Investigating the origination and evolution of a morphological novelty in *Drosophila* genitalia

by

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The origination of new morphological structures has been one of the most fascinating problems in the field of evolutionary developmental biology. Like any anatomical feature, morphological novelties are controlled by unique developmental programs. In particular, developmental signaling pathways that are conserved throughout an organism's development and are responsible for specifying fields of cellular identities have been frequently implicated in the evolution of novelties. Yet, we lack a solid understanding of the evolutionary history of the signaling centers associated with novelties. Namely, what roles did they have prior to the appearance of the novelty, and how did their novel activities emerge? Here, I examine the evolutionary origins of a morphological novelty. Utilizing a newly evolved complex morphology in an organism possessing an arsenal of genetic tools, I investigate 1) the regulation of a signaling center associated with the development of a novelty 2) the pre-existing roles of the novelty-associated signaling center, and 3) how this signaling center exerts its downstream effects. Specifically, I investigated the origin of the posterior lobe, a recently evolved cuticular projection on male fruit fly genitalia unique to the *Drosophila melanogaster* clade. During posterior lobe development, Delta, a ligand of the Notch signaling pathway, is expressed in a spatially expanded pattern which is essential for posterior lobe development. I explored the posterior lobe associated regulation of *Delta* and discovered that this signaling center becomes active days before its involvement in posterior lobe development. I identified an early-acting role essential for genital

development— the eversion of the genital disc. I then examined the mechanism by which Delta orchestrates this conserved process, and determined a role for the apical extracellular matrix (aECM) protein Dumpy in genital disc eversion. This work demonstrates that complex morphological novelties may develop from pre-existing programs in the context of already intricate developmental processes, emphasizing the importance of uncovering the ancestral roles of genetic programs associated with novelties. Furthermore, through discovering an ancestral role for this signaling center, I uncovered a critical function for Notch signaling in genital disc eversion, contributing to our understanding of this convoluted and vital process for *Drosophila* genital development.

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Preface

I must express my deepest gratitude to my advisor, Dr. Mark Rebeiz, who has been most influential in my academic journey and the origins of this project. Mark's mentorship and unmatched enthusiasm for exploring the enchanting mysteries of evo-devo has created a scientific environment where curiosity and nuanced views are welcomed and celebrated, which I feel lucky to have been a part of. I also thank the many members of Rebeiz lab, past and present, for their genuine support and always being willing to share their unique insights. I am grateful to my committee, Dr. Karen Arndt, Dr. Gerard Campbell, Dr. Lance Davidson, and Dr. Miler Lee, for their valuable discussions and thoughtful insights on my project as well as their continued support.

Finally, I would like to thank my family. My appreciation to my mother, father, and sister for being a source of inspiration and for their unwavering support throughout the years goes beyond words.

1.0 Introduction

Throughout evolutionary history, organisms have developed a wide array of anatomical structures commonly referred to as “morphological novelties”. Such features are defined as individualized body parts that are non-homologous to any anatomical structure in their ancestral lineage (Muller & Wagner, 1991; Wagner & Lynch, 2010). Like any other body part, novel morphologies arise during development through orchestrated events governed by gene regulatory networks (GRNs), interconnected circuits of transcription factors and signaling pathways that ultimately control specific cell fate decisions (Levine & Davidson, 2005; Peter & Davidson, 2015; Rebeiz & Tsiantis, 2017). Thus, comprehending how morphological novelties originate requires an understanding of the networks that participate in the novelty, and the evolutionary changes that altered the genetic program. Further, true apprehension of an origination event is not possible without investigating the ancestral roles that the novelty-associated program may have had prior to the emergence of its novel function. It is only by uncovering whether the network in question had previous developmental roles or if roles were assembled *de novo* that will allow us to truly ascertain the origins of a novelty. Work on morphological novelties such as the turtle’s shell, bat wings, bird’s feathers, or cephalopod’s beak, have frequently implicated a prominent role for developmental signaling pathways in their development (Cooper & Sears, 2013; Harris et al., 2005; Harris, Fallon, et al., 2002; Hockman et al., 2008; Loredo et al., 2001; Navet et al., 2009; Weatherbee et al., 2006). However, how these signaling pathways have evolved new expression patterns and what roles they had prior to them being tinkered with and the emergence of novelties has remained unexplored.

Below, I will introduce several leading systems of morphological novelty, and detail how signaling pathways have been found to be involved. I will describe the barriers that hinder our ability to fully interrogate the origins of novelties, and also introduce a system that is uniquely positioned to fill key gaps in our understanding of complex morphological novelty.

1.1 Changes to gene regulatory networks underlie morphological evolution

1.1.1 Developmental genes are conserved across metazoa

Multicellular organisms exhibit a vast array of morphological diversity across the animal kingdom. Yet, the majority of the genes that pattern the development of such distinct anatomies are highly conserved (Carroll et al., 2005). One of the first notions that illuminated the high conservation of developmental genes was studies of the body plan patterning *Hox* genes (Gellon & McGinnis, 1998; McGinnis et al., 1984). Hox transcription factors are famously known to specify segments along the anterior-posterior body axis, instructing early tissue identities. Their ability to establish the architecture of the body plan was first described by homeotic transformations in *Drosophila* as a result of *Hox* gene mutations, including the antenna-to-leg transformation caused by the *Antennapedia* mutation (McGinnis et al., 1984). The coding sequences of Hox genes and their genome organization was later found to be deeply conserved spanning the animal kingdom (Duboule, 2007; McGinnis et al., 1984). Further, Hox genes display a deep functional conservation between distantly related species. For example, the mouse Hox-2.2 was shown to be a functional homolog of the *Drosophila Antennapedia*, as its misexpression in *Drosophila* induced a homeotic transformation mimicking the antenna-to-leg phenotype observed

in the *Antennapedia* mutant (Malicki et al., 1990).

The functional conservation of developmental genes between metazoans is not limited to Hox (Nitta et al., 2015). Commonly known as 'master control genes', many genes encode transcription factors that act as developmental switches with the ability to orchestrate the differentiation of multiple cell types to a certain fate. The *Drosophila eyeless* gene shares strong sequence identity with its mouse homolog *Small eye (Pax6)*, and its misexpression in *Drosophila* can induce ectopic eyes on various tissues (Halder et al., 1995). Furthermore, a common characteristic of multicellular organisms, the ability for cells to communicate with each other and induce fates of neighboring cells, is coordinated by deeply conserved molecules of intracellular signaling pathways (Pires-dasilva & Sommer, 2003). The role of signaling pathways in specifying territories of cells is prevalent throughout all stages of development. These pathways are not only evolutionarily conserved across metazoa, but are also frequently re-used in different developmental contexts throughout an organism's life-span (Gerhart, 1999). Hence, signaling pathways are highly pleiotropic, serving multiple roles during animal development in different tissues (see section 1.2). The highly conserved and pleiotropic reservoir of developmental genes across metazoa, or the 'genetic toolkit', suggests that alterations to protein coding regions would have deleterious effects and present a less-frequently traveled route of morphological evolution.

1.1.2 Developmental programs are controlled by gene regulatory networks

The apparent paradox that diverse organismal complexity across animal phyla is controlled by a deeply conserved genetic toolkit has led to the proposal that changes to the expression of genes in time and space contribute to morphological evolution in favor of coding changes that are predicted to be highly pleiotropic (Carroll, 2008; Carroll et al., 2005; Davidson, 2006; Peter &

Davidson, 2011). This hypothesis has been borne out by many studies documenting regulatory changes altering developmentally induced morphologies (Averof & Patel, 1997; Burke et al., 1995; Carroll et al., 1994; Deutsch & Mouchel-Vielh, 2003; Warren et al., 1994). Thus, understanding the evolution of forms relies on an understanding of how gene expression patterns are regulated. Central to developmental patterning and tissues taking on form are gene regulatory networks (GRNs), sets of interacting signaling pathways and transcription factors that regulate the expression of their target genes to control specific cell fates. Signaling pathways are often at high tiers of a GRN, patterning development through cell-cell communication most often by activating other transcription factors in signal-receiving cells. These interactions occur through *cis*-regulatory elements (CREs), non-coding DNA sequences which when bound by a combination of transcription factors, spatiotemporally regulate the expression of a gene (Levine, 2010; Levine & Davidson, 2005; Veitia, 2008).

When considering an altered expression pattern of a particular gene, causative changes could be explained as *cis*- or *trans*- regulatory modifications (Rebeiz & Williams, 2017). Mutations in CREs (*cis*-regulatory), disrupting or generating new binding sites or altering the spatial distances between interacting proteins that bind to the CRE, can modify the expression of a gene in a spatiotemporal manner without affecting its other functions (Rubinstein & Souza, 2013). *Trans*- regulatory changes are explained by altered expression of an upstream factor whose gain or loss of expression in the tissue of interest affects the activity of downstream genes (Wittkopp & Kalay, 2011). Hence, changes in a given GRN could occur at multiple levels; in the expression of upstream factors, intermediate nodes, or downstream of the network. The impact that a mutation could have on a GRN is strongly dependent on where in this chain of regulatory interactions the alteration occurs.

Gene regulation by transcription factors follows a combinatorial logic, wherein a combination of transcription factors that are expressed in a certain spatial domain at a given time affect the expression of their target genes. Hence, CREs often include binding sites for a number of transcription factors that can repress or activate a gene. Accordingly, CREs can either promote or repress transcription of their target gene(s), in which case they are referred to as enhancers or silencers, respectively. The dynamic interplay between activators and repressors is critical for carving out precise gene expression patterns. A foundational example of this combinatorial logic is the expression of the *Drosophila even-skipped* (*eve*) gene in the embryo (Small et al., 1992). The product of this gene is expressed in seven transverse stripes with sharp boundaries along the embryo which function in segmentation (Macdonald et al., 1986). Each stripe is formed by receiving inputs from gap genes, which are top regulators of the embryonic segmentation GRN that determines the identity and position of each segment (Jaeger, 2011). These are both activators and repressors that are spatially patterned such that they sculpt thin stripes of eve expression in a restricted manner (Small et al., 1992). The stripe 2 enhancer, for example, is activated by Bicoid and Hunchback, and repressed by Kruppel and Giant, resulting in a distinct stripe in the embryo (Ludwig & Kreitman, 1995; Small et al., 1992). Of the seven eve stripes, individual or pairs of stripes are driven by separate CREs, each with a specific combinatorial logic. While stripes 1, 2, and 5 are each regulated by a separate CRE (i.e. have modular enhancers), stripes 3 and 7, as well as stripes 4 and 6, share a regulatory element in pairs (Harding et al., 1989; Small et al., 1996; Veitia, 2008). This property of enhancers — known as “modularity”— has important evolutionary implications, as mutations in one enhancer will most likely not change the expression of a gene in other regions of the organism (Carroll, 2008).

Another attribute of gene regulation is the existence of redundant or "shadow" enhancers. This is the notion that specific expression patterns can be regulated by two or multiple enhancers, which may allow for sequences to be altered without dire consequences (Barolo, 2013; Cannavo et al., 2016). Shadow enhancers are a well-documented feature in *Drosophila* development, and there is an increasing amount of evidence describing their pervasiveness in other organisms as well (Cannavo et al., 2016; Frankel, 2012). Shadow enhancers drive completely or partially overlapping expression patterns and may confer robustness in gene expression. They are posited to buffer against genetic and environmental perturbations. When mutated, it has been shown to lead to greater variability in phenotypes when subjected to sub-optimal environmental or genetic states (Barolo, 2013; Hobert, 2010; Perry et al., 2010). For example, over a decade of work on the *Drosophila shavenbaby* (*svb*) gene identified seven larval enhancers that regulate its expression in the late embryo (Frankel et al., 2010; McGregor et al., 2007; Stern & Frankel, 2013). *Svb* encodes a transcription factor that controls the production of larval trichomes — hair-like projections of the epidermal cells that predominantly aid in larval locomotion. When a regulatory region of the *svb* locus containing three of the seven enhancers was deleted, loss of trichomes was only observed under sub-optimal conditions, including extreme temperatures and reduction of a *svb* regulator, suggesting that the redundant enhancers offer a buffering mechanism under stressful conditions (Frankel et al., 2010). In *Drosophila* photoreceptor neurons, *defective proventriculus* (*dve*) is regulated by two CREs. One of these enhancers is normally repressed, but becomes active in the absence of its other enhancer, serving as an inducible back up element (Yan et al., 2017). In mice, *Shh (Sonic Hedgehog)* expression in the developing teeth and tongue is regulated by three enhancers with overlapping domains of activity, and deletion of each enhancer independently does not affect tooth development (Amano, 2020).

Owing to their buffering mechanisms, enhancer redundancy may be a source of morphological evolution. Having multiple enhancers may allow for mutations to occur that are involved in generating novel expression patterns with minimal consequences to their original function (Hong et al., 2008). This may render the GRN more tolerant to modifications, as new patterns evolve while old ones are maintained.

1.2 Signaling pathways specify cell fates

Cell-cell interactions are crucial to organismal development across all metazoa (Pires-dasilva & Sommer, 2003). Signal-transduction pathways, or signaling pathways, are responsible for cells to communicate during the entire lifespan of an organism, from early embryonic stages throughout adulthood. Among seventeen intercellular signaling pathways, seven major pathways are recognized to be repeatedly utilized throughout development (Gerhart 1999). These pathways include Hedgehog (Hh), the wingless related (Wnt), Janus kinase (JAK/STAT), transforming growth factor-β (TGF-β), receptor tyrosine kinase (RTK), and Notch signaling (Gerhart, 1999; Pires-dasilva & Sommer, 2003). While diverse in their inductive mechanisms, they share commonalities all with the end result of activating specific genes through their transcriptional effectors. Signal induction is typically initiated by the binding of a ligand to a transmembrane receptor on a signal-receiving cell, leading to a cascade of events that ultimately result in the activation of a pathway's specific transcription factor which will mediate the expression of target genes through their CREs. As a result, cells that receive a signal will adopt a fate that differs from their neighboring signal-sending cells, creating spatially distinct territories of cells with varying identities (Barolo & Posakony, 2002). Signaling ligands can either be secreted from the cell and

act as a morphogen —transmitting signals over long distances and forming gradients, or they can be tethered to the signal-sending cell —only affecting cells adjacent to the signal source, *i.e.* juxtacrine signaling. Thus, signal transduction specifically alters transcription in a subset of cells that are in range to receive the signal and express a corresponding receptor for ligand binding, successively partitioning the organism into domains of particular regulatory properties. The sequential signal induction generating territories of differing cell identities is a prevalent source of cell fate diversity in an organism (Peter & Davidson, 2015). It follows that changes to the spatial and temporal expression patterns of a signaling pathway ligand or receptor would have considerable consequences on cellular identities and morphological evolution.

In accordance with the combinatorial nature of transcriptional gene regulation, the transcriptional effectors of signaling pathways also require the presence of other activators to affect gene expression (Barolo & Posakony, 2002; Perrimon et al., 2012; Sagner & Briscoe, 2017) The interpretation of inductive signaling thus is dependent on the unique gene expression profile of a cell (Figure 1.1.1). The re-deployment of signaling pathways to different developmental contexts is therefore key to the development of different cell types within an organism, and changes to the expression domains of these pathways over evolutionary time has strong implications for the development of novel morphologies (Rebeiz et al., 2015; Rubinstein & Souza, 2013).

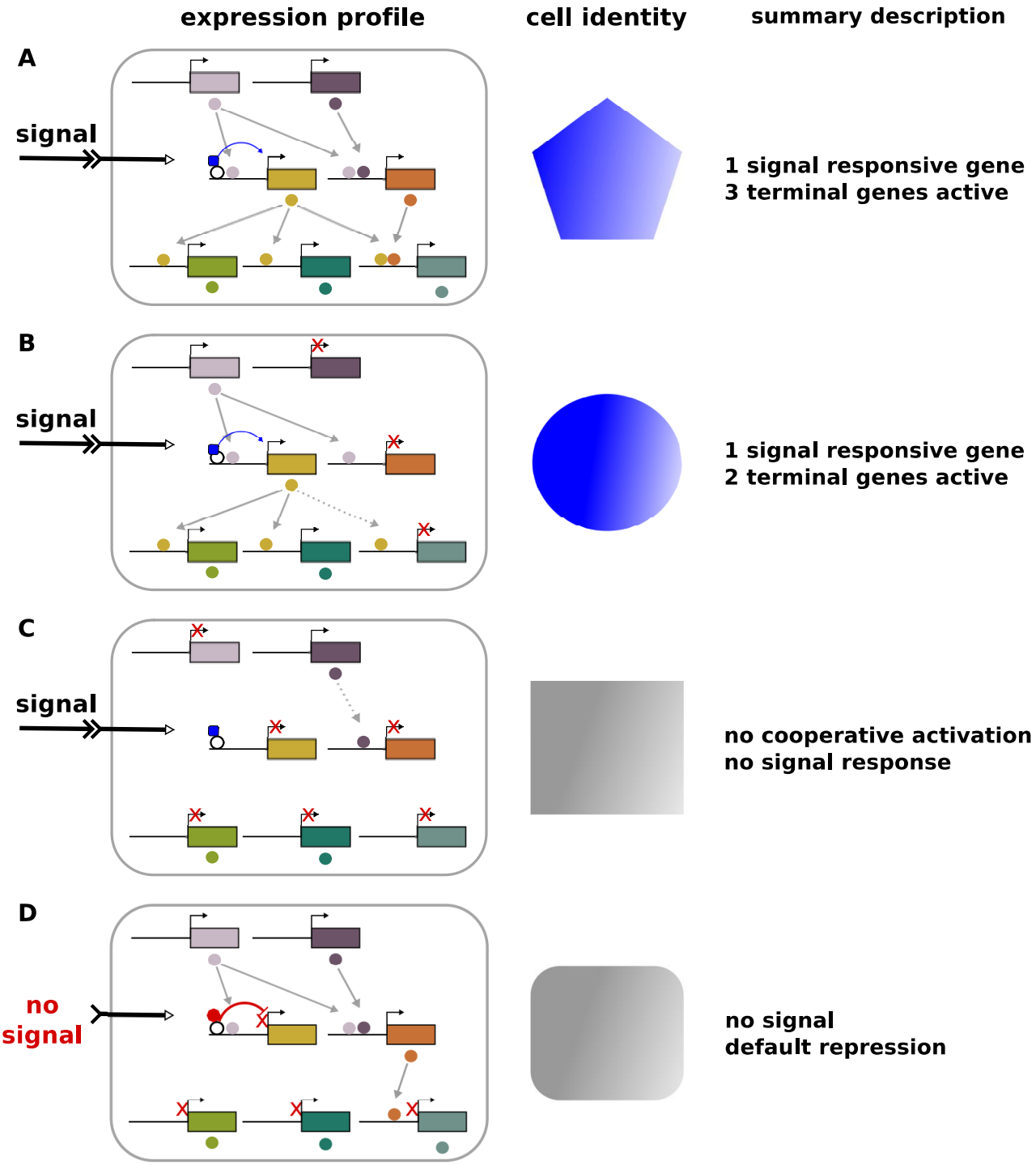

Figure 1.1.1 Signaling pathways produce different cellular outcomes depending on the gene expression profile of their signal-receiving cell.

(Left) A hypothetical GRN architecture, where CREs are represented in black lines adjacent to the transcription start sites for their respective genes. Circles with corresponding colors denote gene products, the open circle denotes the transcription factor of a signaling pathway, blue square indicates signal regulated coactivator, and red octagon denotes a corepressor. The top tier gene products activate middle tier genes, and middle gene products regulate terminal nodes. The yellow gene also has a binding site for a signaling effector, which is always bound to its CRE. When a signal is present, it responds to the signal in combination with the lilac transcriptional activator (A and B). (B) Lack of the top tier purple gene expression changes the expression of downstream genes. The yellow gene only activates a subset of its target genes due to the loss of orange gene expression. (C) A signal is present, but is insufficient to induce the yellow gene due to a lack of cooperative activation. (D) When no signal (ligand) is present, the transcriptional effector is bound by a repressor. Thus, while both top tier regulators are intact, downstream genes are not activated. (Right) Cell fate identities are demonstrated as different shapes. Signal responsive cells are shaded in blue.

1.3 Examples of signaling pathways involved in the evolution of morphological novelties

1.3.1 The evolution and development of the bat wing

Bats are the only mammals that have gained the ability of powered flight. Because of this unique feature, the evolution of bat wings has been of interest to many scientists in the past couple of decades (Sadier et al., 2020). However, it has been a challenging undertaking due to its ancient evolutionary history and lack of informative fossil records. Nevertheless, comparative studies between mice and bat limb development have begun to solve pieces of this puzzle. Bat wings are extended outgrowths of the forelimb with elongated skeletal elements possessing flight membranes. Two major signaling centers of the limb bud, the zone of polarizing activity (ZPA) and the apical ectodermal ridge (AER), are three times larger in the developing bat forelimb compared to that of mice, which contributes to an accelerated growth rate and enlargement of the

bat forelimb (Cooper & Sears, 2013). Conserved in limb development, Shh signaling in the ZPA and Fgf (fibroblast growth factor) signaling in the AER interact in a positive feedback loop to coordinate limb bud outgrowth. The striking difference in the signaling centers of bats and mice correlates with a major spatio-temporal shift in Shh and Fgf8 expression in the ZPA and AER, respectively(Sadier et al., 2020). At early stages of limb bud development in both bats and mice, the anterior-posterior patterning gene Shh is active in the ZPA, albeit in a more extended pattern in the bat limb bud (Cooper & Sears, 2013). Later, when the limb bud of both species takes on a paddle form, *Shh* expression turns off. After this stage, while Shh expression remains off in mice, a second wave of *Shh* is re-activated later in the interdigital tissue of bats (Hockman et al., 2008) (Fig1.2.2). Thus, the developing tissue in bats becomes exposed to the same signal later in development. It has been proposed that the re-initiation of Shh expression in bats became possible through re-activation of the positive Fgf-Shh feedback loop, as Fgf8 also gains a novel expression pattern in the interdigital tissue that precedes the second wave of Shh expression (Hockman et al., 2008). The re-deployment of Shh and Fgf8 in the interdigital regions is critical for wing membrane formation. In both mice and bat limbs, BMP, a promoter of apoptosis, is active in the interdigital tissues. In bats, however, the extended expression of Shh induces Gremlin, inhibiting the apoptotic activity of Bmp (Weatherbee et al., 2006). Furthermore, the extended expression of Fgf8 in the interdigital tissues is thought to increase cell proliferation, leading to growth of the bat wing membranes.

Thus, due to the expanded spatio-temporal pattern of signaling molecules in bats, the early enhancement of the Fgf-Shh interaction has played an important role in limb bud enlargement. Further, the re-initiation of this feedback loop that was re-deployed in a novel expression domain played a critical role in wing membrane formation. The evolutionary history of the Fgf-Shh signal

re-initiation however remains unexplored. Does the re-activation of this signaling source share ancestry with the primary wave of Fgf-Shh, or is the second wave deployed through a novel regulatory mechanism? Tackling this question requires identifying the limb enhancer(s) of Shh, an effort that is becoming possible in the recent decade with genome-wide analysis of putative bat limb enhancers and transgenic assays (Booker et al., 2016; Eckalbar et al., 2016). Comparative genomics has identified multiple regulatory regions that display rapid sequence change in the bat lineage, but are highly conserved in other vertebrates. The well-characterized mouse *Shh* limb enhancer, the ZPA regulatory sequence (ZRS), was among these rapidly evolving regions. Using a mouse transgenic assay, the bat orthologous sequence of ZRS was shown to be active in the limb bud (Booker et al., 2016). However, only early stages were analyzed and no differential activity was observed between the mouse and bat sequences. Thus, whether this sequence with a pre-existing role drives signal re-initiation remains to be examined. If the bat ZRS does not exhibit a second wave of activity in a mouse transgenic assay, it would still remain unclear whether it is due to changes in an upstream regulator or that the second wave of *Shh* expression is driven by a separate regulatory element. Hence, development of transgenesis in bats would be a major step forward in our understanding of the evolution of the bat wing GRN.

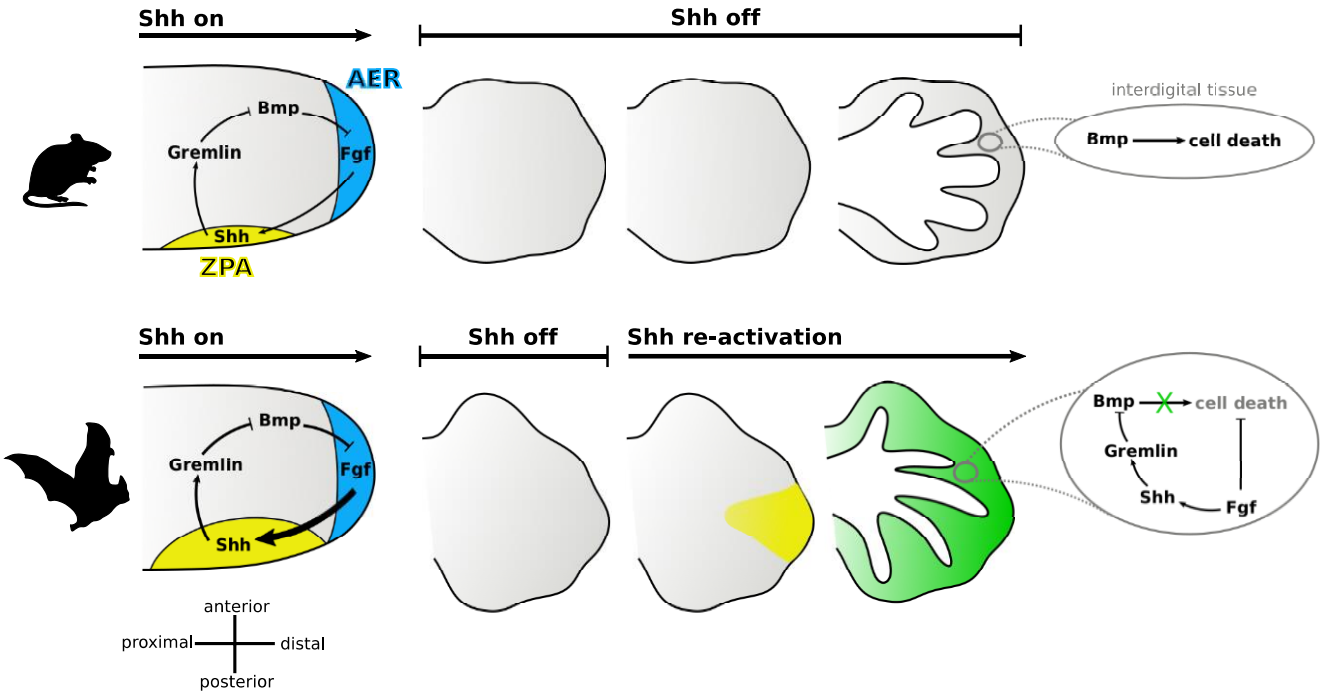

Figure 1.2 2 Schematic comparison of forelimb signaling centers between a mouse (top) and a bat (bottom). In bats, the larger expression domain of ZPA and AER, and an enhanced Fgf-Shh interaction (denoted by thicker arrow) contribute to the increased lengthening of forelimbs. Shh expression is turned off in the limb paddle stage of both species, but re-activates its expression only in the bat limb bud. Re-initiation of the Shh-Fgf feedback loop in the interdigital tissues of the bat wing promotes cell proliferation and inhibits cell death.

1.3.2 Evolution of decidualization in eutherian mammals

The evolution of mammalian pregnancy has lent itself well to studies investigating the origination of novel cell types. The placenta in particular has gained much attraction as it is one of the most variable mammalian organs (Gundling & Wildman, 2015). Eutherian mammals, like humans, develop a placenta during pregnancy which forms in the uterus after conception and is maintained throughout the full course of embryonic development. Marsupials —such as kangaroos, koalas, and opossums— on the other hand, have short term pregnancies and give birth

to developmentally immature joeys who complete their development outside of the mother's body (Renfree, 2010). A key feature that distinguishes eutherian placenta from that of marsupials is the evolution of decidual stromal cells (DSCs), specialized cell types that facilitate implantation and modulate the uterine environment for the progression of pregnancy (Chavan et al., 2016).

In a process called decidualization, DSCs differentiate from a population of uterine cells called endometrial stromal fibroblasts (ESFs), which also exist in non-eutherian mammals. DSC differentiation is induced by decidualizing signals from ovarian hormones including progesterone, and cyclic-AMP/protein kinase A (cAMP/PKA) signaling from the embryo. In response to these signals, a GRN of decidualization is activated to confer DSC identity (Griffith & Wagner, 2017). Comparative studies between human ESFs and those of the opossum have shed light into the evolutionary history of decidualization. The majority of the core decidualization GRN genes exists in opossum ESFs. When experimentally exposed to decidualizing signals, opossum ESFs —used as a proxy of ancestral ESFs— exhibit a response. However, instead of activating downstream effector genes that specify decidual fates, the opossum ESF trans-regulatory landscape elicits an apoptotic and oxidative stress response (Erkenbrack et al., 2018). Thus, it can be postulated that the placental cAMP/PKA signals could have played an ancestral role in stress response, and that a novel cell type GRN evolved through changes to downstream network connections. Upstream changes to the network cannot be ruled out without the identification of signal response CREs, as they may have evolved required inputs from other transcription factors to activate the decidualizing GRN.

1.3.3 The evolution and development of bird's feathers

The bird's feather represents one of the most charismatic examples of morphological novelties. Feathers are morphologically complex and diverse structures which contribute to a variety of functions such as communication, insulation, and flight (C. F. Chen et al., 2015). Along with avian and alligator scales, feathers are integumentary appendages thought to be derived from ancestral archosaur body scales (Harris, Fallon, et al., 2002; Sawyer et al., 2003). However, feathers exhibit a unique developmental process wherein the initial feather placode grows outwardly to form a conical bud, which eventually forms a central rachis, primary branches called barbs, and numerous barbules attached to each barb. Comparative gene expression analysis between the primordia of feathers and scales in chick, duck, and alligator —extant archosaur species— demonstrated a conserved expression pattern of *Shh* and *Bmp2* in the early developing placodes of these structures (Harris, Fallow, et al., 2002). These findings suggested that the early expression of *Shh* and *Bmp2* preceded the origination of feathers and is ancestral to archosaurian integumentary appendages. Later in developing feathers, however, *Shh* and *Bmp2* have evolved a novel expression pattern that correlates with the timing of the conical bud outgrowth. After this stage Shh and Bmp2 express in longitudinal stripe patterns along the proximo-distal axis of the developing feather, which mark the presumptive barb ridges (Harris, Fallon, et al., 2002). The dynamic interplay of the Shh and BMP signaling pathways has been shown to have a critical role in feather development and diversification. In chicks, Shh-Bmp2 signaling exhibit an activator-repressor mechanism, wherein Shh activates itself as well as Bmp, and Bmp negatively modulates Shh. This modulation is important for fusion of barb ridges, which forms the central ridge of the feather (Harris et al., 2005). Collectively, extensive work on feather development has illuminated

that spatial and temporal modifications to ancient signaling centers plays a pivotal role in the development of these complex structures.

1.3.4 The evolution and development of the turtle shell

The shell of turtles and tortoises distinguishes them from all other reptiles. This enchanting morphological novelty is an exoskeleton comprised of two main components: a dorsal carapace and a ventral plastron. The dorsal carapace forms from the unique development of the rib precursors, which instead of moving ventrally to form a ribcage, migrate in a dorsolateral manner to enter the dorsal dermis (Kuraku et al., 2005). The dorsolateral region of the embryonic dermis where the forming ribs enter is called the carapacial ridge (CR), which will later form the outer margin of the dorsal carapace (Cebra-Thomas et al., 2005). These ridges begin to form on the lateral surfaces at an early embryonic stage, which is the first major distinguishing factor between a turtle and chick embryo (Nagashima et al., 2005). FGF signaling has been shown to play an important role for carapace formation (Loredo et al., 2001). Particularly, Fgf10 expression in CR mesenchyme maintains the CR itself, and Fgf8 expression at the distal tips of the ribs directs the migration of the ribs into the dorsal dermis (Cebra-Thomas et al., 2005). Experiments testing the function of FGF signaling in shell formation demonstrated that FGF10 can redirect the growth of ribs in dorsal explants of chick embryos (Cebra-Thomas et al., 2005). At a later stage, BMP signaling from the ribs induces their ossification, and also initiates a signaling cascade that induces the ossification of the surrounding dermal cells. Thus, the ribs seem to act as an organizing center to aid in the generation of the outer carapace plates, the bony plates that make up the majority of the dorsal shell (Cebra-Thomas et al., 2005). Collectively, these studies demonstrate that a novel deployment of FGF signaling to the turtle embryo drastically shifts the direction of rib growth in

turtles. The changes underlying this novel expression as well as its origin prior to its incorporation into the CR tissue, however, remains an enigma

In addition to FGF signaling, the Wnt target Lef1 was found to be involved in carapace development, suggesting a role for the Wnt signaling pathway in its origination (Kuraku et al., 2005). More recently, comparative transcriptomics, gene expression analysis, and *in-vitro* knockdown studies identified Wnt5a as a regulator of CR development (Wang et al., 2013; Zhang et al., 2021). These studies have also yielded a list of candidate downstream genes, which their further investigation *in vivo*, in combination with an advancement in our knowledge of vertebrate rib development, will be pivotal in deciphering the development and evolution of the turtle shell.

1.3.5 The evolution and development of the *Drosophila* egg dorsal appendages

The Drosophila egg contains respiratory appendages on its dorsal-anterior side, providing oxygen to the egg (Hinton, 1969). These tube-like appendages are mainly regulated by two signaling pathways, Dpp and EGFr, which provide anterior-posterior and dorsoventral information in the epithelium, respectively (Berg, 2005; Peri & Roth, 2000). In *Drosophila melanogaster*, Dpp and EGFr activate a number of transcription factors including *mirror (mir)*, which positively regulates the expression of *broad* (*br*), a gene that specifies the dorsal appendage primordia (Fuchs et al., 2012). Mirr also negatively regulates *pipe* (*pip*) in the dorsal follicle cells, rendering *pip* critical for the dorsoventral polarity of the embryo. Comparisons between *D. melanogaster* and *Ceratitis capitata*, a dipteran that does not form dorsal appendages, revealed interesting patterning differences in the eggshell (Vreede et al., 2013). While the expression of Dpp, EGFr, and the downstream gene, *pip*, are conserved in *C. capitata*, *mirr* and the dorsal appendage specific expression of *broad* was not detected in the follicular epithelium of this species. The partial

conservation of the dorsal appendage network, specifically genes involved in specifying polarity, suggested that this network may have carried out an ancestral role involved in providing positional information to the follicular epithelium. Furthermore, the change in *mirr* expression suggested that changes in intermediate nodes on the GRN was a key alteration for the evolution of dorsal appendages (Vreede et al., 2013).

1.4 Challenges in studying the origins of novelties

Despite the many instances where signaling pathway re-deployment has been implicated in the evolution of a novel morphology, how the expression patterns associated with novelties have emerged has remained a puzzle. Two major factors impede our ability to dissect the origination of many morphological novelties and truly uncover causative changes in expression patterns. First, the majority of novelties under investigation involve macroevolutionary changes that have evolved over long evolutionary timescales (Griffith & Wagner, 2017; Harris, Fallon, et al., 2002; Hockman et al., 2008; Loredo et al., 2001; Tarazona et al., 2019). This causes an insufficient phylogenetic coverage which obscures our understanding of the ancestral context in which the novelty emerged from. As a consequence, studies rely on comparisons involving taxa that are far too diverged, such that an accumulation of changes distributed throughout the relevant GRNs will likely obfuscate the causative changes (Liu et al., 2019). Second, some of the most elaborate morphological novelties have evolved in organisms that do not lend themselves well to genetic manipulations, or are even difficult to be reared and maintained in a laboratory. Having a shortage of genetic tools — *i.e.* lack of molecular markers, the means to generate transgenic animals and direct genetic perturbations in a precise spatio-temporal manner— hampers our ability to investigate

evolutionary changes at the level of gene regulatory elements and the function of genes in the relevant developmental contexts (Arnosti, 2003). Without identifying relevant CREs, our interpretations are often limited to the level of correlation of gene expression with the developing tissue of interest (Rebeiz et al., 2015). Due to these obstacles, there is a demand for studying morphological novelties that are more recently evolved and exist in genetically tractable organisms.

1.5 *Drosophila* external male genitalia as a model for studying novelty

Genitalia are one of the most rapidly evolving anatomical structures in the animal kingdom (Eberhard, 1985, 2010). The prevalent divergence of genital traits among animals is so widespread that it is often used as a distinguishing characteristic between closely related species (Kopp & True, 2002). The genitalia of closely related *Drosophila* species provide an ideal system to study novel morphologies, as they exhibit remarkable morphological diversity due to sexual selection (Kopp & True, 2002). Notably, the posterior lobe is a morphological novelty in the male genitalia of *D. melanogaster*. This cuticular outgrowth is unique to the melanogaster clade, and is necessary for copulation (Boll & Noll, 2002a). The posterior lobe projects from an ancestral genital tissue called the lateral plate (also known as the epandrial ventral lobe (Rice et al., 2019)), and is a result of changes to the shape of cells specified to generate the lobe (Smith et al., 2020). This structure evolved approximately 35 million years ago (Russo et al., 2013; Tamura et al., 1997), and the genitalia of lobed and non-lobed species are composed of similar structures otherwise. This permits us to perform comparative analyses of gene expression and gene regulation during development between *D. melanogaster* and *Drosophila* species that lack a lobe, such as *D.*

ananassae, *D. biarmipes*, and *D. pseudoobscura*, which serve as a developmentally accessible proxy for the ancestral ground state.

Exploiting the advantages of the posterior lobe as a model for studying morphological novelties, elegant research by the Rebeiz group has paved the way for uncovering its origins (Glassford et al., 2015; Smith et al., 2020). Work on the evolutionary history of the posterior lobe identified a GRN for this structure, an ancient network that was co-opted from the embryonic posterior spiracles (Glassford et al., 2015). In this study, Glassford et al. (2015) examined a known factor required for posterior lobe development, *Pox neuro (Poxn)* (Boll & Noll, 2002a). Comparative transgenic reporter analysis revealed that the posterior lobe enhancer of *Poxn* from non-lobed species are functionally conserved, suggesting that the enhancer predated the emergence of the posterior lobe (Glassford et al., 2015). In search for pre-existing roles of this regulatory element, the posterior lobe enhancer of *Poxn* was found to be active in the developing larval breathing structures, the posterior spiracles. Owing to the well-characterized posterior spiracle network, many other genes from this ancient structure were examined and identified as important factors for posterior lobe development. Importantly, the shared activity of these genes between the posterior spiracle and the novel posterior lobe were reported at the enhancer level, providing a strong case of network co-option (Glassford et al., 2015). This work has illuminated that an ancient developmental program has been re-deployed to a different development context, resulting in the formation of a morphological novelty. However, the causative alterations leading to this co-option event remain unclear. Furthermore, while perturbing the expression of characterized genes affected the posterior lobe, none of the phenotypes exhibited a drastic reduction in the size of the posterior lobe. This may suggest that a critical factor upstream of the network is yet to be identified.

More recently, work on the morphogenesis of the posterior lobe uncovered the cellular behaviors that underlie the formation of this novelty (Smith et al., 2020). Using a comparative approach, this study discovered that changes to cell shape is the major driver of posterior lobe formation. Specifically, single cell labeling of the posterior lobe revealed that this structure is a single cell tall, indicating that the precursor of the posterior lobe cells dramatically increase in height (Smith et al., 2020). In exploring the external forces that may be involved in increasing the height of posterior lobe cells, Smith et al. (2020) identified a striking association of the apical extracellular matrix (aECM) with the developing posterior lobe. In particular, an aECM protein, Dumpy, was deposited on the lateral plate, the precursor of the posterior lobe, and maintained its association with the developing posterior lobe cells. These bundles of Dumpy displayed a connection to the structures positioned in the center of the genitalia, potentially providing structural support or a pulling force (Smith et al., 2020). Importantly, *dumpy* mRNA expression in the lobed *D. melanogaster* was observed in a spatially expanded manner compared to non-lobed species. Furthermore, while an aECM was observed in non-lobed species, connections to the lateral plate were not detected. Lastly, reduction of Dumpy expression decreased the size of the posterior lobe. These findings suggested that the expansion of an ancestral aECM network and its novel cellular connections played an important role for generating the unique posterior lobe. How this expansion occurred, and what pre-existing roles it carried out, remain intriguing questions to be explored. Uncovering the genetic changes that regulate the expansion of Dumpy will be a critical step in understanding how it became integrated in the posterior lobe network.

2.0 Elaborating upon an ancient landmark: The temporal and spatial extension of an ancestral signaling center underlies a genital novelty in *Drosophila*

The origin of morphological novelties has long fascinated biologists. Signaling pathways play important roles in the formation of novelties, however, the history of such pathways prior to their integration into new developmental programs remains unclear. Here, I investigated the evolution of a novel structure in the male genitalia of *Drosophila melanogaster*. I describe that a developmental signal required for the formation of this novelty had expanded from a pre-existing role crucial for a much earlier process in genital development. This analysis provides a case in which a novelty was built upon an older signaling function, suggesting that novelties could be formed by minor modifications to already intricate developmental programs.

2.1 Introduction

The evolutionary origin of new morphological structures ("morphological novelties") remains an enigmatic process that has captivated the imagination biologists for centuries (Darwin, 1859). Despite astounding anatomical diversity in the animal kingdom, genes that govern the formation of novelties are often conserved even between distantly related taxa (Rudel & Sommer, 2003; Rubinstein & Souza, 2013; Carroll et al., 2005), and recurrently used in multiple tissues over the course of an organism's development (Carroll, 2008; Rebeiz et al., 2015). Specifically, a core set of signaling pathways are frequently re-used throughout development to regulate the formation

of different tissues (Pires-dasilva & Sommer, 2003; Perrimon et al., 2012). Though elegant work on complex morphological novelties such as the turtle's shell (Kuraku et al., 2005; Loredo et al., 2001), butterfly eyespots (Carroll et al., 1994; Keys et al., 1999), the bat's wing (Hockman et al., 2008; Sears, 2007; Weatherbee et al., 2006), and birds' feathers (Harris, Fallon, et al., 2002) have implicated a prominent role of signaling pathway re-deployment in their origination, we currently lack a molecular picture of how these roles first appeared and were shaped into complex genetic programs. In particular, we lack a detailed history of how important developmental signals became expressed to pattern these structures, and how downstream cellular responses emerged.

To study the origination of a novel structure, two factors have hindered progress towards developing a satisfying molecular history of how novelties were assembled. First, many novel morphologies of interest have evolved over long evolutionary times, such that the ancestral context from which the novelty first emerged cannot be traced with any confidence (Loredo et al., 2001; Clark-hachtel, Courtney M. and Tomoyasu, 2020). Indeed, many developmental evolutionary studies of novelty have focused on the development of single organisms without comparing to outgroup species that lack the structure (Wasik & Moczek, 2011; Harris, Fallow, et al., 2002; Harris et al., 2005). This is an understandable approach for macroevolutionary changes which arose in the distant past, as conserved landmarks are often completely absent. However, this strategy may overlook multiple layers of change that erased informative intermediate steps of the evolutionary trajectory. Thus, evaluating novelties across a wide range of timescales may offer multiple unique insights concerning their beginnings. A second barrier is that most systems lack the genetic and developmental tools necessary to assess gene regulatory changes and the function of genes in the relevant tissue (Kuraku et al., 2005; Tarazona et al., 2019; Emlen et al., 2006). Both of these barriers have necessitated the study of more recently evolved morphological novelties that

exist in genetically tractable model organisms (Vreede et al., 2013; Glassford et al., 2015; Smith et al., 2020; Vargas-Lowman et al., 2019; Martin et al., 2012; Mazo-Vargas et al., 2017; Livraghi et al., 2021; Werner et al., 2010; Arnoult et al., 2013; Brunetti et al., 2001). Doing so allows us to trace changes within the gene regulatory networks underlying these novelties.

Gene regulatory networks (GRNs) control development by integrating spatial and temporal information from transcription factors and signaling pathways to pattern the expression of target genes (Levine & Davidson, 2005). In the context of morphological novelty, the study of enhancers uniquely provides access to study mutations that may have contributed to the evolution of these traits through comparative reporter assays (Werner et al., 2010; Glassford et al., 2015). By comparing the activities of enhancers from two or more species in a common genetic background, one can attribute activity differences to the tested regulatory elements. Furthermore, reporter assays can detect co-option and pleiotropy (Glassford et al., 2015; Murugesan et al., 2022; Preger-Ben Noon et al., 2018). When a program downstream of a signaling pathway is deployed in a new context, the responsible enhancer will be pleiotropic for both ancestral and novel tissues (Glassford et al., 2015). Finally, enhancer elements are particularly useful for tracing cellular lineages through complex morphogenetic movements that may be difficult to disentangle.

The genitalia of the model organism *Drosophila (D.) melanogaster* offers a system in which a relatively recent morphological novelty can be examined in a genetically tractable system. Genital traits are noteworthy for their rapid evolution (Eberhard, 2010), and are often the distinguishing characteristic between closely related species (Kopp & True, 2002). Notably, the posterior lobe is a morphological novelty in the male genitalia of *D. melanogaster* (Kopp & True, 2002) (Figure 2.1A*)*. This cuticular outgrowth is unique to the *melanogaster* clade, and is necessary for copulation (Kopp & True, 2002; Polak & Moehring, 2015; Frazee & Masly, 2015).

The posterior lobe projects from an ancestral genital tissue called the lateral plate, and is a result of changes to the shape of cells specified to generate the lobe (Smith et al., 2020).This structure evolved approximately 35 million years ago (Tamura et al., 1997), and the genitalia of lobed and non-lobed species are composed of similar structures otherwise. This permits us to perform comparative analyses of gene expression and gene regulation during development between *D. melanogaster* and *Drosophila* species that lack a lobe, such as *D. ananassae* (Figure 2.1A), *D. biarmipes*, and *D. pseudoobscura*, which serve as a developmentally accessible proxy for the ancestral ground state.

Here, I investigated how a signaling pathway became associated with the novel posterior lobe. We found that Notch signaling plays an important role for posterior development. Specifically, the spatial expansion of the Notch ligand, Delta, in a zone adjacent to the posterior lobe is required for its development, and that this expansion is unique to *D. melanogaster*. I dissected the regulatory elements involved in the deployment of Delta to the lobe-forming region, and investigated its ancestral function in the development of genital structures. Surprisingly, this analysis revealed that the Delta/Notch signaling center becomes active days before the posterior lobe forms, serving a role in the development of conserved genital tissues. In particular, I've implicated an early-acting role for this signal in controlling genital disc eversion – a process in which the epithelium underlying these structures turns inside out. This work demonstrates that novelties may be formed in the context of ancestrally complex developmental programs, by adding new roles to pre-existing signals to connect a new program to well established ancestral ones.

2.2 Results

2.2.1 Species-specific expression of *Delta* is essential for development of the posterior lobe

In a screen of major signaling pathway ligands during posterior lobe development, we found that the Notch ligand, Delta, is expressed in multiple male genital structures, including a region adjacent to the developing posterior lobe at the base of the lateral plate and clasper (Figure 2.1B-D). Importantly, this expression precedes posterior lobe development in the early pupal genitalia (Figure 2.1B). As the posterior lobe initiated its development at mid pupal stages, the expression pattern of Delta was spatially expanded along the lateral plate and clasper boundary (Figure 2.1C). At a later stage once the posterior lobe had formed, Delta's expression retracted dorsally towards the anal plate (Figure 2.1D). The early expression of Delta preceding posterior lobe development, and its expansion corresponding to the developmental timing of the posterior lobe made it a strong candidate regulator of the posterior lobe gene regulatory network. To determine whether this pattern is unique to lobe-bearing species, we next examined the expression of Delta in *Drosophila* species that do not form a posterior lobe (non-lobed). Immunofluorescent staining of Delta using a polyclonal antibody that is cross-reactive in multiple species as well as *in situ* hybridization of *Delta* mRNA in the non-lobed genitalia of *D. biarmipes* and *D. ananassae* revealed that while Delta is expressed in a small area at the base of the claspers and lateral plates, its expression is limited to a much smaller zone compared to *D. melanogaster* (Figure 2.1G and Figure 3.8B). These results suggested that the expansion of *Delta* is specific to the posterior lobe forming species, *D. melanogaster*, and correlates with the timing of posterior lobe development.

To determine whether the posterior lobe associated expanded pattern of Delta plays a role in posterior lobe development, we utilized the GAL4-UAS system to knock down its expression

by RNAi. A *Delta*-directed short hairpin RNA was driven by a genital-specific driver of *Pox neuro* (*Poxn)* (Boll & Noll, 2002a), which is active in a broad pattern in the genitalia, including the base of the lateral plate and claspers where *Delta* is also expressed (Figure 2.5B) (Boll & Noll, 2002; Glassford et al., 2015). Comparisons of adult phenotypes revealed that reduction of *Delta* expression interferes with posterior lobe development, resulting in smaller and defective posterior lobes (Figure 2.1L, M. and Figure 2.5C). Importantly, reduction of *Delta* expression using this driver results in a pattern that resembles *Delta* expression in non-lobed species, suggesting that some aspect of the spatially expanded pattern of Delta is necessary for posterior lobe development (Figure 2.1L). To further investigate the role of Delta-Notch in the development of the posterior lobe, we stimulated Notch pathway activity by expressing a constitutively active form of Notch (Notch intracellular domain) (Zacharioudaki & Bray, 2014; Go et al., 1998) under the control of the aforementioned *Poxn* driver. These animals developed a larger posterior lobe compared to the controls (Figure 2.1M and Figure 2.5C), suggesting that Notch signaling plays a role in modulating posterior lobe development. These data showed that not only is the spatially expanded expression of *Delta* required for posterior lobe development, but that the size of the posterior lobe is sensitive to the amount of Notch signaling, suggesting that expansion of this pathway could have been an important evolutionary step in the origins of this structure.

To ascertain how the Notch pathway contributes to posterior lobe development, we assessed its activation in the developing male genitalia. Delta is a transmembrane ligand of Notch, sending signals to adjacent cells (Henrique & Schweisguth, 2019; Yamamoto, S., Schulze, K. L., & Bellen, 2014). This is noteworthy because the expression of Delta is expanded between the lateral plate and clasper, which is adjacent to the developing posterior lobe. Thus, we expect the cells of the posterior lobe to be Notch responsive, receiving signals from the adjacent Delta pattern.

To identify an appropriate readout of Notch activity, we tested the expression of canonical Notch targets of the *Enhancer of split* (*E(spl)*) complex by *in situ* hybridization (Zacharioudaki & Bray, 2014), and found that the bHLH repressor *E(spl)mβ* is expressed adjacent to Delta expressing regions throughout the genitalia, acting as an appropriate marker for Notch activity (Figure 2.1 I). Notably, in *D. melanogaster*, *E(spl)mβ* is expressed in two patches of cells adjacent to each side of the expanded Delta pattern, including the posterior lobe developing from the lateral plate (Figure 2.1 F, I). To directly compare *E(spl)m*β with Delta, we employed an *E(spl)mβ*-GFP transcriptional reporter transgene which contains the proximal 1.4 kilobase (kb) of sequence adjacent to its promoter, a region known to recapitulate *E(spl)mβ* in other imaginal tissues. I observed that Delta and *E(spl)mβ*-GFP are indeed active in mutually in exclusive regions, and that the developing lobe showed apparent Notch pathway activation (Figure 2.1 E, H). To investigate Notch activity in non-lobed species, I tested the expression of *E(spl)mβ* in pupal genitalia of the non-lobed species *D. biarmipes* and *D. ananassae* by *in situ* hybridization. Similar to *D. melanogaster*, we found *E(spl)mβ* expression adjacent to Delta expressing cells. However, in these species, the expression *of E(spl)mβ* was limited to a small ring-like pattern at the base of the lateral plates and claspers (Figure 2.1 J and Figure 3.8 A). The region of this ring-like pattern of Notch-responsive activity is in concurrence with *Delta*'s spatially restricted expression pattern observed in non-lobed species (Figure 2.1 G and Figure 3.8 B). These observations indicate that 1) the cells of the posterior lobe are indeed Notch responsive, and that 2) Notch/Delta signaling has a conserved ancestral pattern of downstream pathway activity during the development of genital structures. I examine this role in the following sections.

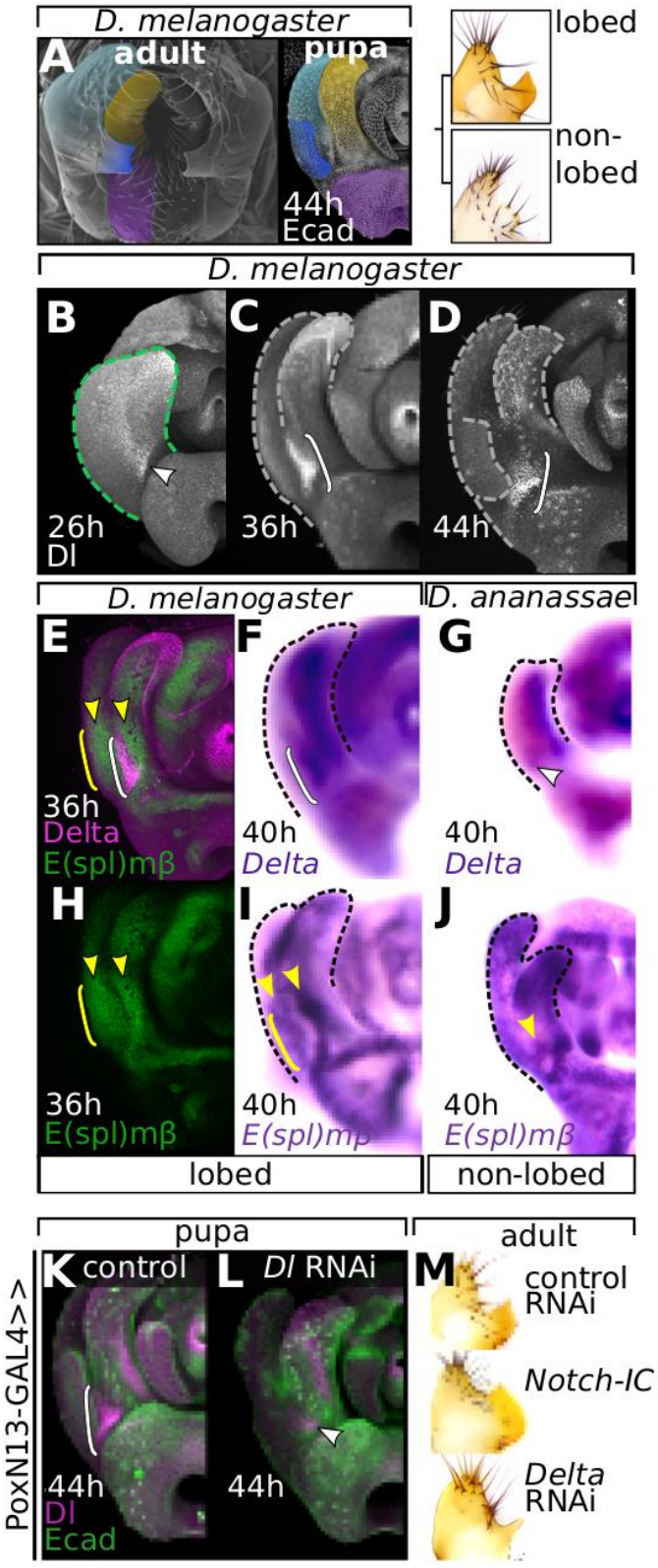

Figure 2.1 The spatially expanded expression of *Delta* is necessary for posterior lobe development in *D. melanogaster*.

(A) Left: *D. melanogaster* adult and pupal (44h APF) male genitalia. The novel posterior lobe (royal blue) protrudes and an ancestral structure called the lateral plate (light blue), adjacent to another structure conserved between lobed and non-lobe species, the clasper (yellow). The analia is highlighted in purple. Right: Phylogenetic tree with brightfield images of adult lateral plate of a lobed and non-lobed species. (*B-D*) Immunofluorescent time-course of Delta expression. Represented images display half a pupal genitalia. At 26h APF prior to the initiation of posterior lobe development the lateral plate and clasper are fused, and Delta is expressed at the dorsal base of the lateral plate/ clasper (arrowhead, B). As the lobe initiates its development at 36h APF, the expression of Delta spatially expands adjacent to the developing posterior lobe (bracket, C), and at 44h APF when the lobe has formed, the lobe-associated expression of Delta retracts (bracket, D). (*F,G*) Comparison of *Delta* mRNA expression between *D. melanogaster* (F) and the non-lobed species *D. ananassae* (G). The expression of *Delta* expands in *D. melanogaster* (bracket), but is localized to a smaller region in non-lobed species (arrowhead, G). (E, H) An *E(spl)mβ*-GFP reporter reveals Notch activity adjacent to Delta expressing cells (yellow arrowheads) and the cells of the developing posterior lobe (bracket) and acts as a readout of Notch signaling. (*E, H-J*) Comparison of a canonical Notch target, *E(spl)mβ,* mRNA expression between *D. melanogaster* (I) and and *D. ananassae* (J). The cells of the posterior lobe are Notch-responsive (yellow bracket). Two regions adjacent to either side of Delta expressing cells respond to Notch signaling (yellow arrowheads) (I). In non-lobed species, Notch activity is limited to a small circular pattern at the base of the lateral plate and clasper (yellow arrowhead in J). (K-M) Knockdown of *Delta* reduces the size of the posterior lobe, and over-activation of the Notch pathway increases the size of the posterior lobe (M) compared to the control. (L) Knockdown of *Delta* with the PoxN13-GAL4 driver reduces the expression of Delta, resembling its ancestral expression pattern.

2.2.2 Partially redundant transcriptional enhancers regulate the lobe-associated expression of *Delta*

Considering the unique association of Delta's expanded expression with lobe development, we sought to discover how this novel deployment occurred. To determine whether changes in *Delta* expression were encoded by *cis*-regulatory evolution, we first identified the enhancer

sequences regulating *Delta* specifically in the lobe-forming region. To this end, we carried out a screen of ~ 125 Kb of DNA at the *Delta* locus for pupal genital enhancers. We utilized transgenic reporters from the Janelia GAL4 collection (Pfeiffer et al., 2008), which spanned ~64 Kb of non-coding DNA upstream and intronic sequences of *Delta*. In addition, we cloned ~95 Kb of non-coding DNA downstream of *Delta* into transgenic GFP (Green Fluorescent Protein) reporter constructs. Among these constructs, we identified two elements which recapitulate the endogenous posterior lobe-associated expression of Delta (Figure 2.2 A-C). Enhancer1 is located ~36 kb downstream of the *Delta* transcription start site (Figure 2.2 A and B), and enhancer2 is ~ 60 Kb downstream of the *Delta* coding unit (Figure 2.2 A and C).

Having located the posterior lobe enhancers of *Delta*, I next investigated their functions and necessity in posterior lobe development. CRISPR/Cas9-mediated homology directed repair was employed to delete each enhancer individually. For each deletion, the enhancer region was replaced with a Ds-RED marker driven by a 3X-P3 promoter, which permitted the detection of integration events by red eye fluorescence. To our surprise, neither enhancer deletion (2.4 kb Δenhancer1 or 5.5 kb Δenhancer2) reduced the size of the posterior lobe (Figure 2.6 A) or noticeably affected the expression of *Delta* (Figure 2.6 B). These results suggested that the two enhancers may act redundantly in regulating *Delta* near the posterior lobe

2.2.3 The Delta/Notch signaling center is active days before the posterior lobe forms

While the two enhancers of *Delta* have redundant activities and both overlap the endogenous expression of Delta, they exhibit slightly different spatial activities. In contrast to enhancer1 which fully recapitulates the endogenous expression pattern of *Delta* during posterior lobe development, enhancer2 is active in cells located more ventrally, between the lateral plate

and clasper (Figure 2.2 B and C). Considering that expression patterns appear over developmental time, we were also curious to know if the two posterior lobe-associated enhancers of *Delta* showed differences in their timing in addition to their contrasting spatial activities. Thus, I investigated the relative developmental timing of each enhancer's activation. To capture reporter activity more directly than GFP protein which persists for days after expression, I performed *in situ* hybridization to detect *GFP* transcripts in the transgenic reporter lines (Figure 2.7 A). This confirmed that the late activity of both enhancer1 and enhancer2 in the pupal genitalia represented active transcription rather than perdurance of GFP from an earlier stage. At earlier stages of pupal development, when the precursor of the posterior lobe had not yet differentiated, stronger GFP mRNA signal was detected from enhancer2 compared to enhancer1 (Figure 2.7 A). Considering that the posterior lobe develops during mid-pupal stages, the earliest pupal stages we had previously assessed were at 24h APF. The reasoning behind this was that at 24h APF, the lateral plate (also known as the epandrial ventral lobe (Rice et al., 2019)) which is the precursor of the posterior lobe has not fully separated from the clasper. Additionally, the developing genitalia of lobed and non-lobed species at this time point look quite similar, with the presumptive lateral plate and clasper still being fused together. Lastly, we were unable to detect obvious differences in Delta expression between lobed and non-lobed genitalia at 24h APF. However, the striking difference of enhancer1 and enhancer 2 activity at 24h APF prompted us to assess even earlier pupal time points. To our surprise, enhancer2 was active throughout pupal development. To find the onset of enhancer2 activation, we decided to test its activity in a completely different stage of the *Drosophila* life cycle, the third instar larvae (L3), when the genital disc is still at the imaginal disc stage. Surprisingly, enhancer2 drove GFP expression in the male genital primordium of the imaginal disc in a bilateral pattern (Figure 2.2 G), while no GFP was detected in the larval genital discs of the enhancer1-GFP

reporters (Figure 2.2 F). We confirmed that the larval disc activity of enhancer2 recapitulates the endogenous expression of *Delta* by *in situ* hybridization and immunofluorescence (Figure 2.2 J). The early imaginal disc activity of Delta and enhancer2 was a particularly unexpected finding, as it is three days prior the transition to the pupal stage, and about three and a half days before the initiation of posterior lobe development (JM Tennessen, 2011). Not surprisingly, this early activity is conserved between *D. melanogaster* and the non-lobed *D. ananassae* (Figure 2.2 K), suggesting that an early ancestral role of *Delta* exists that has been expanded upon and modified in *D. melanogaster* into a pattern required for the formation of the novel posterior lobe.

Having identified an early larval imaginal disc role for enhancer2, we reasoned that deletion of this enhancer would eliminate the early conserved activity of Delta and thus illuminate the ancestral role of this signaling center. However, as mentioned earlier, deletion of this enhancer did not affect Delta expression or the development of the posterior lobe. Furthermore, the early ancestral pattern of Delta was unaffected in the male genital primordium in Δenhancer2 animals (Figure 2.6 B). The lack of an obvious phenotype in this deletion line motivated us to further examine the locus of *Delta* for additional enhancers, however this time with a focus on the larval genital disc. Utilizing the Janelia Gal4 collection (Aurélie et al., 2012), we identified seven additional upstream and intronic regions that drive larval disc activity (Figure 2.8 A-I). While the expression domain of some of these reporters are quite broad, they all overlap Delta's expression in the male genital primordium bilaterally on each side of the disc. Thus, it is likely that some of these enhancers work together or redundantly to regulate the early larval disc activity of Delta, suggesting that an early conserved role of Delta is controlled by a robust regulatory mechanism.

Given that enhancer1 and enhancer2 drove strong pupal activity resembling Delta's expression, we next examined the evolutionary history of these two enhancers.

2.2.4 Changes within the *Delta* locus, as well as the trans-regulatory environment contributed to the expansion of *Delta*

The expansion of *Delta* in lobed species could be explained by two main mechanisms. First, mutations within the *cis*-regulatory elements may have led to *Delta*'s deployment in a larger population of cells in lobed species. Second, it is possible that expression changes of transcription factors regulating *Delta*'s enhancers have caused this spatial expansion. To understand the evolutionary history of *Delta*'s posterior lobe associated expansion and test these two possibilities, we examined the ability of each DNA fragment from a non-lobed species to drive GFP in a common *D. melanogaster trans*-landscape. We assessed the orthologous sequences of enhancer1 and enhancer2 from the non-lobed species, *D. ananassae* and *D. biarmipes*, in a transgenic reporter assay. Each DNA fragment was cloned into a GFP reporter construct and inserted into the same landing site in the genome of *D. melanogaster*. We found that the orthologous sequence of enhancer1 from each species tested was able to drive GFP in a pattern resembling *Delta*'s expanded expression (Figure 2.2 D and Figure 2.7 B -D). This suggests that enhancer1 is functionally conserved and that changes within the *trans*-regulatory landscape have contributed to the expansion of *Delta*. In contrast to enhancer1, the orthologous sequence of enhancer2 from non-lobed species did not activate GFP in a *D. melanogaster* background, suggesting evolutionary modifications to this element (Figure 2.2D and Figure 2.7).

Interestingly during larval development, unlike *D. melanogaster* where enhancer1 was not active in the larval disc, enhancer1 sequences from non-lobed species drove strong larval disc activity (Figure 2.2H and Figure 2.7 E). Enhancer2, on the other hand, drove very weak activity, such that GFP was only detected with high laser power (Figure 2.2 I and Figure 2.7 E). These results suggest that while the genital imaginal disc activity of *Delta* is ancestral, the functionally

conserved enhancer1 has lost an ancient role while the modified enhancer2 has taken over this conserved role. Collectively, comparative analysis of both enhancers indicates that a combination of *cis*- and *trans*- regulatory changes were responsible for the expansion of *Delta* to form the posterior lobe, and that the two enhancers exhibit temporal shifts in the responsibility to drive the early conserved activity of *Delta*.

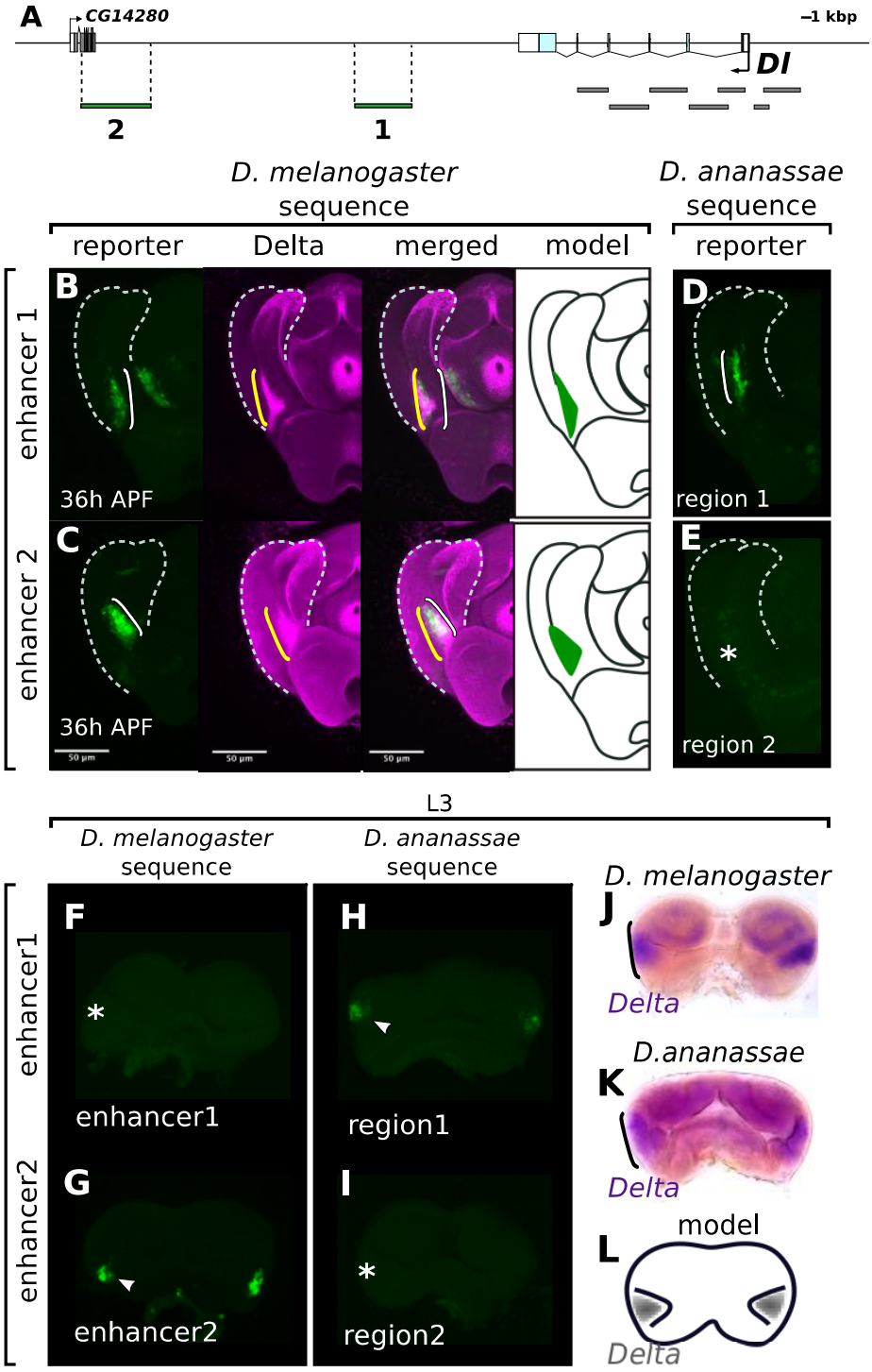

Figure 2.2 Two posterior lobe-associated enhancers of *Delta* are temporally stratified and have partially redundant spatial activities in the pupal genitalia.

(A) schematic of the *Delta* locus, with two enhancer regions displayed by green bars downstream of *Delta*. (B-C) Activity of the posterior-lobe associated enhancers of *Delta* (green, white bracket) relative to the endogenous expression of Delta (magenta, yellow bracket). Enhancer1 overlaps the full expansion of Delta (B) whereas enhancer2 recapitulates the more ventral portion of the pattern (C). (D-E) changes both in *cis* and *trans* contribute to the expanded expression of *Delta*. The orthologous region of enhancer1 from *D. ananassae* drives expression in an expanded pattern (bracket, D) similar to *D. melanogaster* (bracket, B) suggesting this element is functionally conserved. The orthologous segment of enhancer2 from *D. ananassae*, however, is not active in a *D. melanogaster* background (asterisks, E) suggesting changes to the element itself. (F-G) enhancer2, but not enhancer1, is active in the larval genital disc. (H-I) the orthologous sequence of enhancer1 but not enhancer2 of *D. ananassae* drives larval disc activity in a *D. melanogaster* background. (J-L) *in situ* hybridization of *Delta* (black brackets) confirms *Delta* is expressed in the larval genital disc of *D. melanogaster* (J) and *D. ananassae* (K). Cartoon model highlights the relative regions of the lateral Delta expressing epithelial cells of the male genital primordium (L).

2.2.5 Delta plays an ancestral role in the eversion of the genital disc

We hypothesized that *Delta* is responsible for an ancestral function because of the following reasons: 1) *Delta* was expressed in the imaginal genital discs of both lobed and non-lobed species, 2) *Delta*'s expression persisted in the developing pupal genitalia of non-lobed species, and 3) Notch signaling was active in regions adjacent to *Delta* expressing cells in the developing non-lobed genitalia. Furthermore, there is not a clear relationship between larval disc folds and pupal genital structures, and we were uncertain about which pupal structures the Delta expressing lateral regions of the L3 primordium corresponded to. Thus, we sought to investigate the ancestral function of *Delta* to understand the origin of this signaling center, and reasoned that tracing the lineage of *Delta* expressing cells would provide insight into its ancestral role. We performed a developmental time-course of enhancer2 activity from the genital imaginal disc

through pupal development. Each enhancer2-GFP transgenic genital sample was stained with an antibody against E-cadherin to mark the apical surface of epithelial cells. Importantly, the genital imaginal disc starts inside out, with the apical surfaces facing each other and the basal lamina surrounding the outside of the disc. As the disc develops, it undergoes morphogenetic movements and eversion at the posterior edge to form the external anal and genital structures, such as the lateral plates and claspers (Epper, 1983). At 0h APF (after pupal formation), enhancer2 is active in the male genital primordial folds on each side of the disc (Figure 2.3 A). The folds in which enhancer2 is active correspond to regions that begin to evert outward from the opening at the posterior stalk at early pupal stages (Figure 2.3 A and B). At around 20 hAPF, the cells marked by enhancer2 have partially everted, which form the precursor of the lateral plate and clasper (Figure 2.3 A and B. 4A). These results demonstrated that *Delta*'s expression tracks with cells that undergo eversion to form the lateral plates and claspers, structures that are ancestral to the posterior lobe. Importantly, tracking the early larval disc expression of Delta with enhancer2 in *D. melanogaster* suggested that it is the same signaling center that persists through pupal development that later becomes associated with the posterior lobe.

To elucidate the ancestral role of *Delta*, we genetically perturbed the early expression of *Delta* in the larval imaginal disc. We utilized the publicly available Flylight Image Database (Aurélie et al., 2012) to select GAL4 drivers that are active in the lateral male genital primordium of the larval disc, and carried out a screen with the goal of knocking down *Delta* early and strongly enough only in the lateral clusters to disrupt the ancestral function. From this UAS-GAL4 *Delta*-RNAi screen, we successfully identified a GAL4 driver in the *MSR1* locus (GMR64C05-GAL4) that specifically knocked down *Delta* in the lateral clusters of the male genital primordium without affecting *Delta*'s expression in other regions (Figure 2.3 C-E). As a result, 100% of the early *Delta*

knockdown animals exhibited defects in genital eversion (Figure 2.3 F and G and Figure 2.9 C). At a pupal time point when the precursor of the lateral plates and claspers have everted in the control animals, the genitalia of *Delta* knockdown animals were still encapsulated within the tissue that should form the 8th tergite (Figure 2.3 G). Interestingly, 22% of male adults completely lacked an external genitalia, which we postulate is due to failure of eversion (Figure 2.9 A and B). Due to the incomplete and variable nature of RNAi, *Delta* was not completely knocked down in some animals. Nonetheless, the adults that formed an external genitalia had defects in either claspers, lateral plates, posterior lobes, or all three structures (Figure 2.3 H-I and Figure 2.9 D). These results indicate that Delta had an early function important for the eversion of the genital disc, a complex process which is presumably necessary for genital development of all *Drosophila* species and predates the evolution of the posterior lobe (Figure 2.4).

2.2.6 Delta is upstream of a vast apical extracellular matrix network

Our previous work on the cellular development of the posterior lobe had uncovered the mechanism by which the cells of the posterior lobe behave (Smith et al., 2020). This study revealed that the cells of the posterior lobe drastically increase in height to project from their lateral plate precursor, and implicated an important role for the apical extracellular matrix (aECM) in shaping the posterior lobe cells. A gigantic protein of the aECM, Dumpy, covers the lateral plate of *D. melanogaster* in an expanded pattern compared to non-lobed species, and is essential for proper posterior lobe development (Smith et al., 2020). It is speculated that Dumpy provides structural support as the intrinsic factors of the posterior lobe cells drive elongation, or that Dumpy tethers create a mechanical force to pull the cells of the posterior lobe (Smith et al., 2020). Given that Dumpy is a cellular effector molecule and likely downstream of the posterior lobe gene regulatory

network, we were curious to know if there is a potential link between Delta and *dumpy*. Similar to *Delta*, *dumpy* expression is spatially expanded in *D. melanogaster* compared to non-lobed species, resembling that of Notch responsive regions (Smith et al., 2020). Thus, I examined whether Dumpy is downstream of Delta, and whether it also has an early role in genital eversion. First, utilizing a line in which the Dumpy protein is endogenously tagged with a Yellow Fluorescent Protein (Dumpy:YFP) (Lye & Naylor, 2014; Lowe et al., 2014), I detected Dumpy deposition within the epithelial folds of the larval genital disc (Figure 2.3 K.). Dumpy tethers within the genital disc clearly lined the surface of the male genital primordium in the lateral regions, overlaying the Delta expressing epithelial cells (Figure 2.3 K and Figure 2.10). To test whether Dumpy has a role in genital disc eversion, I examined whether Dumpy is disrupted under larval disc *Delta* RNAi conditions. In *Delta* RNAi animals with an eversion defect, Dumpy lost clear connections to the apical surface of the everting cells compared to the control which form organized connections (Figure 2.3. L and M). Dumpy deposition in the larval genital disc was also decreased compared to controls (Figure 2.10 B), suggesting that the early activity of Delta is necessary for the presence of Dumpy on the surface of the male genital primordium. Together, these data suggested that Dumpy acts downstream of Delta, providing a likely explanation for how Delta exerts its effect at the cellular level.

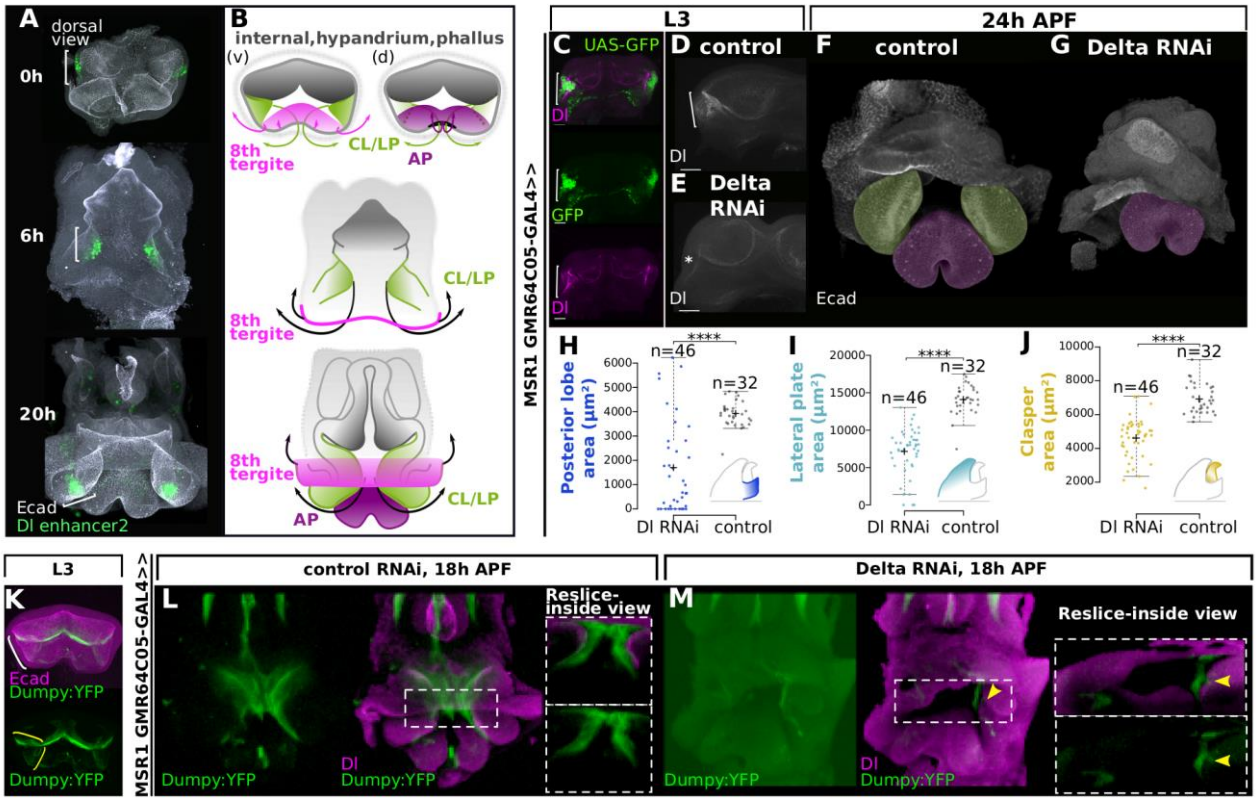

Figure 2.3 The posterior-lobe associated signaling center has an early ancestral role involved in genital disc eversion.

(A) The activity of enhancer2 tracks with the cells of the genital disc that undergo evagination. At 0h APF, enhancer2 is active in the male genital primordium in a bilateral pattern. As the genital disc elongates at an early pupal stage (6h APF), enhancer2 tracks with the epithelial folds which will be a point of evagination. At 20h APF as the genitalia is mid-eversion, the signaling center marks a patch of cells between the prospective lateral plates and claspers. (B) cartoon description of panel A. The male genital primordium on the lateral sides of the L3 disc where *Delta* is active is denoted by green shading through development.The 8th tergite on the ventral side of the disc is colored in pink. This tissue everts along with the future clasper/lateral plate (CL/LP) and eventually fuses dorsally . The anal pate is colored in purple. Grey color marks tissue that will form the internal genitalia as well as the hypandrium, phallus, and branches.(C) Reporter activity of the MSR1-GMR64C05-GAL4 driver (green) in the L3 genital disc overlaps Delta expression (magenta) in the lateral folds of the male genital primordium. (D,E) Reduction of *Delta* by RNAi in the L3 genital disc (D, asterisks) compared to control (E, bracket). (F,G) Early knockdown of *Delta* in the L3 genital disc causes defects in genital eversion (G). Prospective lateral plate/claspers (green false color) fail to evert in upon early

Delta knockdown (G) compared to a control (F). The anal plate is false colored in purple. (H-J) Reduction of Delta early in the L3 genital disc causes severe defects in the development of the posterior lobe (H), lateral plate (I), and clasper (J). Asterisks denote significant differences (two tailed Student's t-test, ****$p<0.0001$). (K) Dumpy is deposited within the L3 genital disc and covers the epithelial folds of the lateral male genital primordium. The lateral fold is marked by a white bracket (top) and Dumpy deposition is marked by yellow brackets (bottom). (L,M) Dumpy deposition is disrupted in genitalia with genital disc defects. Early knockdown of *Delta* with an L3 genital disc driver reduces and disrupts the organization of Dumpy tethers (compare G to F).

2.3 Discussion

Despite strong implications for the role of signaling pathways in the formation of many novel morphologies, efforts to understand how their pivotal roles were established have lagged far behind. Here, we identified a signal source, Delta, that is crucial to a novelty that formed on an intermediate timescale, permitting an opportune glance at its developmental and evolutionary past. Our comparative analysis of lobed and non-lobed species revealed that the Delta signal predated the posterior lobe novelty, suggesting roles preceding its evolution. The Delta signal initiates at a remarkably early developmental time, where it is required for the fundamental process of disc eversion, common to all species analyzed in this study. I placed this role in eversion upstream of the terminal effector Dumpy, which also participates directly in posterior lobe development. These findings suggest that morphological novelties that originate on macroevolutionary scales may evolve through elaborations of ancestral signaling centers whose beginnings may have been obscured by the passage of time. Because of their antiquity, the genetic architecture of these ancestral systems may be quite intricate and robust. I discuss below the implications of such "ancestral complexity" in understanding the origins of elaborate morphological structures.

The phenotypes we observed for *Delta* knockdown are some of the most dramatic posterior lobe defects we have found, and overexpression of the Notch intracellular domain is the only treatment that has been shown to dramatically increase lobe size to date. Considering the expanded expression of *Delta* near the developing lobe of *D. melanogaster,* this motivated our search for enhancers to trace its evolutionary history. While a combination of *cis* and *trans* differences accounts for the expanded expression we observed, the reporters notably allowed us to lineage trace the developmental trajectory of this signaling center back to the third instar genital disc. Prior to this study, our knowledge regarding the morphogenetic processes that occur during early genital disc development at pupal stages was sparse. Valuable studies had been carried out in larval genital discs identifying genes and signaling pathways involved in the anterior and posterior compartmentalization and patterning (E. H. Chen & Baker, 1997; Sánchez et al., 1997; Estrada et al., 2003). While the eversion of the genital disc had previously been described (Epper, 1983) how this phenomena occurred remained largely uninvestigated. The results presented here highlight how novelties may depend upon signals that were initially deployed to pattern ancestral structures at earlier stages of development. Work in the bat limb also hints at this possibility with the re-initiation of Shh signaling (Hockman et al., 2008) specific to the bat wing after Shh in the zone of polarizing activity has ceased. Such findings underline the importance of considering a wide range of developmental times that may bear upon evolving traits, rather than focusing solely on stages proximate to the developmental appearance of the trait.

Our survey of *Delta* regulatory elements also revealed a redundant architecture which controls a broad domain of ligand expression that shifts over time. Redundant enhancers are quite common, and are thought to foster robustness to environmental and genetic variation (Barolo, 2013; Perry et al., 2010; Frankel et al., 2010). The enhancer elements I found in the genital disc

cover a wider spatial domain than was observed for the pupal enhancers. This suggests that the lobe-patterning signal center may have emerged from a portion of the total genital disc signaling center (Figure 2.11). Furthermore, our *Delta* knockdown experiments revealed a counterintuitive relationship between *Delta* expression and lobe patterning. When *Delta* RNAi is driven by the early enhancer, the ventral expansion is completely ablated, but lobe morphology is unaffected (Figure 3.7). This suggests that the dorsal expansion (near the anal plate) represents the portion of *Delta* signal relevant to the formation of this novelty. These results underscore the importance of fine-scale genetic manipulations in determining which portion of an expression pattern is most necessary to the developmental events under study.

Previous work had implicated a critical role of the aECM in the development of the posterior lobe (Smith et al., 2020). Here, I demonstrated that Dumpy deposition was affected when Delta was knocked down early in the larval genital disc, suggesting that Dumpy may serve an early Delta-dependent role in disc eversion and a late role downstream of Delta in the posterior lobe network. A plausible model is that *dumpy* was regulated by Delta in the larval genital disc of both lobed and non-lobed species and carried out an ancestral function of genital disc eversion. As *Delta* evolved an expanded expression pattern in *D. melanogaster*, the expression of *dumpy* may have expanded as a result. According to this model, a pre-existing target of Delta became involved in the development of the posterior lobe by becoming active in a broader expression domain. Crucial to examining this model is identifying the regulatory elements that regulate *dumpy* in the larval genital disc as well as the posterior lobe. It is also possible, if not likely that in addition to bringing along pre-existing downstream targets, Delta has also gained novel targets in lobed-species that contribute to posterior lobe development.

Previous work on the evolution of the posterior lobe had shown that an ancestral network of the embryonic posterior spiracle was co-opted in the genitalia (Glassford et al., 2015). The connection between the Delta signaling center and co-opted posterior spiracle network however is yet to be explored. An interesting question is whether the expansion of *Delta* is downstream of the co-opted network or vice-versa. Our preliminary data suggests that the expansion of *Delta* during pupal stages is downstream of Pox Neuro (Poxn), a key component of the co-opted network (Bill Glassford, personal communication). In a Poxn mutant, the ancestral (not-expanded) pattern of Delta remains unaffected, however this pattern does not expand. This is in agreement with our data showing that Delta has a much earlier onset of expression than Poxn. Thus, it is possible that the derived, expanded expression of *Delta* is mostly attributed to *trans*-regulatory changes that occurred as a result of co-opted spiracle network, and the co-option of the spiracle network may have ignited the redeployment of transcriptional regulators rather than terminal effectors.

The complexity of ancestral systems has been an important concept in developmental evolution. As we learned from sequencing the human genome (Craig Venter et al., 2001), and an increasing array of basally branching organisms (Srivastava et al., 2010; Martinez et al., 2006; Chapman et al., 2010) it is the case that most transcription factors and signaling pathways are conserved within animals and beyond rather than each clade having an abundance of lineage specific genes and gene families. Our work here causes us to appreciate how ancestrally complex signaling centers may apply to newly formed novelties. These centers may show faint signs of relation to signals deployed much earlier in development. The regulatory architecture of their participating loci may show unexpected intricacies such as redundancy that resulted from the evolutionary refinement of their ancestral roles. Our efforts to resolve these faint connections and

complex developmental systems are crucial to developing a sophisticated understanding of what would otherwise appear to have arisen through inexplicable events.

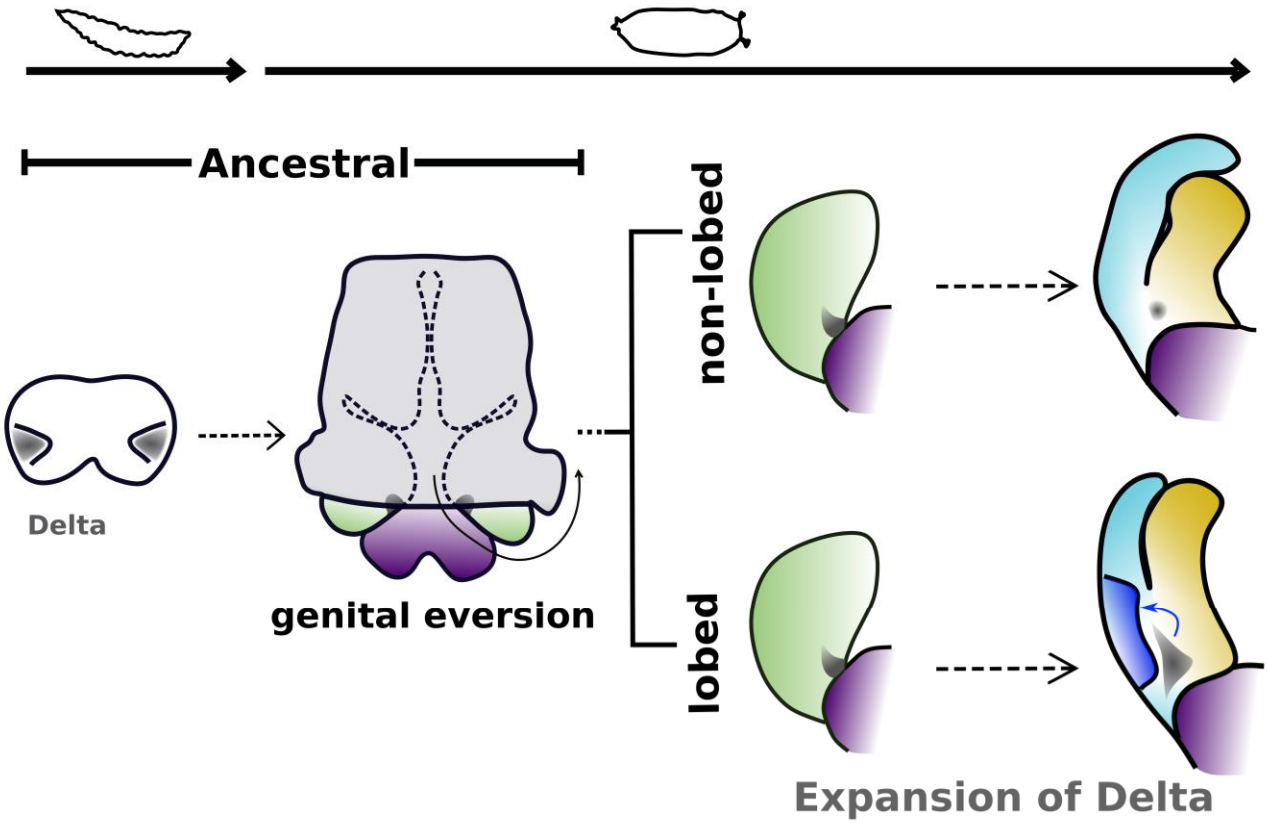

Figure 2.4 Cartoon model of an ancient signaling center underlying the origination of the posterior lobe.

(From left to right) A Delta/Notch signaling center (grey) is active in the larval genital disc with an ancestral function of genital disc eversion. This signaling center persists through pupal development in non-lobed species, where it carries out potential ancestral roles in later pupal development. In lobe-forming species, The ancestral pattern at the base of the lateral plate/clasper precursor (green) later spatially expands along the two structures (lateral plate in light blue, clasper in yellow) forming the novel posterior lobe (royal blue).

2.4 Supplementary Figures

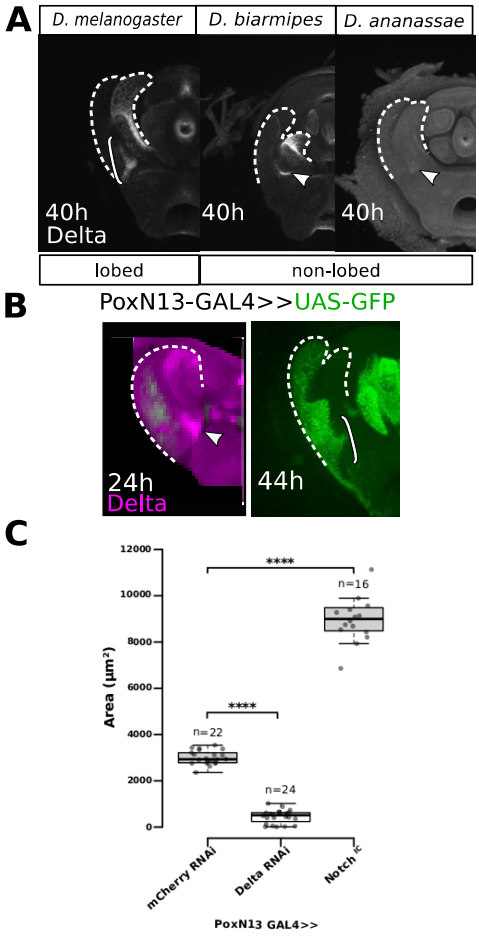

Figure 2.5 . The expanded expression of Delta is unique to the lobed D. melanogaster and is necessary for posterior lobe development.

(A) Pupal genitalia of *D. melanogaster*, *D. biarmipes*, and *D. ananassae* stained with a cross-reactive polyclonal antibody against Delta. Expanded lobe-associated expression is marked with a bracket, and the ancestral pattern is indicated by arrowheads. (B) Reporter activity of the PoxN13-GAL4 driver (green) at 24h and 44h APF. At 24h APF while the driver is active in the presumptive lateral plate (bracket), its activity does not overlap Delta (magenta, white arrowhead). (C) Quantification of *Delta* RNAi and constitutive activation of the Notch pathway using the PoxN13-GAL4 driver. Asterisks denote significant differences (two tailed Studen'ts t-test, ****$p<0.0001$). Whiskers extend 1.5 times the interquartile range above and below Q1-Q3.

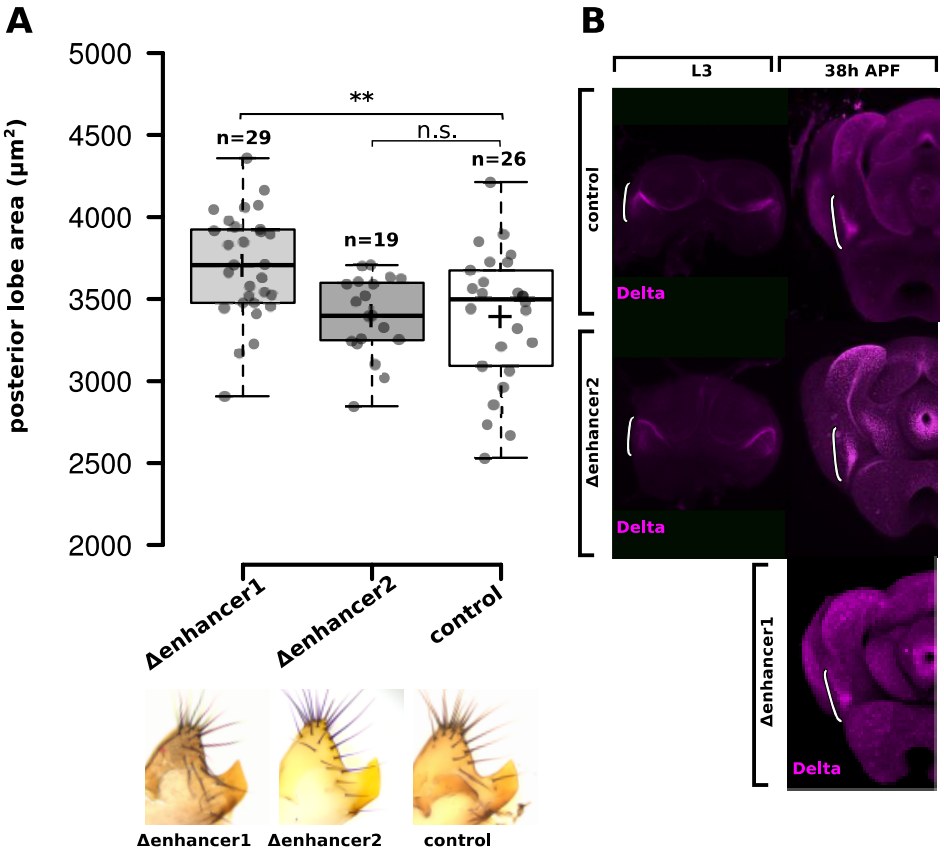

Figure 2.6 Neither deletion of enhancer1 nor enhancer2 reduces the size of the posterior lobe.

(A) Quantification of each enhancer deletion. Asterisks denote significant differences (two tailed Studen'ts t-test, **p<0.01, n.s. denotes not significant). (B) Delta expression in L3 genital disc and 38h APF pupal genitalia of control (top) compared to Δenhancer2 (middle) indicates Delta does not display qualitative difference in the absence of enhancer2 which drives both larval and pupal activities. In the absence of the pupal enhancer1, Delta is still expressed in an expanded pattern at 38h APF (bottom).

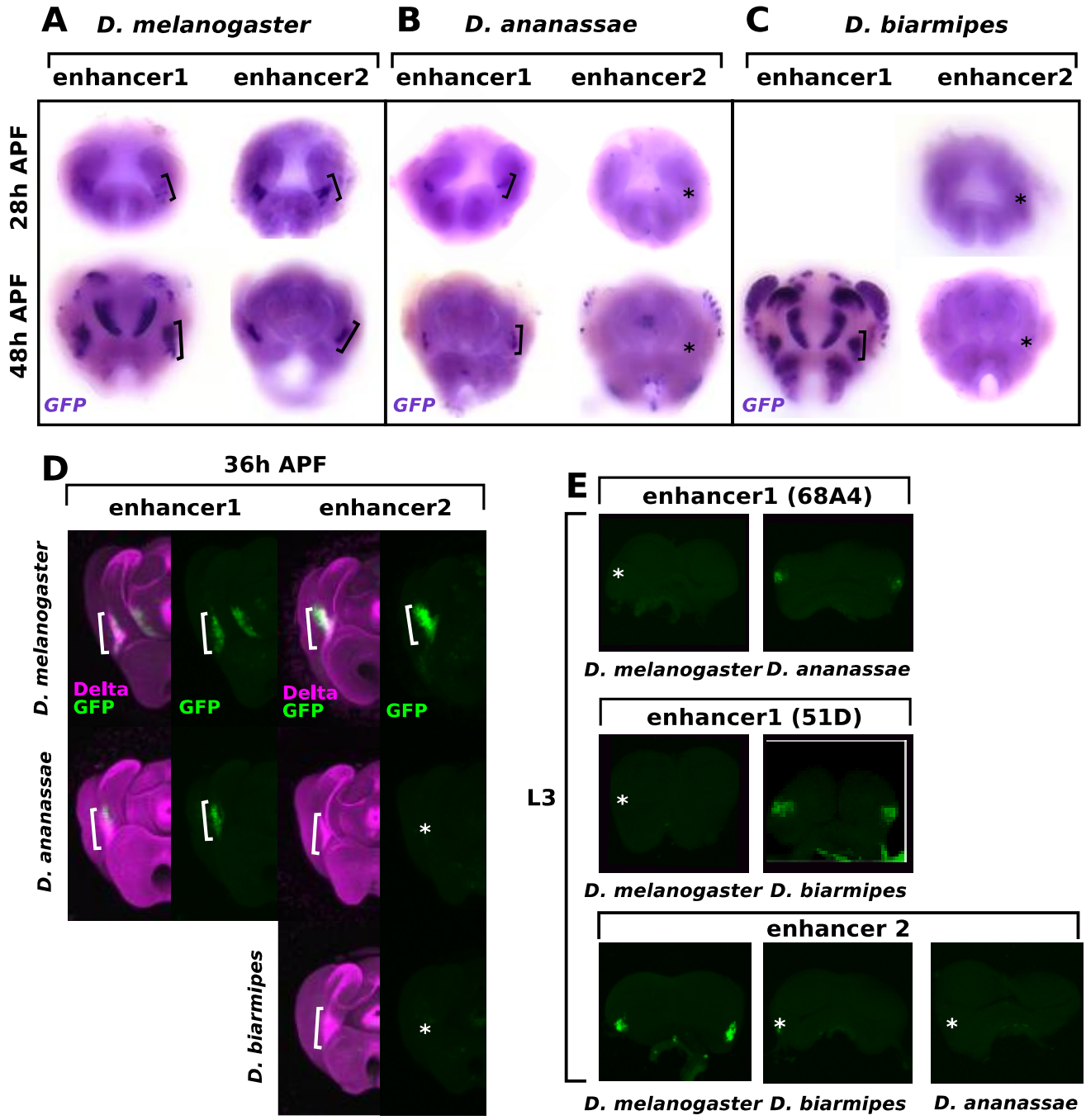

Figure 2.7 Temporal and spatial activity of Delta enhancer1 and enhancer 2.

(A-C) *GFP in situ* hybridization of the lobe-associated reporters of *Delta*. The lobe-associated enhancers of *Delta* have differences in their spatial and temporal activities in *D. melanogaster* (A). In *D. melanogaster*, enhancer2 drives stronger activity at an early pupal stage (28h APF). As the lobe has developed at 48h APF, both enhancers still drive

activity, albeit enhancer1 in a broader spatial pattern compared to the more ventrally active enhancer2. (B and C) The orthologous sequences of enhancer1 from *D. ananassae* (B) and *D. biarmipes* (C) drive pupal expression in a *D. melanogaster* background, suggesting the element is functionally conserved. However, the orthologous region of enhancer2 from these species fails to drive activity in the pupae (asterisks), suggesting that enhancer2 has been modified. (D) Comparison of orthologous reporters by GFP fluorescence in the developing pupal genitalia at 36h APF. Samples were stained against a monoclonal antibody against Delta. (E) Comparison of orthologous reporters by GFP fluorescence in the L3 genital disc. 68A4 and 51D denote landing sites in the *D. melanogaster* genome.

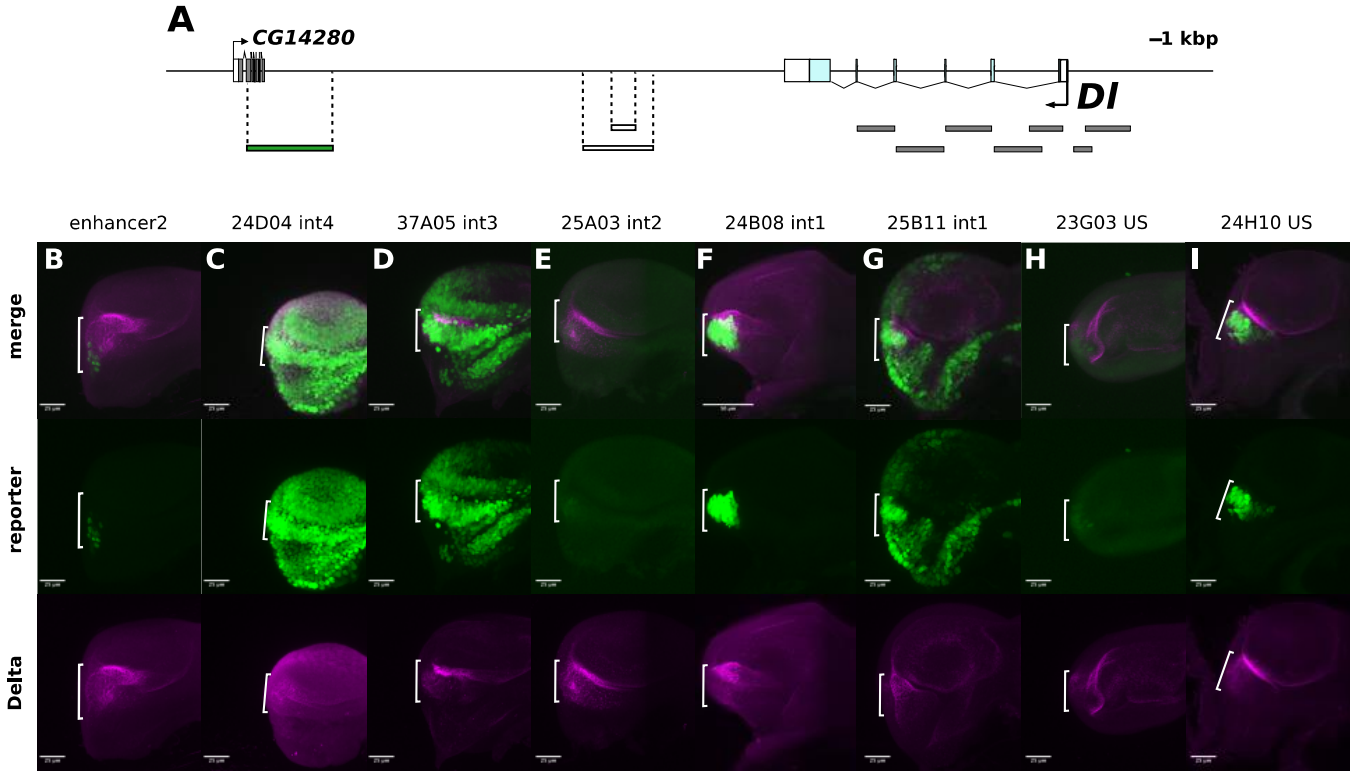

Figure 2.8 Highly redundant regulatory elements overlap the larval disc activity of *Delta*.

(A) schematic of the *Delta* locus, displaying the relative region of *D. melanogaster* regulatory elements driving larval disc activity. Green bar indicates activity in both L3 and pupal stages, white bar indicates pupal activity only, and grey bars indicate L3 activity all in *D. melanogaster*. (B-I) Confocal images of reporters in the male L3 genital disc (green) overlapping Delta expression (magenta) in the male genital primordium

located laterally on the disc (brackets). Images correspond to the order of annotated elements in panel A from left to right. Represented images are half of the L3 disc. confocal images were taken at 63x magnification.

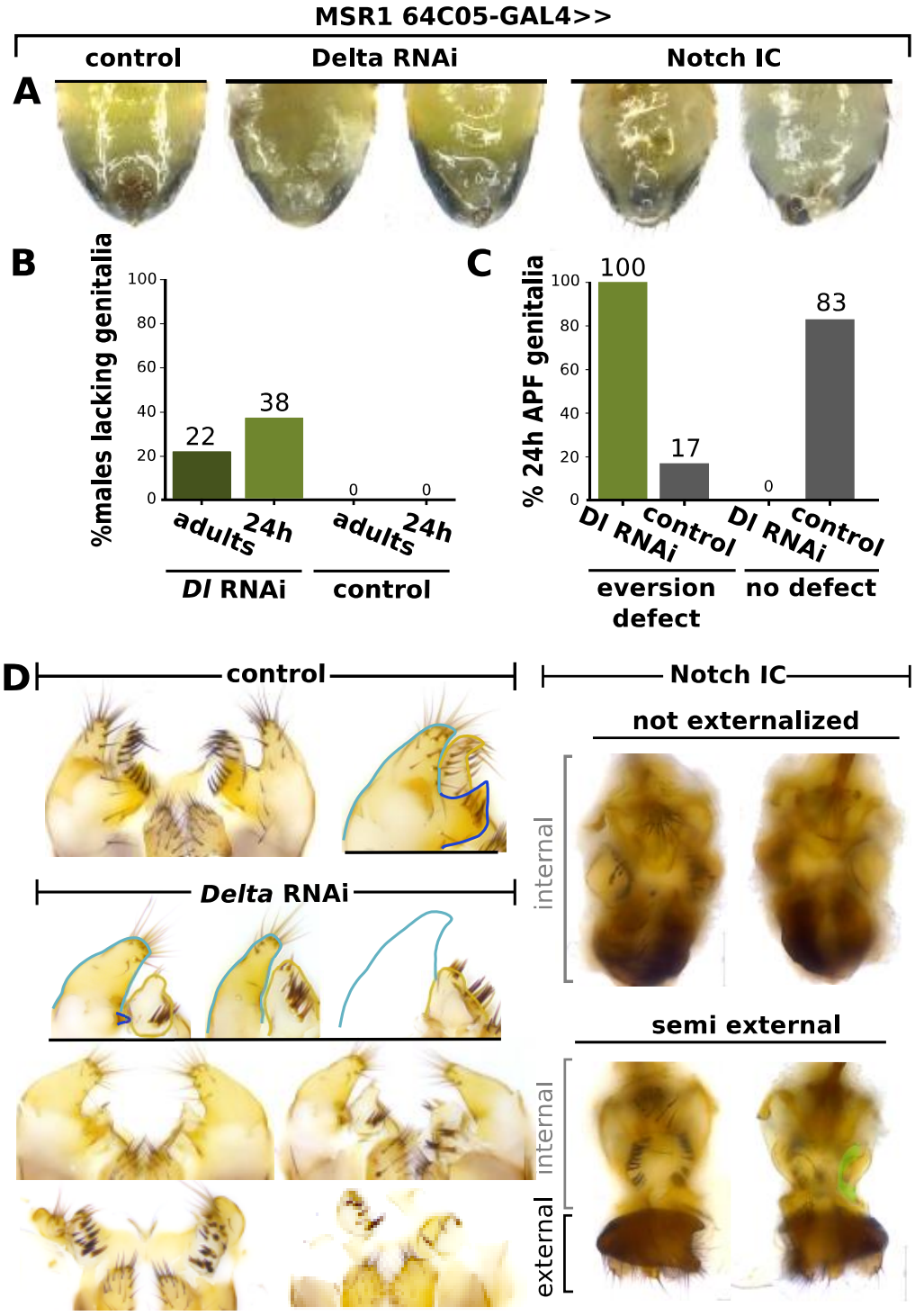

Figure 2.9 phenotypes of early reduction of *Delta* by RNAi

(A-C) animals with severe genital eversion defects fail to develop genitalia. (A) Adult abdomens of control (left) Delta RNAi (middle), and Notch over expression(right). Abdomens form *Delta* RNAi and Notch overexpression display lack of an external genitalia. (B) 22% of *Delta* RNAi adults and 38% of pupae dissected at 24h APF lack a recognizable genitalia. (C) 100% of *Delta* RNAi animals display a defect in genital eversion at 24h APF. (D) A range of adult genital defects represented in *Delta* RNAi genitalia that complete eversion. Over-activation of the Notch pathway in the L3 genital disc causes severe eversion defects. Represented adult genital cuticles were dissected from inside the abdomen, as they failed to externalize. An adult genitalia that has partially externalized from the abdomen resembles a wildtype developing pupal genitalia undergoing early stages of eversion (green shading).

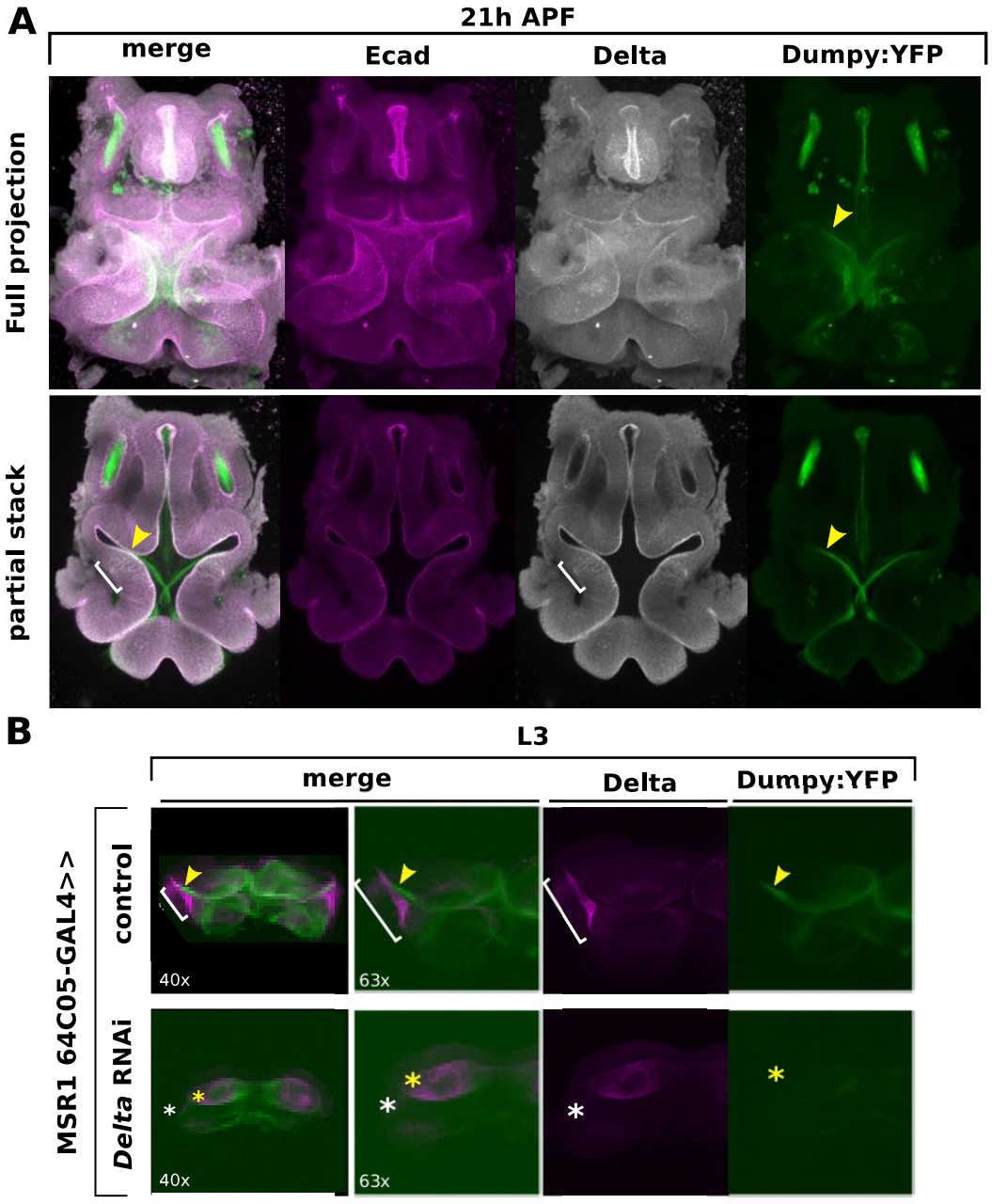

Figure 2.10 Dumpy is deposited near Delta expressing cells at early stages.

(A) Dumpy is deposited in an organized manner in the early everting pupal genitalia. A 21h APF reporter of Dumpy:YFP (green) stained with antibodies against Delta (grey) and Ecadherin (magenta). Top panel displays full projection of z-stacks, bottom panel displays a partial stack in order to visualize the inside of the everting pupal genitalia. Dumpy is tethered to the Delta expressing epithelial folds (yellow arrowhead and white bracket,

respectively). (B) Knockdown of *Delta* in the L3 genital disc also reduces Dumpy:YFP surrounding the male genital primordium bilaterally (bottom, compare to control). Reduction of Delta and Dumpy are indicated by white and yellow asterisks, respectively.

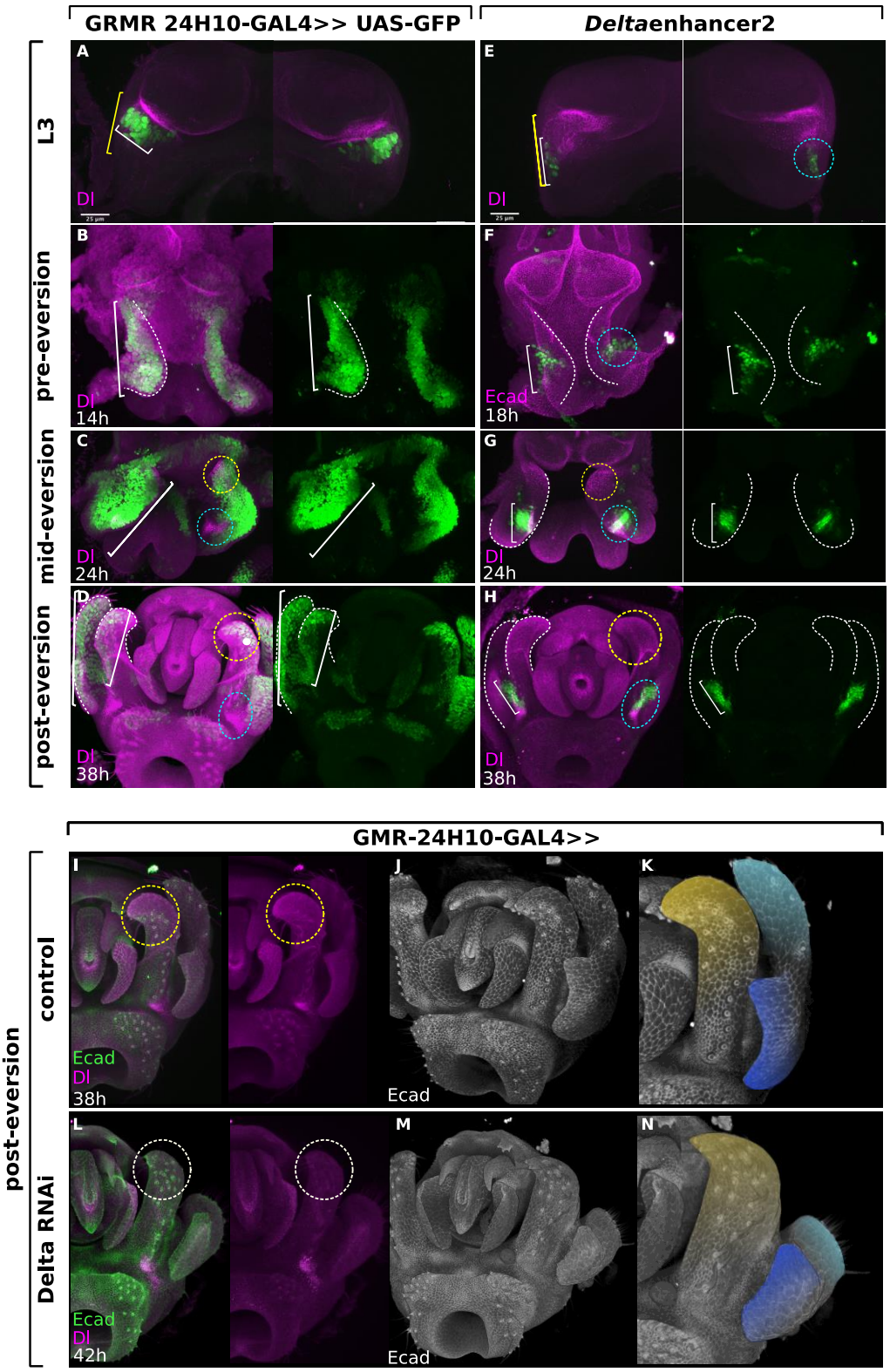

Figure 2.11 The posterior lobe patterning signal emerges from a portion of a broader Delta pattern in the larval genital disc.

Activity of a larval enhancer of Delta (GMR24H10) (A-D) compared to the activity of Delta enhancer2 at four developmental stages (E-H). GFP marks reporter activity (white bracket), and Delta antibody is shown in magenta (yellow bracket in A and E). (A) GMR24H10 drives GFP activity in the lateral folds of the larval disc and overlaps Delta expression in this region. (B) At a pre-eversion stage, this activity is observed in a broad region which corresponds to the lateral plate/clasper (LP/CL) precursor (white bracket). (C) Mid-eversion, the LP/CL activity of the reporter overlaps the ventral expression of Delta at the tip of the future clasper (yellow dotted circle), but excludes the posterior lobe associated Delta pattern (blue dotted circle). (D) At a later stage post-eversion, GFP corresponds to the more ventral regions of the lateral plate and clasper, and does not overlap the lobe-associated pattern of Delta (blue dotted circle). (E) While *Delta* enhancer2 drives activity in the lateral folds of the larval genital disc (left:white bracket, right: blue dotted circle), this pattern overlaps only a portion of Delta's endogenous expression domain (yellow bracket). (F) At a pupal stage pre-eversion, enhancer2 is only active in a subset of evaginating cells of the future LP/CL (white bracket and blue dotted circle). (G) Mid-eversion, enhancer2 only overlaps the lobe-accosiated expression of Delta (blue dotted circle), and does not overlap the ventral LP/CL activity of Delta (yellow dotted circle). (H) This is also observed post-eversion after the LP and CL have differentiated. (I-N) Knockdown of Delta using the GMR24H10-GAL4 driver affects the clasper and lateral plate (compare I-E with L-N). (I) In the control, Delta is expressed at the ventral tip of the clasper (yellow dotted circle), whereas knockdown of Delta strongly reduces this pattern (L, white dotted circle). (M, N) 3D rendering of post-eversion *Delta*-RNAi demonstrates defects in clasper and lateral plate development (compare to J and K). Claspers (false colored in yellow) of *Delta*-RNAi animals are shorter and less curved, and the lateral plates (false colored in light blue) are truncated.

3.0 Conclusions and future directions

3.1 Conclusions

Here, I have investigated the evolutionary origins of a signaling source critical for the development of the posterior lobe. I examined how changes to its regulation contributed to the expansion of this signaling center, which was key to providing inroads to its ancestral role. I discovered an unexpectedly prolonged temporal activity of the signaling center, and identified an early acting ancestral function — the eversion of the genital disc. This work highlights the importance of studying the ancestral roles that signaling centers associated with novelties were built upon. In this case, I illustrated that a novel function was added to a pre-existing signaling center with robust regulation involved in an intricate and conserved developmental process. Thus, novelties may arise through what may seem like minor changes to expression patterns that occur in the context of complex programs with ancient roles.

In this chapter, I will present efforts in identifying upstream regulatory changes causing the expansion of *Delta* expression. Further, I will discuss the downstream consequences that follow the elaboration of this signaling center, and present preliminary experiments to investigate regulatory interactions between Delta and the aECM protein Dumpy and identify additional targets important for posterior lobe development. I will present data that highlight the importance of a detailed dissection of the patterns associated with novelties and describe how even what may seem like slight alterations to expression patterns can be broken down into functional sub-patterns. Finally, I will speculate on the roles of Delta in non-lobed species and outline a rationale for exploring other conserved roles this signaling center may have at later stages of development.

3.2 Future directions

3.2.1 Identify upstream regulators of Delta

As presented in chapter 2, the functional conservation of *Delta* enhancer1 suggests that one or more changes to the *trans*-regulatory landscape in *D. melanogaster* has played a major role in the expanded expression of *Delta*. Identifying *trans*-regulatory changes that account for the expansion of *Delta* entails finding regulators of *Delta* enhancer1 which do not exhibit an expanded expression pattern in a homologous region of lobed-species. To generate a list of candidate regulators, it was important to first narrow down the regulatory region to the smallest sub-fragment possible that could recapitulate the enhancer1 expression pattern. This would allow me to identify important regions of enhancer1 where critical transcription factors bind. I subdivided the 2 kb enhancer1 into 6 overlapping fragments. Each fragment was approximately 700-800 bp long and overlapped adjacent fragments by ~400bp on each side. These segments were cloned into a GFP reporter and injected into the genome of *D. melanogaster* (Figure 3.1, A). By screening these reporters, I identified 3 important sub-regions: enhancer1-N with lobe-associated activity, enhancer1-O drove sheath and lobe associated activity, and enhancer1-P drove only sheath activity (Figure 3.1, B-G). Given that enhancer1-O best recapitulated the full 2kb enhancer, I utilized this fragment as a minimal enhancer1 of *Delta*.

Having a minimal reporter in hand, I next sought to identify regions containing important binding sites spanning the enhancer. To this end, I generated 15 mutant enhancer1-O reporters in which I've introduced a different ~60bp of sequence that was scrambled. In each scrambled block, every other base pair possesses a non-complementary transversion mutation. The 15 scrambled blocks span the entire ~730 bp of the minimal enhancer, all with ~10 bp overlap (Figure 3.1, H).

If mutations spatially decrease the domain of reporter activity to an appreciable extent, it will suggest that the mutated regions contain binding sites for important activating inputs into the enhancer. Conversely, mutations that increase the spatial expression domain could contain binding sites for important repressors. It is possible that the expansion of *Delta* occurred through the gain of activating inputs, the loss of repressive inputs, or both. Thus, reporters that fail to drive a wild-type pattern of GFP expression provide smaller stretches of sequences with potential binding sites to prioritize a search for upstream activators and repressors. Thus far I have identified seven regions with noticeable effects on reporter activity when mutated. Regions 6, 7, 8, and 9 considerably decrease or modify spatial activity (Figure 3.1, O-R), regions 5 and 14 moderately reduce expression (Figure 3.1, N and V), while region 11 nearly ablates reporter activity (Figure 3.1, T). Region 6 was of particular interest as its activity resembled that of Delta expression in non-lobed species. Preliminary searches for putative binding sites in this region using the JASPAR database (Fornes et al., 2020) identified potential candidate regulators which should be further investigated for their expression, function, and connection to *Delta*.

Below, I present data on two candidate regulators that I considered, which provides a launching point for future studies of *trans*-regulatory changes upstream of Delta.

3.2.1.1 Drop as a candidate regulator of Delta

To narrow down the list of candidate regulators obtained from the mutant reporters, I used two main criteria: 1) I selected genes that are expressed in the pupal genitalia determined by previous RNA-sequencing experiments, and 2) genes that have known functions in the development of the genitalia or morphologically similar tissues, or in the regulation of Delta/Notch signaling. One such candidate gene is the homeodomain transcription factor *Drop* (*Dr*) (D'Alessio & Frasch, 1996), which is necessary for external male genitalia development (Chatterjee et al.,

2011). Knockdown of Drop using an early genital disc driver causes defects in posterior lobe development (Figure 3.2 H) (Chatterjee et al., 2011), making it a strong candidate regulator of *Delta*. Two putative *Dr* binding sites reside in region 6 of enhancer1. Mutation of both binding sites simultaneously reduced reporter activity, suggesting that these binding sites are critical to drive a wildtype pattern of expression (Figure 3.2 A and B). That being said, the possibility that mutation of the putative binding sites disrupts the activity of other transcription factors that recognize a similar sequence cannot be discounted. *In situ* hybridization of *Dr* in the male pupal genitalia detected expression between the lateral plate and clasper in the region of *Delta'*s lobe associated expression. However, this pattern was also observed in *D. ananassae* and thus is not unique to *D. melanogaster* (Figure 3.2 D and E). These data suggest that while Drop may be an upstream regulator of *Delta*, it is likely not a *trans*-regulatory change that accounts for the expansion of *Delta*. Nevertheless, I have identified an enhancer of *Drop* that recapitulates its lateral plate/clasper activity, which can be examined for its temporal activity and evolutionary conservation (Figure 3.2 F). It is possible that minor spatial and temporal changes of multiple trans-regulators have contributed to the expansion of *Delta*, in which case would only be detected through an exhaustive investigation of their spatiotemporal patterns.

3.2.1.2 Doc2 as a candidate regulator of Delta

One way to identify upstream regulators of an enhancer is to exploit its pleiotropic activities as a window into other functions of the element that may share regulation with the novel aspect of its activity (Glassford et al., 2015). In addition to the genitalia, enhancer1 of *Delta* exhibits activity in the embryo's longitudinal visceral musculature (LVM) (identified by Bill Glassford) (Ismat et al., 2010). It is thus possible that a similar set of *trans* regulators activate

enhancer1 in both tissues. Thus far, we have identified a strong candidate regulator of *Delta* in the posterior lobe, *Dorsocross2* (*Doc2)*. *Doc2* is a member of the *Dorsocross* family of transcription factors expressed in the LVM between embryonic stages 10 and 12 (Bae et al., 2017; Reim et al., 2003; Reim & Frasch, 2005). In *D. melanogaster*, *Doc2* is expressed along the lateral plate and clasper, favoring clasper expression (Figure 3.3 C). I identified 9 putative *Doc2* binding sites in the minimal enhancer1, one of which is conserved between *D. melanogaster* and *D. virilis*. Lack of binding site conservation in this minimal region is not surprising; one explanation is that in other species due to high binding site turnover, Doc2 binding sites are distributed outside the minimal enhancer region (Venkataram & Fay, 2010). Mutation of all 9 binding sites strongly reduced reporter activity (Figure 3.3 B), suggesting that the mutated sequences carry important information for *Delta* regulation. Comparisons of orthologous sequences of the *Doc2* genital boarder enhancer (GBE) however, suggests that this regulatory element is functionally conserved between *D. melanogaster* and the non-lobed species, *D. ananassae* and *D. pseudoobscura* (Figure 3.3 D-F). Thus, similar to Drop, it is possible that Doc2 regulates the lobe associated expression of *Delta*, however is unlikely a major *trans* regulatory change to account for the expansion of *Delta*. Furthermore, due to a potential functional redundancy with its paralogous genes, Doc1 and Doc3, it remains unclear whether Doc2 is functionally relevant to posterior lobe development, as reduction of *Doc2* expression produced no noticeable phenotypes (data not shown). One way to circumvent this challenge would be to investigate the functionality of the *Doc* GBE enhancer using CRISPR genome editing. However, as my work in Chapter 2 on the regulation of *Delta* showed, redundant elements may exist for this activity of *Doc2* and such experiments may therefore not yield phenotypes.

Overall, further investigation of the mutant reporters of enhancer1 can shed light into upstream changes that regulate an expanded pattern of *Delta*. Additional candidate regulators of note include *C15, Trl, B-H1,* B-H2, *tailup, Nk7.1, mirror*, *caupolican* (de Navascués & Modolell, 2007; Hackett et al., 2016), and *escargot* (Vincent et al., 2019). This chapter also highlights the challenges in identifying *trans*-regulatory changes when studying network evolution in highly polygenic traits. Having a well characterized *trans*-regulatory landscape of the precursor tissues that underlie a novelty can greatly facilitate the identification of causative changes. Advances in single cell RNA-sequencing has made this attainable, as transcriptomic atlases provide a reliable roadmap to complex cellular contexts. Ongoing work by our collaborators has made significant progress in generating single-cell transcriptomes of *Drosophila* male genital tissues (Ella Preger-Ben Noon, personal communication). Utilizing this gene expression atlas generated across multiple developmental stages will undoubtedly direct the search in *trans*-regulators of *Delta* leading to its expansion, and will provide a strong starting point for further functional tests.

3.2.2 Downstream targets of Delta

A critical step in deciphering the evolution of *Delta*'s role in posterior lobe development is to understand how Delta exerts its lobe-associated effects downstream. Namely, to uncover which genes become integrated downstream of the posterior lobe network, and examine whether they are expressed through pre-existing regulatory links, or if new connections have been established to express novel targets.

As presented in Chapter 2, I've demonstrated that the apical extracellular matrix protein Dumpy acts downstream of the posterior lobe Delta/Notch signaling center. This connection is established early in the larval imaginal disc, as reduction of *Delta* in the L3 disc disrupted dumpy

deposition and genital eversion. These results suggest that the connection between Delta and Dumpy is ancestral to the formation of the posterior lobe. The expansion of Delta then likely provides a context for which its pre-existing target, Dumpy, can expand to adopt a novel role in posterior lobe development. It is currently unclear, however, if Dumpy is a direct or indirect target of Notch. An important step in determining this relationship entails identifying the enhancer(s) of *dumpy*. To this end, I've examined the regulation of *dumpy* and have detected candidate regions that recapitulate the endogenous patterns of *dumpy* expression (Figure 3.4.) Thus far, 3 coarsely mapped (~5kb each) regulatory regions drive activity in a lobe-associated manner (Figure 3.4 A, E, and F). While each reporter displays a unique pattern, they also partially overlap in their domains of activity (reporters A, L, and N). An intronic region, fragment L, drives strong activity in the clasper adjacent to Delta, as well as in the developing posterior lobe (Figure 3.4, E). Construct N, another intronic regulatory region, partially shares the clasper activity of reporter L, and is also active in the L3 disc (Figure 3.4 F). The pupal activity of this reporter is also adjacent to the expanded *Delta* pattern (not shown). Reporter A, ~21.4 kb upstream of the transcription start site of *dumpy*, best recapitulates the expression of dumpy in a patch of cells on the clasper and the tip of the developing posterior lobe (Figure 3.4 A). This region also exhibits strong activity in the L3 genital disc in lateral folds of the male genital primordium (personal communication with Catarina Colmatti Bromatti). Importantly, region A contains a strong binding site for the Notch pathway-activated transcription factor Su(H) that is conserved between lobed and non-lobed species (Figure 3.4 G). Mutating this binding site in the context of a reporter would provide insight as to whether *dumpy* responds to Notch directly or through intermediate factors. In addition, deletions of the enhancers of *dumpy* using CRISPR/Cas9 genome editing could illuminate their necessity for posterior lobe development.

In addition to posterior lobe associated activities of dumpy, I've identified potential enhancers that drive expression in the central structures of the genitalia, the hypandrium, phallus, and sheath (Figure 3.4 B, C, and D). There is a strong localization of aECM covering these structures in both lobed and non-lobed species, which forms connections to the clasper (Smith et al., 2020). The connections of Dumpy from the center of the genitalia to the clasper are established early in development, from the larval stage (see chapter2). As this localization likely represents an ancestral network of aECM which expanded to the lateral plate, the respective enhancers can be further investigated for their evolutionary history as well as function. My data suggests that early in development, these connections are important for the eversion of the genital disc. Later in development, these connections may play a key ancestral role in clasper development, which was then adjusted to integrate into posterior lobe development (discussed in section 3.2.4).

Collectively, whether Dumpy responds to Notch signaling directly or through intermediate factors, my data indicates that there is both an early and a late response of Dumpy to the signaling center that persists through development. This presents an interesting case where the downstream mechanism of Notch signaling has been preserved throughout development to carry out vastly different functions, the eversion of the genital disc and the formation of the posterior lobe. However, these elements remain coarsely mapped, and may be further subdivided in future studies. In addition to targeting terminal effector genes, Notch may exert its downstream effects through other signaling pathways. The JAK/STAT signaling pathway has been shown to play a role in posterior lobe development (Glassford et al., 2015). Specifically, the ligand of the JAK/STAT pathway, *unpaired* (*upd*), displayed a temporal expansion in species that develop a posterior lobe compared to non-lobed species (Glassford et al., 2015). This activity is driven by a functionally conserved enhancer (Sarah Smith, personal communication), which I anticipate act downstream

of the Notch signaling pathway for the following reasons: 1) *upd* is active in a Notch responsive domain (Figure 3. 5 A and B), 2) a JAK/STAT responsive reporter displayed reduced activity in a *Delta* RNAi background (Sarah Smith, personal communication), 3) the lobe associated expression pattern of Delta was not noticeably affected in an *upd* enhancer deletion background (Figure 3.5 C and D) (*upd* enhancer CRISPR line generated by Sarah Smith), and 4) the posterior lobe phenotype of *Delta* RNAi is considerably more dramatic than that of the knockdown of any of the JAK/STAT pathway components (Glassford et al., 2015) (Sarah Smith, personal communication), suggesting that the Delta/Notch signaling center is a top regulator of the posterior lobe. To test whether the *upd* enhancer is directly regulated by Notch signaling, I have mutated two Su(H) binding sites by introducing a non-complimentary transversion to every other base in the context of the *upd* enhancer reporter (transgenic flies in preparation). If these mutations reduce the temporal expansion of the *upd* enhancer, it will indicate that the lobe-associated role of the JAK/STAT signaling center directly responds to the Delta/Notch signaling center. The activity of *upd* in lobed species persists through late stages of pupal development. In non-lobed species, however, *upd* expression comes to a halt around the time that the posterior lobe initiates its development in *D. melanogaster* (Glassford et al., 2015). This timing correlates with the spatial expansion of *Delta*. It is possible then, that the spatial expansion of *Delta* in lobed species has allowed for *upd* to extend its temporal window of activity and gain a role in posterior lobe development.

Another potential signaling pathway downstream of the Delta/Notch signaling center is Wnt (Bejsovec, 2018; Lento et al., 2012). The wingless (wg) ligand is expressed adjacent to the lobe-associated pattern of *Delta*, closer to the ventral extension on the clasper. This expression overlaps the activity of the Notch-responsive *E(spl)mβ*-GFP reporter, making it a likely Notch

target (Figure 3.6 A-C). That being said, further investigations are required to uncover whether the Wg morphogen has a role in posterior lobe development. To date, reduction of the pathway components, Legless and Armadillo, transcriptional co-activators of the Wg pathway, have not yielded a posterior lobe phenotype (Gavin Rice, personal communication). Testing additional drivers that better cover the spatiotemporal activity of Wg, as well as reducing the expression of additional components of the pathway may be necessary to uncover the function of Wg in this context. Armless, a positive regulator of Wg signaling which stabilizes Armadillo may be one such component (Bejsovec, 2018). In further efforts of examining the role of Wg expression adjacent to the expanded pattern of Delta, I deleted the clasper regulatory element of Wg using CRISPR/Cas9 genome editing. This enhancer is a 3.5 kb element upstream of *Wg*, which recapitulated the clasper activity of Wg in a reporter assay (Identified by Gavin Rice, personal communication). Deletion of this enhancer did not noticeably affect Wg expression, nor did it produce an appreciable phenotype in the posterior lobe, lateral plate, or clasper (Figure 3.6 D-F). As discussed in chapter 2, redundant enhancers looming in the locus may hamper our ability to deduce with any confidence whether this pattern of Wg is functionally relevant to the development of the posterior lobe or its ancestral precursors.

The downstream targets mentioned above are likely a small subset of targets that may respond to the posterior lobe associated Delta/Notch signaling center. Finding additional targets of this signaling center would not only fill in the gaps of our knowledge on how the posterior lobe forms, but will also offer insight into consequences that follows the deployment of a signaling source to a new context that has not seen the signal before. Targeted DamID (TaDa) has been shown to be a reliable cell-type specific chromatin profiling technique in *Drosophila* melanogaster to identify downstream targets (Marshall et al., 2016; Southall et al., 2013). In this method, the

genomic interaction sites of a protein that is fused to a DNA marking enzyme are mapped by methylation of the surrounding DNA (Mcclure & Southall, 2015). Methylated sites are then cleaved by restriction enzymes, and subjected to sequencing. TaDa utilizes a tissue-specific expression control system to drive the activity of the Dam-fusion proteins only in the cells of interest, allowing measurements of specific cells from whole-tissue collections. Identification of Notch targets in the posterior lobe using TaDa would require two transgenic lines: a driver line that can initiate tissue-specific expression in the cells which respond to *Delta* expression (e.g. *upd* enhancer-GAL4), and a TaDa-fusion line that will express the Su(H) in the domain of the driver line (UAS-Dam-Su(H)). Once Notch targets are identified, their expression patterns and necessity for lobe development must be examined. Whether these genes differ in expression between lobed and non-lobed species will determine whether they are novel targets of the Notch pathway, or pre-existing targets that are now expressed in a broader pattern associated with posterior lobe development.

An end goal experiment that would suggest we have gained a near complete understanding of posterior lobe development, is to genetically engineer a posterior lobe in a non-lobed species. This would require expressing a gene in a precise spatiotemporal manner in a non-lobed species that would be sufficient to induce posterior lobe development in its respective *trans*-landscape. Due to shifts in the *trans*-regulatory environment and regulatory connections, success in such an experiment would be more plausible by expressing important genes at the terminal nodes of the GRN rather than top regulators. This would entail generating a construct that directs cDNA expression of a downstream gene using an enhancer that drives an expanded pattern on the lateral plate of a non-lobed species. Thus far, Dumpy is one of the strongest terminal effector genes of the posterior lobe GRN. As described earlier, the aECM is a pre-established network covering

ancestral male genital structures (Smith et al., 2020). At the larval stage, Dumpy is downstream of Delta, suggesting that it is an ancestral target of Notch signaling. My current working model suggests that with the expansion of Delta, a context was provided for Dumpy to expand along with it. While the enhancers of *dumpy* described here have not yet been tested from non-lobed species, I anticipate that they are functionally conserved and that the expansion of Dumpy mostly results from changes in Delta expression. Thus, it is unlikely that the *dumpy* enhancers would drive expanded activity in non-lobed species. Since the TaDa experiment suggested above identifies regulatory DNA, it would be a good starting point to find enhancer elements that could drive an expanded lateral plate activity in non-lobed species. If such a driver is found, it would be key for testing the sufficiency of Dumpy and other downstream genes of the posterior lobe GRN to induce posterior lobe development. However, I fully expect that additional terminal effector genes will be required to generate a posterior lobe. For example, multiple other Zona Pellucida (ZP) domain containing genes (Jaźwińska & Affolter, 2004) have been found expressed in different parts of the genitalia (Ben Vincent, personal communication). These genes and other aECM components or modifying enzymes may be important for building aECM connections and determining to which cells they connect to.

3.2.3 Dissecting the expanded pattern of Delta expression to functional sub-patterns

The lobe-associated expression pattern of *Delta* is a spatial expansion of an ancestral pattern between the base of the lateral plate and clasper (see chapter 2). This expansion occurred in two opposite directions along the dorsoventral axis: 1) a dorsal expansion where Delta expression extends towards the anal plate, and 2) a ventral expansion where Delta activity extends between the lateral plate and clasper (Figure 3.7 A and C). Our initial investigations viewed the

entire expanded pattern of Delta as one domain carrying out a unified function of directing posterior lobe development. My knockdown experiments using drivers with different domains of activity however, have challenged this view. Reduction of *Delta* by RNAi hairpins directed by the PoxN13-GAL4 driver, affects both the ventral and dorsal expression of Delta (Figure 3.7, G-I). This leaves only a small pattern restricted to the base of the clasper near the lateral plate, resulting in a drastic reduction in the size of the posterior lobe (Fig 3.7 I, Figure 2.2 M, and Figure 2.5 C). In this treatment, the Delta expression which remains resembles the ancestral pattern observed in non-lobed species at a similar stage of development (Figure 3.8). However, reduction of *Delta* directed by a *Delta*-enhancer2 driver only affected the ventral expansion of the pattern (Figure 3.7 D-F), as enhancer2 does not overlap the dorsal extension of this signaling center (Figure 2.2 C). Surprisingly, this reduction does not noticeably reduce the size of the posterior lobe. These data suggest that the ventral expansion of Delta may be dispensable for the formation of the posterior lobe, and that counter to our initial speculations, the dorsal expansion is important for the formation of this novelty.

The functional consequence of the ventral expansion of Delta still remains unclear. It is possible that this domain of the signaling center is important for morphological aspects of the posterior lobe that have been overlooked by measuring its area in 2D. Hence, the ventrally expanded domain may be critical for the overall shape of the posterior lobe, which could be uncovered using morphometric analysis to quantitatively detect changes in shape. It is also possible that the ventral expansion may affect other structures (e.g. the clasper) in ways beyond our current measurement tools. Furthermore, it is unclear whether the dorsal expansion of *Delta* is sufficient for posterior lobe development, or if both domains are required for its formation. Given that reduction of both the dorsal and ventral extensions of Delta expression yielded an ancestral-

like pattern and strong defects in posterior lobe development, I postulate that both domains of the expansion are critical for aspects of posterior lobe development. Identifying a driver that only targets the dorsal extension of the signaling center would provide insight to this conundrum.

Collectively, these data showcase the complex relationship of pattern to phenotype. When novel expression patterns are observed in correlation with the emergence of phenotypes, their precise functional relevance is oftentimes invoked (see Chapter 1.3). In studies of novelties, particularly involving non-model organisms, functional tests if performed are often limited to pharmacological treatments with broad effects, or genetic perturbations with little control over the spatial and temporal window of application (Cebra-Thomas et al., 2005; Harris, Fallow, et al., 2002; Navet et al., 2009; Weatherbee et al., 2006). As a consequence, the intricacies of a developmental pattern and the functional relevance of each sub-domain may be overlooked, which skews our interpretation of how patterns may be associated with novel forms. The work presented here exemplifies how this may be the case, and suggests that associating new expression domains to novel morphologies requires a meticulous evaluation and breakdown of the relevant expression pattern that goes beyond our initial biases.

3.2.4 Examining the role of Delta in non-lobed species

In this thesis, I identified an ancestral role of which the posterior lobe signaling center was built upon—the eversion of the genital disc. This function is conserved across all *Drosophilids*, and precedes the evolution of the derived posterior lobe. As described in chapter 2, however, this signaling center persists in the pupae of non-lobed species throughout later stages. While I have uncovered an early ancestral role, the functions that this signaling center carries out post-eversion in non-lobed species remains unknown. I postulate that a late ancestral role of Delta at the base of

the lateral plate and clasper may be crucial for shaping the clasper through Dumpy and other downstream targets. Like *D. melanogaster*, the aECM is also deposited on the claspers of non-lobed species, albeit to a lesser extent (Smith et al., 2020). Considering the role of the aECM in anchoring the cells of the clasper and lateral plate to the center of the genitalia, it is possible that the aECM connections are critical for pulling the cells of the clasper upward or preventing them from collapsing. Testing this hypothesis requires targeting *Delta* at a later pupal stage post-eversion in a very specific spatial manner. My perturbation experiments reducing *Delta* expression later in development using the PoxN-GAL4 driver did not completely eliminate the signaling center at the base of the clasper and lateral plate. Instead, it yielded a spatially reduced expression pattern of Delta that resembled that of non-lobed species (Figure 3.8, B and D). Thus, while these experiments reduced the expanded pattern of Delta such that it affected the posterior lobe, the late ancestral pattern persisted. Identifying an appropriate GAL4 driver that fully targets the expression of Delta at the base of the lateral plate and clasper would be key to answering this question. Notably, a driver that ubiquitously drove *Delta*-RNAi resulted in lethal phenotypes (not shown) necessitating future searches for more specific but sufficiently broad drivers. If such a driver is also active in the *trans*-landscape of non-lobed species, it would allow testing the function of Delta by transgenic RNAi injection in non-lobed species as well. Future work can also use CRISPR/Cas9 genome editing in non-lobed species to remove the pupal enhancer1. However, this must be done with caution that the highly redundant regulatory mechanism of *Delta* may also apply to non-lobed species.

In conclusion, my work has provided insight into the origins of novel structures. It highlights the importance of comparative studies and assessing a broad range of developmental times that may not seem directly relevant to the structure of interest. The future work I proposed

here will help paint a clearer picture of what occurs downstream of a signaling pathway deployment event. Moreover, it will provide an additional explanation of the origins of the most critical signaling center involved in posterior lobe development that is known to date. These experiments will further showcase the power of comparative studies when investigating novelties that have evolved over short evolutionary timescales. A commonality of most past examples of novelties, as discussed in chapter1, is the lack of appropriate comparisons due to long divergence times and inability to fathom the foundational state from which the novelty emerged from. Also, in these examples, an absence of reporter assays presents a grave barrier in tracking the activity of causative genes, as it is difficult to trace whether the expression of a gene through dramatic morphogenetic changes is from the same source or not. This is a critical issue for understanding the origins of novelties. As described in chapter2, uncovering the early ancestral role of *Delta* became possible by tracking its enhancer, without which we would not have been able to deduce with any confidence that the same signaling source was active from the larval stage throughout pupal development.

It is critical that novelties are studied across a wide range of evolutionary time scales to broaden our perspectives on the unique events that could occur during a structure's origination. Detailed dissections of novelty-associated patterns in time and space will also become more prevalent with the growing success of CRISPR/Cas9 genome editing *in vivo* as well as reporter assays, all of which are ingredients for a veracious report on the origins of a morphological novelty.

3.3 Figures

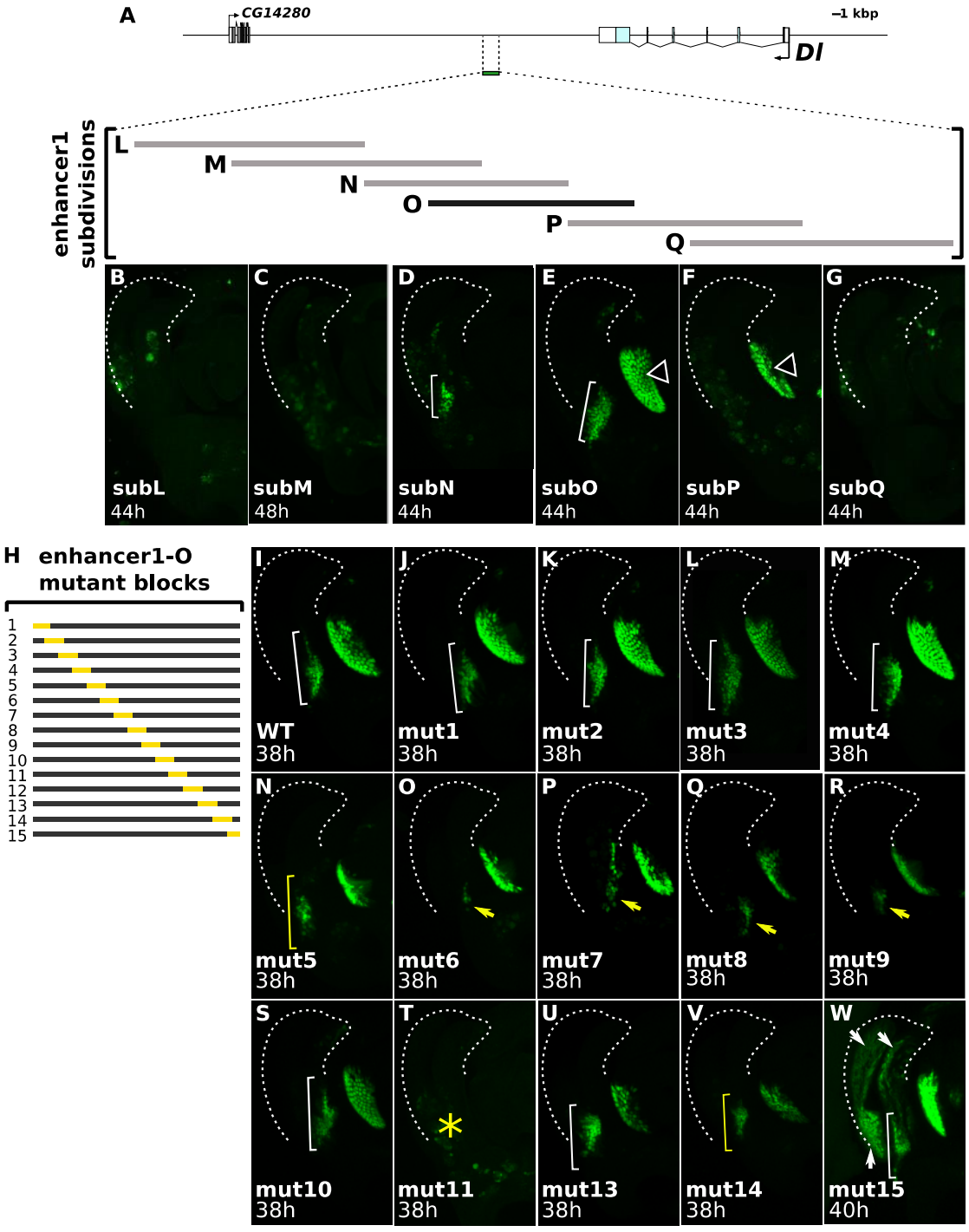

Figure 3.1 Subdivisions and mutational analysis of *Delta* enhancer1 to identify its *trans*-regulators.

(A) Subdivisions of Delta enhancer1. Fragments (denoted as grey or black bars) are ~700-800 bp long with ~400 bp of overlap. (B-G) The elements were tested in a reporter assay. Subdivision enhancer1-O best recapitulates the full-length activity (E). Brackets denote posterior-lobe associated pattern, arrowhead points to the sheath. (H) Schematic represents blocks scrambled sequence spanning the minimal enhancer of Delta. Yellow bars denote ~60 bp of scrambled DNA. (I-W) Reporters of mutated blocks of DNA in the minimal enhancer1 of *Delta*. White brackets indicate posterior lobe-associated activity similar to wildtype. Yellow bracket suggests moderate reduction of the pattern. Yellow arrow indicates notable reduction of reporter activity. Yellow asterirsks in panel T denotes loss of reporter activity. White arrows in panel W suggest ectopic acitivity.

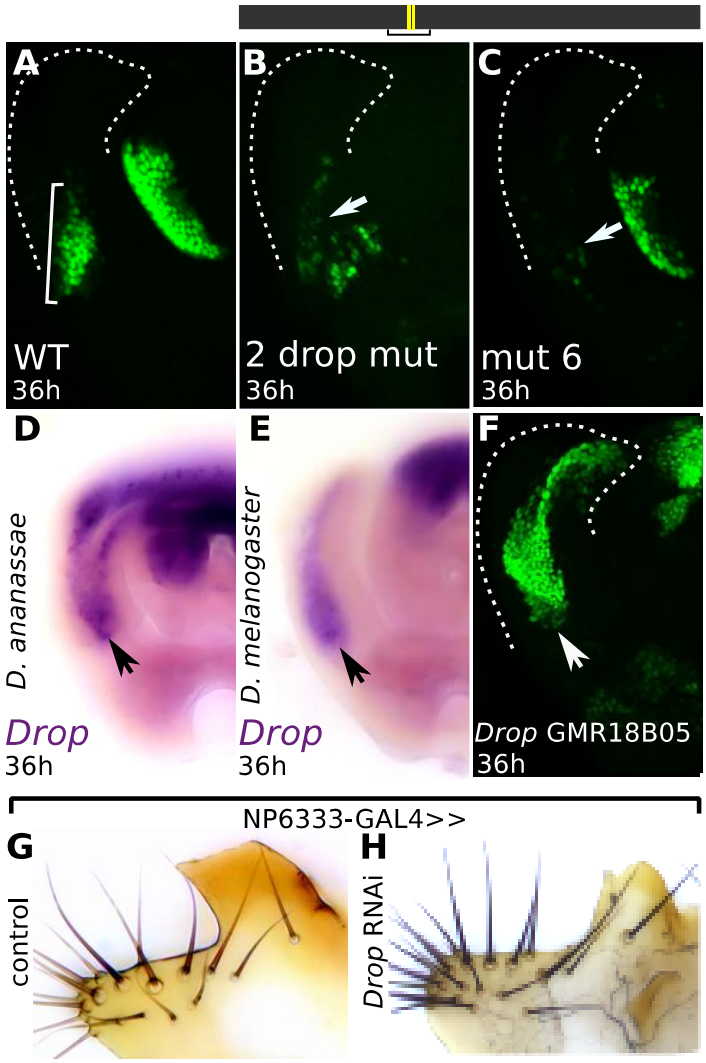

Figure 3.2 Drop is a candidate regulator of *Delta*.

(A) WT minimal reporter of *Delta* enhancer1-O. (B) mutation of two Drop binding sites in enhancer1-O reduces the spatial domain of GFP activity. These two binding sites reside in the 6th region of scrambled DNA of the minimal enhancer (mut6) (C). The black bar denotes enhancer1-O, yellow stripes mark the mutated Drop binding sites, and the black bracket marks region 6. (D,E) *in situ* hybridization of *Drop* shows expression between the lateral plate and clasper of both *D. ananassae* (D) and *D. melanogaster* (E). (F) Regulatory region of *Drop* that recapitulates its endogenoues lateral plate/clasper activity.(G, H) RNAi knockdown of *Drop* reduces the size of the posterior lobe.

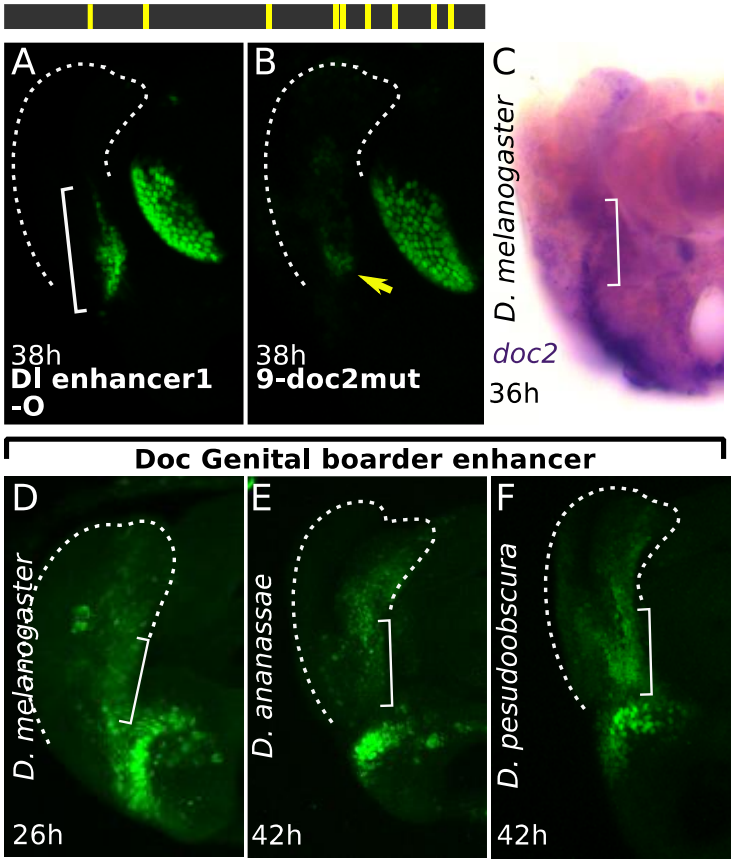

Figure 3.3 Doc2 is a candidate regulator of *Delta*.

(A,B) Mutation of 9 binding sites of Doc2 in the minimal enhancer1 of *Delta* reduces reporter activity (compare yellow arrow in panel B to white bracket in panel A). (C) In situ hybridization of *doc2* shows mRNA expression on the clasper near the lateral plate in a region that may overlap *Delta*. (D-F) The Doc genital boarder enhancer sequence is functionally conserved between lobed (D) and non-lobed (E,F) species.

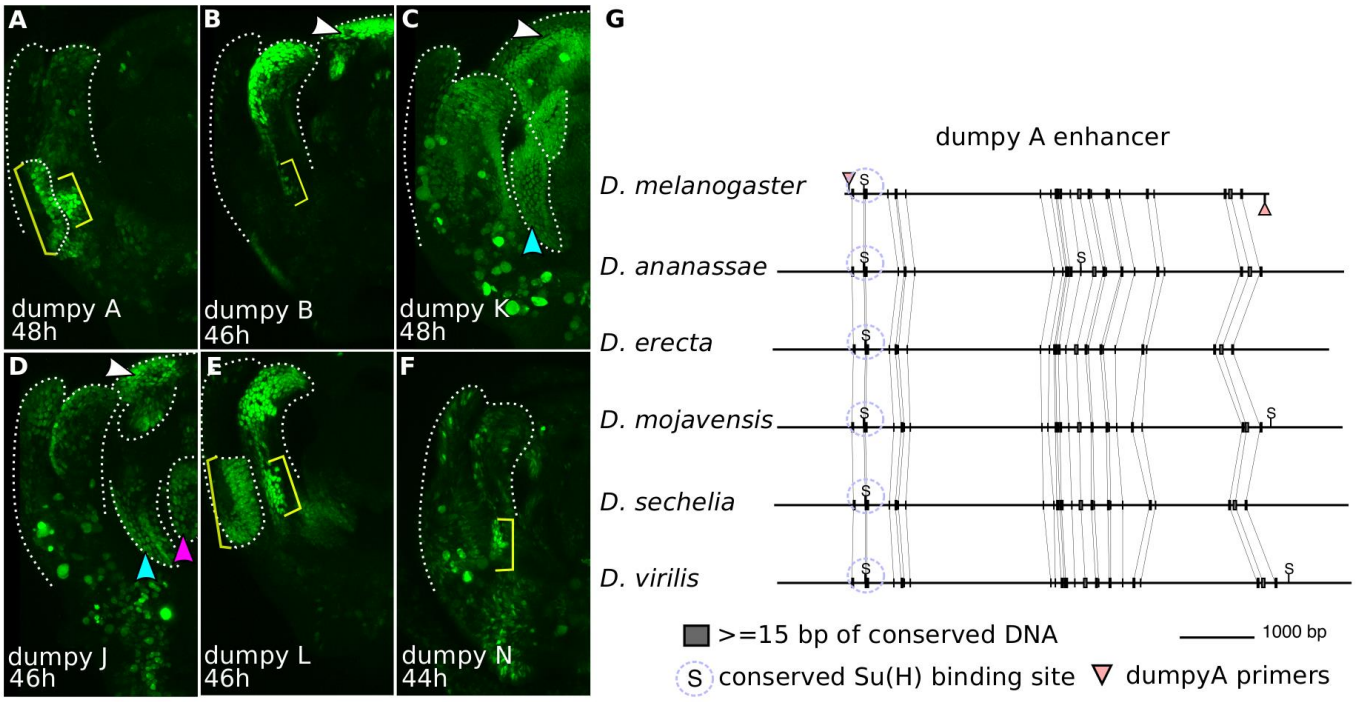

Figure 3.4 Reporter assays of *dumpy* enhancers.

(A) reporter A best recapitulates the endogenous activity of dumpy in the posterior lobe and clasper at this time point. (dumpy *in situ* published by (Smith et al., 2020)) (B) dumpy B shows weak activity in the clasper near the posterior lobe (yellow bracket) and strong activity at the tip of the clasper and hypandrium (arrowhead). (C) dumpy K is a candidate sheath (blue arrowhead) and hypandrium (white arrowhead) enhancer of dumpy. (D) Reporter J drives activity in the hypandrium, sheath, and phallus (white, blue, and pink arrowheads, respectively). (E) Reporter L drives strong activity in the posterior lobe and in the clasper. (F) Reporter N drives activity in a small patch on the clasper near the lateral plate, which may overlap the clasper activity of reporters A, B, and L. (G) alignment of *dumpy* A across six *Drosophila* species and a conserved Su(H) site within the enhancer.

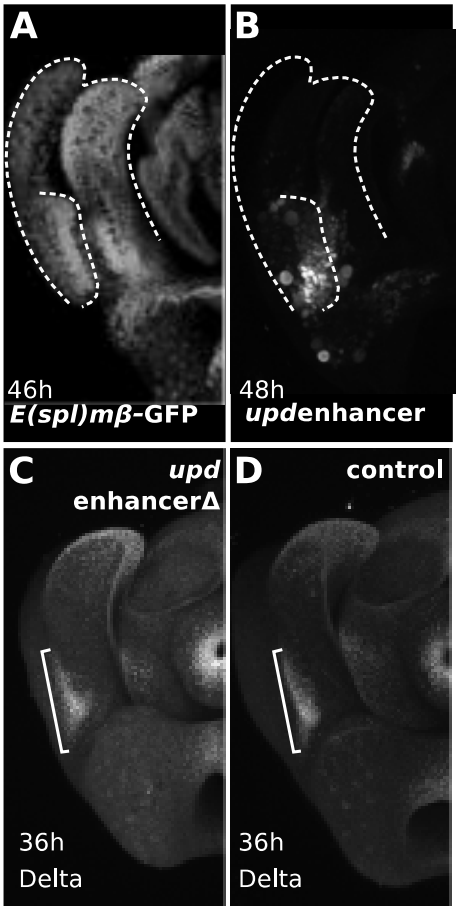

Figure 3.5 The JAK/STAT pathway is a candidate direct target of Notch signaling.

(A-B) the upd posterior lobe enhancer (B)is active in Notch responsive regions where the E(spl)mβ-GFP reporter is expressed (A). (C-D) Delta expression is not noticeably affected in an *upd* enhancer deletion background (white bracket, compare C and D).

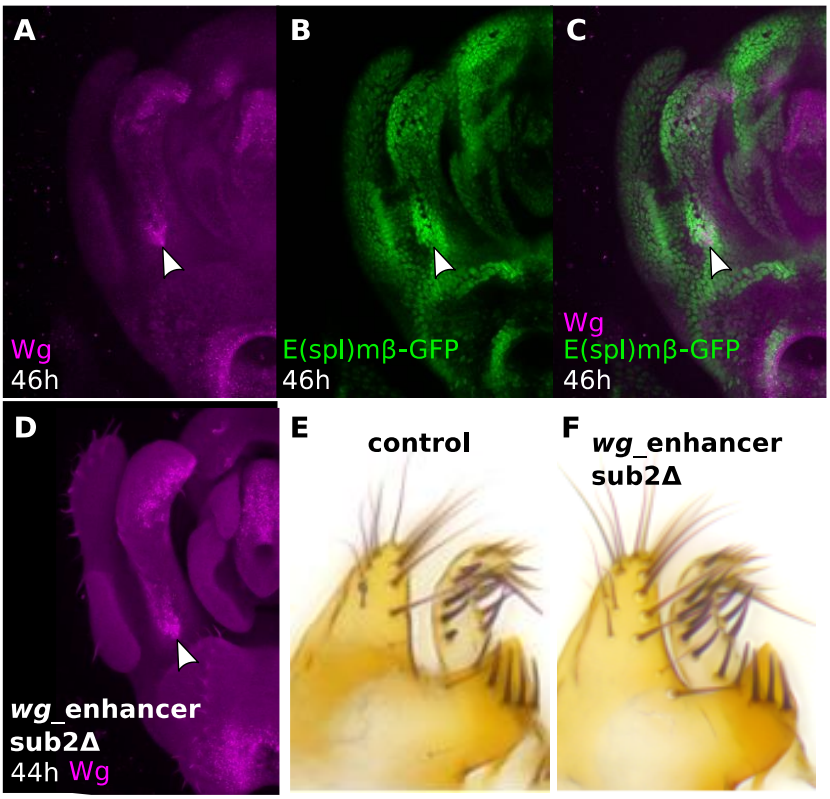

Figure 3.6 Wg is a candidate target of Notch signaling.

(A-C) The Wg ligand is expressed in Notch-responsive domains (B) on the clasper near the lateral plate. (D-F)) Deletion of a wg regulatory element driving this pattern of expression does not affect Wg expression (D) or noticeably affect the posterior lobe, clasper, and lateral plate (compare E and F)

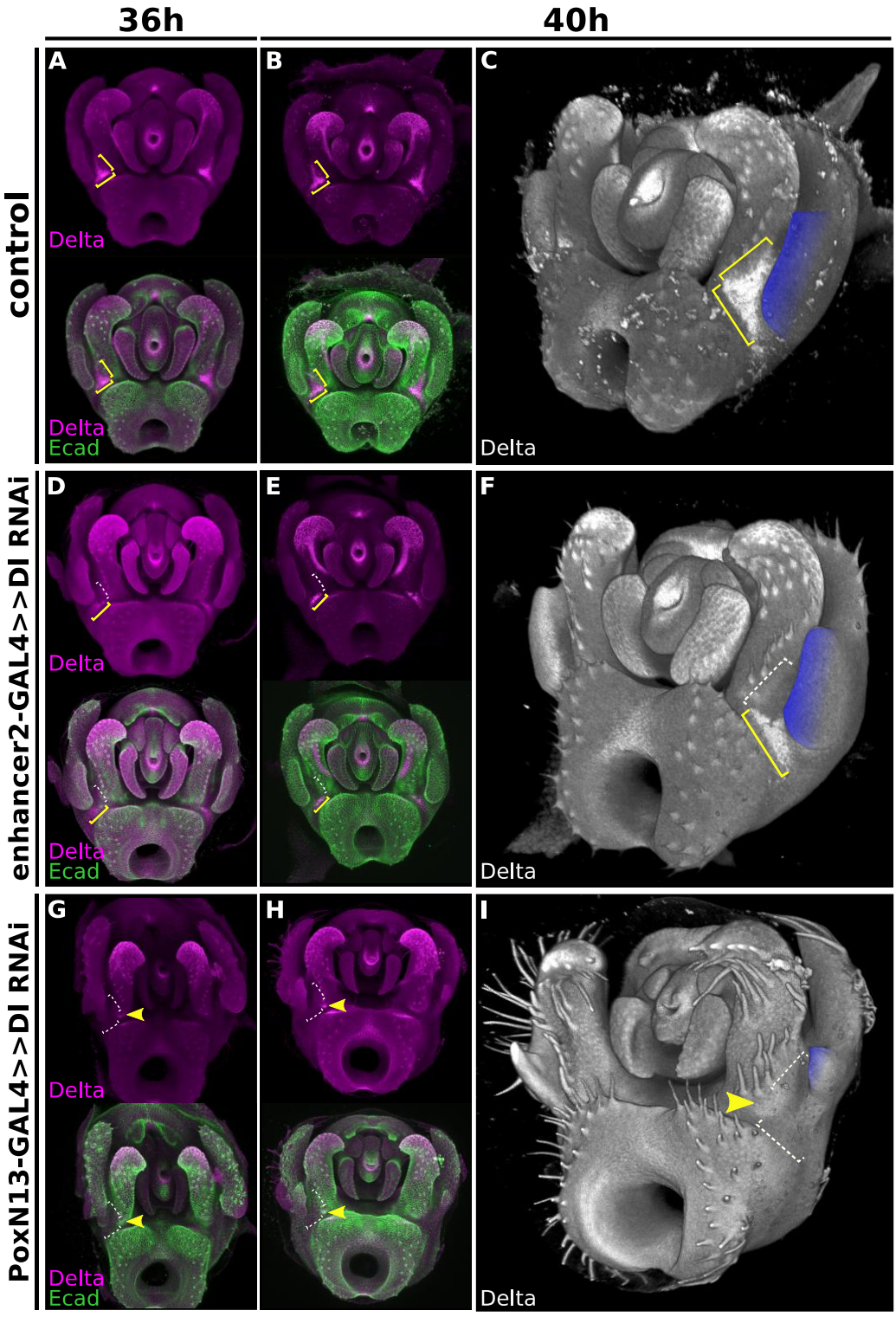

Figure 3.7 Dissecting the expanded pattern of Delta to its functional sub-patterns.

(A-B) WT Delta expression at two timepoints by immunofluorescence. The expanded expression of Delta can be broken down into a ventral and dorsal extension (yellow brackets). Dorsal extension is towards the anal plate. Bottom panels overlap with Ecadherin antibody to visualize the apical surface of the cells. (C) 3D rendering of a 40h APF pupa stained with Delta (yellow brackets). The developing posterior lobe is false colored in blue. (B-F) RNAi knockdown of *Delta* using a GAL4 line driven by the early enhancer2 of Delta affects the ventral extension of Delta (white bracket indicates loss of ventral extension) but not the dorsal extension (yellow bracket). Posterior lobe development is not noticeably affected in these animals. (G-I) RNAi knockdown of *Delta* driven by PoxN-GAL4 affects both the dorsal and ventral expansions of Delta (white brackets) and causes a severe posterior lobe defect. Yellow arrowheads point to the remaining Delta activity at the base of the clasper.

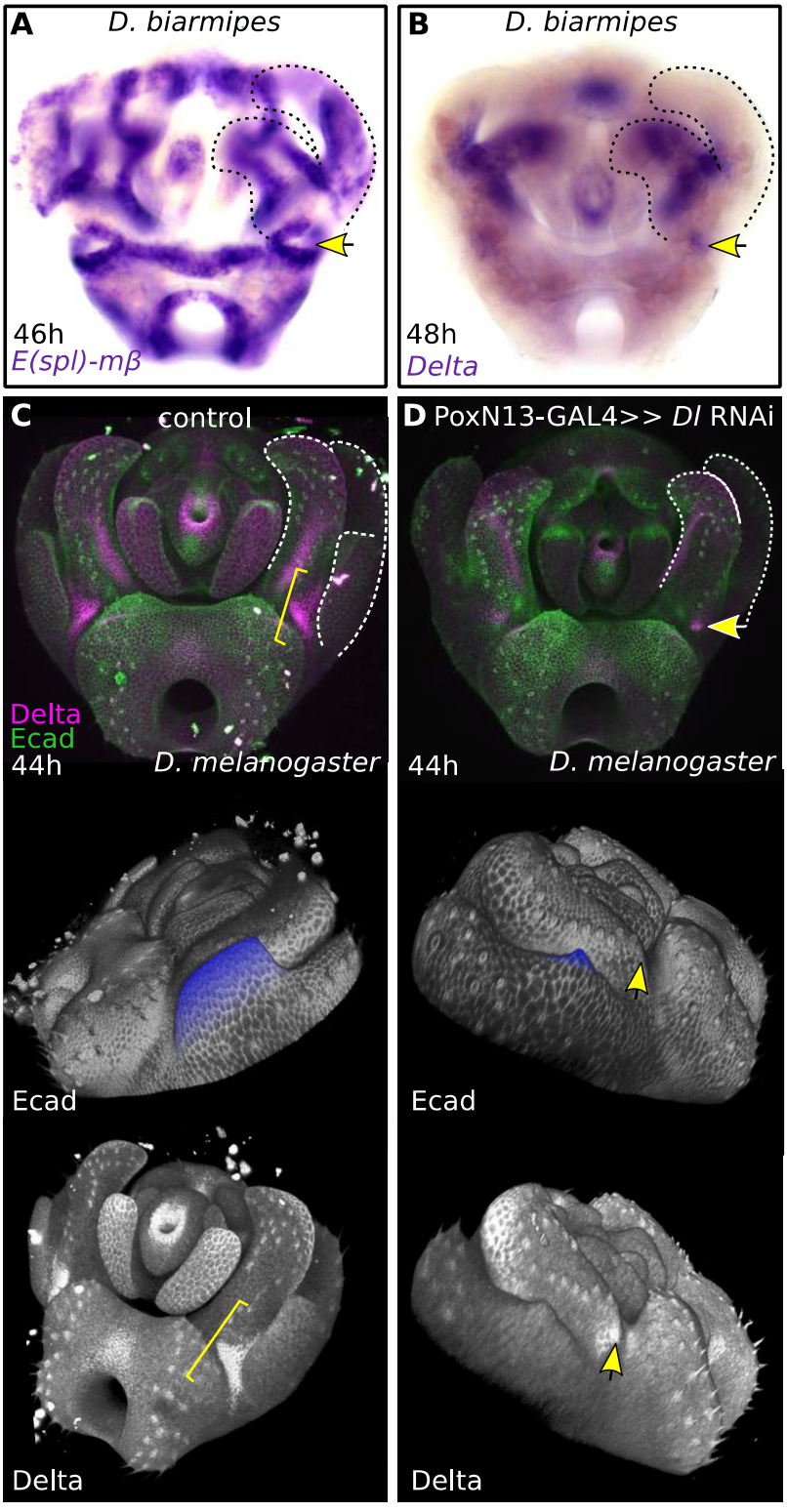

Figure 3.8 A late-ancestral role of Delta is yet to be discovered.

(A) mRNA expression of the canonical Notch target *E(spl)-mβ* in the non-lobed species *D. biarmipes* used as a proxy of the Notch pathway activity. Yellow arrow points to a ring-like pattern of *E(spl)-mβ* where the base of the lateral plate and clasper meet near the anal plate. This pattern surrounds where Delta is expressed (B).(B) *Delta* mRNA expression in *D. biarmipes* is localized to a small region at the base of the clasper near the lateral plate (yellow arrow). (D) knockdown of Delta by RNAi reduces Delta expression to the base of the clasper near the lateral plate, resembling the non-lobed *Delta* pattern (yellow arrow) (compare to B and C). 3D rendering shows this remaining activity of Delta on the clasper is above the crevis.

Appendix A Key resources and methods

Appendix A.1 Methods

Appendix A.1.1 Drosophila stocks and husbandry

Fly stocks were reared at room temperature on standard cornmeal agar media. For RNAi experiments, flies were reared at 29 °C. The *Drosophila melanogaster* line used in this study is mutant for the *yellow* and *white* genes and was isogenized for eight generations (y^1w^1, Bloomington Stock Center #1495). Outgroup species that lack a posterior lobe *(Drosophila ananassae #*0000-1005.01, *Drosophila biarmipes #*0000-1028.01) were obtained from the University of California, San Diego Drosophila Stock Center (now called The National Drosophila Species Stock Center at Cornell University).

Appendix A.1.2 Transgenic constructs

To generate GFP reporter flies, regulatory elements were PCR amplified using primers listed in Table 2, and cloned into a vector containing GFP and a minimal hsp70 promoter (pS3aG) (Williams et al., 2008). Primers were designed using the GenePalette software tool (Rebeiz & Posakony, 2004). *AscI* and *SbfI* restriction sites were added to the primer sequences (Integrated DNA Technologies) to insert the amplified region into the multiple cloning site of the vector. Regions of interest were amplified from genomic DNA prepared by the DNeasy Blood & Tissue Kit (QIAGEN). *D. melanogaster* transformant lines were created by phiC31 mediated site specific

recombination into the 68A4 “attP2” landing site on the third chromosome(Groth, A. C., Fish, M., Nusse, R., & Calos, 2004) or the 51D site on the second chromosome(Bischof, J., Maeda, R.K., Hediger, M., Karch, F., and Basler, 2007) by Rainbow Transgenics. For each GFP reporter, 2-5 independent insertion lines were analyzed.

Appendix A.1.3 Larval and pupal genital sample preparation

Pupal genital samples were prepared for *in situ* hybridization according to Glassford et al. 2015 (Glassford et al., 2015) (Vincent et al., 2019). In short, to standardize aging, male white pre-pupae were incubated at 25°C until ready for dissection. Samples from RNAi crosses were incubated at 29°C with appropriate controls. Pupae were cut in half in cold PBS, fat bodies were flushed out, and pupal case was removed. Samples younger than 20 hours APF (after pupal formation) and larval samples were cut in half and turned inside out at the posterior end to remove fat bodies to prevent damaging or dislodging the delicate early genitalia. All samples were fixed in PBS with 0.1% Triton-X and 4% paraformaldehyde (PBT-fix) for 30 min at room temperature. Samples containing fluorescent labels or being prepared for immunostaining were then washed twice in PBT. Samples to be used for in situ hybridization were rinsed twice in methanol and twice in ethanol, and stored at -20°C in 100% ethanol.

Appendix A.1.4 Generation of CRISPR/Cas9 mutants

Deletion of the enhancers of Delta was accomplished by CRISPR/Cas9 homology directed repair using gRNA targets flanking the enhancer boundaries (Table 2). For deletion of enhancer1, *D. melanogaster* embryos were injected by Rainbow Transgenics with a mixture of 250 ng/μL

nos-Cas9 vector, 100 ng/μL of each gRNA vector (pCFD3-dU63gRNA), and 500 ng/μL donor plasmid (Generated by Sarah Smith). The donor plasmids for homology directed repair contain a *3XP3::DsRed* cassette flanked by approximately 1kb of genomic DNA. Transformants were identified by the expression of DsRed in the eyes of the progeny of the injected flies. Embryo injections to delete *Delta* enhancer2 were performed in house. A mixture of 200 ng/μL per gRNA and 500 ng/μL donor vector was injected into nos-Cas9 expressing embryos (Bloomington #78781). gRNAs were generated by *in vitro* transcription. Briefly, a double stranded DNA template containing a T7 promoter was amplified by PCR. In vitro transcription was then carried out using the MEGAscript T7 Transcription kit (Invitrogen).

Appendix A.1.5 *In situ* hybridization

To detect mRNA localization, in situ hybridization was performed following the protocol described in Rebeiz et al., 2009. Modifications were made according to Glassford et al., 2015 to utilize the InsituPro VSi robot (Intavis Bioanalytical Instruments)(Vincent et al., 2019). Briefly, fixed samples were washed in methanol, rehydrated in PBT (PBS with 0.1% Triton-X), fixed in PBT-fix, and incubated in hybridization buffer for 1 hour at 65°C. Prehybridized samples were then incubated with digoxygenin riboprobes (primers for amplifying mRNA probes listed in Table X) for 16 hours at 65°C, and subsequently washed in hybridization buffer followed by PBT washes to remove unbound riboprobes. To reduce background noise, samples were blocked in PBT with 1% bovine serum albumin for 2 hours. Blocked samples were then incubated with anti-digoxigenin antibody Fab fragments conjugated to alkaline phosphatase (Roche Diagnostics) diluted in PBT at 1:6000 overnight at 4°C. After several PBT washes, alkaline phosphatase color reactions were performed by incubating samples in nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3’-

indolyphosphate p-toluidine salt (NBT/BCIP) (Promega) and monitored under a dissecting microscope. Color reactions were stopped by PBT washes when purple stain was detected. Samples were mounted on a glass slide coated with Poly-L-Lysine in an 80% glycerol 0.1 M Tris-HCL (pH 8.0) solution.

Appendix A.1.6 Immunostaining

To detect the expression patterns of proteins, genital samples removed from the pupal membrane were incubated in primary antibody diluted in PBT at 4°C overnight. The following primary antibodies were used: monoclonal mouse anti-Delta 1:100 (DSHB, #C594.9B0s), polyclonal goat anti-Delta 1:100 (Santa Cruz Biotechnology, Inc.), rat anti-Ecadherin 1:100 (DSHB, #DCAD2). To remove unbound primary antibody, the samples were washed in PBT 3-5 times over the course of an hour. To detect bound primary antibody, the samples were subsequently incubated in a fluorescent-dye conjugated secondary antibody diluted in PBT and incubated either at 4°C overnight or at room temperature for 4 hours. The following secondary antibodies were used: donkey anti-mouse Alexa 488 1:400 (A21202, Thermo Fisher Scientific), donkey anti-goat Cy2 1:400 (705-225-147, Jackson ImmunoResearch), donkey anti-rat Alexa 647 (A78947, Thermo Fisher Scientific), and donkey anti-rat Alexa 594 1:400 (A21209, Invitrogen). Samples were then washed in PBT, incubated in 50% PBT and 50% glycerol solution, and mounted on a glass slide in an 80% glycerol 0.1 M Tris-HCL (pH 8.0) solution. Mounting wells were made with a single or double layer of double sticky tape on the glass slides. To avoid rotation of the sample during mounting, the wells were coated with Poly-L-Lysine solution. Glass cover slips were placed on the samples to seal the wells.

Appendix A.1.7 Microscopy and image analysis

Cuticles of adult genitalia and stained in situ hybridization samples were imaged on a Leica DM2000 with a Leica DFC540 camera at 20x magnification. Fluorescently labeled samples were imaged using an Olympus Fluoview 1000 or a Leica TCS SP8 confocal microscope at 40x magnification. Larval samples were imaged at 63x magnification where indicated. Images were processed with Fiji (Schindelin et al., 2019).

Appendix A.2 Key resources

Table 1. Key resources

name	sequence	notes
T9Large_HArmL1_R	gtgcatatgtccgcggccgcAACGGAACATTCAAGTCAGACCC	enhancer2 CRISPR primers
T9Large_HArmL_F	tcttgcatgctagcggccgcAACACCACCACCTGAACCAGCT	
T9Large_HArmR1_F	ctccatgcataaggcgcgccACGGGGTTAAACGGTTGTTGAC	
T9Large_HArmR_R1	gcagaaggcctaggcgcgccCTATGAGTTCCACTGGGCTGAC	
T9Large_HArmR_R2	gcagaaggcctaggcgcgccGTTGGCGTTAGCTAACGGCACA	
T9Large_L1_gRNA1	aaaataatacgactcactataggGACTTGAATGTTCCGTTGTTgttttagagctag	Enhancer2 5548 bp deletion with L1R1 guides.
T9Large_R1_gRNA	aaaataatacgactcactataggAGGCAGTCAGGTTGTTCACGgttttagagctag	
Tile9large_Left_F_seq1	GCATGATAATGGAGGGCATTGG	180 bp upstream of the left

		homology arm (of F1)
Tile9large_Left_F_seq2	GACTTTGTGACAATGGGCGATC	118 bp upstream of the left homology arm
Tile9large_Right_R_seq1	GCCTGGCTTCTGCATTTTGCTTC	184 bp downstream of the right homology arm (of R1)
Tile9large_Right_R_seq2	ATTGCCACACGCGTCTTTCCAT	220 bp downstream of the right homology arm (of R1)
pHD-dsRed_rightF_dns1	GACTACACCATCGTGGAGCAGT	enhancer2 CRISPR validation primers
pHD-dsRed_leftR_dns1	TGAACTCCTTGATGACGTCCTC	
Tile9_Right_outArm_R	ATTGACGCTGACAAGGCTTGGC	
Tile9_Right_inArm_R	GCAATTTCGCCCTTGGCAATTG	
Tile9_Left_inArm_F	GGCTTAGATGCACAGTGGTA	
Cas9-RT-F	GCATAAAGAAGAACCTCATTGG	
Cas9-RT-R	GAAAGAGTCATCCACCTTAGC	
Actin5c-RT-F	ATACTCCTCCCGACACAAAGC	
Actin5c-RT-R	CAGGTAGTCGGTCAAATCGC	
bia_Dl-DS-Tile9_F1	TTCCGggcgcgccCACCCACAGAGGTACCACGTATA	
bia_Dl-DS-Tile9_F2	TTCCGggcgcgccCAGCCACGGACTCCATGATGAT	
bia_Dl-DS-Tile9_R1	TTGCCcctgcaggTACTCCGCTGTCCAAGTGTGTC	
bia_Dl-DS-Tile9_R2	TTGCCcctgcaggATGCAGATTAACGTCGCCTG	
ana_Dl-DS-Tile9_F1	TTCCGggcgcgccTAGCCCAACAACAGGCGGAAGAT	
ana_Dl-DS-Tile9_F2	TTCCGggcgcgccTCCTATTCGGATCGCCCATCGA	

ana_Dl-DS-Tile9_R1	TTGCCcctgcaggCCCTCTGGCATATATGTATAGC	
ana_Dl-DS-Tile9_R2	TTGCCcctgcaggCGTCTGCCCAAAAGGCTTATC	
pse_Dl-DS-Tile9_F1	TTCCGggcgcgccACGATACCCATGCGCACGACAT	
pse_Dl-DS-Tile9_F2	TTCCGggcgcgccACGGATGAGATGATGCCCAGTC	
pse_Dl-DS-Tile9_F3	TTCCGggcgcgccGGCTTTCTCAGTCTGTTTGAGG	
pse_Dl-DS-Tile9_R2	TTGCCcctgcaggATGGCTGGCAGTGTCAACAACC	
pse_Dl-DS-Tile9_R3	TTGCCcctgcaggCCGTCAATACCCCATCAGATCT	
DlPLE_O_Dr-mut-F	GTAAAGATCGTGGgTgCgAcTgAcAgCACTATCGTCTACCTGCTGCG	
DlPLE_O_Dr-mut-R	CGCAGCAGGTAGACGATAGTGcTgTcAgTcGcAcCCACGATCTTTAC	
upd-MA5-Su(H)mut1-F	TATGGGTATGGGCATCGGCATGGGgAgGtGcTGTGGAGCGGATGACG	
upd-MA5-Su(H)mut1-R	CGTCATCCGCTCCACAgCaCcTcCCCATGCCGATGCCCATACCCATA	
upd-MA5-Su(H)mut2-F	CTCAATTTTGGAAATGCaTaAaAaACGACGAAAGCGAAC	
upd-MA5-Su(H)mut2-R	GTTCGCTTTCGTCGTtTtTtAtGCATTTCCAAAATTGAG	
MA5-CD4-F	GGCCAGCACCAGAGAATCAACT	Sarah Smith
MA5-CD4-R	TCGCACATTTTGTGGCATGAGG	Sarah Smith
Dl PLE-O-1-56-F	TTCCGggcgcgccTTCCCTATTCCCTACGCTTAGC	Outside primer for enhancer1 scrambles
Dl PLE-O-15-58-R	TTGCCcctgcaggCAAGATGATCGAATTCAAACCGAGTG	
Dl PLE-O-doc2mut-R	TTGCCcctgcaggCCATCGTCGATCCGTAACAACT	
Dl PLE L.F	TTCCGggcgcgccGAAGTCAGCTGCTCTAGGCGATTAG	806 bp
Dl PLE L.R	TTGCCcctgcaggCATGGTGCAAGTGGTTCCATCTCA	
Dl PLE M.F	TTCCGggcgcgccCACATCTGGAACACGCCTCCGATTG	879 bp
Dl PLE M.R	TTGCCcctgcaggGACCTGCCAACCTGTACCAATCAAC	
Dl PLE N.F	TTCCGggcgcgccGAGATGGAACCACTTGCACCATGTG	721 bp

Dl PLE N.R	TTGCCcctgcaggCACTGGCCAAAGTTTCAACTCTCTG	
Dl PLE O.F	TTCCGggcgcgccGTACATCTGCACGAAGATGATCTG	728 bp
Dl PLE O.R	TTGCCcctgcaggCCATAGGCTAGCAGTAACAACT	
Dl PLE P.F	TTCCGggcgcgccCAGAGAGTTGAAACTTTGGCCAGTG	826 bp
Dl PLE P.R	TTGCCcctgcaggCACAACTGCGTGAGAAGTTGCC	
Dl PLE Q.F	TTCCGggcgcgccGAATAAACTACATGGAGGCGGCGGAG	931 bp
Dl PLE Q.R	TTGCCcctgcaggGTTGAGGCGACTGAAAACACTAACAG	
Dl-PLE-Lrg-F	TTCCGggcgcgccTGCGAATTGCTTGTACGTGACACG	mel, ana, pse
Dl-PLE-Lrg-F1	TTCCGggcgcgccACGACCATGTCCAGTCAACCACT	
Dl-PLE-Lrg-F2	TTCCGggcgcgccTGAGCGAAGCGTGAAGTTGACT	
Dl-PLE-Lrg-R4	TTGCCcctgcaggATCGACACGCTTAACTGGCA	
Dl-PLE-Lrg-R3	TTGCCcctgcaggGAGCGCAAAAATGTTGATGCAATCG	
mbeta-F'5	CCCAGCGAGGCCAGCTCCA	Probe primer-ana,mel.bia,pseu
mbeta-F1	GACCTATCAGTACCGCAAG	ana,mel,pseu, bia
mbeta-F2	GTGATGAAGCCCATGCTGGA	ana,mel,pseu, bia
mbeta-F3	GAGCACATGAAGAAGCTGCG	ana, mel, pseu,bia
mbeta-F4	CTCAACTACCTGCAAGTGGTGGT	ana,mel,bia
mbeta-R'2	taatacgactcactatagggagaTGGAGCTGGCCTCGCTGGG	ana,mel,bia,pseu
mbeta-R1	taatacgactcactatagggagaCGCAGCTTCTTCATGTGCTC	ana,mel,pseu, bia
mbeta-R3	taatacgactcactatagggagaACCACCACTTGCAGGTAGTTGAG	ana,mel,bia
mbeta-R4	taatacgactcactatagggagaTGAGGCTCTGCTAGAGA	ana,mel,bia
Doc2.1.f	GTTGGCGATGAAGACATCTT	mel, ana, bia, pse
Doc2.1.r_T7	taatacgactcactatagggagaAGGCCTTTGTGTTCGCCGAGA	
Doc2.1.rb_T7	taatacgactcactatagggagaTCATACGCACCGCTGATCTG	
Doc2.2.f	GCAAACGGATTGTTGTCGAT	
Doc1_F_melanabia	TTTCCCGGAGACGGAGTTCGT	Doc1_F_melanabia

mel_Doc1_R_T7	taatacgactcactataggGCAACTGGATGAGTTGCTCCAT	mel_Doc1_R_T7
ana_Doc1_R_T7	taatacgactcactataggGCACACGTTGCTCACAAATCAC	ana_Doc1_R_T7
bia_Doc1_R_T7	taatacgactcactataggCTCAAGTAGGTTGCTCCATGGT	bia_Doc1_R_T7
Doc3_F	AGCAGCTTCAGCATCTCGGACA	mel,ana,bia,pse,sim
Doc3_R_T7	taatacgactcactataggCCTGGAAATCCTAATTAACTGC	mel,ana,bia,pse,sim
Doc3_F1	TCGACAACAATCCCTTTGCCAAGG	mel,ana,bia,pse,sim
Doc3_R1_T7	taatacgactcactataggTGTCCGAGATGCTGAAGCTGCT	mel,ana,bia,pse,sim
mel_Doc1_GBE_F	TTCCGggcgcgccTATCAGGCCACCGCCAAACTTCCTC	
mel_Doc1_GBE_R	TTGCCcctgcaggGTTTGTCACGGCATAGATTGTGTTC	
ana_Doc1_GBE_F	TTCCGggcgcgccTGGTGGAGCAACTGCATCGTCA	
ana_Doc1_GBE_R	TTGCCcctgcaggTTCGATTCGGACTGACAGACTG	
bia_Doc1_GBE_F	TTCCGggcgcgccCATTGCAATTGCGTGGCCCTT	
bia_Doc1_GBE_R	TTGCCcctgcaggTGCTTATCCTCGGTCCTATCTG	
pse_Doc1_GBE_F	TTCCGggcgcgccGTGGGTATAGAGGTATGGGGTA	
pse_Doc1_GBE_R	TTGCCcctgcaggATAATGAGGCGCAGTCGTGCTG	

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