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FlyBook

The wing imaginal disc

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Abstract

The *Drosophila* wing imaginal disc is a tissue of undifferentiated cells that are precursors of the wing and most of the notum of the adult fly. The wing disc first forms during embryogenesis from a cluster of ~30 cells located in the second thoracic segment, which invaginate to form a sac-like structure. They undergo extensive proliferation during larval stages to form a mature larval wing disc of ~35,000 cells. During this time, distinct cell fates are assigned to different regions, and the wing disc develops a complex morphology. Finally, during pupal stages the wing disc undergoes morphogenetic processes and then differentiates to form the adult wing and notum. While the bulk of the wing disc comprises epithelial cells, it also includes neurons and glia, and is associated with tracheal cells and muscle precursor cells. The relative simplicity and accessibility of the wing disc, combined with the wealth of genetic tools available in *Drosophila*, have combined to make it a premier system for identifying genes and deciphering systems that play crucial roles in animal development. Studies in wing imaginal discs have made key contributions to many areas of biology, including tissue patterning, signal transduction, growth control, regeneration, planar cell polarity, morphogenesis, and tissue mechanics.

Keywords: wing; *Drosophila*; imaginal disc; FlyBook

Introduction

In holometabolous insects like *Drosophila*, which undergo complete metamorphosis, the precursors of most adult structures of the head, thorax, and genitalia are maintained during larval stages as distinct clusters of undifferentiated cells called imaginal discs. The name refers to the final, adult stage of insect development, which is classically known as the imago. The wing imaginal disc (henceforth, wing disc) gives rise to the wing and wing hinge, and also the dorsal half of the body wall in the second thoracic segment (T2, also known as the mesothorax), which in *Drosophila* comprises the bulk of the thorax (Fig. 1, a and b). This includes the back of the thorax, the notum, and part of the lateral sides of the thorax, the pleura.

The imaginal discs are easily recognizable within the body cavity of the larva (Fig. 1c), which facilitated classical approaches involving transplantation, as well as more recent approaches incorporating both analysis of dissected fixed discs and live imaging. The relatively flat morphology of the wing disc, with most cells in a single epithelial layer, has also facilitated imaging-dependent approaches and contributed to their popularity as an experimental model. The imaginal discs undergo extensive growth during the larval stages of *Drosophila*, with the wing imaginal disc increasing in size over 1,000-times. In contrast to many larval tissues, which become polyploid, the imaginal disc cells remain diploid, and discs grow by increasing cell numbers. The extensive growth of the wing disc has contributed to its utility as a model for studies of organ size control, and to the identification in wing discs of genes that play key, conserved roles in controlling growth of animal tissues. The growth of wing discs has also facilitated methods for creating genetic mosaics through induction of recombination and growth of mitotic clones, thus enabling analysis of requirements for genes that are also required at earlier stages of development, and distinguishing autonomous from nonautonomous effects.

During wing disc development, distinct cell fates are assigned to different regions, and the wing disc transitions from a simple sac of cuboidal epithelial cells to an organ with complex epithelial morphology that includes regional differences in cell shape and a pattern of local folding around the future wing hinge. Patterning of the wing discs is mediated by several highly conserved signaling pathways, and many of the components of these pathways were first identified and characterized based on their roles in wing discs. Classical genetic studies identified and characterized many mutations that affect wing size, shape, and posture Waddington (1940) and Lindsley and Grell (1968), in part because flies do not need their wings to survive or reproduce in laboratory culture. These mutations have contributed to the identification of genes that play important roles in wing discs. Indeed, some of the genes that are crucial for normal wing development were first identified over 100 years ago (Morgan and Bridges 1916; Bridges and Morgan 1919). Even today, investigators studying the effects of genetic alterations on wing discs often examine adult wings as the final outcome of processes that occurred during wing disc development. Studies in wing discs take advantage of the many sophisticated genetic techniques

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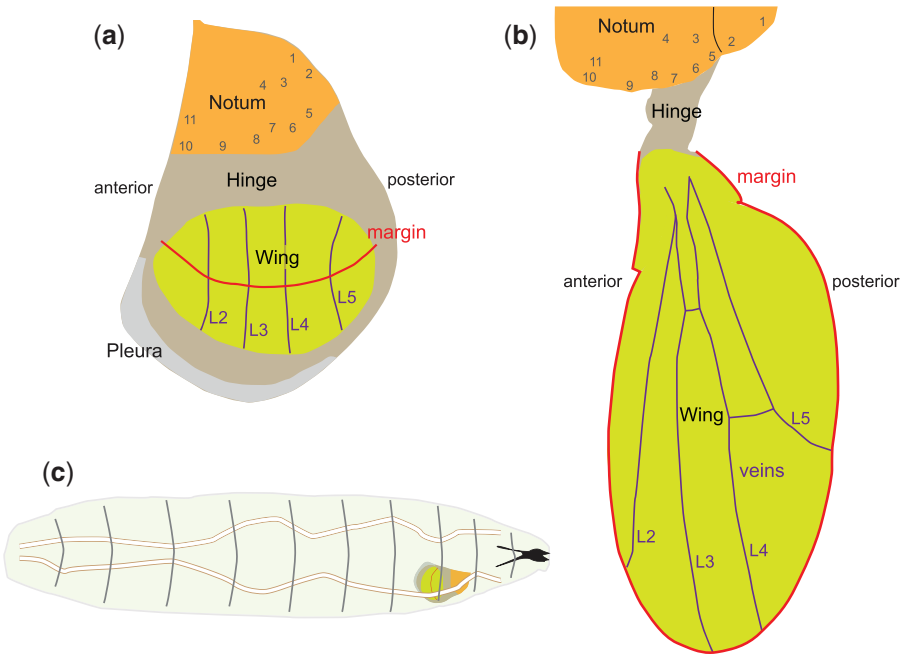

Fig. 1. The wing imaginal disc. a) Schematic of late third instar wing disc, with approximate locations of notum, hinge, wing, and pleural regions identified by distinct coloring, and approximate locations of precursors for macrochaete identified by numbers, as indicated by Bryant (1975a): 1, 2—scutellar bristles; 3, 4—dorsocentral bristles; 5, 6—postalar bristles; 7, 8—supraalar bristles; 9, 10—notopleural bristles; 11—presutural bristle. Approximate location of wing margin (red) and longitudinal veins L2–L5 (purple) are also indicated. b) Schematic of adult wing, hinge, and half notum, marked as in (a) to illustrate relationship between wing disc and adult derivatives. c) Schematic of third instar larva with approximate location of the wing disc indicated.

available in *Drosophila*, including diverse methods for generating mutations, creating genetic mosaics, manipulating and analyzing gene expression patterns, and labeling cells (St Johnston 2002; Blair 2003; del Valle Rodriguez *et al.* 2012; Hales *et al.* 2015; Caygill and Brand 2016; Bier *et al.* 2018; Germani *et al.* 2018).

In this review, we describe the structure and development of the wing disc and provide an overview of how studies of wing discs have made key contributions to many areas of biology.

Embryonic origin of the wing disc

The wing disc is thought of as a larval structure, but the initial specification of wing discs occurs during embryogenesis. The 2 wing discs of each developing fly (left and right) arise from cells in the lateral epidermis of T2 around embryonic stages 11–13 (Bate and Arias 1991; Cohen *et al.* 1993; Requena *et al.* 2017). The imaginal disc primordia can be identified in the embryo by their distinct cellular morphology (Madhavan and Schneiderman 1977; Bate and Arias 1991), but analysis of their specification has been greatly aided by the identification of genes that are specifically expressed in these cells, together with the cis-regulatory modules that drive expression in disc primordia (Williams *et al.* 1991; Cohen *et al.* 1993; Fuse *et al.* 1996; Requena *et al.* 2017). Examination of molecular markers, together with lineage analysis, has revealed that 2 adjacent populations of cells together give rise to wing discs (Requena *et al.* 2017) (Fig. 2). Around embryonic stage 11 thoracic imaginal disc primordia (TP) are specified, recognizable by expression of the transcription factor Distal-less (Dll) (Cohen 1990; Cohen *et al.* 1993) (Fig. 2c). The TP give rise to both leg and wing disc cells (Cohen *et al.* 1993; Requena *et al.* 2017). Around stages 12–13, wing disc primordia (also referred to as dorsal primordia) become recognizable by expression of the transcription factors Snail (Sna) and Vestigial (Vg) (Williams *et al.* 1991; Cohen *et al.* 1993; Fuse *et al.* 1996; Requena *et al.* 2017) (Fig. 2). The wing primordia appear on the dorsal sides of Dll-expressing TP cells, and include both cells from the TP and cells just dorsal to the TP. The close association of the wing and leg imaginal disc primordia is consistent with lineage studies showing that individual marked clones created at early embryonic stages can contribute to tissue from both leg and wing discs (Wieschaus and Gehring 1976; Lawrence and Morata 1977). Around embryonic stage 14, the T2 imaginal disc primordia become physically separated into distinct leg and wing primordia, as the wing primordia cells migrate dorsally (Fig. 2). A similar process in T3 separates leg and haltere disc primordia.

The dual origin of wing disc cells has implications for the evolutionary origin of the insect wing. Two main hypotheses have been suggested (Clark-Hachtel and Tomoyasu 2016). The gill-exite hypothesis proposes that wings evolved from an outgrowth at the base of the leg that functioned as a gill in aquatic insects, whereas the paranotal hypothesis proposes that wings evolved from an extension of the notum. The observation that the wing disc primordium includes populations of cells that are both shared and distinct from the leg disc primordium has been interpreted as supporting a unified hypothesis that combines the paranotal and gill-exite hypotheses (Niwa *et al.* 2010; Clark-Hachtel and Tomoyasu 2016; Requena *et al.* 2017; Linz and Tomoyasu 2018).

Several different approaches have been used to estimate the number of cells in an embryonic wing disc primordium. By direct examination of the morphologically identifiable wing disc primordia in the embryo after their separation from the leg primordia, Bate and Arias (1991) estimated that a primordium has 24 cells, whereas counting of Vg-expressing cells around this stage led (Cohen *et al.* 1993) to estimate a primordium contains around 30 cells. A similar analysis in the first instar larva yielded an

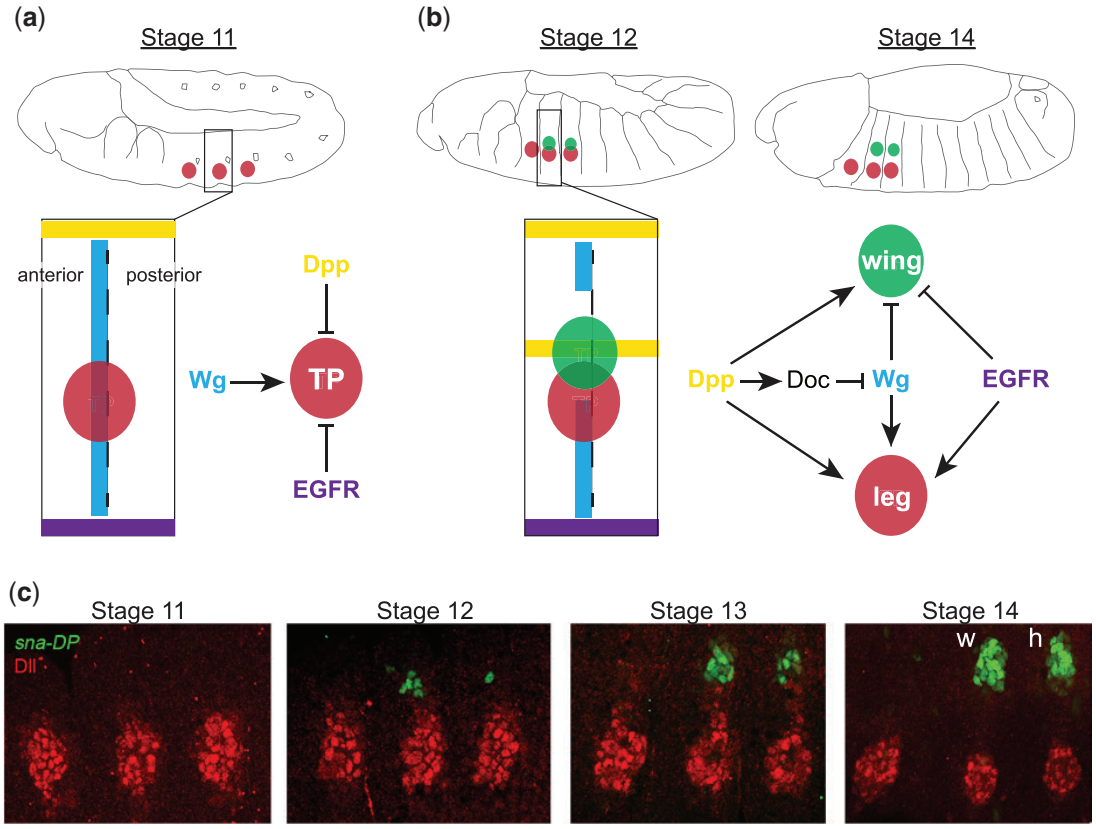

Fig. 2. Embryonic origin of the wing disc. Schematics showing approximate embryonic locations of thoracic imaginal disc primordia above, and signaling inputs that control their specification below, based on Requena *et al.* (2017). a) At stage 11, formation of the TP (red oval) is promoted by Wg (blue line) signaling near the A–P compartment boundary (dashed line) and suppressed in more dorsal and more ventral cells by Dpp and EGFR signaling, respectively. b) At stage 12, the wing primordia begins to form, comprising cells from both the TP and more dorsal cells (green). The Wg stripe is interrupted by Doc-mediated repression, and formation of the wing primordia is promoted by Dpp and inhibited by Wg and EGFR signaling. At stage 14, the wing primordia migrate dorsally, separating from the leg primordia. c) Images of the thoracic/leg primordia (Dll stain, red) and wing/haltere primordia (*snail-DP-lacZ* reporter, green) in embryos at the indicated stages (gift of Carlos Estella). Cells of the TP that will become wing/haltere primordia lose expression of Dll. w marks wing primordium, h marks haltere primordium.

estimate of 38 cells (Madhavan and Schneiderman 1977), but this could have included associated tracheal, nerve, or adepithelial cells. Martín *et al.* (2009) inferred a founder size of 55 cells from measurements of clone sizes and the number of cells in late third instar wing discs, however, they induced their clones around the time when Madhavan and Schneiderman (1977) estimated that wing disc cells begin dividing, which may have skewed the estimate. By estimating the frequency of mosaicism in clonal analysis experiments using a single marker (Lawrence and Morata 1977) estimated a wing primordium has ~20 cells. By using a method (Tie-Dye) that generates multiple clone labels and examining frequencies of labeled clones (Worley *et al.* 2013) estimated with 73% confidence that it derives from 25 to 31 cells. Thus, a variety of approaches suggest that the initial number of wing disc primordium cells is likely around 25–30. Around embryonic stage 14, the wing primordia invaginate to form small epithelial sacs that remain connected to the embryonic epidermis. They undergo little or no cell division until near the end of the first larval instar (Madhavan and Schneiderman 1977; Bate and Arias 1991).

The specification of the wing disc primordium requires signals that inform cells of their segmental identity, and their anterior–posterior and dorsal–ventral location within the segment. Segmental identity is provided by Hox genes, and formation of the thoracic and wing primordia is repressed in more anterior segments by *Sex combs reduced* and in more posterior segments by *Ultrabithorax*, *abdominal A*, and *Abdominal B* (Vachon *et al.* 1992; Carroll *et al.* 1995; Gebelein *et al.* 2002; Requena *et al.* 2017). The Hox gene expressed in T2, *Antennapedia*, is not required for formation of TP but does enhance Dll expression in the TP (Uhl *et al.* 2016). The TP is specified around the anterior–posterior (A–P) compartment boundary, such that it includes both anterior and posterior cells from its inception. This can be explained by the role of the *Drosophila* Wnt protein Wingless (Wg), which is expressed along the anterior side of the A–P boundary in the embryo. Wg is required for specification of the thoracic imaginal discs, as revealed by the requirement for Wg for expression of Dll in the TP (Cohen 1990; Cohen *et al.* 1993) (Fig. 2a). The lateral location of the TP is set by repression in more dorsal cells from Decapentaplegic (Dpp) signaling, and repression in more ventral cells from epidermal growth factor receptor (EGFR) signaling (Goto and Hayashi 1997).

As the TP splits into leg and wing primordia, distinct effects of Wg, Dpp, and EGFR signaling are observed, which act in concert to specify distinct thoracic imaginal disc primordia (Fig. 2b). Dpp becomes expressed in a lateral stripe just dorsal to the TP, and promotes wing primordium fate (Goto and Hayashi 1997; Hamaguchi *et al.* 2004; Requena *et al.* 2017), as well as, at lower levels, proximal leg fate (Goto and Hayashi 1997). Conversely, EGFR signaling from more ventral cells represses wing primordium fate, while promoting leg fate (Kubota *et al.* 2000; Requena *et al.* 2017). Dpp signaling also leads to local repression of Wg expression through upregulation of Dorsocross (Doc) transcription

factors. This results in lower levels of Wg signaling, which favors wing disc fate over leg disc fate (Kubota *et al.* 2003; Requena *et al.* 2017). Thus, dynamic integration of positional cues results in specification of 2 bilaterally symmetric clusters of ∼25–30 embryonic cells as wing disc primordia.

Cell biology of the wing disc

Although the wing disc is sometimes treated as a simple sheet of epithelial cells, it has a complex morphology, including heterogeneities in cell shape, type, and organization.

Wing disc epithelial cells

The early wing disc is a flat sac of cuboidal epithelial cells, with the apical sides toward the lumen. As the wing disc begins to grow, differences in cellular morphology appear. Cells on one side flatten, forming a thin squamous epithelium called the peripodial membrane or peripodial epithelium (PE) (Auerbach 1936; McClure and Schubiger 2005) (Fig. 3a). The peripodial cells ultimately constitute a small fraction of wing disc cells, roughly 5% at the end of larval development (McClure and Schubiger 2005), and contribute correspondingly little to the cuticle of the adult fly (Milner *et al.* 1984). Nonetheless, they may interact with other cells (Gibson and Schubiger 2000; Pallavi and Shashidhara 2005), and they play essential roles in the morphogenetic transformation of the disc that occurs during metamorphosis (Milner *et al.* 1984; Pastor-Pareja *et al.* 2004; Aldaz *et al.* 2013). Cells on the other side elongate apico-basally as their density increases, forming a columnar epithelium (Fig. 3, a and b). Most of the growth, and ultimately most of the cells, of the wing disc are in the columnar epithelium, also referred to as the disc proper (DP), and consequently most studies effectively treat the wing disc as an epithelial monolayer. Near the edge of the wing disc there is a transition zone with roughly cuboidal cells (McClure and Schubiger 2005; Aldaz *et al.* 2010) (Fig. 3, a and b). By late third instar columnar cells become densely packed and increasingly tall, particularly in the central region of the disc that will give rise to the wing, where cells can be ∼40 μm tall and ∼2 μm wide (Fig. 3, b and d). These columnar cells become pseudostratified (the nuclei are not all in the same plane), a physical necessity as the widths of the cells become less than the width of the nucleus (Fig. 3, b, c, and e). Division of these pseudostratified cells involves a process of interkinetic nuclear migration, in which nuclei migrate to the apical region of the cell, which transiently expands to enable the planar divisions that maintain the disc epithelium as a monolayer (Meyer *et al.* 2011; Chanet *et al.* 2017) (Fig. 3, c and d). Planar divisions of wing disc cells also require the *Drosophila* NuMA protein Mushroom body defect (Mud) and proteins that help localize Mud (Nakajima *et al.* 2013; Bergstralh *et al.* 2016).

Wing disc epithelial cells, like other insect epithelia, are physically connected through cell–cell junctions near their apical side (Fig. 3, c–e). Adherens junctions, which are connected to the actin cytoskeleton, provide mechanical coupling between cells, and regulate apical cell shape (Farhadifar *et al.* 2007). Apical cell sizes and shapes differ across the wing disc, reflecting local differences in cell behavior and mechanics (Aegerter-Wilmsen *et al.* 2012; Legoff *et al.* 2013; Mao *et al.* 2013; Pan *et al.* 2018; Dye *et al.* 2021). A paracellular diffusion barrier between apical and basal surfaces is formed by septate junctions (Tepass *et al.* 2001), which are just basal to the adherens junctions (Fig. 3c). Apical to the adherens junctions, a region known as the marginal zone or subapical region includes transmembrane proteins that participate in the regulation of polarity and intercellular signaling (Tepass 2012; Thompson 2013). Wing disc microtubules can be found both oriented parallel to the plane of the epithelium near the apical or basal surface, as well as extending from the apical to the basal side of the cell (Fristrom and Fristrom 1975; Eaton *et al.* 1996). Microtubules appear more apically concentrated in columnar cells, but basally concentrated in cuboidal cells or within folds (Sui *et al.* 2012). F-actin networks are found throughout wing disc cells, with some of the highest levels near the adherens junctions (Eaton *et al.* 1995) (Fig. 3, b and e). There is also actomyosin-mediated tension along the basal surface of wing pouch cells, which contributes to the curvature of the wing pouch (Nematbakhsh *et al.* 2020). Wing disc cells have filopodial extensions near their basal side, which can extend over a few cell diameters and have been implicated in Notch signaling (de Joussineau *et al.* 2003). A distinct type of long thin, actin-rich cellular extension, termed cytonemes, were first described in wing disc cells and have been implicated in intercellular signaling (Ramírez-Weber and Kornberg 1999; Hsiung *et al.* 2005; Roy *et al.* 2011; Bischoff *et al.* 2013). Cytonemes can extend for several cell diameters and are often oriented toward the sources of signaling molecules.

The wing disc has a basal extracellular matrix (ECM), or basement membrane (BM), including the conserved proteins collagen, laminin, perlecan, and nidogen (Hynes and Zhao 2000; Ramos-Lewis and Page-McCaw 2019; Bonche *et al.* 2021) (Fig. 3, c and e). Rather than being secreted by wing disc cells, collagen, nidogen, and a substantial fraction of perlecan are secreted by other tissues, principally the fat body, and then spread to the wing disc through the hemolymph, which baths all larval organs (Pastor-Pareja and Xu 2011; Dai *et al.* 2018; Bonche *et al.* 2021). However, functionally important laminin expression is provided by wing disc cells (Urbano *et al.* 2009). Cellular attachments to BM influence the morphology of the wing disc, as when integrin function is disturbed, BM formation is impaired, or BM proteins are degraded, columnar cells flatten toward a cuboidal shape (Domínguez-Giménez *et al.* 2007; Pastor-Pareja and Xu 2011; Ma *et al.* 2017). The BM also influences the distribution of extracellular signaling molecules (Ma *et al.* 2017).

Nonepithelial cells in the wing disc

In addition to the epithelial cells that make up the bulk of the wing disc, the wing disc also contains smaller numbers of other cell types. Neurons and associated glia are embedded within the wing disc epithelium. They form from sensory organ precursor cells that are selected through a lateral inhibition process mediated by Notch signaling, and they will form sensory bristles in the notum and along the anterior edge of the wing in adult flies (Huang *et al.* 1991; Gómez-Skarmeta *et al.* 2003). These neurons send axons between the epithelium and basal lamina of the wing disc.

Adult muscle precursor cells (AMP, originally described as adepithelial cells) underly the notum and are located between the columnar disc epithelium and the BM (Madhavan and Schneiderman 1977) (Fig. 3, a and b). These cells are mesodermal in origin, and they will form the flight muscles of the adult fly (Bate *et al.* 1991; Fernandes *et al.* 1991). Their location, proliferation and patterning depend upon signals from wing disc epithelial cells, including Wg, Notch, fibroblast growth factor and hedgehog (Hh) (Gunage *et al.* 2014; Hatori and Kornberg 2020; Everetts *et al.* 2021).

The wing disc is also tightly associated with tracheal cells (Inoue and Hayashi 2007), which provide oxygen to wing disc cells and form a primordium for the air sac that will deliver oxygen to

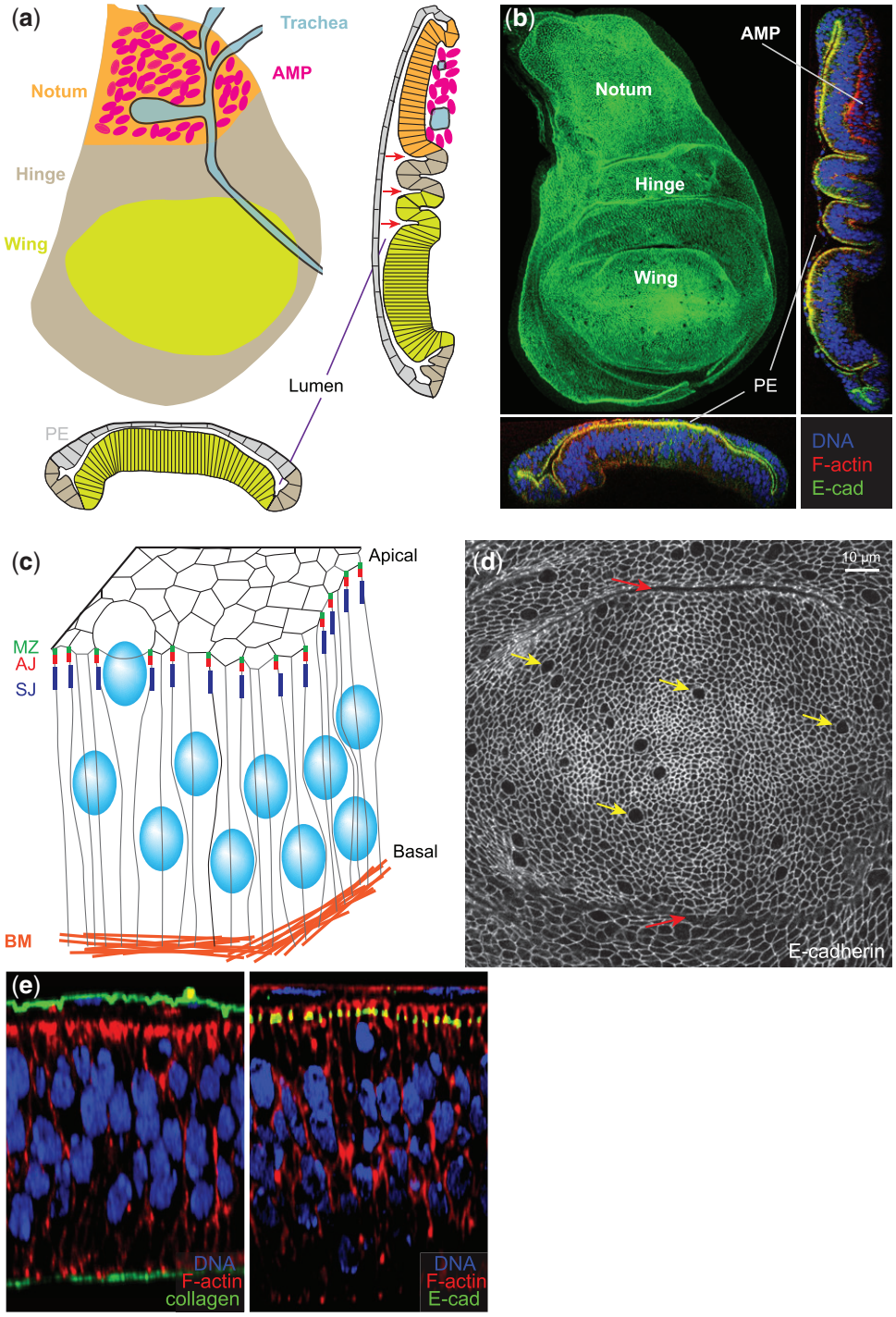

Fig. 3. Cell biology of the wing disc. a) Schematics of a horizontal view of the wing disc (top left) and sections across the length and width of the disc to illustrate different cell types, including the squamous PE (gray) columnar cells of the wing (olive), hinge (brown) and notum (orange), and AMP (pink) and tracheal (blue) cells underlying the notum. Red arrows point to the hinge–notum (top), hinge–hinge (middle), and hinge–pouch (bottom) folds. b) Confocal micrographs of late third instar wing disc. Top left panel displays a maximum projection of E-cad staining (green). Panels at right and bottom show slices across the length and width of the disc, and include staining for DNA and F-actin as well as E-cad. c) Schematic section of columnar wing disc epithelia, illustrating pseudostratification, with nuclei in blue, and relative locations of marginal zone (green), adherens junctions (red), and septate junctions (dark blue) indicated. BM is indicated in orange at bottom. d) Extracted surface of confocal micrograph of apical surface of the wing region of the wing disc, with cells outlined by E-cadherin staining. Note the variations in apical cell size. Red arrows highlight the fold at the edge of the wing pouch; yellow arrows highlight a few examples of mitotic cells, which are transiently enlarged as they round up. e) Confocal micrographs of vertical sections through the wing disc, showing DNA (blue), F-actin (red), and at left collagen (encoded by *viking*, green) and at right E-cad (green).

flight muscles in the adult (Fig. 3a). Wing disc tracheal patterning also depends upon signaling from disc cells, mediated at least in part through cytonemes (Roy *et al.* 2014; Du *et al.* 2018; Hatori and Kornberg 2020). Thus, although the wing disc is often thought of as a simple epithelial organ, the tight association and exchange of signals with mesodermal and tracheal cells emphasize that

the wing disc, like more complex vertebrate organs, includes a diversity of cell types that are organized by signaling interactions between epithelia and neighboring nonepithelial cells.

Patterning of the wing disc

Intensive studies of how cells in different regions of the wing disc acquire distinct fates have yielded fundamental insights into conserved mechanisms of tissue patterning and the signaling networks responsible for establishing it.

Cleavage of dissected third instar wing discs and transplantation of disc fragments back into larval hosts about to undergo metamorphosis was used to create the first detailed fate maps of the wing disc (Bryant 1975a) (Fig. 1a). This was possible because by the end of the third instar, the characteristic shape of the wing disc makes distinct regions readily identifiable, and disc fragments corresponding to morphologically identifiable regions differentiate into consistent adult structures. In broad terms, the 4 main regions of the late third instar wing disc are the wing pouch, an oval-shaped region that gives rise to the wing blade; the proximal wing and wing hinge, a folded region that gives rise to structures at the base of the wing; the roughly triangular notal region, which gives rise to most of the back of the fly in the thorax, and the PE, which gives rise to some of the pleura. The asymmetry of the wing disc also makes it possible to distinguish anterior from posterior sides.

Expression profiling approaches, including microarrays, enhancer mapping, and single cell RNA sequencing, have revealed intricate and complex patterning of gene expression in wing disc cells by the end of the third larval instar (Butler *et al.* 2003; Jory *et al.* 2012; Bageritz *et al.* 2019; Deng *et al.* 2019; Everetts *et al.* 2021). The distinct fates of different regions of the wing disc are specified through a process of progressive refinement as disc development proceeds. Beginning with an initial subdivision of the wing disc into broad regions, the disc then becomes further subdivided as a series of transcription factors, signaling molecules, and their targets become expressed in distinct patterns.

Establishment of wing disc patterning

The wing disc contains regionally distinct cell types from its inception in the embryo, as it forms straddling the A–P compartment boundary, and thus includes both anterior and posterior cells (Fig. 2, a and b). Posterior cells are defined by expression of the transcription factor Engrailed (En) (Morata and Lawrence 1975; Kornberg 1981) (Fig. 4a). Although some wing disc cells originate as part of an early, initially Dll-expressing TP that also gives rise to the leg imaginal disc and others originate from a slightly more dorsal primordium (Fig. 2), lineage analysis shows that cells from either primordia can contribute to each of the main regions of the wing disc (Requena *et al.* 2017).

During the first and second larval instars, the wing disc becomes subdivided into distinct appendage (wing) vs body wall regions. This subdivision is largely dependent upon the regionalized expression of 3 secreted signaling molecules: Vein (Vn), Wg, and Dpp (Fig. 4b). Vn, a ligand for the EGFR, is expressed in more dorsal cells of the early wing disc, whereas Wg is expressed in more ventral cells of the early wing disc (Couso *et al.* 1993; Williams *et al.* 1993; Ng *et al.* 1996; Simcox *et al.* 1996). Vn and Wg signaling antagonize each other, helping to maintain distinct dorsal and ventral territories (Baonza *et al.* 2000; Wang *et al.* 2000). Jak-Stat signaling, which is elevated in the ventral half of the wing disc, is required to maintain restriction of EGFR signaling to more dorsal cells at late second and early third instar (Recasens-Alvarez *et al.* 2017). The origin of the dorsal vs ventral differences in Vein and Wg expression in the early wing disc is not entirely clear. It has been reported that Vn expression is induced de novo in the first instar wing disc by Dpp signaling across the lumen from the overlying, future peripodial cells, and then maintained by a positive feedback loop through EGFR signaling (Wang *et al.* 2000; Paul *et al.* 2013), although this then raises the question as to what localizes Dpp expression to these overlying cells.

Notum fate is promoted by expression of the 3 homeodomain transcription factors of the Iroquois complex (Iro-C), Araucan, Caupolican, and Mirror (Diez del Corral *et al.* 1999). Loss of the

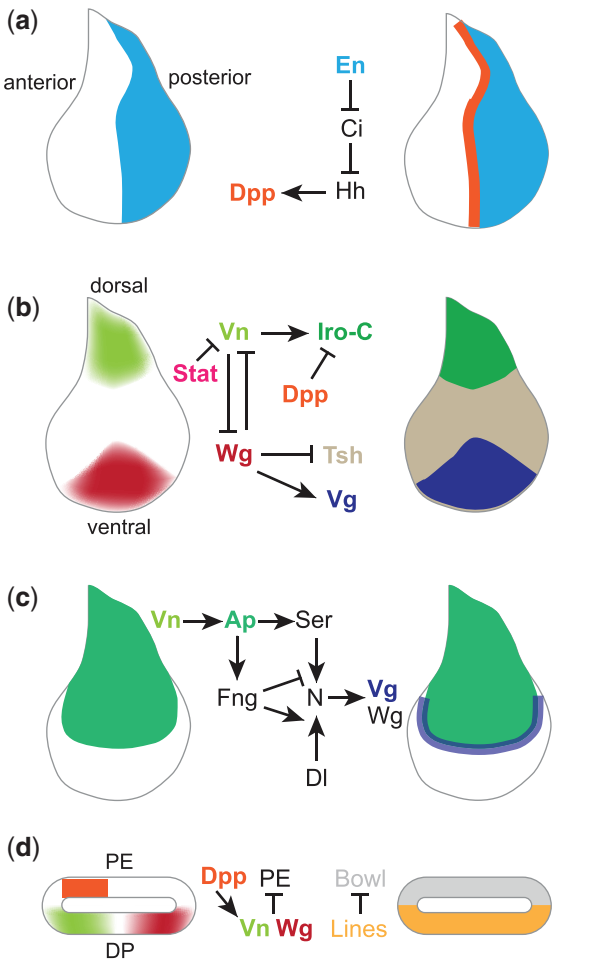

Fig. 4. Early wing disc patterning. Schematics of wing discs illustrating key early steps in patterning. a) Expression of En in the posterior of the wing disc creates distinct anterior and posterior compartments. En represses expression of the Hh pathway transcription factor Ci. Ci directly and indirectly represses Hh, restricting Hh expression to posterior cells. The complementary expression of Ci and Hh limits Hh to signaling to anterior cells, where it induces transcription of Dpp. b) The wing disc is subdivided into appendage-body wall regions by the differential expression of Wg in ventral cells and Vn in dorsal cells, which mutually antagonize each other. Vn promotes notum fate by activating expression of Iro-C genes, while Wg promotes wing fate by promoting expression of Vg and suppressing expression of Tsh. c) Expression of Ap in the dorsal half of the wing disc creates distinct dorsal and ventral compartments. Ap regulates activation of Notch (N) along the D–V boundary by promoting transcription of Fng and Ser in dorsal cells. Notch activation along the D–V boundary leads to upregulation of Vg, from its boundary enhancer, as well, in the future wing region, Wg. d) Schematic cross-section illustrating subdivision of the wing disc into DP and PE cell layers. Expression of Vn, Wg, and Lines promotes DP fate and represses PE fate and Bowl.

Iro-C transforms notum cells into wing hinge cells. Vn is required to promote Iro-C expression in dorsal cells of the wing disc (Wang *et al.* 2000; Zecca and Struhl 2002a) (Fig. 4b). Conversely, Wg contributes to the transcriptional program that defines the future wing, which is first distinguished by elevated expression of Vg and reduced expression of Teashirt (Tsh) (Williams *et al.* 1993; Couso *et al.* 1995; Wu and Cohen 2002) (Fig. 4b). This is soon followed by repression of Homothorax (Hth) expression, and activation of Nubbin (Nub) expression (Ng *et al.* 1996; Azpiazu and Morata 2000; Zirin and Mann 2004). Dpp signaling has a complex role in this initial subdivision of the wing disc. After the initial contribution of Dpp in peripodial cells to activating Vn expression, Dpp, which becomes more highly expressed in the ventral half of the DP, plays a key role in repressing notal fates by repressing expression of Iro-C genes (Cavodeassi *et al.* 2002).

Around the same time that the wing disc is subdivided into wing vs body wall regions, it also becomes subdivided into peripodial vs DP regions. This subdivision also depends upon Wg and EGFR signaling, but in this case both of these pathways suppress peripodial fate (Fig. 4d) (Baena-López *et al.* 2003). Thus, it appears that different combinations of the same signals differentiate wing, notum, and PE, with EGFR promoting notum, Wg promoting wing, and absence of both signals resulting in PE. It has also been reported that formation of PE requires nonautonomous contributions from Hh and Dpp signaling (McClure and Schubiger 2005). The requirement for Dpp might now potentially be explained by its role in promoting Vn expression (Paul *et al.* 2013).

A key transcription factor in the initial specification of PE is Bowl (Fig. 4d) (Nusinow *et al.* 2008). Bowl protein is detected specifically in peripodial cells, and not in DP cells, due to the action of Lines, which is active in DP cells and promotes degradation of Bowl. Broad and early loss of Bowl can convert PE into DP cells, and complementarily, loss of Lines can convert DP cells into PE cells. However, later maintenance of distinct PE and DP fates does not depend upon Bowl. The mechanism that establishes the differential activity of Lines has not been described but would seem likely to be downstream of Wg and EGFR signaling. Elongation of columnar cells in the future wing is promoted by Dpp and Wg signaling (Widmann and Dahmann 2009a,b). Consistent with this, misexpression of Spalt (Sal) complex genes, which are targets of Dpp signaling, can alter the morphology of PE cells toward that of columnar cells (Tang *et al.* 2016). Moreover, misexpression of Lines in PE cells allows expression of Sal, and this contributes to a peripodial to columnar cell transformation, because the transformation is suppressed if Sal expression is knocked down (Tang *et al.* 2016).

Compartmentalization of the wing disc

Development of the wing disc is critically dependent upon its subdivision into orthogonal A–P and dorsal–ventral (D–V) regions called compartments, as communication between cells in these compartments establishes signaling centers that direct further wing patterning and growth. A compartment boundary forms when a mechanism for separating cells is coupled to heritable control of gene expression that defines positional identity. This creates distinct populations of nonintermixing cells. Conversely, noncompartmental subdivisions form when the maintenance of spatially distinct gene expression profiles depend upon cells' position rather than their lineage. For example, in the wing disc, the A–P and D–V subdivisions are compartmental, but the subdivisions into notum vs wing, or peripodial vs columnar epithelium, are not.

Compartments were first discovered in the *Drosophila* wing but subsequently identified in a variety of invertebrate and vertebrate tissues (Irvine and Rauskolb 2001; Dahmann *et al.* 2011). The discovery of compartments was made possible by the development of techniques for genetically marking individual cells and their descendants through induction of mitotic recombination. This led to the observation that the spatial distributions of clones of cells respect a boundary along the middle of the wing (Garcia-Bellido *et al.* 1973). For example, individual clones are always composed of only anterior or only posterior cells. Moreover, in most of the wing clone boundaries are irregular, but along the middle of the wing they form a straight line that demarcates the A–P compartment boundary. Remarkably, this boundary does not correspond to any distinct morphological features that can explain how cells are separated. Moreover, the boundary is maintained even when cells in 1 compartment are given a growth advantage compared with cells in the other compartment by using dominant slow-growth mutations called *Minutes* (Garcia-Bellido *et al.* 1976). The anterior–posterior compartmental subdivision of the wing begins during embryogenesis even before the disc primordia form (Garcia-Bellido *et al.* 1973, 1976; Wieschaus and Gehring 1976). A–P compartmentalization is dependent upon the posterior-specific expression of En (Morata and Lawrence 1975; Kornberg 1981), which is established in the embryo and then maintained by stable inheritance of the chromatin state (Moazed and O'Farrell 1992; Breen *et al.* 1995; DeVido *et al.* 2008).

Around the beginning of the second larval instar, the wing disc is further subdivided into distinct dorsal and ventral compartments (Garcia-Bellido *et al.* 1976), specified by dorsal expression of the transcription factor Apterous (Ap) (Cohen *et al.* 1992; Diaz-Benjumea and Cohen 1993; Blair *et al.* 1994) (Fig. 4c). The dorsal-specific expression of Ap is promoted during the second instar by EGFR signaling (Wang *et al.* 2000; Zecca and Struhl 2002b). Ap expression initially overlaps Iro-C expression, but during second instar Ap becomes expressed in a broader domain that encompasses the dorsal half of the wing primordia as well as the notal region (Wang *et al.* 2000; Zecca and Struhl 2002a). This is thought to occur as a consequence of *ap* transcription being only transiently dependent upon EGFR signaling, and then heritably maintained independent of EGFR through autoregulation and maintenance of the chromatin state (Zecca and Struhl 2002b; Oktaba *et al.* 2008; Bieli *et al.* 2015). As the wing disc grows, this results in Ap being expressed in a broader domain than Iro-C, which continues to require EGFR signaling (Zecca and Struhl 2002b). The separation of the Ap domain from the Iro-C domain is essential for allowing the formation and growth of the future wing (Zecca and Struhl 2002a; Rafel and Milan 2008).

Signaling between compartments

Along the A–P compartment boundary, P cells signal to A cells through the Hh pathway. Hh is expressed specifically by posterior wing disc cells and can only productively signal to anterior wing disc cells because En represses expression of the Hh pathway transcription factor Cubitus interruptus (Ci) in posterior cells (Eaton and Kornberg 1990; Zecca *et al.* 1995) (Fig. 4a). Repression of Ci by En also plays a key role in establishing the posterior-specific expression of Hh, as Ci represses Hh in anterior cells both directly, and indirectly, through regulation of scribbler (sbb, also known as mtv) (Methot and Basler 1999; Apidianakis *et al.* 2001; Bejarano *et al.* 2007; Bejarano and Milán 2009). The complementary expression of Hh and Ci result in Hh pathway activation in a stripe of cells along the anterior side of the A–P compartment border, with the width of this stripe corresponding to the distance

over which Hh can productively spread. Hh signaling is best known in the wing disc for inducing expression of the BMP family member Dpp (Basler and Struhl 1994). Dpp has profound effects on wing patterning and growth, consequently manipulations of Hh signaling can have similarly dramatic effects (Basler and Struhl 1994; Tabata and Kornberg 1994).

Signaling across the D–V boundary is mediated by the Notch pathway, which is activated along both sides of the boundary (Fig. 4c). Notch is activated in dorsal boundary cells by signaling from the Notch ligand Delta (Dl) and in ventral boundary cells by signaling from the Notch ligand Serrate (Ser) (Diaz-Benjumea and Cohen 1995; de Celis *et al.* 1996; Doherty *et al.* 1996). The differential signaling of Dl and Ser is regulated by Fringe (Fng) (Irvine and Wieschaus 1994; Kim *et al.* 1995; Fleming *et al.* 1997; Panin *et al.* 1997; Klein and Arias 1998b), which inhibits Ser binding to Notch and enhances Dl binding to Notch by glycosylating the Notch extracellular domain (Brückner *et al.* 2000; Moloney *et al.* 2000; Xu *et al.* 2007). The dorsal-specific expression of Ser and Fng, established by Ap, combine to limit Ser to signaling to ventral wing cells, while the presence of Fng and the cis-inhibition of Notch ligands leads Dl to preferentially signal to dorsal, Fng-expressing cells (de Celis and Bray 1997; Panin *et al.* 1997; LeBon *et al.* 2014). Targets of Notch activation at the D–V boundary, including Wg and Vg (Kim *et al.* 1995; Rulifson and Blair 1995; Kim *et al.* 1996), play key roles in wing patterning and growth, and consequently interactions between dorsal and ventral cells are essential for wing development (Diaz-Benjumea and Cohen 1993; Irvine and Wieschaus 1994).

Separating cells into distinct compartments

Compartmentalization requires a mechanism for separating cells, and early hypotheses suggested that transcription factors that specify compartmental identity might also regulate cell affinity, and thereby sort cells into distinct populations. However, the hypothesized cell affinity molecules proved elusive. A breakthrough in understanding compartmentalization then came with the realization that signaling between compartments plays a key role in separating them. For example, anterior cells that cannot receive the Hh signal can cross the A–P compartment boundary (Blair and Ralston 1997; Rodriguez and Basler 1997). The mechanism by which Hh signaling maintains the boundary remains only partially understood. One key factor appears to be the levels of Interference hedgehog (Ihog) and Brother of ihog (Boi) proteins, which are downregulated by Hh signaling. These 2 proteins act redundantly as Hh coreceptors, but can also mediate cell adhesion and contribute to A–P cell segregation in wing discs independently of their function as Hh receptor components (Hsia *et al.* 2017). Nonetheless, Ihog/Boi cannot completely explain the segregation of cells to A and P compartments, and there is also evidence for a contribution of Dpp signaling (Shen and Dahmann 2005), and a role for actomyosin-mediated tension (Landsberg *et al.* 2009; Rudolf *et al.* 2015).

Multiple mechanisms also contribute to separation of dorsal and ventral cells. The LRR proteins Capricious (Caps) and Tartan (Trn) are expressed specifically by dorsal cells during second and early third instar, and their expression contributes to segregation of dorsal and ventral cells, presumably by mediating cell adhesion (Milán *et al.* 2001). Intercompartmental signaling is also essential to maintenance of the D–V compartment boundary (Micchelli and Blair 1999; Rauskolb and Irvine 1999). Notch signaling across the D–V boundary establishes a line of elevated cytoskeletal tension, including elevated levels of F-actin and myosin (Major and Irvine 2005, 2006; Aliee *et al.* 2012). This upregulation of cytoskeleton tension keeps cells separated and maintains the straightness of the boundary. Cytoskeletal regulation by Notch cannot be explained by the canonical Notch transcriptional pathway, and the mechanism by which Notch signaling regulates cytoskeleton tension at the D–V boundary remains unknown. Differential expression of Caps and Trn can also contribute to elevated tension along the D–V boundary (Michel *et al.* 2016). At late third instar additional mechanisms, including a zone of nonproliferating cells (ZNC) established downstream of Notch signaling, also contribute to maintenance of the D–V boundary (O'Brochta and Bryant 1985; Becam *et al.* 2011).

Wing disc morphogens

Compartment boundaries play a fundamental role in wing disc patterning by acting as sites of production for Dpp and Wg, which spread from compartment boundary cells to direct the expression patterns of genes throughout the developing wing disc. Since the shape of the boundary can affect the distribution of these signals, it has been suggested that the relatively straight and smooth compartment boundaries are important in part because they provide a reproducible morphogen distribution (Dahmann and Basler 1999). The concept of a morphogen was first proposed by Turing (1952), who suggested that specification of different cell types in different places could be explained by molecules that would exhibit spatial differences in concentration and specify different fates according to their concentration. A simple way to produce a concentration gradient is to have a localized source, such as the stripes of Wg or Dpp expression along compartment boundaries, together with a "sink" that removes molecules and thus prevents them from accumulating to uniformly high levels throughout the tissue (Crick 1970). This mechanism also correlates the concentration of the morphogen to distance from the source, and so provides a means for specifying position within a tissue, a concept which has become central to our understanding of morphogens (Wolpert 1969; Sharpe 2019). Some of the first compelling tests arguing for the existence of morphogens in a cellular system were performed on Wg and Dpp within the wing disc. The importance of Dpp and Wg signaling to wing development, combined with the tractability of the wing disc as a model, has stimulated decades of study centered on their roles in wing patterning, including whether they act as morphogens, how they spread through tissues, and how they promote growth. In parallel, numerous investigations have taken advantage of the wing disc to identify and characterize components of these and other signaling pathways that play key roles in wing disc patterning.

The Dpp morphogen gradient

Dpp regulates the expression of genes that are activated or repressed in broad domains surrounding the stripe of Dpp transcription, including *optomotor-blind* (*omb*) and the Sal complex genes *spalt major* (*salm*) and *spalt-related* (*salr*) (Fig. 5a). The long-range action of Dpp on downstream target genes was demonstrated by contrasting the nonautonomous effects of Dpp to the cell-autonomous effects of loss or activation of the Dpp receptor Thickveins (Tkv) (Nellen *et al.* 1996; Lecuit and Cohen 1998). Moreover, a gradient of Dpp protein decreasing away from the A–P boundary has been visualized using Dpp:GFP transgenes (Entchev *et al.* 2000; Teleman and Cohen 2000). A gradient of Dpp pathway activity can be visualized using an antibody against phosphorylated Mad, a key transcription factor of the Dpp pathway, although this gradient differs in shape from the Dpp protein gradient due to modulation of Dpp receptor levels (Tanimoto

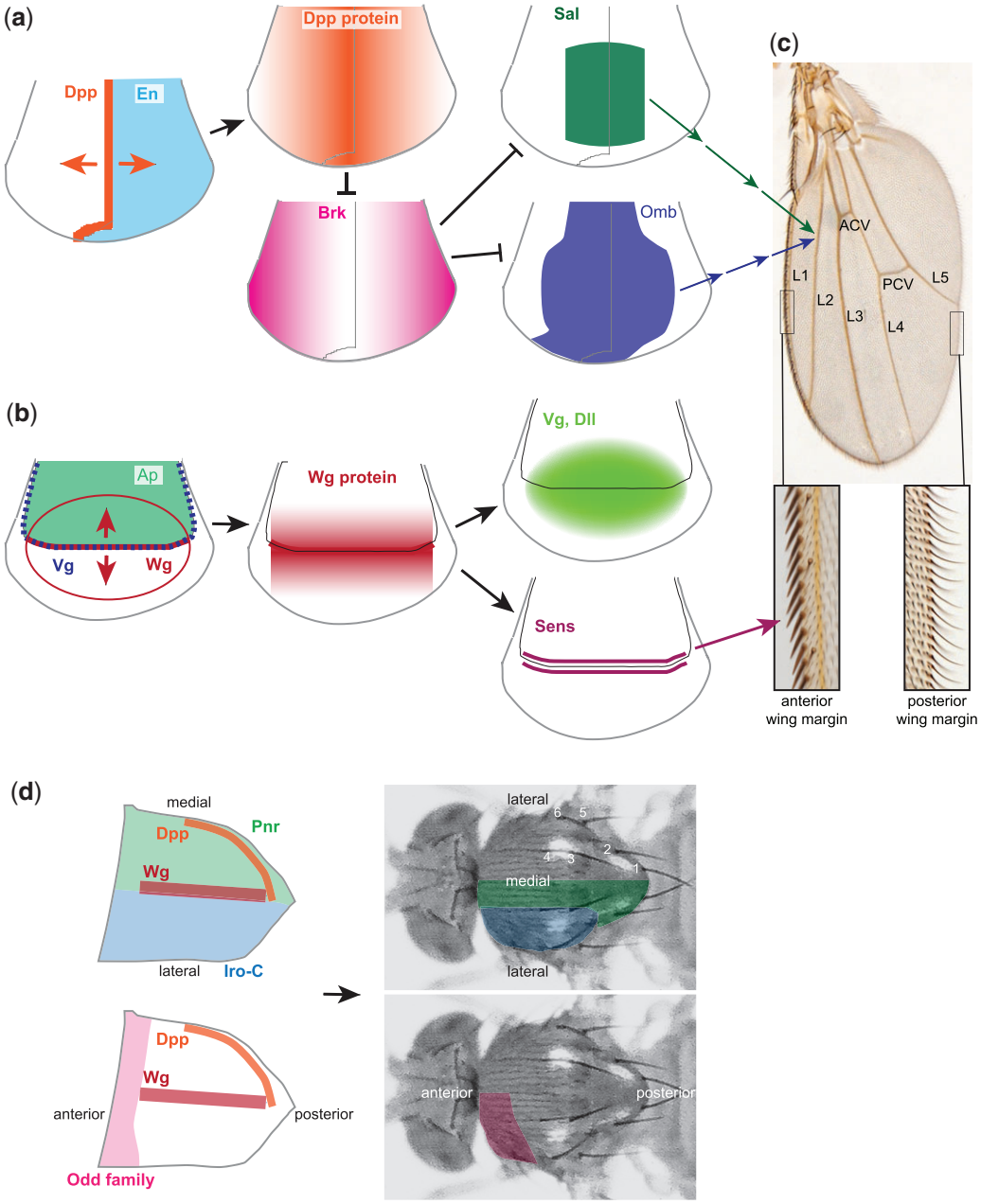

Fig. 5. Patterning by wing disc morphogens. Patterning of the wing region. a) Dpp spreads from the A–P boundary, forming a protein gradient that leads to graded repression of Brk. Brk represses expression of Sal complex genes and Omb, which are expressed in different domains due to different sensitivities to Brk levels. Several genes expressed in different A–P domains downstream of Dpp ultimately act in concert to position L2 and L5 wing vein primordia. b) Wg spreads from the D–V boundary, forming a protein gradient that promotes expression of Sens at high levels, and Vg and Dll at lower levels. Expression of the proneural gene ac (data not shown) overlaps *sens* but is only found in anterior cells, where sensory bristles will form. c) Adult wing patterning established by wing disc patterning includes positions of the wing veins and the wing margin bristles and hairs, shown at higher magnification in the boxes at bottom. d) Patterning of the notal region includes subdivision into a medial Pnr-expressing region and a lateral Iro-C-expressing region; differences amongst the different members of the Iro-C also occur during third instar (Ikmi *et al.* 2008). Multiple members of the odd protein family are expressed in the more anterior region of the notum. The adult notum is formed by fusion of the heminota from the left and right wing discs. Notal patterning defines regions where mechanosensory bristles form; some of the macrochaete are identified as in Fig. 1.

et al. 2000; Teleman and Cohen 2000). The argument that Dpp acts as a morphogen in the wing disc was further supported by observations that lower levels of Dpp pathway activity are needed to promote expression of Omb, whereas higher levels of pathway activity are needed to promote expression of Sal, which can explain why Omb is normally expressed in a broader domain than Sal (Nellen *et al.* 1996). Regulation of Omb and Sal by Dpp is mediated through creation of an inverse gradient of the transcriptional repressor Brinker (Brk) (Fig. 5a), which is repressed by Dpp signaling (Campbell and Tomlinson 1999; Jaźwińska *et al.* 1999; Minami *et al.* 1999; Müller *et al.* 2003).

How does Dpp spread through the wing disc?

The wing disc has provided an outstanding system for investigating how long-range secreted signals spread through tissues. Models that have been proposed for how Dpp spreads from the A–P boundary to more lateral regions of the wing disc include transcytosis, transport through cytonemes, and diffusion

(Matsuda et al. 2016). Transcytosis was suggested by observations that endocytosis is required for formation of the Dpp gradient (Entchev et al. 2000). However, others have argued that the effects of endocytosis could be explained by altered levels of cell surface receptors (Lander et al. 2002), and direct analysis did not reveal requirements for endocytosis or Tkv in the spread of Dpp (Belenkaya et al. 2004; Schwank et al. 2011a). It has also been proposed that long-range Dpp signaling could be mediated through cytonemes (Ramírez-Weber and Kornberg 1999; Hsiung et al. 2005; Roy et al. 2011, 2014). This is suggested by observations that cytonemes from lateral cells orient toward and contact Dpp-expressing cells, and that Tkv can be observed moving along cytonemes. However, the contribution of cytonemes to Dpp gradient formation and signaling in wing disc epithelial cells remains unclear, as experimental tests of the consequences of cytoneme disruption (Roy et al. 2014) rely on manipulations that could also affect other processes. The most popular explanation for how Dpp spreads through the wing disc is that of extracellular diffusion, although there remains disagreement over whether Dpp gradient formation is best explained by free diffusion or by diffusion that is restricted through binding to receptors and glypicans. Glypicans are heparan sulfate proteoglycans attached to the cell-surface through glycosylphosphatidylinositol (GPI) anchors. They regulate developmental signaling pathways by binding secreted signaling molecules, and they have been implicated at various steps of signaling including control of movement, stability, signaling, and intracellular trafficking (Yan and Lin 2009). It is clear that both levels of Tkv (Lecuit and Cohen 1998; Tanimoto et al. 2000), and levels of the 2 *Drosophila* glypicans, Dally and Dally-like (Dlp) (Fujise et al. 2003; Belenkaya et al. 2004; Akiyama et al. 2008), influence the shape of the Dpp gradient, although it has also been argued that this could reflect effects on steps other than diffusion, and that the best explanation for how Dpp spreads through tissue is simply free diffusion (Zhou et al. 2012).

An elegant recent test of parameters that influence the shape of morphogen gradients used GFP and synthetic GFP-binding proteins based on nanobodies to mimic the Dpp morphogen gradient in the wing disc (Stapornwongkul et al. 2020). These experiments revealed that free diffusion could generate a gradient in the wing disc, however, a gradient approximating the shape of the normal Dpp gradient required a combination of high affinity, signaling receptors, and low affinity, nonsignaling, GPI-anchored binding proteins, presumably mimicking the contribution of glypicans. The low affinity binding proteins limit leakage of free GFP outside of the wing disc and may also contribute to gradient formation by diffusing within and between cells. Thus, observations of synthetic GFP gradient formation suggest that restricted diffusion plays a key role in Dpp gradient formation.

Dpp gradient scaling

The Dpp gradient is maintained over days during larval development, during which its relative size adapts to the increasing size of the wing disc. This raises the question of how the size and shape of the gradient is adjusted to match the altered dimensions of a growing disc, a process referred to as scaling. Three key factors have been identified that influence the shape of the Dpp gradient and are regulated by Dpp signaling, thus providing potential mechanisms for scaling: Dpp receptors, glypicans, and a secreted, feedback regulator of Dpp signaling, Pentagone (Pent). Pent, which is repressed by Dpp signaling, interacts with and promotes endocytosis of glypicans to broaden Dpp distribution in the wing disc, and has been suggested to play an essential role in gradient scaling (Vuilleumier et al. 2010; Ben-Zvi et al. 2011; Hamaratoglu et al. 2011; Norman et al. 2016). However, a more recent study reported that Pent could not explain scaling throughout the entire wing disc and proposed instead that feedback regulation of Dpp receptors and glypicans is also required to account for gradient scaling (Zhu et al. 2020).

The Wg signaling gradient

Although Wg is initially broadly expressed in the ventral region of the second instar wing disc, by early third instar this broad expression disappears and Wg transcription in the distal wing becomes concentrated along the cells straddling the D–V compartment boundary, where Notch is active (Couso et al. 1993; Williams et al. 1993; Diaz-Benjumea and Cohen 1995; Rulifson and Blair 1995) (Fig. 5b). Expression of downstream targets of Wg signaling like Vg and Dll can be detected up to 15–20 cells away from the D–V boundary in late third instar wing discs. A Wg protein gradient declining away from the boundary can be directly visualized by antibody staining (Neumann and Cohen 1997; Strigini and Cohen 2000), and higher levels of Wg expression are needed to induce expression of genes or reporter constructs that are normally expressed closer to the D–V boundary, consistent with a concentration-dependent response to Wg (Zecca et al. 1996; Neumann and Cohen 1997). Direct, long-range effects of Wg were demonstrated by comparing the nonautonomous effects of Wg expression to the cell autonomous effects of transgenes that activate or block the Wg signaling pathway (Zecca et al. 1996; Neumann and Cohen 1997). In addition, although Wg is normally a secreted protein, an active, membrane-tethered form (Nrt-Wg) could be created, which rather than acting at long range only activates Wg signaling in neighboring cells (Zecca et al. 1996). While these observations implied that Wg acts as a morphogen in the wing disc, subsequent studies called this into question. Most strikingly, it was found that replacing endogenous Wg with the membrane tethered Nrt-Wg could still support development of nearly normal (though smaller) wings, apparently arguing against the importance of long-range diffusion or formation of spatial gradients for wing development (Alexandre et al. 2014). However, more recently studies have revealed that Nrt-Wg can actually be detected several cells away from its site of synthesis on the D–V boundary (Chaudhary et al. 2019). In addition, a feedback loop with the Wg receptor Frizzled 2 (Fz2), which is downregulated by Wg signaling, contributes to long-range signaling even in the absence of detectable Wg (Chaudhary et al. 2019), and the authors' experiments implied that Wg could directly signal up to 11 cells away from the D–V boundary, but longer range effects were dependent upon Fz2. However, it is not yet clear if the effects of Fz2 on cells apparently outside the range of Wg secreted from D–V boundary cells reflect true ligand-independent signaling, a persistence of response to earlier exposure to Wg, or a heightened response to undetectably low levels of Wg.

Investigations into how Wg spreads through the wing disc have paralleled investigations of how Dpp spreads, including suggestions of spread by transcytosis, cytonemes, free diffusion, or restricted diffusion. An added complication for Wg is that due to a lipid modification that is essential for its activity (Willert et al. 2003), it is not very soluble as a free protein, and associates with lipid binding proteins (Panáková et al. 2005). As for Dpp, levels of both signaling receptors and glypicans modify the Wg gradient (Baeg et al. 2001; Franch-Marro et al. 2005; Schilling et al. 2014). However, the 2 *Drosophila* glypicans have distinct roles in Wg signaling, with Dally contributing to active signaling as a coreceptor and Dlp required for spread of Wg through the wing disc (Lin and

Perrimon 1999; Baeg *et al.* 2001; Franch-Marro *et al.* 2005; Han *et al.* 2005; McGough *et al.* 2020). An explanation for the distinct role of Dlp in the spread of Wg has been provided by the discovery that Dlp associates with Wg through its lipid moiety (McGough *et al.* 2020). As for Dpp, the key role of Dlp in the long-range spread of Wg would seem to favor an important contribution of restricted diffusion to the spread of Wg through the wing disc, although this does not exclude the possibility that some fraction of Wg spreads by other mechanisms, and it has been reported, for example, that disruption of HSPG synthesis compromises the formation or stability of cytonemes (Bischoff *et al.* 2013).

Proximal–distal wing patterning

In addition to A–P and D–V patterning, appendages like the wing also have a proximal–distal (P–D) axis, with distinct cell fates specified at different distances from the body. Wg and Dpp act combinatorially in the wing disc to regulate P–D patterning of the developing wing by promoting expression of distally expressed genes and repressing expression of proximally expressed genes (Williams *et al.* 1993; Ng *et al.* 1995; Kim *et al.* 1996; Klein and Arias 1998a; Azpiazu and Morata 2000; Wu and Cohen 2002; Weihe *et al.* 2004; Zirin and Mann 2004). Indeed, the intersection of the A–P and D–V compartment boundaries defines the center of the developing wing pouch, and ultimately, the distal tip of the adult wing (Fig. 6a). The main subdivision of the wing field is between the distal wing and proximal wing regions. The distal wing forms the wing pouch in the larval disc and the wing blade in the adult. The proximal wing and wing hinge form structures at the base of the wing. The terms proximal wing and wing hinge are often used interchangeably, although formally they are distinct, with the proximal wing cells in between the distal wing and the wing hinge (Bryant 1975a; Diez del Corral *et al.* 1999). The distal wing is characterized by expression of Vg and Scalloped (Sd) (Campbell *et al.* 1992; Williams *et al.* 1993), whereas the proximal wing is characterized by expression of Teashirt (Tsh), Hth, and Zn finger homeodomain 2 (Zfh2) (Azpiazu and Morata 2000; Casares and Mann 2000; Wu and Cohen 2002; Whitworth and Russell 2003). The expression patterns of these genes are partially overlapping, and additional genes including *rotund* (*rn*), *nab*, *nub*, *elbow B* (*elB*), *no ocelli* (*noc*), and *defective proventriculus* (*dve*) have been identified that are expressed in distinct proximal–distal domains, establishing different subregions of the developing wing (Ng *et al.* 1995; St Pierre *et al.* 2002; Weihe *et al.* 2004; Terriente Félix *et al.* 2007) (Fig. 6a). Proximal–distal patterning of the wing also depends upon mutually repressive interactions between distally and proximally expressed genes (Azpiazu and Morata 2000; Casares and Mann 2000; Wu and Cohen 2002; Whitworth and Russell 2003; Weihe *et al.* 2004).

Vg plays a key role in linking signaling from compartment boundaries to wing development. Vg is a transcriptional coactivator that partners with the DNA-binding protein Sd to specify future wing blade cells (Williams *et al.* 1991; Campbell *et al.* 1992; Williams *et al.* 1993; Halder *et al.* 1998; Paumard-Rigal *et al.* 1998; Simmonds *et al.* 1998). Vg and Sd are required for survival of wing pouch cells, and misexpression of Vg can transform cells in other imaginal discs toward wing blade fate (Kim *et al.* 1996; Liu *et al.* 2000). Regulation of Vg expression involves input from compartment boundary signals, including Dpp, Wg, and Notch (Couso *et al.* 1995; Kim *et al.* 1995, 1996; Neumann and Cohen 1996b; Kim *et al.* 1997; Klein and Arias 1998a), which act through 2 distinct enhancers: a boundary enhancer that responds to Notch activation, and a quadrant enhancer that requires Dpp and Wg signaling (Kim *et al.* 1996, 1997) (Fig. 6b). Activation of Vg from either enhancer also requires auto-regulation from Vg-Sd (Campbell *et al.* 1992; Williams *et al.* 1993; Halder *et al.* 1998; Paumard-Rigal *et al.* 1998; Simmonds *et al.* 1998; Klein and Arias 1998a; Zecca and Struhl 2007a), although at the quadrant enhancer Yorkie (Yki) can substitute for Vg (Zecca and Struhl 2010).

During early wing disc development, proximal wing fate is promoted by Wg (Klein and Arias 1998a; Casares and Mann 2000; Whitworth and Russell 2003). Cells that receive Wg rather than Vn, and fail to receive Notch activation, form proximal wing. While early specification of proximal wing fate depends upon the broad ventral expression of Wg, after this fades Wg becomes expressed in 2 concentric circles in the proximal wing: an inner ring induced during early third instar, and an outer ring induced during mid-third instar (Couso *et al.* 1993; Williams *et al.* 1993) (Fig. 6a). The inner ring is established by signaling from Vg-expressing distal wing cells (Ng *et al.* 1995; Liu *et al.* 2000; del Alamo Rodríguez *et al.* 2002) (Fig. 6b). This signaling is mediated by the Ds-Fat pathway and is regulated by the differential expression of Four-jointed (Fj) and Dachsous (Ds), which are activated and repressed, respectively, downstream of Vg (Cho and Irvine 2004; Zecca and Struhl 2010). These rings of Wg expression in the proximal wing are maintained through a positive regulatory loop with Hth (Azpiazu and Morata 2000; Casares and Mann 2000; del Alamo Rodríguez *et al.* 2002), and contribute to proximal wing and hinge patterning and growth. Hinge patterning also involves local activation of the Jak-Stat pathway, mediated by localized expression of the Jak-Stat pathway Unpaired (Upd) ligands (Ayala-Camargo *et al.* 2013; Johnstone *et al.* 2013). Dorsal hinge cells also express Drop (Dr, also known as Msh), which contributes to repression of Iro-C complex genes, thereby maintaining separation of wing hinge from notum (Villa-Cuesta and Modolell 2005).

Although different proximal–distal domains are not separated by strict lineage restrictions, cells in different regions tend not to intermix. This is evident when the expression of transcription factors that define distinct regions is altered. Thus, for example, clones of cells that are mutant for Iro-C genes within the notum tend to sort out from neighboring, Iro-C expressing cells (Villa-Cuesta *et al.* 2007), and clones of cells forced to express Vg in the proximal wing will sort out from neighboring cells that lack, or express only low levels of, Vg (Liu *et al.* 2000).

Patterning of the late third instar wing disc

The patterning of the wing disc established by Wg, Dpp and other signaling molecules is ultimately manifest in the placement of distinct structures at precise locations in the adult wing and notum. The cellular-level resolution needed to achieve this begins to appear around the end of larval development.

Wing margin

By late third instar, gene expression patterns characteristic of distinct cell types that will form along the edge of the wing, the wing margin, have been established. The margin is maintained by continued Notch activation along the D–V boundary, but at this stage Notch is activated by a feedback loop between D–V boundary cells, which express Wg in response to Notch activation, and flanking cells, which express Notch ligands in response to Wg signaling (de Celis and Bray 1997; Micchelli *et al.* 1997). Wing margin hairs and bristles are formed by these flanking cells. Anterior wing margin cells form mechanosensory and chemosensory bristles, while posterior wing margin

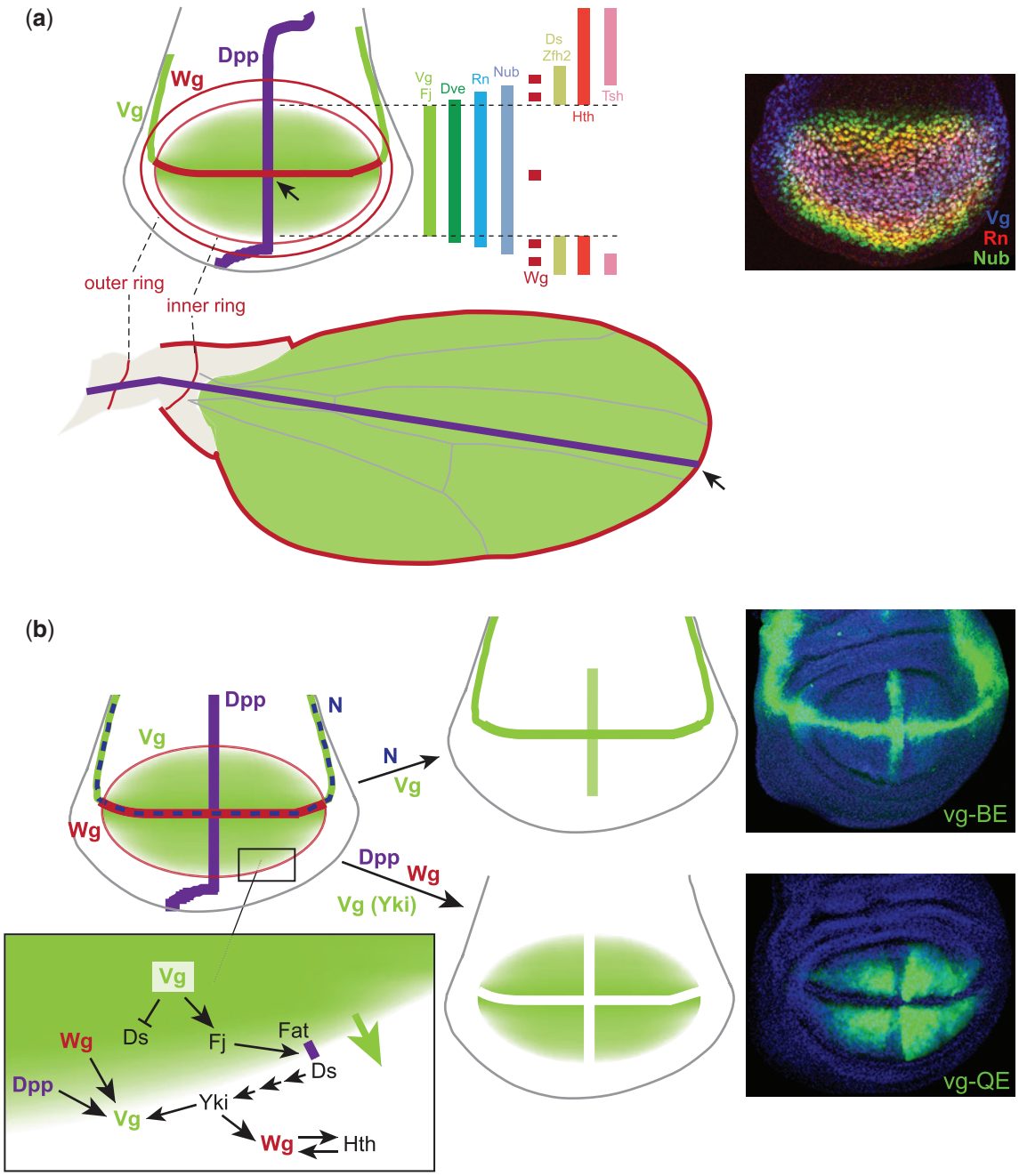

Fig. 6. Proximal-distal patterning in the wing. a) At left, schematics of the wing region depicting expression of Vg, Wg, and Dpp at top left in a disc and at bottom in a wing. The Wg and Dpp compartment boundary stripes intersect in the middle of the wing pouch, which will later correspond to the distal tip of the adult wing (arrows). Right of the disc schematic, the relative expression domains at late third instar of several genes involved in P–D patterning are indicated [adopted from Cho and Irvine (2004)]. Approximate location in the adult of Wg expression in the inner and outer proximal wing rings has been determined by staining *lacZ* enhancer trap lines (Neumann and Cohen 1996a; Liu *et al.* 2000). At far right, a mid-third instar disc stained for expression of Vg, Rn, and Nub is shown to illustrate P–D differences in gene expression [reproduced with permission from Cho and Irvine (2004)]. b) Illustration of Vg regulation. Vg expression in the wing pouch is mediated by distinct boundary (BE, responding to Notch) and quadrant (QE, responding to Wg and Dpp) enhancers, as shown in schematic form, and, at far right, staining of reporters. Notch is activated along the D–V boundary throughout third instar and then also along the A–P boundary at late third instar. Vg expression mediated by the quadrant enhancer can spread into more proximal cells through Ds-Fat signaling, illustrated in the box at bottom left. Ds-Fat mediated signaling from wing pouch cells also establishes the inner ring of Wg expression in the proximal wing.

cells form long, noninnervated, hairs (Fig. 5c). Proneural genes like *achaete* (*ac*) and *senseless* (*sens*) are upregulated by Wg signaling in cells adjacent to the Wg stripe on the D–V boundary (Fig. 5b) (Phillips and Whittle 1993; Couso *et al.* 1994; Rulifson and Blair 1995; Jafar-Nejad *et al.* 2006). These stripes of proneural gene expression then resolve into clusters of cells that give rise to the sensory organ precursor cells that will later form wing margin bristles.

Wing veins

The wing blade is formed from 2 main cell types—vein and intervein (Fig. 5c). The wing veins provide rigidity to the adult wing,

and tubes for tracheae, nerves and hemolymph (Blair 2007). Vein cells are more densely packed than intervein cells, and secrete thicker, more darkly pigmented cuticle. The *Drosophila* wing has 5 main longitudinal veins (L1–L5), which run along the length of the wing, and 2 main cross-veins (ACV and PCV), which run perpendicular to the longitudinal veins and connect L3 to L4 (ACV) and L4 to L5 (PCV). The longitudinal veins are specified in the late third instar wing disc, while the cross-veins are specified in the pupal wing disc. Vein formation is promoted by EGFR signaling, and expression of Rhomboid, which promotes activation of the EGFR ligand Spitz, is one of the earliest markers of longitudinal veins in the wing disc (Sturtevant *et al.* 1993). Intervein cells are defined by expression of Blistered (Bs), which promotes intervein fate and suppresses vein fate (Fristrom *et al.* 1994; Montagne *et al.* 1996).

The position of most longitudinal veins is specified downstream of the anterior–posterior patterning established by Hh and Dpp signaling. Distinct networks of genes regulate the positioning of each longitudinal vein, but they follow a common logic wherein veins are specified along the borders of genes expressed in different A–P domains. The most central veins, L3 and L4, which form straddling the A–P boundary, are positioned primarily by Hh signaling, which acts through regulation of Collier (Col). Cells receiving high levels of Hh signaling express Col and become intervein cells, whereas cells bordering Col expression form L3 and L4. (Mullor *et al.* 1997; Strigini and Cohen 1997; Biehs *et al.* 1998; Vervoort *et al.* 1999; Mohler *et al.* 2000). The more peripheral veins, L2 and L5, are positioned by Dpp signaling, which acts through regulation of several genes expressed in broad domains, including the Aristaless, Brk, Omb, Optix, Salm, and Salr transcription factors, which then define along their borders stripes of gene expression that will become L2 and L5 (Gómez-Skarmeta and Modolell 1996; Sturtevant *et al.* 1997; Lunde *et al.* 1998; de Celis and Barrio 2000; Cook *et al.* 2004; Sugimori *et al.* 2016; Martin *et al.* 2017a). Each of the provein stripes express distinct genes, including *knirps* and *knirps-related* in L2, *abrupt* in L5, and Iro-C genes in L3 and L5. The L1 vein forms along the anterior wing margin.

The veins are restricted to narrow stripes of cells by a negative feedback loop with Notch signaling, which promotes intervein fate and inhibits vein fate (de Celis and Garcia-Bellido 1994; Sturtevant and Bier 1995; de Celis *et al.* 1997; Huppert *et al.* 1997). EGFR signaling upregulates expression of Notch ligands in vein cells, which then signal to neighboring cells to repress vein fate. During pupal development, Dpp becomes upregulated along vein cells, and Dpp signaling plays an essential role in maintaining veins through a positive feedback loop with EGFR signaling (de Celis *et al.* 1997; Sotillos and De Celis 2005). Thus, an interplay between EGFR, Notch, and Dpp signaling positions the wing veins. Several genes that encode core components of these pathways were first identified through the effects of mutations on wing veins.

Notum

As for the wing, much of the patterning of the notum in the wing disc is initiated during larval stages, and Dpp and Wg play key roles (Calleja *et al.* 2002). The notum becomes subdivided into medial and lateral regions by the expression of the transcription factor Pannier (Pnr) in medial notum cells, while Iro-C complex genes become preferentially expressed in lateral notum cells (Calleja *et al.* 2000; Ikmi *et al.* 2008) (Fig. 5d). Iro-C gene expression is positioned through a combination of EGFR and Dpp signaling (Letizia *et al.* 2007), and there are some differences in expression pattern amongst the 3 family members (Ikmi *et al.* 2008). Pan and Ush expression are promoted by Dpp signaling in the medial notum (Tomoyasu *et al.* 2000). Dpp is expressed from the A–P compartment boundary, but most of the notum is anterior compartment, and Dpp levels are low in the lateral notum. Wg is expressed in a central stripe that is regulated downstream of Dpp, together with inputs from Pnr and Ush (Sato and Saigo 2000; Tomoyasu *et al.* 2000). Patterning of the notum along the A–P axis is mediated by multiple members of the odd-skipped family of genes, which are expressed in the anterior notum (Del Signore *et al.* 2012) (Fig. 5d).

Dpp and Wg, together with transcription factors they regulate in different regions of the notum, then determine the positioning of large mechanosensory bristles (macrochaetae) by regulating the expression patterns of both proneural genes of the achaete-scute complex (Haenlin *et al.* 1997; Garcia-Garcia *et al.* 1999; Calleja *et al.* 2000), together with genes that antagonize achaete-scute complex gene activity (Usui *et al.* 2008). The number and positioning of macrochaete is sufficiently precise that each has a unique name (Figs. 1 and 5d) (Stern 1955). Small mechanosensory bristles (microchaetae), which form in rows along the notum (Fig. 5d) arise from stripes of proneural gene expression that are patterned by Notch signaling during pupal development (Corson *et al.* 2017; Couturier *et al.* 2019). For both macro- and microchaete, a single sensory precursor (SOP) cell is selected from a cluster of cells expressing proneural genes; the SOP then then undergoes stereotyped divisions to form the mechanosensory organ. Patterning of the notum downstream of Wg and Dpp also determines sites of attachment for flight muscles by regulating the expression pattern of the *stripe* gene (de Celis *et al.* 1999; Ghazi *et al.* 2003).

Planar cell polarity of wing disc cells

Wing disc epithelial cells, like many other tissues, exhibit planar cell polarity (PCP). PCP is the polarization of cells within the plane of the tissue, perpendicular to apical–basal polarity, and is readily visible in derivatives of the wing disc through the orientation of hairs and bristles in the adult wing and notum (Fig. 7) (Mlodzik 2020). Indeed, the *Drosophila* wing has long been one of the primary models for discovery of PCP components and analysis of their functions (Gubb and García-Bellido 1982). Each epidermal cell makes a single hair, the location and orientation of which is controlled by PCP pathways (Wong and Adler 1993). Insect hairs are nonsensory actin-rich cellular extensions; it has been proposed that the wing hairs serve an aerodynamic role by guiding air flow over the surface of the wing (Wootton 1992). Insect bristles are multicellular mechanosensory and chemosensory organs; their orientation is also controlled by PCP pathways.

Two main PCP pathways have been described, the canonical, or Fz-dependent, PCP pathway and the Ds-Fat PCP pathway (Strutt and Strutt 2021) (Fig. 7, a and b). Components of each pathway localize near apical junctions, and their localization becomes polarized in conjunction with establishment of PCP. In the wing region of the wing disc, polarization occurs along the proximal–distal axis, and some components of each pathway localize to the proximal sides of cells, whereas other components localize to the distal sides. Proximally localized transmembrane proteins in 1 cell physically interact with distally localized proteins in the neighboring cells, which helps to establish, maintain, and propagate PCP.

Overall orientation of polarity in the Ds-Fat system is governed by expression gradients of 2 of the components: Ds, which is a large cadherin family protein that binds to Fat, and Fj, which is a Golgi-localized kinase that modulates binding between Fat

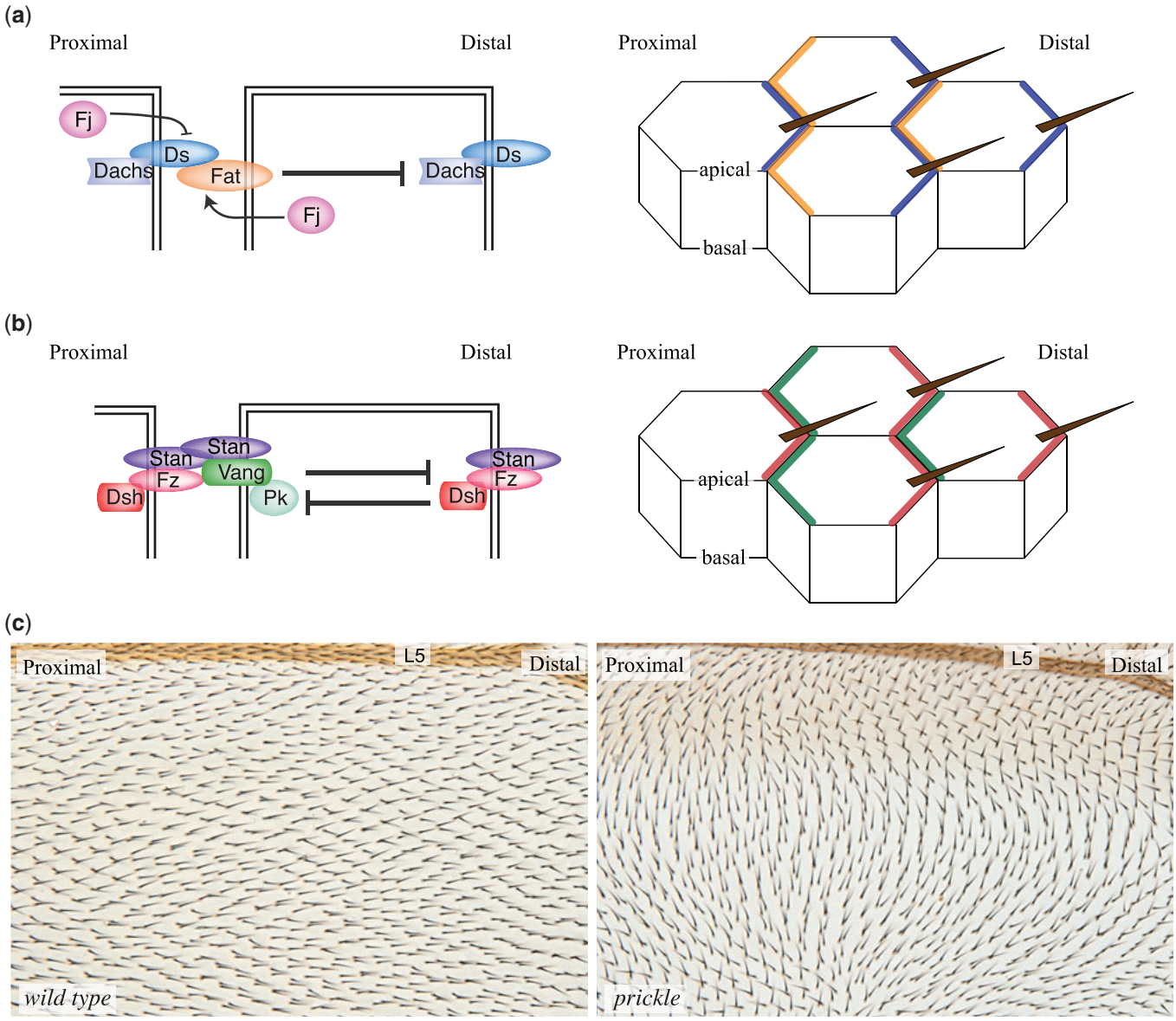

Fig. 7. PCP in the wing disc. a) Schematics illustrating polarization of proteins of the Ds-Fat PCP pathway in the wing disc. Left panel shows schematic cross-section through cells, right panel shows 3D perspective. At pupal stages, wing hairs (brown triangles), pointing distally, will form near the distal vertices of each cell. b) Schematics illustrating polarization of proteins of the Fz PCP pathway in the wing disc. Left panel shows schematic cross-section through cells, right panel shows 3D perspective. The Stan (also known as Flamingo) protein accumulates on both distal and proximal cell membranes. c) Example of PCP in the adult wing, illustrated by distally pointing hairs in wild-type, and misoriented hairs in a *prickle* mutant. Portions of this figure reproduced from Ambegaonkar and Irvine (2015).

and Ds by phosphorylating their extracellular domains (Matakatsu and Blair 2004; Ishikawa *et al.* 2008; Brittle *et al.* 2010; Simon *et al.* 2010). The Ds gradient, from proximal to distal in the wing, and the Fj gradient, from distal to proximal, combined with binding between Fat and Ds, result in cellular polarization of Ds and Fat with Ds accumulating distally and Fat accumulating proximally (Clark *et al.* 1995; Villano and Katz 1995; Ma *et al.* 2003; Cho and Irvine 2004; Matakatsu and Blair 2004; Strutt *et al.* 2004; Ambegaonkar *et al.* 2012; Brittle *et al.* 2012). Polarization of Ds and Fat leads to polarization of the Dachs protein, which is removed from apical membranes by Fat (Mao *et al.* 2006). The Ds and Fj gradients in the wing are established downstream of the Wg and Dpp gradients, at least in part through Vg. Ds and Fj expression is graded across the distal wing at early third instar, but flattens by the end of third instar, except near the edge of the wing pouch (Cho and Irvine 2004). Nonetheless, polarization is maintained, likely due to an ability to maintain polarization through cell division, combined with the propagation of polarization from expression boundaries at the edge of the wing pouch (Ambegaonkar *et al.* 2012; Wortman *et al.* 2017).

The mechanisms that direct overall orientation of polarity in the Fz system remain unclear (Sagner *et al.* 2012). Since 2 of the key components, Fz and Dsh, also participate in Wnt-β-catenin signaling, it had been thought that Wg or other Wnt proteins, which are expressed at the D–V boundary of the wing, might play a role in orienting Fz-PCP. Evidence for this was reported (Wu *et al.* 2013), but more recent studies have ruled out the possibility of Wnts contributing to orientation of Fz-PCP in the *Drosophila* wing disc (Ewen-Campen *et al.* 2020; Yu *et al.* 2020).

The 2 PCP pathways can act independently, but also cross-talk with each other (Adler *et al.* 1998; Strutt and Strutt 2002; Ma *et al.* 2003; Merkel *et al.* 2014; Olofsson *et al.* 2014). They are linked in some contexts by the expression of a particular isoform of the *prickle-spiny legs* (*pk-sple*) locus, which is one of the key components of the Fz-PCP pathway (Gubb *et al.* 1999). The Sple isoform can bind to Dachs and Ds, and when this form predominates the Ds-Fat pathway can direct the orientation of the Fz pathway (Ayukawa *et al.* 2014; Merkel *et al.* 2014; Ambegaonkar and Irvine 2015). Recognition of the genetic interaction between these pathways had led to the suggestion that Ds-Fat could be responsible for orienting Fz-PCP (Adler *et al.* 1998; Strutt and Strutt 2002; Ma *et al.* 2003; Matakatsu and Blair 2004), however, subsequent studies have revealed that this normally only occurs under conditions where Sple is the predominant isoform (Ayukawa *et al.* 2014; Merkel *et al.* 2014; Ambegaonkar and Irvine 2015). In the wing, for example, Pk is the predominant isoform (Olofsson *et al.* 2014), so the Fz pathway must be oriented by other, as yet unidentified, cues.

Although hair polarity is typically visualized in the adult, and hairs first form during pupal development, imaging, and conditional knock down experiments have revealed that PCP is actually established in the wing disc by the middle of the third instar, as revealed by the polarization of PCP proteins (Sagner *et al.* 2012). In the wing, there is a remarkable reorientation of polarity that occurs during pupal development. In the larval wing disc, PCP is largely oriented toward the D–V boundary, but in the adult wing it is largely oriented toward the distal tip of the wing. This reorientation is associated with oriented cell elongation, cell division, and cell rearrangement that occur during pupation as a result of contraction of the wing hinge, which generates an anisotropic tension that realigns PCP along the proximal–distal axis (Aigouy *et al.* 2010). PCP also orients ridges that form between cells in the adult wing (Doyle *et al.* 2008; Hogan *et al.* 2011). In the posterior wing, these ridges to not realign during pupation, and consequently there is a discordance between the orientation of PCP for wing hairs and wing ridges. In the notum, PCP is oriented along the anterior–posterior axis of the fly, and there are again roles for both Fz and Ds-Fat PCP pathways in orienting hairs and bristles (Gho and Schweisguth 1998; Lu *et al.* 1999; Adler 2012).

Growth of the wing disc

The wing disc undergoes extensive growth during larval development (Fig. 8), and studies in wing discs have yielded fundamental insights into diverse factors that control cell proliferation and organ size, including components of key growth-regulating pathways, and the roles of tissue patterning, metabolism, and mechanics on regulation of organ growth.

Parameters of wing disc growth

The wing disc normally grows through increases in cell number, rather than increases in cell size, and imaginal disc cells remain diploid. Notably, however, the size of the wing disc can be uncoupled from cell number through experimental manipulations of cell cycle regulators, which yield a relatively normally size wing disc or compartment even with altered cell sizes (Weigmann *et al.* 1997; Neufeld *et al.* 1998). There is relatively little apoptosis during wild-type wing disc development (Milán *et al.* 1997), so size regulation is achieved primarily through controlling cell proliferation rather than cell death.

Direct counts of labeled nuclei in the DP led to an estimate of 30,350±1,400 cells at end of third instar (Martín *et al.* 2009), although this did not include the peripodial cells. McClure and Schubiger (2005) estimated that at late third instar the DP has 39,200±1,170 cells, and the PE had 2,099±236 cells. Some cell division continues during pupal development (Milán *et al.* 1996a, 1996b), and by counting hairs in the adult derivatives of the wing and notum, Garcia-Bellido and Merriam (1971) estimated that the adult derivatives of the wing disc comprise ~52,000 cells, although this is a rough estimate as wing hinge cells were not counted. Considering that the wing disc starts from a population of ~25–30 cells in the embryo, we can infer that it undergoes a more than 1,000-times increase in cell number during larval development, which corresponds to roughly 10 cell divisions.

Both temporal and spatial variations in growth rates occur over these 10 cell divisions. Growth rates gradually decline as the

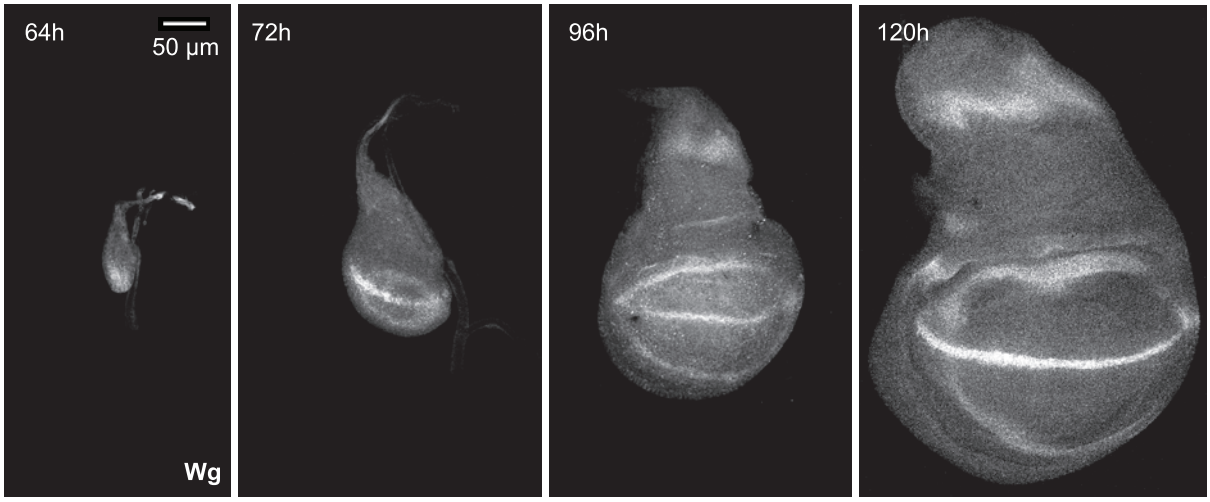

Fig. 8. Wing disc growth. Wing discs stained for Wg are shown at different times after egg laying to illustrate the growth of the wing disc. Embryogenesis, first instar and second instar each take ~1 day at 25°C, while third instar takes just over 2 days, so the disc shown correspond to mid second instar (64 h after egg laying), late second/early third instar (72 h), mid-third instar (96 h) and late-third instar (120 h). All discs are shown at the same magnification.

wing disc ages, with a doubling time of ~6 h during second instar increasing to ~30 h by the end of third instar (Garcia-Bellido and Merriam 1971; Bryant and Levinson 1985; Bittig *et al.* 2009; Martín *et al.* 2009). Small scale local variations in growth of clones or DNA replication can be detected, although for most of wing development growth rates are similar across different regions of the wing disc (González-Gaitán *et al.* 1994; Milán *et al.* 1996a, 1996b). However, there are exceptions to this. At late third instar, the ZNC occurs near the dorsal–ventral compartment border (O'Brochta and Bryant 1985), established downstream of Notch and Wg signaling (Johnston and Edgar 1998; Duman-Scheel *et al.* 2004; Herranz *et al.* 2008). Careful analysis of growth patterns at different stages of development led to realization that cells in the center of developing wing transiently grow at a faster rate than more proximal cells during early phases of wing disc growth (Mao *et al.* 2013). Quantitation of EdU labeling or clones sizes at different time points throughout wing disc development further revealed that this reverses in older wing discs, with proliferation rates slightly lower in the more distal parts of the wing disc when compared with more proximal regions (Johnston and Sanders 2003; Pan *et al.* 2018). Growth is also relatively lower in the hinge during early stages of larval development (Tozluoğlu *et al.* 2019). At the pupal stage, additional heterogeneities in cell proliferation appear, including between vein and intervein cells (Milán *et al.* 1996a, 1996b).

Transplantation experiments in which wing discs were dissected out of third instar larvae of different ages and then cultured in female abdomens for several days implied that wing discs grow to a preferred size regardless of the amount of time allowed for growth (García-Bellido 1965; Bryant and Levinson 1985). Similar conclusions have been reached without transplantation by genetically delaying pupariation (Parker and Struhl 2020; Strassburger *et al.* 2021). Thus, while the final size of an organ is a function of both the rate and duration of growth, there is a size control mechanism in wing discs that arrests growth when an appropriate size has been reached. Clonal analysis experiments have further implicated compartments as units of size control. Using *Minutes*, it is possible to create wing discs in which cells in the anterior or posterior compartment grow at a much different rate than cells in the complementary compartment. Remarkably, normal wings form, as the faster-growing compartment essentially stalls to let the slower growing compartment catch up near the end of larval development (Garcia-Bellido *et al.* 1976; Martín and Morata 2006).

Hippo signaling in wing discs

The Hippo signaling network contributes to the regulation of growth and cell fate throughout the metazoa (Misra and Irvine 2018; Zheng and Pan 2019). Many of the studies that first identified Hippo pathway components and deciphered their roles in the pathway were performed in wing discs, where mutations of pathway components can have dramatic effects on growth. Hippo signaling regulates the activity of the Yki transcriptional coactivator protein, which partners with Sd to activate target genes. Increased Yki activity in the wing disc stimulates growth, whereas loss of *yki* suppresses growth (Huang *et al.* 2005). Yki is inhibited by the upstream kinase Warts (Wts), which phosphorylates Yki to promote its cytoplasmic localization (Dong *et al.* 2007; Oh and Irvine 2008). Wts is activated by Hippo and related kinases, and Wts and Hippo are regulated by a wide variety of upstream cues. Indeed, a defining feature of the Hippo network is its sensitivity to diverse inputs, which enable it to integrate information about the physical environment, metabolism, and local patterning to modulate organ growth.

One key regulator of Hippo signaling in the wing disc is the Ds-Fat pathway. Regulation of the levels and localization of Dachs by gradients of Fj and Ds not only polarizes cells, it also, together with Dachs ligand with SH3s (Dlish, also known as Vamana), downregulates Wts and an additional upstream regulator of Yki, Expanded (Bennett and Harvey 2006; Cho *et al.* 2006; Silva *et al.* 2006; Willecke *et al.* 2006; Vrabioiu and Struhl 2015; Misra and Irvine 2016; Zhang *et al.* 2016). Consequently, manipulations of the Fj or Ds expression patterns can locally alter cell proliferation in the wing disc and influence wing size (Rogulja *et al.* 2008; Willecke *et al.* 2008). Complete loss of Dachs reduces the wing to less than half its normal size (Mao *et al.* 2006), whereas loss of Ds or Fat throughout the wing disc can increase its size (Bryant *et al.* 1988; Mahoney *et al.* 1991; Clark *et al.* 1995; Matakatsu and Blair 2006).

The Hippo pathway is also regulated in the wing disc by cytoskeletal tension. Even before a link to the Hippo pathway was made, theoretical considerations suggested that the crowding of cells in the center of the developing wing might inhibit growth, and act as a counter to growth-stimulating effects of wing disc morphogens (Aegerter-Wilmsen *et al.* 2007; Hufnagel *et al.* 2007). Artificial stretching of wing discs can stimulate cell proliferation (Schluck *et al.* 2013). Subsequent studies established that tension at AJ inhibited Wts by recruiting the Wts inhibitor Jub (Rauskolb *et al.* 2014; Sun *et al.* 2015). This recruitment is mediated through α-catenin, which can undergo a tension-dependent change in conformation (Yonemura *et al.* 2010; Rauskolb *et al.* 2014; Alégot *et al.* 2019; Sarpal *et al.* 2019). Experimental manipulations have also demonstrated suppression of Yki activity by growth-induced crowding (Pan *et al.* 2016), and correlated reductions in cytoskeletal tension that normally occur as cells become more crowded with decreasing Yki activity (Pan *et al.* 2018; Borreguero-Muñoz *et al.* 2019). In addition to changes in tension at AJ, it has also been proposed that cell shape could influence Hippo signaling by concentrating or diluting upstream regulators associated with cell–cell junctions, and consistent with this idea high levels of nuclear Yki have been observed in wing disc PE cells (Borreguero-Muñoz *et al.* 2019). Spectrins are also required for normal Yki activity in the wing disc (Deng *et al.* 2015; Fletcher *et al.* 2015), raising the possibility of additional modes of cytoskeletal regulation.

The Hippo pathway can also stimulate growth in wing discs in response to tissue damage or loss of cell polarity (Parsons *et al.* 2010; Grusche *et al.* 2011; Sun and Irvine 2011). These effects are mediated at least in part through activation of the Jnk pathway, which has also been proposed to contribute to normal wing disc growth through cross-regulation of Hippo signaling (Willsey *et al.* 2016). Loss of cell polarity or cell–cell contact may also modulate Hippo signaling through transmembrane proteins that participate in homophilic binding and acts as upstream regulators of the Hippo pathway, including Echinoid and Crumbs (Chen *et al.* 2010; Grzeschik *et al.* 2010; Ling *et al.* 2010; Robinson *et al.* 2010; Yue *et al.* 2012).

Metabolic pathways also cross-talk with Hippo signaling in multiple ways (Ibar and Irvine 2020). In wing discs, Insulin signaling can regulate the Hippo pathway (Straßburger *et al.* 2012), Akt, which is a key downstream factor in Insulin and mTor pathways, can influence Yki activity through a mechanism that appears to involve a novel phosphorylation of Hippo (Borreguero-Muñoz *et al.* 2019), and mTOR has been reported to regulate Yki activity independently of Hippo signaling (Parker and Struhl 2015).

Influence of A–P and D–V patterning on growth of the wing disc

A link between the patterning of the wing disc and its growth was first suggested by regeneration experiments (Bryant 1975b; Haynie and Bryant 1976). If part of a wing disc is excised, and the remaining part is cultured in a female abdomen, the disc fragment can grow until the missing part is regenerated (or, in the case of smaller fragments, duplicated) (Fig. 9a). If 2 disc fragments are fused, growth will "fill in" the missing tissue. Together with similar studies of intercalary regeneration in other models, this suggested that developing appendages possess positional information that controls their growth (French *et al.* 1976; Bryant *et al.* 1981).

The link between patterning and growth was solidified by the discovery that genes that play key roles in the patterning of the wing disc also control its growth. When cells with different compartmental identities are juxtaposed, for example by misexpression of En in anterior cells or misexpression of Ap in ventral cells, growth is stimulated, and when these manipulations create a new intersection of A–P and D–V compartment boundary cells, this growth can be organized into partial wing duplications (Diaz-Benjumea and Cohen 1993; Zecca *et al.* 1995). Similar effects can be generated by misexpressing genes that play key roles in signaling between compartments, including Hh and Fng (Capdevila and Guerrero 1994; Irvine and Wieschaus 1994; Tabata and Kornberg 1994; Zecca *et al.* 1995). Genes that participate in intercompartmental signaling are also required for the normal growth of the wing, which reflects the essential roles that induction of Dpp, Wg and Vg along compartment boundaries plays in wing formation.

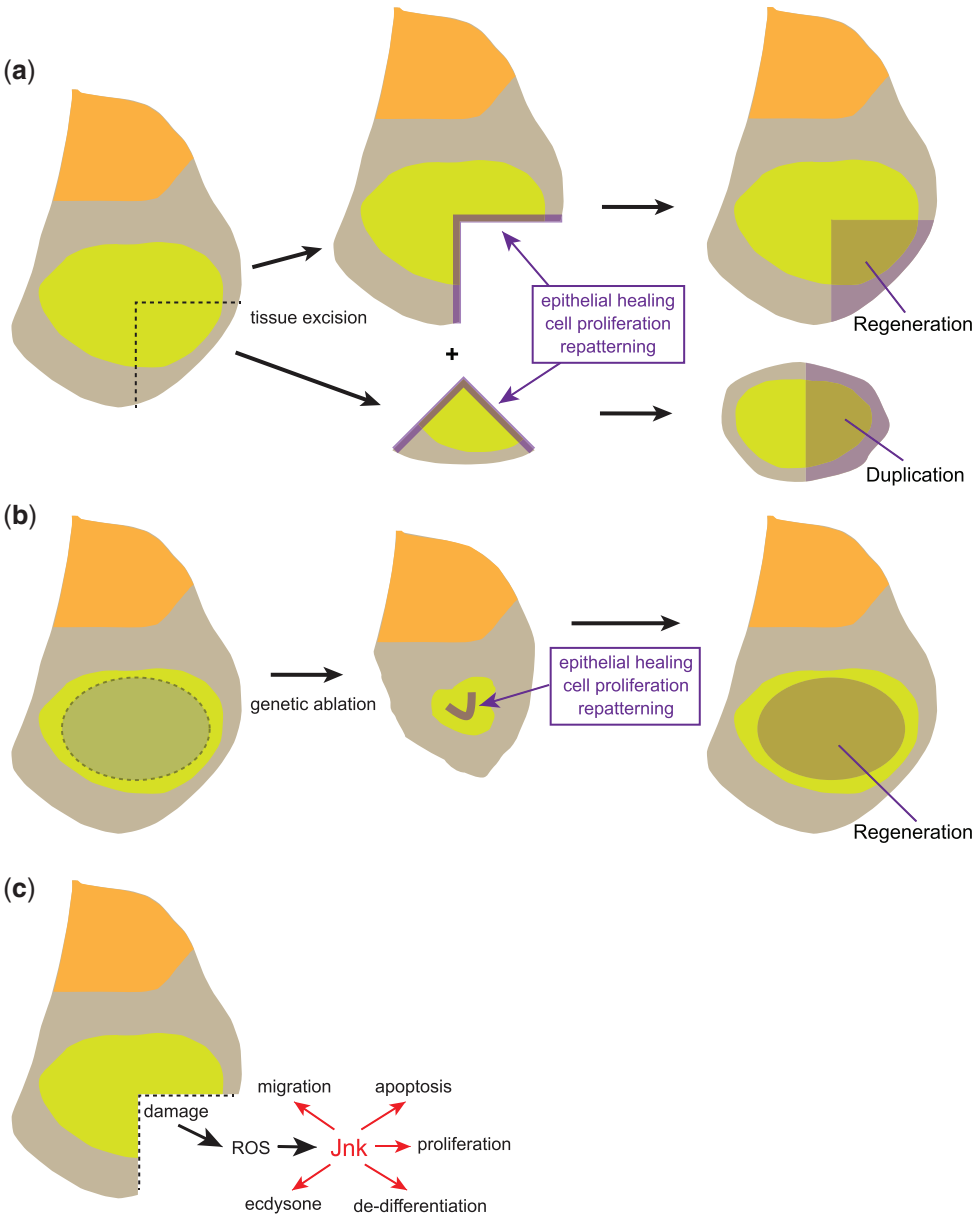

Fig. 9. Wing disc regeneration. a) When a portion of a wing disc is excised, the missing part can be regenerated through a process including healing of the epithelium, stimulation of cell proliferation near the cut edges, and tissue repatterning. Smaller fragments typically generate duplicated structures rather than regenerating. b) Wing disc regeneration can also occur after genetically induced ablation of cells in a defined region of the disc. c) The damage response is coordinated by Jnk, which is activated by ROS and triggers multiple responses that enable regeneration.

Growth control in the wing disc by Dpp

In addition to its central role in patterning the wing disc along the A–P axis, the morphogen Dpp is also required for normal wing disc growth and is able to induce overgrowth when misexpressed or over-expressed (Spencer *et al.* 1982; Capdevila and Guerrero 1994; Zecca *et al.* 1995). Experiments removing functional Dpp receptors, or deleting the expression or spread of Dpp from the A–P boundary, emphasize that Dpp signals directly to cells at a distance from the A–P boundary to promote growth (Burke and Basler 1996; Barrio and Milán 2017; Bosch *et al.* 2017; Matsuda and Affolter 2017). These observations raise a question that has been debated for over 30 years: how is the gradient of Dpp pathway activity interpreted to yield the relatively even distribution of growth that occurs for most of normal wing disc development?

The simplest explanation is the suggestion that the gradient does not matter—there is a threshold level of Dpp activity that promotes growth, above which wing cells continue to grow and below which they fail to grow. Consistent with this, experiments that effectively flatten the Dpp gradient can promote substantial wing growth (Barrio and Milán 2017; Bosch *et al.* 2017). The principal challenge to this has been the observation that increasing Dpp expression, or expressing activated forms of Tkv, stimulates wing overgrowth (Capdevila and Guerrero 1994; Zecca *et al.* 1995; Nellen *et al.* 1996). However, analysis of growth patterns reveals that cells in different regions respond differently to uniform increases in Dpp—in lateral regions, where Dpp levels are normally very low, increasing Dpp pathway activity stimulates growth, whereas in medial regions, where Dpp levels are normally high, uniformly increasing Dpp does not increase growth (Martín-Castellanos and Edgar 2002).

This leads to a second class of models, which suggest that the growth-promoting effects of Dpp are balanced by a growth inhibitor (Serrano and O'Farrell 1997). There is evidence for such inhibitors, although how each contributes remains to be clarified. One key factor is Brk. As for its effects on wing patterning, much of the influence of Dpp on wing growth is mediated through repression of Brk, which itself acts as a repressor of growth (Martín *et al.* 2004; Schwank *et al.* 2008). However, Brk cannot easily explain why the growth response to Dpp differs between medial and lateral cells, because Brk itself is a target of Dpp, and indeed it has been inferred based on examination of hypomorphic *dpp* mutants that the distinct growth responses of medial and lateral cells are not established by Dpp (Schwank *et al.* 2008). Alternatives that have been suggested include differences in Fat activity or Vg expression (Martín-Castellanos and Edgar 2002; Schwank *et al.* 2011b). Differences in cytoskeletal tension are also a factor, as by mid-third instar cells in the center of the wing have lower levels of cytoskeletal tension at adherens junctions than more proximal cells (Aegerter-Wilmsen *et al.* 2012; Pan *et al.* 2018), and increasing cytoskeletal tension preferentially increases cell proliferation in the medial part of the disc where Dpp signaling is highest (Pan *et al.* 2016).

An alternative explanation that has been proposed is that cells need to experience continually increasing amounts of Dpp in order to continue growing. This was suggested by observations that cells across different positions of the Dpp gradient and at different stages of wing disc development experience an ~50% increase in levels of Dpp signaling per cell division (Wartlick *et al.* 2011). However, the significance of this has been questioned based on observations that cells can continue to grow in the absence of Dpp signaling if *brk* is mutant (Schwank *et al.* 2012), and that Dpp can be provided by heterologous, uniform expression from the *tubulin* promoter and wings still grow (Bosch *et al.* 2017).

Models for intercalary regeneration led to the suggestion that it could be the gradient, rather than the absolute level, of Dpp activity that promotes wing growth (Day and Lawrence 2000). This was supported by observations that juxtaposing cells with different levels of Dpp pathway activity transiently stimulates cell proliferation in the medial wing, whereas high uniform levels of activity inhibit growth in the medial wing (Rogulja and Irvine 2005). It has received further support from connections between Dpp signaling and the Ds-Fat pathway, which provides a mechanism for the Dpp expression gradient to regulate growth (Rogulja *et al.* 2008). However, the suppression of medial growth observed with high level uniform activation of Dpp appears to be an indirect consequence of high level proliferation in lateral cells (Schwank *et al.* 2008). Additionally, when the normal source of Dpp along the A–P boundary is eliminated and replaced with a moderate level of uniform Dpp expression, the wing disc can still grow (Bosch *et al.* 2017).

In weighing the evidence for or against various models, we emphasize that there appear to be multiple mechanisms through which Dpp can promote wing growth, and experimental support for 1 model does not necessarily exclude another. Thus, it seems clear that some of the contribution of Dpp to wing growth can be explained by a simple threshold model, but other mechanisms can also contribute, although how much remains subject to debate. In threshold models, the distance over which Dpp can spread and productively signal is a key factor in determining the size of the wing disc (Barrio and Milán 2020; Parker and Struhl 2020; Zecca and Struhl 2021), although one also has to consider differences in responsiveness to Dpp that exist between medial and lateral regions.

Growth control in the wing disc by Wg

Wg expressed by D–V boundary cells is required for growth of the distal wing (Couso *et al.* 1994; Neumann and Cohen 1997). However, expression of Wg cannot substitute for loss of Notch activation at the D–V boundary, because Vg is also an essential Notch target there for promotion of wing growth (Kim *et al.* 1996; Klein *et al.* 1998; Klein and Arias 1998a). Wg signaling is normally graded, but low level uniform Wg expression can support wing growth (Baena-Lopez *et al.* 2009). Ectopic expression of Wg within the wing pouch has relatively little ability to promote growth, and high level Wg activity can actually inhibit growth in the distal wing (Neumann and Cohen 1996a; Klein and Arias 1998a; Johnston and Sanders 2003; Baena-Lopez *et al.* 2009). In contrast, elevated expression of Wg has strong mitogenic effects in the proximal wing, where the rings of Wg expression are also required for normal growth (Neumann and Cohen 1996a). Some reports have suggested that elevated Wg expression can increase growth in the proximal part of the wing pouch (Giraldez and Cohen 2003; Barrio and Milán 2020), but to the extent this has been reported it might be a consequence of the increased cell proliferation induced by Wg in the proximal wing, together with a shifting boundary between distal and proximal wing (Zecca and Struhl 2007b). Thus, most studies suggest that Wg has a potent ability to promote growth in the proximal wing, but in the distal wing its influence is permissive rather than instructive.

Control of wing growth by Vg

Vg, which together with its partner Sd is required for survival and growth of wing pouch cells (Kim *et al.* 1996; Liu *et al.* 2000), has 2

conceptually important roles in controlling wing growth. First, as a key target of Notch, Wg and Dpp, Vg integrates signaling from both A–P and D–V compartment boundaries. Second, Vg participates in a dynamic process that shifts cells from the proximal wing into the distal wing. These roles of Vg depend upon its regulation by its distinct boundary and quandrant enhancers, which maintain Vg expression after an initial transient phase of broad expression early in wing disc development.

Activation of Vg expression by Notch through the boundary enhancer generates a population of Vg-expressing cells in which Wg and Dpp can then activate the Vg quadrant enhancer, thereby maintaining Vg expression even in cells that are pushed away from the D–V boundary by cell proliferation (Kim *et al.* 1996). The maintenance of Vg expression is 1 mechanism by which threshold levels of Wg and Dpp contribute to wing growth, but Wg and Dpp are also required for wing disc growth independently of their role in promoting Vg (Zecca and Struhl 2007b; Barrio and Milán 2020; Parker and Struhl 2020; Zecca and Struhl 2021). Vg may also contribute to growth regulation by the Ds-Fat pathway, as it regulates Fj and Ds expression (Cho and Irvine 2004; Zecca and Struhl 2010). This can influence growth within the wing pouch, and also indirectly growth within the proximal wing, through induction of the inner ring of Wg expression (Cho and Irvine 2004; Zecca and Struhl 2010).

Analysis of Vg regulation led to the discovery that it could contribute to growth of the wing blade not only through autonomous effects on Vg-expressing cells, but also through a "feed-forward" mechanism that recruits neighboring, proximal cells into the future wing blade (Fig. 6b) (Zecca and Struhl 2007b, 2010, 2021). Three factors contribute to this recruitment (1) Vg expression is activated through its quadrant enhancer in the presence of Vg, Wg, and Dpp (Kim *et al.* 1996); (2) boundaries of Fj and Ds expression induce elevated Yki activity (Rogulja *et al.* 2008; Willecke *et al.* 2008); (3) Yki and Vg both partner with the same DNA binding protein, Sd, and while they have distinct activities, Yki can substitute for Vg in the activation of Vg expression through the quadrant enhancer (Zecca and Struhl 2010). This can result in a spread of Vg expression to neighboring cells. Because Vg regulates Fj and Ds expression (Cho and Irvine 2004; Zecca and Struhl 2010), this process can occur reiteratively, expanding the developing wing blade. This mechanism has the capacity to spread over many cells (reflecting the range of Wg and Dpp signaling) under artificial conditions (Zecca and Struhl 2007b, 2010, 2021), although it remains unclear what fraction of normal wing size is dictated by this recruitment process, because Vg and Ds-Fat signaling are also required for normal growth within the wing pouch. We also note that on its own this process would not increase growth of the wing disc, rather it shifts cells from the proximal wing into the distal wing. It has been argued that it promotes disc growth due to the induction of Wg expression in the proximal wing, but Ds-Fat signaling, as revealed by the genetic requirement for *dachs*, is only required for induction of Wg in the proximal wing at early third instar (Cho and Irvine 2004). Additionally, a *wg* allele, spd^{fg}, that eliminates Wg expression in the inner ring, strongly reduces the size of the proximal wing and hinge but has only mild effects on the size of the wing blade (Neumann and Cohen 1996a).

Orientation of growth in the wing disc

The shape of the wing disc, and ultimately the adult wing, depends not only on the pattern and amount of growth but also on the orientation of growth. During normal wing disc development, there is a bias in the orientation of growth along the proximal–distal axis of the distal wing, visible in the elongated shape of marked clones of cells (Bryant 1970; Resino *et al.* 2002). This orientation of growth is dependent upon the Ds-Fat pathway (Baena-Lopez *et al.* 2005; Mao *et al.* 2011). Ds-Fat signaling is also required for the orientation of cell divisions, which are normally biased along the proximal–distal axis, but the bias in cell division orientation is an insufficient explanation for how growth is oriented, because cell division orientation is randomized in *mud* mutant wing discs, yet *mud* does not significantly affect the orientation of growth in the wing disc or shape of the adult wing (Zhou *et al.* 2019). Quantitative analysis of cell behaviors in wing discs growing in ex vivo culture revealed that changes in shape of the developing wing pouch during growth reflect 3 main processes: oriented cell divisions, cell rearrangements, and cell shape changes (Dye *et al.* 2017). However, the relative contributions of these processes to growth orientation amongst individual discs can vary, and loss of division orientation in *mud* mutants could be compensated for by an increased contribution of cell rearrangements (Dye *et al.* 2017; Zhou *et al.* 2019).

In the proximal wing, growth is oriented circumferentially. In this region, growth orientation correlates with the circumferential orientation of mechanical stress. This stems in part from the initially faster growth of distal wing cells, which circumferentially stretches more proximal wing disc cells (Legoff *et al.* 2013; Mao *et al.* 2013). However, circumferentially oriented stress is maintained even after differential growth no longer occurs, and it has been proposed that it is instead driven largely by radially oriented cell rearrangements induced by a mechanosensitive feedback (Dye *et al.* 2021).

Notum growth

Growth of the notum has not been as intensively investigated as growth of the wing, although Dpp is required for normal notum growth, as the notum is much smaller in *dpp* mutant wing discs (Spencer *et al.* 1982). In addition to proliferation of cells initially fated to form notum, part of the growth of the notum comes from a shift of cells from the PE to the notum. This shift has been revealed by lineage tracing experiments (Pallavi and Shashidhara 2003; McClure and Schubiger 2005). It is also consistent with the genetic requirement of peripodial cells for growth of the notum, although part of this effect may reflect a contribution of PE to DP cell survival (Gibson and Schubiger 2000; Pallavi and Shashidhara 2003; Nusinow *et al.* 2008). By counting cells McClure and Schubiger (2005) estimated that at the beginning of the third instar there are 3 times as many cells in the DP as in the PE, but at late third instar the ratio is 20:1. As based on differences in growth rates one would have expected a final ratio of only 12:1, it appears that a substantial fraction of PE cells shift to the DP, and this is particularly important for growth of the notum. During pupal stages, live imaging has revealed that cell numbers in the notum are limited in part by a crowding-induced delamination (Marinari *et al.* 2012; Levayer *et al.* 2016).

Integration of wing disc and organismal growth

The influence of local factors like wing disc patterning must be integrated with systemic effects so that the growth of the wing disc is coordinated with the growth of the rest of the animal. The growth of the wing disc, like all parts of the fly, is influenced by metabolism and metabolic pathways (Mirth and Shingleton 2012; Okamoto and Yamanaka 2015). Starvation can lead to flies that are much smaller than normal, but with relatively normal proportions, although some variations in the effects of nutrition on different body parts, including the wing, have been observed

(Shingleton *et al.* 2009). The effects of starvation can be mimicked by blocking the Insulin or mTor signaling pathways (Chen *et al.* 1996; Böhni *et al.* 1999; Colombani *et al.* 2003). During larval development the imaginal discs are bathed in hemolymph, which provides a mechanism for inter organ communication. A key focus of nutritional regulation during larval stages is the fat body. Activation of mTor signaling in the fat body promotes growth of other larval tissues by stimulating production of *Drosophila* Insulin-like peptides (dILPs) (Colombani *et al.* 2003; Géminard *et al.* 2009). Studies in genetic mosaics have confirmed that Insulin and mTor signaling pathways are also autonomously required within imaginal disc cells for normal growth, and have revealed that these pathways affect cell size as well as cell number (Böhni *et al.* 1999; Weinkove *et al.* 1999; Oldham *et al.* 2000; Zhang *et al.* 2000; Brogiolo *et al.* 2001).

A key external signal controlling the growth and development of the imaginal discs is the steroid hormone 20-hydroxyecdysone (which we will abbreviate as ecdysone). Ecdysone is synthesized by the prothoracic gland under the control of a wide range of developmental and environmental signals (Mirth and Shingleton 2012; Texada *et al.* 2020). Ecdysone is best known for its role in triggering molts between larval instars and the larval to pupal transition. However, small pulses of ecdysone also occur during the third instar (Warren *et al.* 2006), and a low level of ecdysone is required for growth of the larval wing disc (Herboso *et al.* 2015). At least in part, this requirement for ecdysone reflects a role in regulating the expression of developmental patterning genes that are required for normal wing disc growth (Mirth *et al.* 2009; Dye *et al.* 2017; Parker and Struhl 2020). For reasons that are not yet understood, the dosage of ecdysone required for continued growth is proportional to disc size (Strassburger *et al.* 2021). The understanding that ecdysone is required for continued disc growth and normal gene expression has also contributed to success in growing wing discs in culture (Dye *et al.* 2017).

Multiple signals converge to regulate ecdysone production during larval development. Nutritional status, through Insulin signaling, appears to influence ecdysone production by regulating the growth of the prothoracic gland (Mirth *et al.* 2005). Importantly, ecdysone production is also regulated by signaling from the wing and other imaginal discs. It has long been known that certain classes of tumor-inducing mutations, or tissue damage that triggers regeneration, can delay pupariation. A key molecular mechanism for this delay was provided by the discovery that it is in part mediated by induction of the relaxin-like signaling peptide, Dilp8, in damaged or tumorous wing discs (Colombani *et al.* 2012; Garelli *et al.* 2012). Dilp8 signals to its receptor Lgr3 in larval brain and prothoracic gland to downregulate ecdysone production, slowing growth and delaying metamorphosis (Colombani *et al.* 2015; Jaszczak *et al.* 2016). Dilp8 also coordinates the relative growth of wing discs and other organs during development. This function is visible, for example, by the variability in wing size (referred to as fluctuating asymmetry) in flies lacking Dilp8 signaling (Colombani *et al.* 2012; Garelli *et al.* 2012). This coordination reflects the fact that in addition to activation by damage signals, Dilp8 is also regulated by inputs that report the growth status of imaginal discs. Dilp8 is upregulated by Yki (Boone *et al.* 2016); Yki activity normally declines in wing discs as they approach their mature size (Pan *et al.* 2018; Borreguero-Muñoz *et al.* 2019), and deletion of the enhancer that mediates Yki regulation of Dilp8 results in fluctuating asymmetry similar to that of loss of Dilp8 (Boone *et al.* 2016). Dilp8 regulation also contributes to a mechanism through which an individual slow-growing imaginal disc can slow growth of other organs; the transcription factor Xrp1 is upregulated in slow growing imaginal disc cells and upregulates Dilp8 expression (Boulan *et al.* 2019). Dilp8 thus provides a mechanism for sensing, through multiple inputs, when imaginal disc growth is completed and metamorphosis can ensue. While regulation of Dilp8 can occur in other imaginal discs, most studies of Dilp8 have focused on wing discs, which presumably have a key role in Dilp8 regulation as the largest of the imaginal discs. Other signals secreted by imaginal discs, including Dpp and Upd3, can also influence ecdysone production (Setiawan *et al.* 2018; Cao *et al.* 2021; Romão *et al.* 2021). Dpp pathway activation in the prothoracic gland declines in older larvae, suggesting that, as for Dilp8, levels of Dpp released by imaginal discs may help coordinate growth and developmental transitions (Setiawan *et al.* 2018).

Signaling from muscles also influences the growth of wing and other imaginal discs. This signaling is mediated by the activin family member Myoglianin (Myo) (Upadhyay *et al.* 2020), which is expressed by larval muscles. Wing discs in *Myo* mutants are ∼40% smaller than in wild-type larvae. This signaling may help coordinate the size of appendages and cuticular body parts with the size of the larval body and the musculature that will be attached to them.

Regeneration of the wing disc

Wing discs as a model for regeneration

The wing disc has long been recognized as for its regenerative capacity, which provided early insights into the relationship between patterning and growth, and more recently has enabled studies investigating how tissues respond to and repair damage (Worley and Hariharan 2021). Regeneration is a process of reformation of damaged or excised tissue. It typically requires recognition of the damage, followed by regrowth and repatterning of the missing tissue. While adult wings of *Drosophila* cannot regenerate, wing discs can recover from damage or removal of tissue by regenerating the missing cells. Early regeneration studies relied on the fact that transplantation of discs into larval hosts led to differentiation on schedule with metamorphosis of the host, but discs could alternatively be transplanted into female abdomens, where they could grow without differentiating (Hadorn and Buck 1962; Bryant 1971, 1975a, 1975b). During this growth phase, damaged wing discs regenerate missing tissue (Fig. 9a). The success of regeneration was assayed by retransplantation into larval hosts, or examination of cell proliferation and molecular markers of disc cell fates (O'Brochta and Bryant 1987; Mattila *et al.* 2005).

More recently, techniques were developed to excise tissue from imaginal discs without removing them from the larva (Pastor-Pareja *et al.* 2008; Díaz-García and Baonza 2013). Additionally, genetic approaches have been developed to kill cells in defined regions of the disc during larval development and then examine the ability of the disc to regenerate this tissue within the larva (Smith-Bolton *et al.* 2009; Bergantinos *et al.* 2010; Herrera *et al.* 2013) (Fig. 9b). These genetic approaches are amenable to doing genetic and genomic analysis and screens, and have yielded insights into processes, molecules and pathways that contribute to regeneration, as well as how regeneration is coordinated with the development of the rest of the organism.

Jnk activation coordinates wing disc regeneration

Activation of Jnk signaling is essential for regeneration in wing discs and acts through multiple downstream effectors to coordinate elimination of dying cells, tissue remodeling, stimulation of proliferation, modulation of cell fate, and systemic responses

that facilitate effective regeneration (Fig. 9c) (Bosch *et al.* 2005; Mattila *et al.* 2005; Bergantinos *et al.* 2010; Grusche *et al.* 2011; Sun and Irvine 2011; Colombani *et al.* 2012; Garelli *et al.* 2012). Reactive oxygen species (ROS), which are generated by dying cells, are key inducers of Jnk activation, as well as of p38 MAPK activation, which also contributes to regeneration (Santabárbara-Ruiz *et al.* 2015; Fogarty *et al.* 2016). Efficient regeneration requires an appropriate level and duration of Jnk signaling, and genes that modulate ROS levels are required for appropriate Jnk activation and consequently, wing disc regeneration (Brock *et al.* 2017; Khan *et al.* 2017). Jnk activation also feeds back on ROS production to modulate the duration of the response (Khan *et al.* 2017).

Regenerative growth in wing discs

Jnk activation is required for early steps in regeneration including cellular processes that close wounds, modify cell fates and stimulate cell proliferation (Bosch *et al.* 2005; Mattila *et al.* 2005; Bergantinos *et al.* 2010). In wing discs, as in many other contexts, a population of cells near the wound site, often referred to as a blastema, are stimulated to undergo increased proliferation (O'Brochta and Bryant 1987). The majority of blastema cells in wing disc regeneration experiments arise from cells in which Jnk activation was induced (Bosch *et al.* 2008).

The stimulation of proliferation during regeneration shares features with apoptosis-induced compensatory cell proliferation (Huh *et al.* 2004; Pérez-Garijo *et al.* 2004; Ryoo *et al.* 2004; Morata *et al.* 2011), a phenomena in which induction of cell death by irradiation or expression of proapoptotic genes stimulates proliferation of neighboring cells in a Jnk-dependent process. Jnk signaling promotes cell proliferation through cross-talk with other signaling pathways, including Wg, Hippo, and Jak-Stat. Dying cells can also release Dpp, but Dpp is not actually required for the induction of proliferation by dying cells (Pérez-Garijo *et al.* 2009). It has also been reported that Wg is not required for compensatory cell proliferation (Pérez-Garijo *et al.* 2009), however, Wg is required for normal regeneration after genetically induced tissue damage, during which Wg expression is induced near wound edges in a Jnk-dependent process (Smith-Bolton *et al.* 2009).

Jnk activation in regenerating wing discs also leads to activation of Yki, and Yki is required for regenerative growth after tissue damage (Grusche *et al.* 2011; Sun and Irvine 2011). Indeed, regenerative growth in the wing disc is even more sensitive to Yki levels than normal developmental wing growth (Grusche *et al.* 2011; Sun and Irvine 2011; Repiso *et al.* 2013). One mechanism through which Jnk pathway activation increases Yki activity is direct phosphorylation of the Wts inhibitor Jub by Jnk, which promotes binding of Jub to Wts (Sun and Irvine 2013). The Ds-Fat pathway also contributes to Yki regulation during regeneration (Grusche *et al.* 2011; Repiso *et al.* 2013). The Ds-Fat pathway also influences the orientation of growth during regeneration and replacement of dying cells (Li *et al.* 2009; Repiso *et al.* 2013). Growth can be oriented toward dying cells in a Fat-dependent process; this presumably makes regenerative growth more efficient at closing wounds.

The Upd family of Jak-Stat pathway ligands are upregulated downstream of Jnk during wing disc regeneration, and Jak-Stat signaling contributes to promotion of cell proliferation as well as loss of cell fate specification (Pastor-Pareja *et al.* 2008; Katsuyama *et al.* 2015; Santabárbara-Ruiz *et al.* 2015; La Fortezza *et al.* 2016; Ahmed-de-Prado *et al.* 2018). During regeneration of the intestine Upd is upregulated downstream of Yki (Karpowicz *et al.* 2010; Ren *et al.* 2010; Shaw *et al.* 2010; Staley and Irvine 2010), so it is possible that Yki also mediates the upregulation of Upd during wing disc regeneration, although to our knowledge this has not been directly tested in wing discs.

Cancer has been described as a wound that does not heal, and there are notable similarities between the mechanisms that stimulate growth in regenerating wing discs and wing discs with mutations in neoplastic tumor suppressor genes (Mirzoyan *et al.* 2019; Gong *et al.* 2021). Many neoplastic tumor suppressor mutations disrupt epithelial cell polarity, which triggers activation of responses that parallel those that occur during regeneration, including activation of Jnk, activation of Yki, and activation of Jak-Stat signaling. These responses can be modulated by local disc patterning, as for example tumors in wing discs form preferentially in the proximal wing and hinge where Jak-Stat signaling is normally active (Tamori *et al.* 2016).

Repatterning during wing disc regeneration

Wing disc patterning has to be re-established after tissue loss or damage, as even the fundamental separation of wing disc cells into A–P and D–V compartments can be disrupted (Díaz-García and Baonza 2013; Herrera and Morata 2014). Repatterning of regenerating tissue in wing discs is thought to follow a similar process as during normal development, including re-establishment of compartment boundaries (Smith-Bolton *et al.* 2009; Bergantinos *et al.* 2010; Herrera and Morata 2014), and expression of Dpp and Wg along these boundaries (Mattila *et al.* 2004; Díaz-García and Baonza 2013). However, the ability to repattern is limited, as in some cases damaged wing discs are unable to regenerate missing structures. In classic disc fragmentation experiments, it was observed that larger fragments of a wing disc could regenerate the missing parts, but smaller fragments generated duplicated structures rather than regenerating (Bryant 1975a, 1975b) (Fig. 9a). In more recent experiments employing localized induction of cell death, it was observed that the wing has a greater capacity for regeneration than the notum, and that fragments that are exclusively notum or wing are unable to regenerate the complementary region (Martin *et al.* 2017b).

Regulation of chromatin modifiers is important for modifications of cell fate downstream of Jnk, and components of both repressive and activating complexes, as well as chromosome remodeling complexes, are regulated by tissue damage and influence regeneration (Klebes *et al.* 2005; Lee *et al.* 2005; Blanco *et al.* 2010; Skinner *et al.* 2015; Tian and Smith-Bolton 2021). The regulation of chromatin modifiers during regeneration may also explain why regeneration is sometimes associated with transdetermination (Klebes *et al.* 2005; Lee *et al.* 2005), in which cells in a regenerating disc sometimes switch fate toward that of a different imaginal disc.

Modulation of developmental timing during wing disc regeneration

Wing disc damage can delay pupariation, and this delay contributes to efficient regeneration by providing time for regenerative growth and repatterning to occur in the damaged disc (Hackney and Cherbas 2014). A key factor mediating this delay is Dilp8, which is secreted by regenerating imaginal disc cells (Colombani *et al.* 2012; Garelli *et al.* 2012). In the absence of Dilp8, damage-induced developmental delay does not occur and regeneration is compromised. Dilp8 secreted from damaged wing disc cells signals to its receptor, Lgr3, in the brain and prothoracic gland to delay pupariation by inhibiting synthesis of ecdysone (Colombani *et al.* 2015; Garelli *et al.* 2015). Expression of Dilp8 is promoted by multiple pathways upregulated during regeneration, including Jnk, Yki, and Jak-Stat (Colombani *et al.* 2012; Katsuyama *et al.*

2015; Boone *et al.* 2016). In addition to regulating Dilp8, Upd3 cytokines secreted by regenerating or tumorous larval tissues can also act directly on the prothoracic gland to suppress ecdysone production (Cao *et al.* 2021; Romão *et al.* 2021).

The regenerative capacity of wing discs decreases over the course of the third larval instar (Smith-Bolton *et al.* 2009), and multiple factors have been identified as contributing this decline. A damage-responsive enhancer of *wg* is subject to epigenetic silencing as wing discs age, reducing the ability of the wing disc to regenerate (Harris *et al.* 2016). Increases in ecdysone levels also suppress regenerative capacity through upregulation of *broad* and concomitant downregulation of *chinmo* (Narbonne-Reveau and Maurange 2019). Increases in ecdysone during the latter part of the third instar also impair regeneration by regulating the localization of the septate junction protein coracle, and consequently decreasing the permeability of the wing disc epithelium (DaCrema *et al.* 2021). This suppresses the ability of Dilp8, which is secreted into the wing disc lumen (Colombani *et al.* 2012), to reach its receptor Lgr3 in the brain and prothoracic gland, and likely also reduces secretion of other signals from imaginal discs, like Dpp and Upd3, that can influence ecdysone production.

Morphogenesis of the wing disc

During larval stages, the wing disc is a sac-like epithelial monolayer that nonetheless has a complex morphology, formed through variations in cell shape and epithelial folding. Then, during metamorphosis the relative flat epithelium of the wing disc undergoes a remarkable transformation to generate the 3D morphology of the adult wing and notum.

In recent years, genetic and imaging approaches have been combined with physical modeling to provide insights into how this transformation occurs.

Formation of folds in the larval wing disc

The epithelium of the DP is initially flat, but as it grows folds begin to appear at precise locations, such that by mid-third instar the wing imaginal disc exhibits a reproducible pattern of folds separating the wing pouch region of the disc from the notal region (Fig. 3, a and b). Three distinct folds form, referred to as the hinge–notum, hinge–hinge, and hinge–pouch folds. These folds are positioned by genes that pattern the wing disc.

The hinge–notum fold forms at the border of Iro-C gene expression, and genetic manipulations of Iro-C expression show that folds can be induced at ectopic Iro-C borders, with the non-expressing cells undergoing apical–basal shortening and invagination (Villa-Cuesta *et al.* 2007). Omb, which is expressed in the wing pouch and hinge but not the notum, also contributes to hinge–notum fold formation (Wang *et al.* 2016). The Jak-Stat pathway Upd ligands become expressed in cells that will form the hinge, and Jak-Stat signaling is also required for normal formation of the hinge–notum fold (Johnstone *et al.* 2013). The hinge–pouch fold forms in cells expressing Doc proteins, whose expression is delimited by repression by Vg in more distal cells and repression by Hth in more proximal cells (Sui *et al.* 2012). Loss- and gain-of-function experiments established that Doc genes contribute to fold formation. The hinge–hinge fold is positioned in part by Wg expression, which is expressed just distal to this fold and represses fold formation (Sui and Dahmann 2020).

Recent studies have identified cellular mechanisms associated with fold formation. All 3 folds are characterized by an apical–basal shortening of cells, and redistribution of microtubules from predominantly apical to predominantly basal (Sui *et al.* 2012; Wang *et al.* 2016). Local degradation of basal ECM has been described at the hinge–pouch (Sui *et al.* 2012) and hinge–hinge folds (Sui *et al.* 2018). There are also intriguing differences between the folds, however, as the hinge–hinge fold is characterized by decreased basal tension, whereas the hinge–pouch fold is characterized by increased lateral tension (Sui *et al.* 2018). The formation of folds by alterations in basal or lateral tension can be reproduced in computational simulations using 3D vertex models (Sui *et al.* 2018). Computational and experimental analyses suggest that differential growth between different regions of the wing disc also contribute to fold formation (Tozluoğlu *et al.* 2019).

Morphogenesis of the pupal wing disc

During metamorphosis, the wing disc undergoes a complex morphogenesis, triggered by ecdysone, to form the adult wing, hinge, and notum. These processes were first described in classic studies decades ago (Auerbach 1936; Waddington 1939; Fristrom and Fristrom 1975), but new insights have been revealed through application of modern genetic techniques, live imaging, and modeling.

Wing disc eversion

The first step in converting the larval wing disc into the pupal wing is eversion, during which the imaginal discs effectively turn inside out, such that appendages extend out of the body cavity and the apical surfaces are now on the surface of the developing animal rather than facing a central lumen (Fig. 10). At the beginning of this process, the developing wing pouch begins to elongate and flattens along the D–V compartment boundary to generate the bilayered shape of the adult wing (Fristrom and Fristrom 1975; Aldaz *et al.* 2010). The dorsal and ventral surfaces of the wing blade become attached through integrin binding to laminin (Brower and Jaffe 1989; Henchcliffe *et al.* 1993; Brabant *et al.* 1996; Martin *et al.* 1999; Sun *et al.* 2021), but undergo phases of flattening, inflation, and reattachment during pupal development (Waddington 1939; Fristrom and Fristrom 1975; Fristrom *et al.* 1993). Myosin-generated forces in the peripodial cells contribute to the folding of the wing and the eversion of the wing disc (Aldaz *et al.* 2013). Around this time, stalk cells that maintain connection of the disc to the larval epithelium invade the larval epidermis and become migratory, expanding the space through which the disc will evert (Pastor-Pareja *et al.* 2004). The PE then ruptures, and the disc everts through the opening formed by ruptured PE and stalk cells.

Morphogenesis of the wing

After eversion the wing continues to elongate and expand. Elongation of the wing during early pupal development (4–5 h after puparium formation) is accompanied by oriented cell intercalation and cell shape changes (Diaz-de-la-Loza *et al.* 2018). These appear to be directed by anisotropic stresses, visible through the polarization of myosin accumulation along cell–cell junctions. Expansion of the surface area of the developing wing is accomplished by cell flattening, as cells transition from columnar to cuboidal (Fristrom and Fristrom 1975; Diaz-de-la-Loza *et al.* 2018). This flattening is accompanied by degradation of the ECM.

Later in pupal development, the developing wing is shaped by a contraction of the wing hinge region, in concordance with attachment of the distal wing to the pupal cuticle (Turner and Adler 1995; Etournay *et al.* 2015; Ray *et al.* 2015). The attachment of the distal wing is mediated through apical ECM proteins,

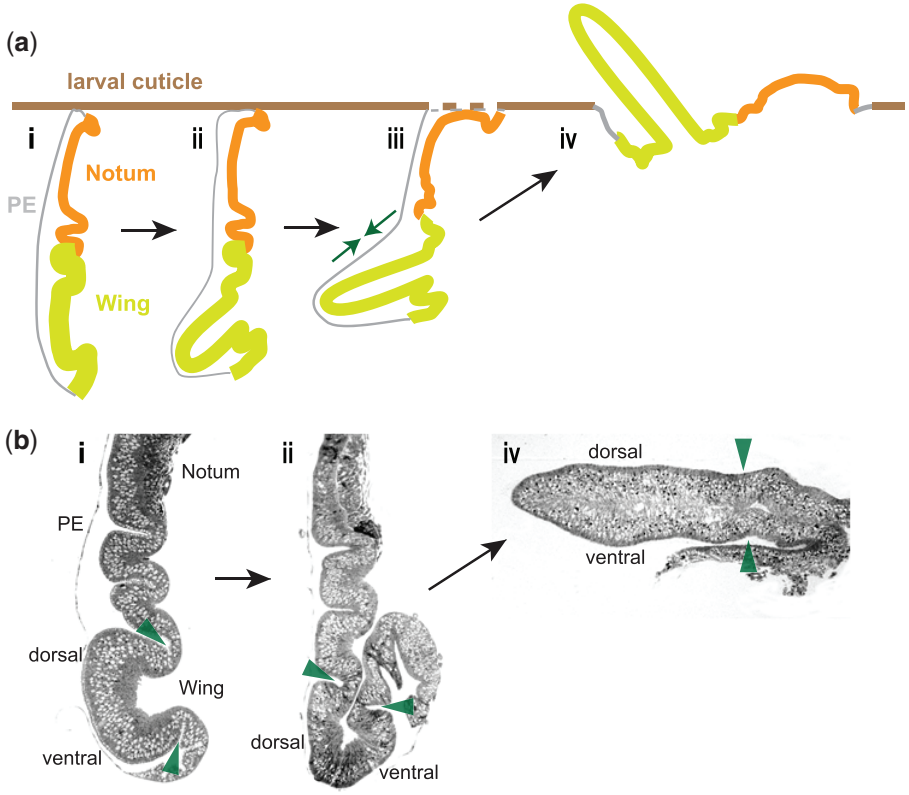

Fig. 10. Wing disc morphogenesis. a) Schematic illustrating steps in wing disc eversion, which occurs during the first half of prepupal development (the ~12-h period that serves as a transition between larval and pupal development). (i) The larval wing disc is attached to the cuticle by a stalk connected to the PE. (ii) Part of the PE attaches to the larval cuticle, and the wing pouch begins to elongate and flatten. (iii) The PE over the notum invades the larval cuticle and ruptures, generating an opening for the disc to evert through. The PE over the wing contracts (green arrows), in part through cells becoming columnar rather than squamous. (iv) The disc has everted through the hole created by invasion and rupture of the PE and larval cuticle. Eversion is driven by contraction of the remaining PE. b) Longitudinal sections of discs at (i) 0, (ii) 2, and (iv) 4 h after puparium formation, roughly corresponding to the same stages in the schematic. Green arrowheads point to the edges of the wing pouch area, which will form the distal wing. Images reproduced with permission from Domínguez-Giménez *et al.* (2007).

including Dumpy. The anchoring of the distal wing, in conjunction with the contraction of the hinge, generates an anisotropic tension that contributes to further wing elongation. This late reshaping of the wing is accompanied by a combination of cell shape changes, cell rearrangements, and cell divisions, all oriented by the anisotropic tension, and reproducible in computational modeling (Etournay *et al.* 2015; Guirao *et al.* 2015; Ray *et al.* 2015). Cells in the late pupal wing blade become more isometric, adopting a relatively even hexagonal packing in a process that depends upon PCP proteins and the physical properties of wing cells (Classen *et al.* 2005; Farhadifar *et al.* 2007). Wing cells secrete cuticle during the middle of pupal development, and then after eclosion undergo an epithelial–mesenchymal transition and apoptosis, such that the mature adult wing is mostly composed of cuticle without underlying cells (Johnson and Milner 1987; Kiger *et al.* 2007; Link *et al.* 2007; Sobala and Adler 2016).

Notum fusion

The notal region of the disc also undergoes morphogenetic changes to spread and replace the larval epidermis, adopt the adult notal shape, and fuse with notal cells from the contralateral wing disc. The fusion of the notal region of the 2 wing discs at the midline of the adult fly is mediated by a subset of peripodial cells at the edge of the notum and requires Jnk pathway activity in these cells (Agnès *et al.* 1999; Zeitlinger and Bohmann 1999; Martin-Blanco *et al.* 2000; Tripura *et al.* 2011).

Conclusion

The *Drosophila* wing disc has been one of the most intensively studied organs in biology. This has resulted in an impressively detailed understanding of many aspects of its development. More broadly, discoveries elucidating fundamental aspects of wing disc development have established paradigms that have informed our understanding of organogenesis throughout the animal kingdom. To highlight just a few examples: Studies in wing discs have provided foundational insights into epithelial cell biology, including how cells divide in pseudostratified epithelia, how PCP is established and maintained, and how local cell shape changes combine to alter the shapes of epithelial tissues. The wing disc has been a particularly important model for understanding how tissues are patterned, beginning with the discovery of compartments, and the logic of developmental patterning that progresses from broad subdivisions to specification of discrete cell types at reproducible locations like bristles and veins. The discovery and characterization of signaling pathways that play key roles in establishing wing disc patterning, like the Notch, Wnt, Hh, and Dpp pathways, has identified mechanisms that play fundamental roles in tissue patterning across diverse animal species, and also established a model for identifying and characterizing components of these conserved pathways. As these signaling pathways are used reiteratively throught the development of all animals, the identification and characterization of their components has had a particularly broad impact. Wing discs

have also been essential to mechanistic investigations of fundamental developmental processes such as how cells are separated into compartments and how long-range signals spread through tissues.

The wing disc has also been a remarkable system for identifying factors that control organ growth and for characterizing their activity and how they are integrated with each other. For example, studies in wing discs, together with the eye disc, led to the discovery of the conserved Hippo signaling network, and laid the foundation of our understanding of its mechanism of action and its control of growth and cell fate. More recently, wing discs have aided investigations of interorgan communication and how systemic factors coordinate the growth and development of different organs within a developing organism. Although there remain gaps in our understanding of how wing disc growth is controlled, in no other organ is there a level of understanding approaching that which exists in the wing disc.

The accumulated foundation of knowledge, together with the many powerful tools available in *Drosophila*, ensure that the wing disc will continue to be fruitful terrain for elucidating fundamental aspects of biology for many years to come. New approaches, including improvements in disc culture that facilitate live imaging, ever more sophisticated genetic, molecular, and genomic techniques, and advances in image segmentation and quantitation, should provide further insights into questions as fundamental as how the size and shape of the wing disc is controlled to how the development of this remarkable organ is coordinated with the physiology of the rest of the animal.

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Conflicts of interest

None declared.

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