each side of a large cell field. Through self-organizing dynamics, additional provein stripes are sequentially activated until five stripes are established. Remarkably, these five stripes are separated by approximately 3 cell diameters, and as each stripe refines to form isolated single proneural cells, the final separation of these cells also measures about 3 cell diameters in any direction. These observations, combined with our findings, suggest that high activation of the Notch signaling pathway within 3-cell width regions might be a characteristic feature of lateral inhibition driven by Delta-Notch feedback.

Why would it be important to achieve high activation of Notch signaling at that particular width in regions adjacent to high Delta expression domains? This arrangement suggests that high Delta regions required active and robust constraint, and that any perturbations in this setup could lead to defects, such as vein overgrowth in the developing pupal wing. On an experimental level, it is worth investigating whether the loss of high Notch signaling across all three cell rows flanking a vein is necessary for vein expansion to occur. Live imaging of Delta and NRE could provide critical insights into this question. Furthermore, examining vein thickening under

conditions that produce this phenotype would be particularly informative. It would be valuable to determine how many additional cell rows are added in response to various conditions and whether this is influenced by the number of flanking cell rows that lose high Notch signaling. The full dynamics of Delta-Notch signaling in response to different perturbations of Notch activity are still be largely unexplored, with much more to discover beyond my initial observations.

5.4 Lessons from pupal wing tissue-level Notch signaling and future work

The findings from Chapter 4 indicate that, although largely undetectable with current signaling reporters, there are widespread low levels of Notch signaling throughout the pupal wing epithelium. We propose that this “baseline” activity may serve as a safeguard mechanism, enabling Delta-Notch signaling to respond more rapidly and effectively when addressing perturbations in vein patterning. In this context, we considered the possibility that low levels of Delta ligand might sustain Notch signaling outside the provein regions and even investigated the potential involvement of Serrate. Our findings indicate that neither is the case, leaving the mechanisms behind Notch activation away from high Delta expression areas unresolved. Two plausible alternative explanations include: (1) a gradient of membrane tension originating from the provein regions, which might extend into the intervein areas and activate low levels of Notch signaling, and (2) activation through endosomal trafficking mechanisms, which are thought to provide a buffering system to set up the field for ligand-driven signaling events.

Perhaps the most significant question arising from the ligand-independent activation of Notch across the entire pupal wing epithelium is whether this represents a general mechanism

utilized in other biological systems. Notch is widely expressed across various species, prompting a longstanding inquiry into why tissues invest resources in expressing this receptor in every cell if its signaling activity is typically confined to a small subset of cells within specific tissue regions. Our findings suggest that maintaining basal levels of Notch activity through ubiquitous receptor expression may be crucial for addressing a diverse range of biological challenges. It is possible that specific thresholds of pathway activation are required to respond to various biological situations, and that having Notch readily expressed with mechanisms in place to sustain low-level activity could facilitate these responses. While our observations do not confirm this, it is conceivable that Notch is continuously poised for activation regardless of ligand exposure, ready to engage fully when biological systems demand its complete functionality.

The limited sensitivity of most current Notch signaling reporters may explain the difficulties in identifying developmental and homeostatic roles for ligand-independent Notch signaling, with much of what we know stemming from studies of aberrant genetic conditions. Therefore, as we improve our ability to detect subtle levels Notch activity, we might be on the verge of launching a new and prolific area of research within the Notch signaling field. A straightforward exploratory experiment, such as examining the highly sensitive NRE-sfGFP reporter across a broad spectrum of biological contexts, could reveal exciting insights into where Notch is activated.

5.5 Lessons from signaling integration during wing vein patterning and future work

A precise balance between vein-inducing and vein-suppressing signals orchestrates venation during *Drosophila* wing development. In this context, our discovery of a ligand-

independent mode of Notch signaling that represses vein fate throughout the wing introduces new questions about the hierarchy and integration of vein developmental cues. Traditionally, the model posits that provein signaling induces Delta expression, which subsequently activates Notch signaling to refine provein domains. However, if Notch signaling can actively repress vein fate independently of ligand exposure, this model becomes significantly more complex.

When does Delta/Serrate-independent Notch signaling first emerge in the developing wing? Does it appear before or after the activation of EGFR and BMP provein signals? If it appears before, this could suggest that epithelial wing cells are directed towards the provein pathway even in the absence of EGFR and BMP. This guidance might be mediated by other instructive signals or, in their absence, could indicate that vein fate is a more stable developmental path compared to intervein fate, necessitating active repression to prevent widespread vein formation. In this scenario, active instructive signals, namely EGFR and BMP, would be required to overcome the default vein-repression mechanism in the appropriate regions. Conversely, if ligand-independent Notch signaling emerges concurrently with or after the activation of EGFR and BMP, it could imply that the vein-promoting signals are sufficiently strong at that stage, requiring Notch to employ additional mechanisms to sustain appropriate levels of vein-repressing activity.

Regardless of the exact sequence of signaling events, the specific transcriptional targets and genetic circuits that mediate the integration of multiple vein developmental cues remain largely unexplored. For instance, while it is established that Notch activity downregulates provein genes and subsequently Delta, it is not clear whether this downregulation occurs through the repression of EGFR and/or BMP signaling or through alternative mechanisms. Similarly, it is uncertain whether EGFR and BMP signaling directly contribute to the repression of Notch

activity within the proveins, or if other provein signals play such role. If these pathways do not directly interact, it might suggest that, beyond initial contributions from upstream cues, the Delta-Notch feedback, along with additional Notch regulatory inputs, could be sufficiently robust to resolve provein versus intervein tissue domains in a self-organizing manner.

I believe that, as new roles for ligand-independent Notch signaling are discovered, it will be imperative to revisit how Notch interacts with other signaling pathways across various developmental contexts.

5.6 Concluding statement

In this dissertation, I demonstrated how an extraordinary variety of regulatory mechanisms, ranging from molecular to cellular and tissue-level, fine-tunes the activity of the Notch signaling pathway during development. Drawing on insights from studying venation in the *Drosophila* wing, I propose that highly elaborate regulatory networks are critical for effectively addressing the complex challenges inherent in Notch-driven biological processes. This work has far-reaching implications. It illustrates how Notch-expressing cells can autonomously employ and adjust their responsiveness to inductive signals while dynamically coordinating pathway use with neighboring cells. Furthermore, it identifies for the first time a developmental role for ligand-independent Notch signaling and suggests potential avenues for discovering further roles of this nature. In addition, this research provides valuable mechanistic insights into how other signaling pathways may be regulated through mechanisms analogous to those governing Notch. Thus, I hope this work will serve as a foundation for advancing novel research directions within the fields of cell signaling, *Drosophila* biology, and developmental biology.

APPENDIX A

AN EFFICIENT METHOD TO HEATSHOCK-INDUCE SOMATIC CLONES IN THE *DROSOPHILA* PUPAL WING

In Chapters 2 and 4, I generate heatshock-driven somatic clones in the *Drosophila* pupal wing to study Notch signaling regulation. This appendix outlines the protocol used, along with the standardization process involved. Specifically, the experimental conditions were optimized by evaluating the frequency of *abl* null clones produced from crossing *y, w, hs>Flp;; ubi-GFP, FRT80B/TM6, Tb* females to *abl², FRT80B/TM6, Tb* males and heatshock-treating their offspring.

Protocol

- 1) Approximately 40 females are crossed to 20-30 males. These parents are allowed to lay eggs overnight for about 16h at 26°C. While the number of eggs laid may be relatively low during the first 1-2 days after setting up the cross, egg production should reach optimal levels by the third day.
- 2) After the egg-laying period, the parents are transferred to a new vial and can be reused for setting up new overnight collections for up to a week. After this period, egg production typically declines significantly.
- 3) Following egg-laying, the offspring are allowed to develop for at least 32h at 26°C before heatshock treatment. Heatshocking earlier than this can result in excessively large clones, which may kill the animals and lead to a reduced clone frequency at the time of

evaluation (see Table A1.1). On the other hand, we observe that heatshocking beyond 32h after egg lay collection reduces clone frequency as well (Table A1.1).

- 4) Larval stage animals are heatshocked in water bath at 37°C for 45min and then returned to 26°C. Longer heatshock treatments are not recommended, as they may overstress the animals or result in excessively large clones, both of which can often be lethal (see Table A1.2).
- 5) If evaluating the vein patterning process, animals are usually dissected during early pupal development, usually at 32h APF. However, if relevant, they are allowed to grow to adulthood for examination of final stage vein patterns.

	Frequency of <i>abl null</i> clones at 32h APF (% of wings with clonal tissue)		
Time after egg lay collection	24h	32h	48h
Heatshock treatment: 45min at 37°C	16% (N=32)	43% (N=153)	25% (N=25)

Table A1.1. Clone frequencies resulting from heatshock treatment at different times after egg lay collection.

	Frequency of <i>abl null</i> clones at 32h APF (% of wings with clonal tissue)		
Duration of heatshock treatment at 37°C	45min	1.5h	30min heatshock + 30min recovery + 30min heatshock
Time after egg collection: 32h	43% (N=153)	12.5% (N=24)	23% (N=30)

Table A1.2. Clone frequencies resulting from different heatshock treatments following 32h after egg collection.

Pilot results

After optimizing the protocol using *abl null* clonal tissue, we assessed its efficiency in generating other somatic mosaics (Table A1.3). Specifically, we evaluated the efficiency of our protocol in producing *Notch*^{55e11}; *Dl*^[RevF10], *Ser*^[RX82]; and *dx*¹⁵² somatic mosaics. To generate *Notch*^{55e11} clones in wing tissue, *Notch*^{55e11}/FM6 females were crossed to *y, w, hs>Flp, His2Av-GFP FRT19A/Y* males. For *Dl*^[RevF10], *Ser*^[RX82] clones, *y, w, hs>Flp;; ubi-RFP, FRT82B/TM6, Tb* females crossed to *Dl*^[RevF10], *Ser*^[RX82], *FRT82B/TM6, Tb* males. To induce *dx*¹⁵² clones, *y, w, hs>Flp, His2Av-GFP FRT19A* females were crossed to *dx*¹⁵² *FRT19A/Y* males. Of note, production *Notch*^{55e11} and *dx*¹⁵² somatic mosaics involves X chromosome recombination using FRT19A, whereas *Dl*^[RevF10], *Ser*^[RX82] clone production requires 3rd chromosome recombination via FRT82B.

	Frequency of clones at 32h APF (% of wings with clonal tissue)		
Genotype	<i>Notch</i> ^{55e11}	<i>Dl</i> ^[RevF10] , <i>Ser</i> ^[RX82]	<i>dx</i> ¹⁵²
32h after egg lay collection, heatshock treatment at 37°C for 45min	64% (N=75)	53% (N=51)	7% (N=54)

Table A1.3. Clone frequencies obtained using the optimized heatshock protocol to generate tissue mosaics for various Notch pathway components.

Although the frequency of *dx*¹⁵² clones was unexpectedly low, the protocol was highly effective in generating a good frequency of clones for all other manipulations. Additionally, this

method has been successfully adopted and adapted by other members of the Rebay Lab to produce heatshock-driven somatic mosaics in other *Drosophila* tissues

APPENDIX B

VEIN PATTERN OUTCOMES OF GENETIC MANIPULATIONS OF THE NOTCH, EGFR, AND BMP SIGNALING PATHWAYS

In the study of the mechanisms regulating wing vein patterning in *Drosophila*, the ability to analyze vein patterns at both the pupal and adult stages and to make appropriate comparisons across different conditions is invaluable. However, a review of the literature reveals that many studies manipulating vein developmental signals, particularly those using the UAS/Gal4 system, employ a wide range of non-uniform conditions: different genetic drivers, distinct wing regions, UAS activation through different temperatures, etc. These differences complicate our ability to make meaningful comparisons across studies.

To address this challenge, I standardized the conditions used in my dissertation work to evaluate vein patterns. Specifically, I consistently raised all flies at 26°C, whether working with mutant alleles or performing UAS-driven manipulations. When using the UAS/gal4 system for my experiments, after initially setting crosses at 18°C, I induce expression of transgenes starting at 0h APF using spatiotemporal control provided by *en>Gal4* and *tub>Gal80^{ts}*. This approach ensures that all manipulations are consistently driven at comparable strength in the posterior compartment of wings. Additionally, by restricting manipulations to pupal development, I bypass any earlier roles of the signaling pathways involved in wing vein patterning, thus preventing any confounding effects.

In this appendix, I present a comprehensive compilation of the vein patterning outcomes resulting from the genetic manipulations conducted throughout my PhD research, all carried out under the standardized conditions outlined previously. Specifically, I provide detailed

descriptions and penetrance data for vein phenotypes induced by 43 distinct manipulations of the Notch pathway, 13 manipulations of the EGFR pathway, and manipulations of the BMP pathway. Adult-stage images are included for many of these conditions, with corresponding pupal-stage images provided in Chapter 3 for most cases.

Results

Genetic manipulations of Notch

GENOTYPE	GAIN/LOSS OF VEINS?	PENETRANCE (% OF WINGS)	DESCRIPTION	REGIONS AFFECTED
Wildtype	-	0% (N=50)	-	-
en>Ab ^l ^{DegradFp} (knockdown) ; <i>abl</i> ^{2/+}	Loss	29% (N=70)	Small vein gaps	PCV
en>Dx	Loss	60% (N=80)	Small- and medium-sized vein gaps	L4, occasionally L5 and PCV
en>Dx ; <i>abl</i> ^{2/+}	Loss	71% (N=44)	Small- and medium-sized vein gaps	L4, occasionally L5 and PCV
en>Dx+Abl	Loss	12% (N=66)	Small gaps	PCV
en>Dx+Ab ^l ^{K417N}	Loss	57% (N=82)	Small- and medium-sized vein gaps	L4, occasionally L5 and PCV
en>Su(dx) ^{RNAi} (BDSC 67012)	-	0% (N=30)	-	-
en>Su(dx) ^{RNAi} (BDSC 3683)	-	0% (N=46)	Occasional loss of pigmentation	Distal tip of L5
en>Nedd4 ^{RNAi} (BDSC 34741)	-	0% (N=40)	-	-
en>Nedd4 ^{RNAi} (BDSC 31687)	-	0% (N=46)	Occasional loss of pigmentation	Distal tip of L5
en>Su(dx) ^{RNAi} 67012+ Nedd4 ^{RNAi} 34741	-	0% (N=38)	-	-
en>Su(dx) ^{RNAi} 67012+ Nedd4 ^{RNAi} 31687	-	0% (N=30)	-	-
en>NICD	Loss	97% (N=90)	Medium- and large-sized vein gaps	L4 and L5, occasionally PCV and ACV
en>DI (BDSC 26994)	Loss	39% (N=49)	Medium- and large-sized vein gaps	L4 and L5, occasionally PCV and ACV
en>DI (BDSC 26995)	Loss	3% (N=36)	Small vein gaps	L4 and L5, occasionally PCV and ACV
en>E(spl) (BDSC 266765)	Loss	6% (N=117)	Small- and medium-sized vein gaps	L4 and L5, occasionally PCV
en>Ser (BDSC 5815)	-	0% (N=54)	-	-
en>GFP	Gain	7% (N=54)	Small ectopic veins	PCV
<i>Notch</i> ^{5419/+}	Gain	94% (N=54)	Vein thickening and/or deltas	Vein thickening at L3 and L5. Deltas at L3, L4 and L5.
<i>Notch</i> ^{5419/+} ; <i>abl</i> ^{2/+}	Gain	80% (N=76)	Vein thickening and/or deltas	Vein thickening at L3 and L5. Deltas at L3, L4 and L5.
en>Abl	Gain	52% (N=65)	Small ectopic veins	PCV
en>Ab ^l ^{K417N} (kinase domain mutant)	Gain	4% (N=48)	Small ectopic veins	PCV
en> Ab ^l ^{W243K} (SH1 domain mutant)	-	-	Tumors in wing blade	-
en> Ab ^l ^{R297K} (SH2 domain mutant)	Gain	53% (N=32)	Small ectopic veins	PCV

en>Abl ^{FABD} (actin binding domain mutant)	Gain	52% (N=50)	Small ectopic veins	PCV
en>Abl+Dx	Gain	17% (N=66)	Small ectopic veins	PCV
en>Abl ; <i>su(dx)</i> ^{32/+}	Gain	50% (N=30)	Small ectopic veins	PCV
en>Su(dx) (BDSC 51664)	Gain	62% (N=40)	Small ectopic veins	PCV, occasionally ACV, L4, L5 and interveins
en>Su(dx) + <i>abl</i> ^{2/+}	Gain	43% (N=63)	Small ectopic veins	PCV
en>Abl+Su(dx)	Gain	85% (N=83)	Ectopic veins	PCV, occasionally ACV, L4, L5 and interveins.
en>Nedd4	Gain	53% (N=30)	Small ectopic veins	PCV, interveins
en>Nedd4S (BDSC 81605)	Gain	9% (N=34)	Small ectopic veins	PCV
en>Nedd4Lo (BDSC 81604)	Gain	90% (N=42)	Large vein thickening, deltas	L4 and L5, occasionally ACV and PCV
en>Abl+Nedd4Lo	Gain	100% (N=31)	Large vein thickening, deltas	L4 and L5, occasionally ACV and PCV
en>Abl+Su(dx) ^{RNAi} 67012	Gain	18% (N=60)	Small ectopic veins	PCV
en>Abl+Su(dx) ^{RNAi} 36836	Gain	10% (N=68)	Small ectopic veins	PCV
en>Abl+Nedd4 ^{RNAi} 34741	Gain	24% (N=54)	Small ectopic veins	PCV
en>Abl+Nedd4 ^{RNAi} 31687	Gain	34% (N=50)	Small ectopic veins	PCV
en>Notch ^{RNAi}	Gain	97% (N=74)	Vein thickening and deltas	L4 and L5, occasionally PCV and ACV
en>Dl ^{RNAi} (BDS 34322)	Gain	100% (N=72)	Large vein thickening, deltas	L4 L5, PCV, ACV
en>Dl ^{DN} (BDSC 26697)	Gain	100% (N=50)	Large vein thickening, deltas	L4 L5, PCV, ACV
en>Dl ^{DN} (BDSC 26698)	-	0% (N=38)	-	-
en>Ser ^{RNAi} (BDSC 34700)	-	0% (N=48)	-	-
en>Ser ^{RNAi} (BDSC 34700)	-	0% (N=68)	-	-
en>Ser ^{DN} (BDSC 52009)	Gain	59% (N=108)	Deltas, ectopic veins, vein thickening	L4, L5, PCV, ACV, interveins

Table A2.1. Description and penetrance of vein pattern phenotypes induced by genetic manipulations of the Notch signaling pathway. All genetic perturbations, except for *Notch*^{5419/+}, *abl*^{2/+}, and *su(dx)*^{32/+}, utilized *engrailed-Gal4* to drive expression of UAS transgenes in the posterior compartment of developing pupal wings. Abl knockdown (Abl^{DegradFP}) was performed in an *abl*^{GFP/abl}² background. L = longitudinal vein. ACV/PCV = anterior/posterior crossvein.

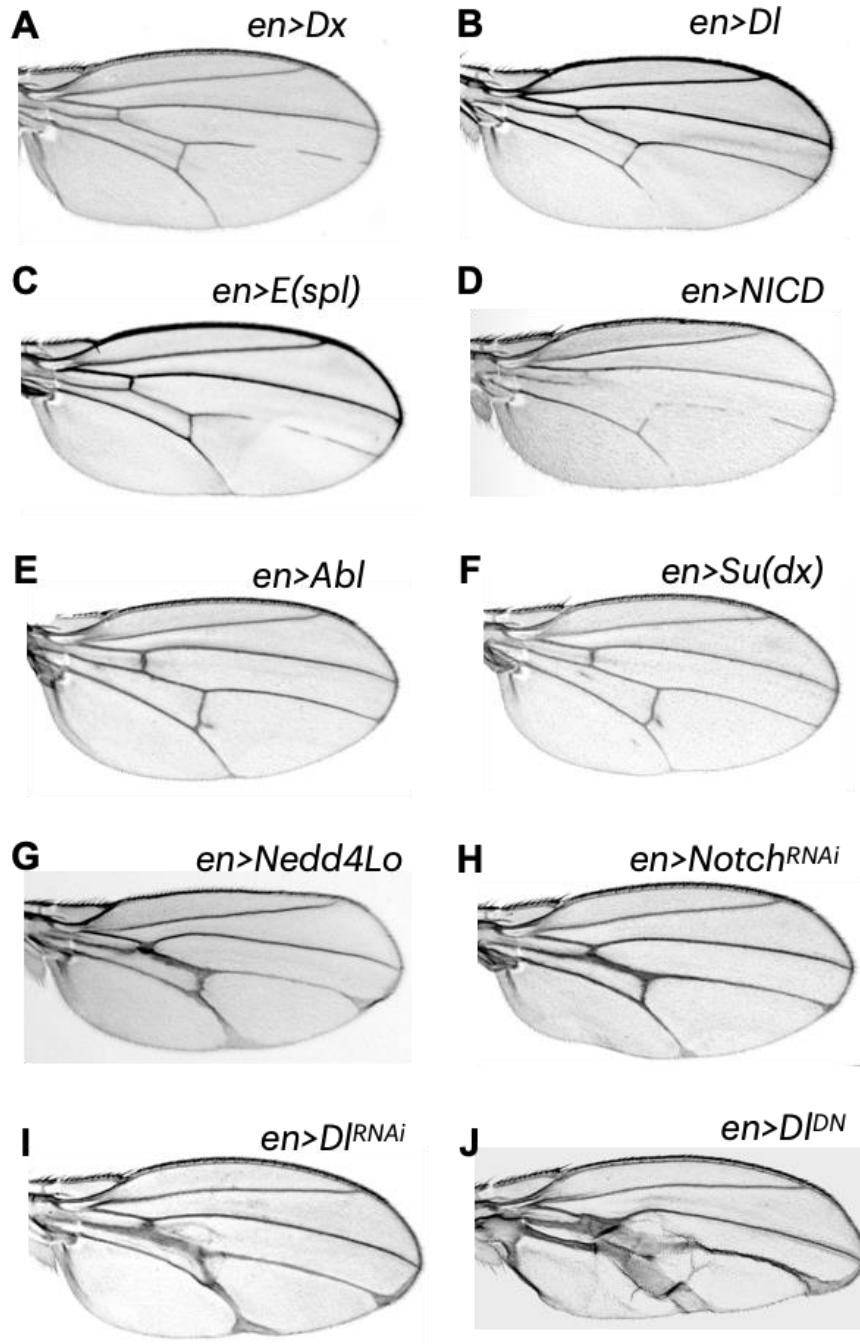


Figure A2.1. Vein loss and vein expansion phenotypes induced by manipulations of the Notch signaling pathway. (A-J) Adult wing phenotypes of the following manipulations: (A) *en>Dx*, (B) *en>Dl*, (C) *en>E(spl)*, (D) *en>NICD*, (E) *en>Abl*, (F) *en>Su(dx)*, (G) *en>Nedd4Lo*, (H) *en>Notch^{RNAi}*, (I) *en>Dl^{RNAi}*, and (J) *en>Dl^{DN}*.

Genetic manipulations of EGFR

GENOTYPE	GAIN/LOSS OF VEINS?	PENETRANCE (% OF WINGS)	DESCRIPTION	REGIONS AFFECTED
Wildtype	-	0% (N=50)	-	-
en>r ^{RNAi} (VDRC 109108)	-	0% (N=56)	-	-
en>r ^{RNAi} (VDRC 43123)	-	0% (N=42)	-	-
en>EGFR ^{RNAi} (BDSC 60012)	Loss	78% (N=40)	Loss of pigmentation and occasionally small vein gaps	L4
en>EGFR ^{RNAi} (BDSC 36770)	-	0% (N=52)	-	-
en>EGFR ^{DN} (BDSC 5364)	Loss	18% (N=40)	Small- and medium-sized vein gaps	L4, L5, PCV
en>Ras17 (in females) (BDSC 4845)	Loss	8% (N=83)	Small vein gaps	PCV
en>r ^{SEM} (BDSC 59004)	Gain	95% (N=44)	Vein branching, ectopic veins	L4, L5, PCV, ACV
en>EGFR ^{ΔTOP} (BDSC 59843)	Gain	100% (N=32)	Dramatic vein branching	L4, L5, PCV, ACV
en>EGFR ^{CA} (BDSC 9533)	Gain	100% (N=86)	Dramatic vein branching, vein thickening, tumors	L4, L5, PCV, ACV
en>EGFR ^{CA} (BDSC 9534)	Gain	100% (N=70)	Dramatic vein branching, vein thickening	L4, L5, PCV, ACV
en>Ras ^{V12} (BDSC 64196)	Gain	96% (N=56)	Dramatic vein branching	L4, L5, PCV, ACV
en>sty ^{RNAi} (BDSC 64196)	Gain	98% (N=42)	Medium and large-sized ectopic veins	L4, L5, PCV, ACV
en>rho (BDSC 8858)	Gain	54% (N=98)	Medium-sized ectopic veins, deltas	L4, L5, PCV, ACV

Table A2.2. Description and penetrance of vein pattern phenotypes induced by genetic manipulations of the EGFR signaling pathway. All genetic perturbations utilized *engrailed-Gal4* to drive expression of UAS transgenes in the posterior compartment of developing pupal wings. L = longitudinal vein. ACV/PCV = anterior/posterior crossvein.

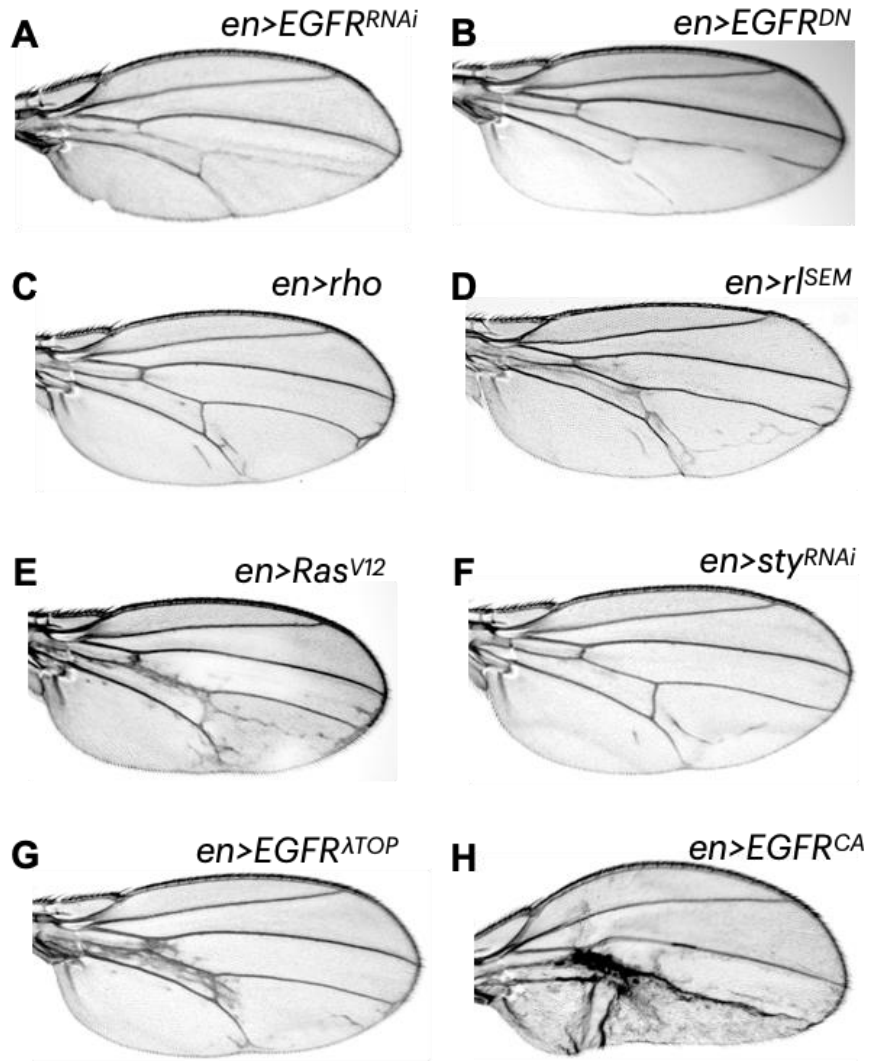


Figure A2.2. Vein loss and vein expansion phenotypes induced by manipulations of the EGFR signaling pathway. (A-H) Adult wing phenotypes of the following manipulations: (A) $en>EGFR^{RNAi}$, (B) $en>EGFR^{DN}$, (C) $en>rho$, (D) $en>r^{SEM}$, (E) $en>Ras^{V12}$, (F) $en>sty^{RNAi}$, (G) $en>EGFR^{\lambda TOP}$, and (H) $en>EGFR^{CA}$.

Genetic manipulations of BMP

GENOTYPE	GAIN/LOSS OF VEINS?	PENETRANCE (% OF WINGS)	DESCRIPTION	REGIONS AFFECTED
Wildtype	-	0% (N=50)	-	-
en>Dpp ^{RNAi} (BDSC 25782)	Loss	6% (N=36)	Small vein gaps	PCV
en>Tkv ^{RNAi} (BDSC 31041)	-	0% (N=44)	-	-
en>Mad ^{RNAi} (BDSC 31315)	Loss	6% (N=98)	Small vein gaps	PCV
en>Dpp (BDSC 1486)	Gain	94% (N=32)	Dramatic vein branching, vein thickening	L4, L5, PCV, ACV
en>Dpp-GFP (BDSC 53716)	Gain	79% (N=32)	Ectopic veins	L4, L5, PCV, ACV, interveins
en>Tkv ^{CA} (BDSC 9533)	Gain	96% (N=55)	Widespread ectopic veins, vein thickening	L4, L5, PCV, ACV
en>Cv (BDSC 42668)	Gain	10% (N=98)	Small ectopic veins	PCV

Table A2.3. Description and penetrance of vein pattern phenotypes induced by genetic manipulations of the BMP signaling pathway. All genetic perturbations utilized *engrailed-Gal4* to drive expression of UAS transgenes in the posterior compartment of developing pupal wings. L = longitudinal vein. ACV/PCV = anterior/posterior crossvein.

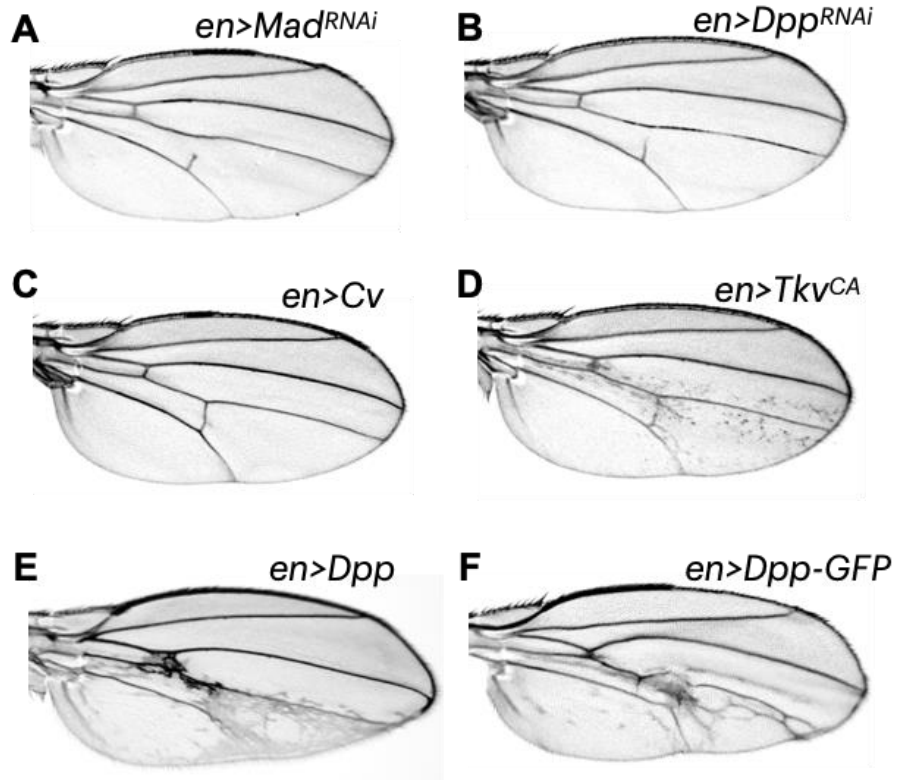


Figure A2.3. Vein loss and vein expansion phenotypes induced by manipulations of the BMP signaling pathway. (A-F) Adult wing phenotypes of the following manipulations: (A) *en>Mad^{RNAi}*, (B) *en>Dpp^{RNAi}*, (C) *en>Cv*, (D) *en>Tkv^{CA}*, (E) *en>Dpp*, and (F) *en>Dpp-GFP*.

Methods

Drosophila genetics

Flies were raised on standard *Drosophila* food at 26°C unless otherwise noted. BDSC and VDRC numbers for UAS lines employed are reported in tables above. In addition, we used *Notch*⁵⁴¹⁹ (a gift from Rick Fehon), *abl1* (Henkemeyer et al., 1987), *abl2* (Henkemeyer et al., 1987), *abl*-GFP (Nagarkar-Jaiswal et al., 2015), *en>Gal4*, *tub>Gal80ts*, UAS-*deGradFp* (Caussinus & Affolter, 2016), UAS-*Abl*-GFP (O'Donnell & Bashaw, 2013), UAS-*Abl*^{K417N}-GFP (O'Donnell & Bashaw, 2013), UAS-*Abl*^{W243K}-GFP (O'Donnell & Bashaw, 2013), UAS-*Abl*^{R297K}-GFP (O'Donnell & Bashaw, 2013), UAS-*Abl*^{K417N}-GFP (O'Donnell & Bashaw, 2013), UAS-*Abl*^{FABD}-GFP (O'Donnell & Bashaw, 2013), UAS-*Flag-Dx* (a gift from Spyros Artavanis-Tsakonas), UAS-*NICD* (Go et al., 1998), UAS-*Notch*^{RNAi} (Zacharioudaki & Bray, 2014), and UAS-*Nedd4* (a gift from Jin Jiang). For UAS-driven genetic manipulations, crosses were established at 18°C and individual animals were transferred to 26°C at 0h APF. They were either dissected at 32h APF or allowed to grow to adulthood. Please see Chapter 3 for images of pupal stage phenotypes.

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