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ADVANCED REVIEW

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Growth control in the *Drosophila* wing disk

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Abstract

The regulation of size and shape is a fundamental requirement of biological development and has been a subject of scientific study for centuries, but we still lack an understanding of how organisms know when to stop growing. Imaginal wing disks of the fruit fly *Drosophila melanogaster*, which are precursors of the adult wings, are an archetypal tissue for studying growth control. The growth of the disks is dependent on many inter- and intra-organ factors such as morphogens, mechanical forces, nutrient levels, and hormones that influence gene expression and cell growth. Extracellular signals are transduced into gene-control signals via complex signal transduction networks, and since cells typically receive many different signals, a mechanism for integrating the signals is needed. Our understanding of the effect of morphogens on tissue-level growth regulation via individual pathways has increased significantly in the last half century, but our understanding of how multiple biochemical and mechanical signals are integrated to determine whether or not a cell decides to divide is still rudimentary. Numerous fundamental questions are involved in understanding the decision-making process, and here we review the major biochemical and mechanical pathways involved in disk development with a view toward providing a basis for beginning to understand how multiple signals can be integrated at the cell level, and how this translates into growth control at the level of the imaginal disk.

This article is categorized under:

Analytical and Computational Methods > Computational Methods
Biological Mechanisms > Cell Signaling
Models of Systems Properties and Processes > Cellular Models

KEYWORDS

Drosophila wing disk, mechanical control, mechanotransduction, organ development, signal integration, signaling pathways

1 | INTRODUCTION

How size, shape and patterning (SSP) of developing tissues, organs and organisms are controlled is one of the major problems in developmental biology, and to solve this we must understand how environmental factors, biochemical signals, and mechanical forces are interpreted and integrated spatially and temporally at all levels.[1] Furthermore, local control of SSP must be synchronized with contemporaneous development at the organism level, and how local signals

Jia Gou and Jay A. Stotsky contributed equally to this review.

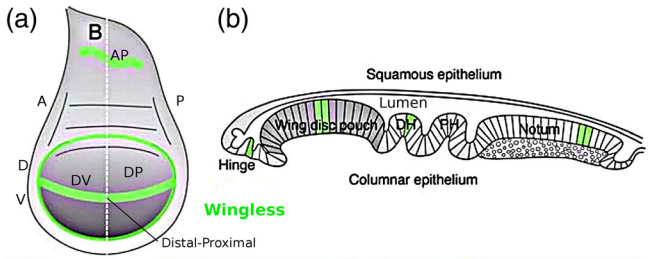

FIGURE 1 (a) The wing disk—A: anterior, P: posterior, D: dorsal, V: ventral, AP and DV: anterior–posterior and dorsal-ventral boundaries, DP: disk pouch. (b) Side view along B in (a). (Reprinted with permission from Widmann and Dahmann (2009))

are integrated with global signals at various levels to produce the appropriate response at the cell level is still an open question in most systems. Some of the underlying questions are (a) what are the local and global factors that control SSP, (b) how are multiple local and global biochemical and mechanical signals integrated at the cell, tissue and organ level, and (c) how are the growth rates and proportions in different-sized organisms of the same species controlled? Answering these is a formidable challenge, for in D'Arcy Thompson's words concerning allometric growth (Thompson, 1942).

> "An organism is so complex a thing, and growth so complex a phenomenon, that for growth to be so uniform and constant in all the parts as to keep the whole shape unchanged would indeed be an unlikely and an unusual circumstance. Rates vary, proportions change, and the whole configuration alters accordingly."

At the local level, cell-to-cell signaling may involve direct exchange of molecules via gap junctions, or it may involve biochemical or mechanical signal transmission to adjacent cells via membrane-embedded molecules that form complexes across the intercellular gaps. Examples include Notch-Delta signaling in numerous systems, the Hippo pathway in both *Drosophila melanogaster* and mammalian systems, and either direct or short-range extracellular signals in cell competition. Global signals include diffusible morphogens in the context of tissue-level pattern formation, tissue-wide mechanical stress in growing tissues, and organism-wide signals carried via the circulatory system. The extracellular signals activate downstream signal transduction networks (STNs) that lead to gene expression or other responses, and because a cell in a tissue rarely receives just one signal, the decision as to how to respond is determined by how the downstream signals are balanced in the network. STNs often involve feed-forward and feedback steps within a pathway, as well as interactions between pathways, and understanding how the response to multiple signals is arbitrated is a fundamental problem in both development and cancer research.

Drosophila melanogaster is currently one of the organisms of choice for studying these problems at the local and global levels, both experimentally and theoretically, and the wing has served as a model organ for such studies. Signaling pathways and other factors involved in SSP of the wing disk (cf. Figure 1) have been identified. The primary morphogens (hedgehog [Hh], decapentaplegic [Dpp], and wingless [Wg]), and many of their interactions in the disk are known (Figure 2), but fundamental questions concerning how their interactions are balanced and how disruption of the balances leads to abnormal outcomes remain unanswered. At the global level, hormones and insulin-like peptides, which are nutrient-dependent, are fundamental for growth at the organismic level, and are transduced via the dTOR[2] pathway at the cell level. Reviews of the global aspects, which are not discussed in detail here, are given in (Andersen, Colombani, & Léopold, 2013; Nijhout et al., 2014). In the remainder of the Introduction we give a broad overview of the biochemical and mechanical aspects of SSP in the wing disk, in the following two sections we delve into the details, and in the final sections we discuss various aspects of a unified model of SSP in the wing disk.

1.1 | Disk development and patterning

An early marker for wing disk cells is expression of vestigial (*vg*), which is active in the embryonic and early larval disk (Williams, Paddock, & Carroll, 1993). The disks (Figure 1a) arise in the embryo as a group of approximately 20–40 cells that evaginate from the embryonic epithelium at approximately 10 hr after egg laying (AEL) (Bate & Martinez Arias, 1991). During the 5 days AEL, the disk completes three larval stages called instars, that terminate at pupation. The disk has two cell layers separated by a lumen (Figure 1b)—a layer of columnar epithelial cells with their apical side at the lumen that develop into the adult wing, hinge and notum, and a peripodial epithelium (PE) overlying the lumen

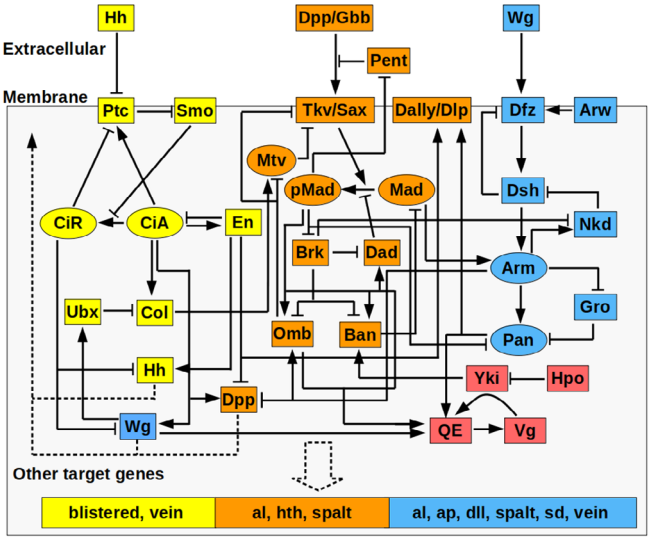

FIGURE 2 The Hh, Dpp, and Wg pathways. Arm: armadillo, Ban: bantam, Brk: brinker, Nkd: naked, Dad (Mad): daughters (mothers) against Dpp, Hpo: Hippo, Dfz: *Drosophila* frizzeled, Dlp: dally-like protein, Gro: groucho, Hth: homothorax, Dsh: disheveled, Mtv: master of thickveins, Tkv: thickveins, Omb: optomotorblind, Pan: pangolin, Sd: scalloped, Smo: smoothened, Ubx: ultrabithorax. Assembled from (Crickmore & Mann, 2008; Fujise et al., 2003; Funakoshi, Minami, & Tabata, 2001; Held, 2002; Rodríguez, 2004; Sopko & McNeill, 2009; Zeng, Rahnama, Wang, Lee, & Verheyen, 2008). See also Table 1

TABLE 1 Additional abbreviations of genes

Gene name	Symbol
Aristaless	*al*
Cubitus interruptus activator (repressor)	*ciA(R)*
Collier	*Col*
Distal-less	*Dll*
Division abnormally delayed	*Dally*
Glass bottom boat	*Gbb*
Pentagone	*Pent*
Patched	*Ptc*
Spalt	*Sal*
Saxophone	*Sax*

(Gibson, Lehman, & Schubiger, 2002). The disk pouch (DP) and notum are specified by Wg and EGFR signaling, resp., while the PE is determined by the absence of these in the early second instar (Baena-López, Carlos Pastor-Pareja, & Resino, 2003). In late-third instar the DP has approximately 10–50 K cells and is approximately 150 μm (DV) × 300 μm (AP) (Blair, 2009).

The first level of spatial patterning in the disk occurs at the embryonic stage via expression of the selector gene engrailed (*en*) in the posterior portion of the disk, where En induces *hh* expression (Albert & Othmer, 2003; García-Bellido, Ripoll, & Morata, 1973). The boundary of *en* expression defines the AP boundary, which cells do not cross (Figure 1). Hh is needed in anterior cells to increase the tension at the boundary and prevent mixing of anterior and posterior cells. The presence of Hh in anterior cells also induces anterior *dpp* expression in a stripe of cells adjacent to the AP boundary (Affolter & Basler, 2007; Basler & Struhl, 1994; Held, 2002), and the resultant Dpp concentration, which is transported throughout the disk (Holtzer, Kicheva, Gonzalez-Gaitan, & Schmidt, 2009; Kicheva et al., 2007), is detected by the Tkv/Punt receptor complex. It is known that Dpp is essential for proper disk development—under- or over-expression of it leads to smaller or larger disks, resp (Hamaratoglu, Affolter, & Pyrowolakis, 2014; Martin-Castellanos & Edgar, 2002), but the level of Dpp needed is ill-defined, as is the mode of transport that establishes the spatial distribution.

The dorsal-ventrol (DV) boundary is established in the second instar by EGFR-controlled expression of *apterous* (*ap*) in the dorsal portion of the disk (Wang, Simcox, & Campbell, 2000; Williams et al., 1993; Zecca & Struhl, 2002), which initiates a cascade of gene expression involving *notch*, *wg*, and *vg* and determines the location of the DV boundary. Wg is expressed throughout the DP in the first and early second instars, but during the third instar expression is restricted to a stripe at the DV boundary (Alexandre, Baena-Lopez, & Vincent, 2014; Swarup & Verheyen, 2012),

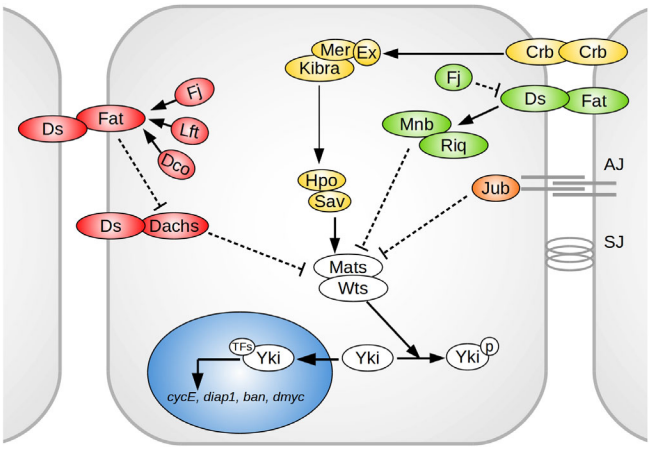

FIGURE 3 The cell-based Hippo pathway. (Reprinted with permission from Gou, Lin, and Othmer (2018)). See also Figure 10

initiated by Delta-Serrate-Notch (DSL) interaction (Zecca & Struhl, 2007a). The Wg signal is transduced via the receptor Dfz and co-receptor arrow, which recruits Dsh to the membrane and inactivates destruction of the arm/β-catenin signaling complex (Bhanot et al., 1996; Mao et al., 2001; Wehrli et al., 2000; Wong et al., 2003). Wg affects growth of DP cells by control of *vg* expression (Terriente, Perea, Suzanne, & Díaz-Benjumea, 2008; Whitworth & Russell, 2003; Zecca & Struhl, 2007b), and the latter leads to recruitment of nonwing cells into the DP at the pouch boundary, which contributes to pouch growth (Zecca & Struhl, 2010). How the gradient of Wg is established is not fully understood (Kreuger, Perez, Giraldez, & Cohen, 2004; Yamazaki et al., 2016), and in particular, whether Wg must be diffusible (Baena-Lopez, Nojima, & Vincent, 2012; Zecca & Struhl, 2007b), or whether a Wg gradient is even necessary for growth (Alexandre et al., 2014; Baena-Lopez, Franch-Marro, & Vincent, 2009), remains open.

While the Hh, Dpp, and Wg STNs have been studied separately, how their interactions affect growth control is not understood. Growth control involves the Hippo pathway (Figure 3), which serves as a major hub for integrating tissue-level morphogen signals at the cell level (Choi, 2018; Hamaratoglu et al., 2014; Misra & Irvine, 2018; Oh & Irvine, 2011). The primary effector in the Hippo pathway is Yorkie (Yki), a co-transcriptional factor whose nuclear localization is controlled by the Yki kinase Warts (Wts)—phosphorylated Yki cannot enter the nucleus. Yki acts by binding to the transcription factor scalloped (Sd) and others to activate the expression of *cyclin E*, *dmyc*, *Diap1*, and *ban* that control proliferation (Rogulja, Rauskolb, & Irvine, 2008), and nuclear Yki can be used as a surrogate for growth. Feedback loops through Yki, such as a short one through dmyc (Neto-Silva, de Beco, & Johnston, 2010), can serve to limit Yki signaling, while other feedback targets include *crumbs*, *ds*, *expanded*, *merlin*, *wts*, *kibra*, and *four-jointed* (*fj*) (Cho et al., 2006; Enderle & McNeill, 2013; Fulford, Tapon, & Ribeiro, 2018; Ikmi et al., 2014; Irvine, 2012; Nolo, Morrison, Tao, Zhang, & Halder, 2006; Oh & Irvine, 2011; Wang, Zhang, & Blair, 2019; Wartlick, Mumcu, Jülicher, & Gonzalez-Gaitan, 2011; Zecca & Struhl, 2010). In addition, Yki modulates morphogen signaling by transcriptional control of the glypicans *dally* and *dlp*—which encode co-receptors or sequestering proteins for Dpp and Wg (Baena-Lopez, Rodríguez, & Baonza, 2008; Fujise et al., 2003; Kreuger et al., 2004)—and Yki also activates myosin at the membrane (Xu et al., 2018). Following establishment of the DV boundary there are four compartments in the wing pouch, each of which evolves independently to a certain degree. Using *minute* mutants in compartments, it was shown that compartments grow at their own rates and regulate expression of *wg* and *vg* independently, but the wing disk grows to the correct size and shape (Martín, Herrera, & Morata, 2009). Thus growth coordination exists at the organ level as well, but not at the cell level—faster dividing cells outcompete slower ones within a compartment (Martín et al., 2009). Moreover, it is known that the mechanism(s) involved in size control regulate the spatial extent or total disk volume, not the cell cycle, and that cell–cell interactions play an important role in controlling cell proliferation (Weigmann, Cohen, & Lehner, 1997).

1.2 | The role of mechanics in growth

However, biochemical signals are not the sole factors of growth control—mechanical effects may provide another layer of control. The lateral membranes of adjacent pouch cells are connected via adherens junctions (AJs) and by septate junctions (SJs) that lie basal to the AJs (Figure 5). The AJs can transmit forces between cells via complexes of E-Cadherins (E-Cads) and adapter proteins, while the SJs separate the extracellular fluid into apico-luminal (AL) and

baso-lateral (BL) layers (Choi, 2018; Gibson & Gibson, 2009; Harris & Tepass, 2010). It is known that compression can reduce the growth rate of tumors (Helmlinger, Netti, Lichtenbeld, Melder, & Jain, 1997), and a cell-based mathematical model of this can reproduce the growth inhibition (Kim, Stolarska, & Othmer, 2007). In the disk high (low) cytoskeletal tension increases (decreases) tissue growth, and growth first slows in the central region of the DP, perhaps due to compression (Pan, Alégot, Rauskolb, & Irvine, 2018). Intracellular forces are transmitted by the cytoskeleton (CSK) and can be transmitted to adjacent cells via mechanical linkages tethered at the AJs (Leckband & De Rooij, 2014; Pinheiro & Bellaïche, 2018). The key constituents of the CSK include F-actin, nonmuscle myosin II (NMII), and cross-linking proteins (Salbreux, Charras, & Paluch, 2012). Most actomyosin—which is cross-linked actin filaments with embedded NNMI—in the disk resides in a fibrous cortical layer located apically. The cortex is comprised of both branched actin and filamentous actin, and therefore can transmit and react to both tangential and normal forces (Kale et al., 2018). The CSK influences the size, shape, and stiffness of individual cells, and these factors must be coordinated in a tissue. It has been suggested that mechanical forces may lead to the more or less uniform cell proliferation observed in the disk, and models based on this have been proposed (Aegerter-Wilmsen, Aegerter, Hafen, & Basler, 2007; Aegerter-Wilmsen et al., 2012; Hufnagel, Teleman, Rouault, Cohen, & Shraiman, 2007; Shraiman, 2005; Vollmer, Casares, & Iber, 2017). In the models, disk size is controlled either by a threshold in a morphogen level (Hufnagel et al., 2007) or by a threshold in a tension gradient (Aegerter-Wilmsen et al., 2012), and when the disk boundary reaches that threshold, proliferation is stopped throughout the disk by a negative feedback of mechanical stress on growth. However, since growth slows in a nonuniform manner in the disk (Pan et al., 2018), this explanation remains tentative, and a more detailed understanding of how biochemical and mechanical signals interact requires a more detailed understanding of the mechanical structure of a cell. This is discussed further in Section 5.

Because there are significant cross-interactions between the biochemical and mechanical pathways, it is the balance between their outputs that determines the outcome, and disruption of the balances can lead to a variety of aberrant results.

2 | BIOCHEMICAL REGULATION OF GROWTH AND PATTERNING

2.1 | The Dpp signaling pathway

While it is known that Dpp is required for proper wing disk growth and patterning (Blair, 2007; Hamaratoglu et al., 2014; Martin-Castellanos & Edgar, 2002), the details of how it regulates development are still not clear. Extracellular Dpp binds to Tkv/Punt and triggers phosphorylation of Mad (Figure 4). Phosphorylated Mad (pMad) alone translocates to the nucleus and forms a complex with nuclear Yki to control transcription of *ban* and other Dpp target genes such as *vg*, *dad*, *omb*, *sal*, and *brk* (Oh & Irvine, 2011). Figure 4 shows that the Dpp signaling pathway is not a simple feed-forward network—various feedback loops exist. For instance, *ban*, is down-regulated by Brk (Martín, Pérez-Garijo, Moreno, & Morata, 2004), but at the same time *ban* represses pMad (Kane, Vora, Padgett, & Li, 2018; Robins, Li, & Padgett, 2005). Another Dpp target gene *dad* binds to Tkv and Sax to inhibit the phosphorylation of Mad (Kamiya,

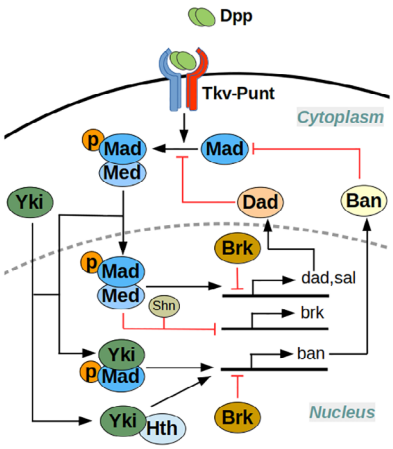

FIGURE 4 The Dpp signaling pathway. The receptor is a complex of two Tkv and two Punt molecules

Miyazono, & Miyazawa, 2008), and this was proposed to play an important role in the maintenance of the robust Dpp activity gradient, as reflected in the level of pMad (Ogiso, Tsuneizumi, Masuda, Sato, & Tabata, 2011).

The secretion of Dpp from a stripe of cells along the AP boundary gives rise to a spatial distribution of Dpp transverse to the AP boundary, but how the distribution is established is still an open question. Three modes of transport have been identified in the disk—diffusion in the extracellular space, transcytosis, and transport via cytonemes (Kicheva et al., 2007; Schwank et al., 2011; Zhou et al., 2012). While cytonemes are important in Hh transport (Bischoff et al., 2013), their role in Dpp and Wg transport is more ambiguous (Akiyama & Gibson, 2015; Matsuda, Harmansa, & Affolter, 2016), and will not be considered here. Diffusion in the extracellular space can be free Brownian motion of a particle in solution, called "free diffusion," or it may involve interactions with other factors such as HSPGs, which is called "facilitated diffusion" or "restricted diffusion" (Restrepo, Zartman, & Basler, 2014; Schwank, Dalessi, et al., 2011; Shimmi, Umulis, Othmer, & O'Connor, 2005). Experimentally measured profiles of Dpp are usually described with a reaction–diffusion model based on "free diffusion" and first-order decay (Kicheva et al., 2007; Wartlick, Mumcu, Jülicher, & Gonzalez-Gaitan, 2011), but the interpretation of the estimated diffusion and decay rates in terms of the underlying processes has been questioned (Lin & Othmer, 2017; Zhou et al., 2012). The governing equations for the evolution of the Dpp concentration in the anterior compartment are

$$\begin{aligned} \frac{\partial c}{\partial t} &= D\frac{\partial^2 c}{\partial x^2} - kc \qquad x \in (0, L) \\ -D\frac{\partial c}{\partial x}(x) &= j \qquad x = 0 \\ -D\frac{\partial c}{\partial x}(x) &= 0 \qquad x = L, \end{aligned} \tag{1}$$

where $x = 0$ ($x = L$) is the AP boundary (pouch boundary). Fitting of FRAP data with this model yielded a diffusion coefficient of $0.1\mu m^2/s$ (Kicheva et al., *2007*), but this leads to a prediction of approximately 70 hr to establish the Dpp gradient in the disk, which may be too long. Zhou et al. (2012) measured a free diffusion coefficient of $20\mu m^2/s$ using FCS, which is more realistic for diffusion in solution, and showed that the previous low value could be understood if receptor-mediated uptake and degradation are incorporated. However their analysis assumes a simple geometry corresponding to the luminal surface in Figure 5, but the extracellular space in the disk is compartmentalized into the apico-luminal and baso-lateral (BL) layers, and recent work shows that spreading in the AL compartment plays a minor role in both growth and patterning—most occurs baso-laterally (Harmansa, Alborelli, Bieli, Caussinus, & Affolter, 2017).

FRAP data reflects Dpp in the lumen and an apical layer of the columnar cells, but some experimental results show that a graded Dpp distribution is found in the BL layer (Harmansa et al., 2017; Melinda, 2013) and the lumenal Dpp is uniformly distributed (Gibson et al., 2002; Harmansa et al., 2017). Others have shown that the majority of Dpp is in the intracellular space and the intracellular apical Dpp forms a long-range gradient (Entchev, Schwabedissen, & González-Gaitán, 2000; Kicheva et al., 2007), but some have observed the opposite (Belenkaya et al., 2004; Müller, Rogers, Shuizi, Brand, & Schier, 2013). If most of the extracellular Dpp resides in the BL domain, a much more complicated transport model would be required to describe the Dpp profile.

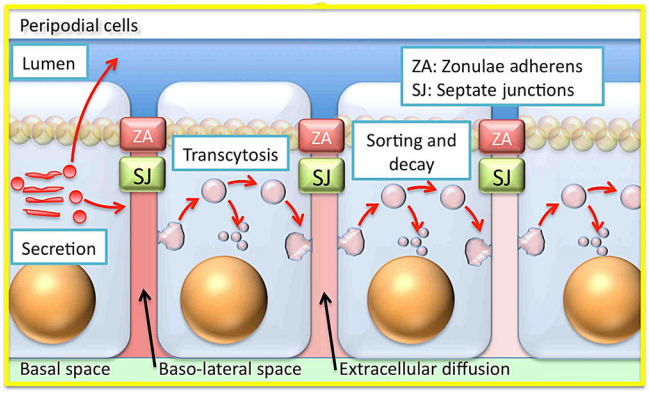

FIGURE 5 A cross-section of a disk showing the transport processes that affect the morphogen distribution (Othmer, Painter, Umulis, & Xue, 2009). ZA are aka AJ

Several kinds of experiments were done to distinguish the different transport models. Some show that Dpp cannot move across dynamin-defective clones, where endocytosis is blocked (Kicheva et al., 2007), but there is contradictory evidence (Belenkaya et al., 2004). While this may support the requirement of transcytosis in the Dpp transport, an alternative interpretation is that the blockage might be caused by the accumulation of surface receptors when the internalization is blocked (González-Gaitán, 2003; Kicheva et al., 2007; Lander, Nie, & Wan, 2002). In any case endocytosis is required to internalize Dpp.

Other questions that must be addressed before a definitive understanding of how the Dpp distribution is established are as follow.

1. Do the columnar cells secrete Dpp to the lumen or the basolateral domain, and how much luminal Dpp originates from the PE? PE cells express *dpp* in a stripe starting from the first instar, while secretion by *dpp*-expressing cells in the pouch stripe begins in mid-second instar (Paul et al., 2013).

2.Is the primary Dpp signaling localized apically or basolaterally? Both the type I receptor Tkv and type II receptor Punt are required for Dpp signaling (Ruberte, Marty, Nellen, Affolter, & Basler, 1995), and Tkv is located both apically and basolaterally, but Punt is only located on the BL side of the pouch cells (Alborelli, 2016; Melinda, 2013). Thus signaling may originate there, while the function of the apical Dpp is to be determined.

While the need for Dpp in growth and patterning is established, whether the spatial gradient plays a role in growth is not, but the fact that uniform low *dpp* expression leads to a normal-sized disk suggests that it does not (Wartlick et al., 2011). Some assert that a low level is needed throughout the disk (Barrio & Milan, 2017; Bosch, Ziukaite, Alexandre, Basler, & Vincent, 2017), but abolishing Dpp transport from the AP source by use of a morphotrap abolishes patterning of the veins, but cells far from the source divide at normal rates (Harmansa, Hamaratoglu, Affolter, & Caussinus, 2015). Thus Dpp may only be needed for growth of cells in the medial region (Harmansa et al., 2015). In addition, proliferation across the disk is quite uniform, despite the fact that Dpp is spatially distributed, and this leads to a major question in the area: how can the growth be uniform when the putativte growth controllers are not (Barrio & Milan, 2017)?

Several Dpp-based models for disk growth control have been proposed (reviewed in Hamaratoglu et al., 2014), but to date most fail to explain all the experimental observations on the effects of OEs and KOs. The simplest one proposes that Brk inhibits growth and Dpp regulates *brk* expression via pMad (Schwank, Restrepo, & Basler, 2008), but it cannot explain the fact that growth at the periphery is enhanced and proliferation of medial cells is reduced in *dpp* OE (Schwank et al., 2008) or in *dpp*/*brk* knock-outs (Schwank et al., 2011; Schwank & Basler, 2010). An alternate model resulted from the observation that the Hippo signaling pathway component Fat inhibits growth in the medial region, which led to the "opposing growth-pathways" model, in which Fat and Dpp signaling counterbalance in the medial region (Schwank, Tauriello, et al., 2011).

Although the proposed mechanism may explain some observations, the fact that Dpp modulates Fat signaling (Rogulja et al., 2008) and that there are interactions between the Dpp pathway and others such as Wg, suggests that a more complex regulatory mechanism is involved. In addition, another BMP homolog, Gbb, which is widely expressed in the wing disk and signals through Mad (Figure 6), may activate the target genes in the lateral region where Dpp is low (Bangi & Wharton, 2006a). In addition to binding with Tkv, Gbb also binds to the ubiquitously expressed type I receptor Sax with relatively high affinity (Bangi & Wharton, 2006b). In distinction with Tkv, Sax can either promote

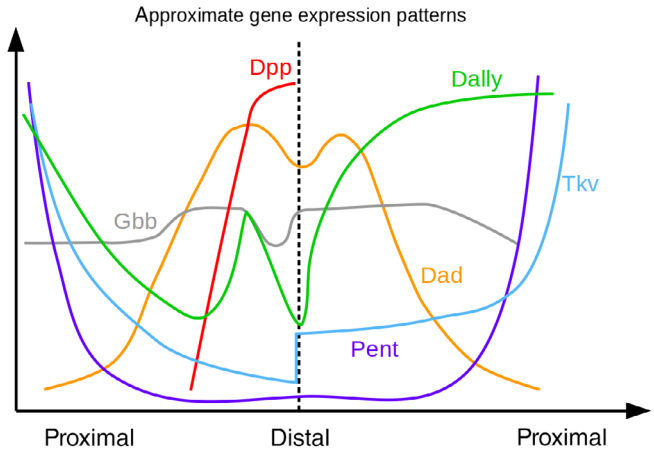

FIGURE 6 Spatial gene expression profiles for genes involved in BMP signaling. Modified from (Raftery & Umulis, 2012)

signaling or block it, depending on its partner type I receptor (Bangi & Wharton, 2006b). A computational model could be used to analyze the interactions of Dpp and Gbb with the receptors and co-receptors to predict their roles in different regions of the disk.

Since flies come in a broad range of sizes, the positioning of veins in the wing must adapt to the size of the wing. The expression borders of the Dpp genes *sal* and *omb* reflect the locations of the veins L2 and L5, resp. (Blair, 2007), which raises the question whether the Dpp gradient adapts to the tissue size (Matamoro-Vidal, Salazar-Ciudad, & Houle, 2015; Matsuda et al., 2016). Some observations find that it does (Teleman & Cohen, 2000) while others do not (Hufnagel et al., 2007). More important is the Dpp activity gradient, as measured by pMad or Dad, which several groups show scales with disk size (Hamaratoglu, de Lachapelle, Pyrowolakis, Bergmann, & Affolter, 2011; Wartlick, Mumcu, Kicheva, et al., 2011). These measures may have different outcomes, since pMad and Dad are downstream of the signal transduction step, and their levels reflect interactions of Dpp with Dally/Dlp and Pent, as well as feedback steps (Akiyama et al., 2008; Norman, Vuilleumier, Springhorn, Gawlik, & Pyrowolakis, 2016). Dally and Dlp affect transport of Dpp, the former as a co-receptor, and the latter by sequestering and stabilizing Dpp (Belenkaya et al., 2004; Fujise et al., 2003; Takeo, Akiyama, Firkus, Aigaki, & Nakato, 2005; Umulis & Othmer, 2013a; Yan & Lin, 2009). Expression of a secreted form of Dally leads to overgrowth by extending the range of Dpp (Takeo et al., 2005), while Dlp has little effect on Dpp signaling. The secreted protein Pent internalizes Dally, thereby modifying the access of Dpp to Tkv and influencing local transduction of the Dpp signal (Norman et al., 2016). Loss of Pent leads to contraction of the pMad gradient and an increase in its amplitude, which changes the scaling of the Dpp gradient and expression of downstream target genes (Ben-Zvi, Pyrowolakis, Barkai, & Shilo, 2011; Hamaratoglu et al., 2011; Vuilleumier et al., 2010). In effect, Pent fine-tunes the balance between local and long-range Dpp signaling, and both *dally* and *pent* expression is repressed by Dpp. It is known how spatial scaling of solutions of reaction-transport models such as Equation (1) can be obtained by modulation of transport or kinetic parameters (Othmer & Pate, 1980; Umulis & Othmer, 2013b), and a model for active modulation of the Dpp gradient based on Dpp, Tkv, Pent, and Dally has been proposed to explain the observed scaling of the Dpp distribution (Ben-Zvi et al., 2011; Umulis & Othmer, 2013a). Further computational studies could serve to predict the profile of other genes under different conditions and compare them with the experimental observations (Bollenbach et al., 2008).

2.2 | The Wg signaling pathway

Unlike *dpp*, *wg* is expressed in the entire pouch in the second instar, and the expression region gradually shrinks to the DV boundary and two rings in the hinge region during the first half of the third instar (del Álamo Rodríguez, Terriente, Galindo, Couso, & Díaz-Benjumea, 2002; Martinez Arias, 2003). In secreting cells, Wg first undergoes apical secretion and dynamin-dependent endocytosis, followed by basolateral release and transport (Yamazaki et al., 2016). In the third instar Wg is usually considered as a morphogen that directs development via its concentration gradient, which is formed by its secretion from the source cells in the DV boundary. Models were proposed to describe the transport of Wg in the extracellular space (Swarup & Verheyen, 2012; Takada, Fujimori, Shinozuka, Takada, & Mii, 2017), in some of which Dally and Dlp were incorporated to facilitate the long-range transport of Wg (Franch-Marro et al., 2005; Marois, Mahmoud, & Eaton, 2006), and in others used to illustrate their role in stabilizing Wg at the cell membrane (Hufnagel, Kreuger, Cohen, & Shraiman, 2006). However, the role of Wg as a morphogen for growth control was challenged by the observation that membrane-tethered Wg generates a normally patterned disk of a slightly smaller size (Alexandre et al., 2014). It was also shown that the prolonged wg transcription could maintain the target gene expression in the interior region of the pouch, which led to the suggestion that the spreading of Wg might be dispensable and that a Wg gradient is not needed for normal disk growth (Baena-Lopez et al., 2009).

While global expression of *wg* recedes in the third instar, local expression of it and its main downstream effector *vg* is turned on at the DV boundary by the vestigial boundary enhancer and DSL (Neumann & Cohen, 1996; Zecca & Struhl, 2007a). In the early third instar, the activity of the Quadrant Enhancer, which activates *vg* expression throughout the pouch, is initialized by inputs from Wg, Dpp, and Notch/Delta (Kim et al., 1996; Klein & Martinez Arias, 1999; Zecca & Struhl, 2007a). *vg* expressing cells also send a short-range signal to upregulate *vg* expression in neighboring cells in conjunction with other inputs (Klein & Martinez Arias, 1999; Zecca & Struhl, 2007b). Thus it was proposed that *vg* generates a feed-forward signal in the wing which is responsible for recruiting nonwing cells into the disk on the proximal boundary (Zecca & Struhl, 2010). As a result, growth of the wing is driven by two mechanisms, either through the signaling-induced wing cell proliferation, or via the incorporation of neighboring undifferentiated cells. The latter

were found to occur between the early and mid-third instar and account for 20% of the wing size (Muñoz-Nava, Alvarez, Chara, & Nahmad, 2019).

Wg and Vg also are involved in other processes that impinge on Hippo signaling. Vg interacts directly with Hippo signaling along the DV boundary, since it competes with Yki to bind with the co-transcriptional factor Sc. This causes the down-regulation of Yki transcriptional activity along the DV boundary (Djiane, Zaessinger, Babaoğlan, & Bray, 2014).

Wg is also required for local cell proliferation in the hinge (Casares & Mann, 2000; Perea, Terriente, & Díaz-Benjumea, 2009). Wg activates the gene *homothorax (hth)* in the hinge, which represses *vg* expression and blocks wing development (Casares & Mann, 2000; Rodríguez, 2004), and it was proposed that it is the mutual inhibition between *vg* and *hth* that determines the boundary of the wing pouch. Thus it is important to understand downstream signaling of Wg in different regions of the wing disk to understand the wing development, and in particular, the transformation of nonwing cells to pouch cells in the feed-forward mechanism. The detailed mechanism, which depends on Fat-Ds signaling as well, is discussed later.

2.3 | The hippo pathway

The Hippo pathway serves as the integrator of several upstream signaling pathways (Figures 2 and 3) and as a controller of entry into the cell cycle. The Hippo module consists of the kinases Hippo, Wts, the adaptor proteins Salvador (Sav), and Mob-as-tumor-suppressor (Mats). The effector of this module is Wts which regulates nuclear entry of Yki by phosphorylating it. Nuclear Yki binds to transcription factors such as Sd to activate the expression of *cyclin E*, *myc*, *DIAP1*, and *bantam*, which control cell proliferation. It also controls expression of genes upstream of the Hippo module, such as *expanded*, *merlin*, *kibra*, and *fj* (Blair & McNeill, 2018; Fulford et al., 2018). Upstream regulators of Yki include Fat (Ft) and Dachsous (Ds), two atypical cadherins that form heterodimers between cells and control pathways which regulate Wts (Figure 3). Both Ft and Ds have intracellular, transmembrane and extracellular domains. The intracellular domains (ICDs) of each can independently modulate Yki levels within a cell, while Ft and Ds on adjacent cell membranes can also associate via their extracellular domains (ECDs) to modulate the cell–cell interaction. Binding between Ds and Ft is modulated by Fj, which phosphorylates the ECDs of Ft and Ds in the Golgi (Hale, Brittle, Fisher, Monk, & Strutt, 2015).

Ft expression is high in the pouch region, especially along the DV boundary, and low in the hinge (Mao, Kucuk, & Irvine, 2009), while Ds and Fj are expressed in a graded manner. Ds expression is low in the wing pouch and is largely confined to the hinge region (Ambegaonkar, Pan, Mani, Feng, & Irvine, 2012; Strutt & Strutt, 2002), while Fj is expressed in a decreasing gradient along the distal-proximal axis (Brittle, Thomas, & Strutt, 2012). Ft signaling via its ICD suppresses growth via Dachs, an atypical myosin whose gene is epistatic to *fat* in terms of its growth effect. Dachs accumulates near the adherens junctions, and membrane-localized Dachs can bind Wts and promote its degradation (Cho et al., 2006), thereby reducing the inhibitory effect of Wts on Yki (Figure 3). Signaling from the ICD of Ds enhances growth by interaction with Riquiqui (Riq), a scaffold protein, and Minibrain (Mnb), a kinase (Degoutin et al., 2013). Ds is required for localization of Riq at the apical junctions, and localized Riq potentiates Mnb phosphorylation of Wts, which reduces its activity (Degoutin et al., 2013).

Experimental results using disk-wide interventions or mutant clones have led to several questions concerning how Ft and Ds collaborate to regulate the Hippo pathway. For example, the effect of Ft on growth is not a strictly decreasing function of the Ft level, as might be expected (Mani, Goyal, Irvine, & Shraiman, 2013). OE of *fat* above wild-type (WT) levels decreases the wing size and complete KO of *fat* increases the size, but a partial knockout of *fat* decreases, rather than increases, the size. Similarly, the effect of Ds is also nonmonotonic: loss of Ds results in enlarged wing disks (Feng & Irvine, 2009), but OE can either reduce (Feng & Irvine, 2009; Rogulja et al., 2008) or enhance growth (Degoutin et al., 2013). The nonlinear regulation of Ft and Ds on Yki was explained by a model that involves subtle competition between the inhibitory and activating branches of the Ft–Ds control network (Gou et al., 2018).

The Ft–Ds–Fj pathway also plays an important role in regulating planar cell polarity (PCP). In addition, other transmembrane proteins affect Hippo dynamics. For instance, Crumbs, which is another upstream regulator of Hippo, is a transmembrane protein whose extracellular domains form homodimers on adjacent cells. In the intracellular space, Crumbs binds with the FERM domain protein Expanded (Ex), Merlin (Mer), and Kibra in the apical domain, which together activate the Hippo kinase cascade (Badouel et al., 2009; Ling et al., 2010). Like Ft/Ds, Crumbs reflects another

mechanism for transmitting cell-to-cell information, and levels of Crumbs can affect cell survival (Hafezi, Bosch, & Hariharan, 2012; Pocha & Knust, 2013).

2.4 | The relationship between the cell cycle and tissue growth

Development of the wing disk from egg laying to pupation involves 9–11 rounds of cell division, with an average cycle time of around 8.5 hr (Martín et al., 2009; Matamoro-Vidal et al., 2015). In addition to the role of the pathways shown in Figure 2 in patterning of the disk, many of their target genes are known to be involved in control of the cell cycle and cell division. It has been shown that Yki and Sd regulate the expression of *cycE*, which is a key regulator of the G1-S transition in *Drosophila* (Lee & Orr-Weaver, 2003). In addition, the Hippo and Dpp pathways target *bantam*, which regulates cell proliferation through the gene *tribble*. Tribble induces the degradation of *cdc25* mitotic activators and regulates proliferation by slowing the G2-M transition (Gerlach, Sander, Song, & Herranz, 2019; Mata, Curado, Ephrussi, & Rørth, 2000). *dmyc*, another Hippo target, acts downstream of insulin and the nutrient-sensing target of rapamycin (TOR) pathway to control growth in the G1 phase of the cell cycle (Gallant, 2013; Parisi et al., 2011).

Cell cycle times in the disk vary both spatially and temporally. For example, wing disk cells show heterogeneous spatial patterns of cell cycle times, in which clusters of cells appear in the same phase due to local cell–cell interactions (Milán, Campuzano, & García-Bellido, 1996a), but cell patches at different locations of the disk show different cell cycle phases and duration (Dubatolova & Omelyanchuk, 2004; Milán, Campuzano, & García-Bellido, 1996b). As mentioned earlier, mechanical forces have been proposed as one of the important controllers of growth, and have also been suggested as mediators of cell cycle control in various systems (Schluck, Nienhaus, Aegerter-Wilmsen, & Aegerter, 2013; Uroz et al., 2018), partially through the regulation of the Hippo components (Madan, Gogna, & Moreno, 2018). These will be discussed further in the next section.

The growth rate of the disk also varies greatly in time (Martín et al., 2009). The majority of cell divisions occurs between second and mid-third instar, and the cell proliferation rate slows down in late-third instar (Nienhaus, Aegerter-Wilmsen, & Aegerter, 2012). This is caused by the cooperation of different growth regulators in the wing disk, but the detailed mechanism of the temporal regulation of the wing disk growth control remains to be elucidated.

However, the cell cycle time and growth rates apparently do not affect the final disk size. In normal WT disks there are about 20–40 cells initially, but the disk can also develop from less than 10 cells, and it may be composed of fewer but larger cells, or increased numbers of smaller cells (Neufeld, de la Cruz, Johnston, & Edgar, 1998). Moreover, as stated earlier, the presence of patches of cells with different growth rates within a compartment does not affect the final size of the disk—faster cells simply outcompete slower cells (Pérez-Garijo, Shlevkov, & Morata, 2009; Worley, Setiawan, & Hariharan, 2013). These observations indicate that there is a tissue level autonomous size control mechanism at work, and a theoretical mechanism for how this may function has been suggested (Gou et al., 2018). In a recent paper, Moss-Taylor et al. showed that Act-β may play the role of a tissue-level coordinator (Moss-Taylor, Upadhyay, Pan, Kim, & O'Connor, 2019).

2.5 | The role of calcium dynamics

Calcium also plays a significant, but poorly understood role in development of the wing disk. Strong phenotypes, including blistered or bent wings, result when Ca^{2+} signaling is perturbed (Balaji et al., 2017; Brodskiy et al., 2019; Dahal et al., 2012; Dahal, Pradhan, & Bates, 2017). Ca^{2+} is a second messenger, and Ca^{2+} signaling networks often exhibit a "bow-tie" structure in that the integrated effect of many inputs is governed by a small number of molecules in the calcium pathway, and they affect many outputs (Brodskiy & Zartman, 2018), similar to the way that many growth-affecting factors impinge upon the Hippo kinase cascade. Like Hippo, growth and mechanical signals both affect Ca^{2+} (Antunes, Pereira, Cordeiro, Almeida, & Jacinto, 2013; Balaji et al., 2017; Brodskiy et al., 2019; Narciso et al., 2015; Narciso, Contento, Storey, Hoelzle, & Zartman, 2017; Restrepo & Basler, 2016). Ca^{2+} signaling in the wing disk exhibits a rich variety of dynamical behaviors. Four distinct modes of signaling exist: spiking, intercellular transients, intercellular waves, and fluttering (Brodskiy et al., 2019). The contribution of each signal type to the overall calcium signal levels varies over time in an overall-decreasing trend as the disk ages (Brodskiy et al., 2019). A recent study has linked spiking with insulin activity, which suggests that it promotes growth, whereas intercellular waves inhibit growth

(Paravitorghabeh, Soundarrajan, & Zartman, 2019). An associated mathematical model in which a Hopf bifurcation parameter governs cellular Ca^{2+} signaling is able to reproduce many features of the observed Ca^{2+} dynamics.

Calcium is particularly interesting in the context of morphogenesis as it affects cytoskeletal behavior and morphogen signaling. Knockdown of the sarco/endoplasmic reticulum calcium pump, SERCA, has been shown to alter cytoskeletal organization (Balaji et al., 2017), and calcium flashes that occur after mechanical wounding are necessary for coordinated actomyosin activity in the wound healing process (Antunes et al., 2013; Narciso et al., 2015; Restrepo & Basler, 2016). A further study has shown that the release, but not the onset, of mechanical compression in ex vivo disks stimulates calcium signaling (Narciso et al., 2017). It would be interesting to determine if *increased* tension can also induce intercellular Ca^{2+} waves, and if these effects occur in vivo (Balaji et al., 2017). Calcium ions are also crucial for cell–cell adhesion; E-Cad, and possibly Ft and Ds, exist in different conformations depending on the number of bound Ca^{2+} ions (Courjean et al., 2008; Tsukasaki et al., 2014).

2.6 | Pathway crosstalk

Growth control of pouch cells involves several signaling pathways, and the existence of crosstalk between different pathway components makes the interpretation of the growth regulatory mechanisms of the disk difficult. As shown in Figure 2, the Dpp and Wg pathways interact at different levels of the signaling cascade, some of which may be crucial for the cooperative function of the two pathways in growth regulation. For instance, Brk, which is negatively regulated by Dpp and required for Wg target gene expression, represses Naked, which is a feedback inhibitor of Wg signaling (Yang, Meng, Da Ma, & Fang, 2013) (Figure 2). pMad transduces Dpp signals, while unphosphorylated Mad interacts with the Pan-Arm transcriptional complex to regulate the expression of Wg target genes (Eivers, Demagny, Choi, & De Robertis, 2011; Eivers, Demagny, & De Robertis, 2009). Thus the two pathways compete for available Mad in the signaling process.

The feed-forward mechanism alluded to earlier also involves several pathways. In the distal region of the pouch, high Wg, together with an auto-regulatory circuit, drives *vg* expression. The authors propose that Vg promotes the expression of *fj*, which enhances Ft signaling at the expense of Ds signaling (Zecca & Struhl, 2010). Conversely, in nonwing cells the absence of Vg and a low level of active Yki lead to low levels of Fj and high levels of Ds, thus promoting Ds signaling at the expense of the Ft signaling. At the interface, the polarized Dachs localization that results from the opposed Ft and Ds gradients elevates the Yki activity, which initiates the auto-regulatory circuit of Vg, and the Wg-dependent Vg regulation that leads the transformation from nonwing to wing cells (Zecca & Struhl, 2010).

This postulated mechanism is intriguing and it explains the transformation of nonwing to wing cells in terms of the spatial distribution of various factors. However, several questions arise. (a) Ft and Ds form heterodimers on opposing cell membranes and their abilities to regulate the downstream Yki activity is coupled through the interactions with Dachs and Riq (Gou et al., 2018). Thus it is not clear how their signaling effects can be separated so cleanly. (b) The propagation of the recruitment process depends on the decaying gradient of Wg from distal to proximal. But in the third instar, Wg is expressed both along the DV boundary and in two rings around the pouch lying in the hinge region (Rodríguez, 2004). What role does expression of *wg* in the hinge play in the recruitment mechanism, and is cell recruitment to the pouch observed in experiments with membrane tethered-Wg?

Several other signaling pathways also have been shown to influence disk development. Hh signaling stimulates Dpp expression and determines vein location (Matamoro-Vidal et al., 2015). Another TGF-β superfamily protein, activin, also plays a minor role governing cell proliferation and is required for larval development (Brummel et al., 1999; Peterson & O'Connor, 2013). The activin type I receptor Babo can phosphorylate Mad (Peterson et al., 2012) and activin signaling competes with BMP signaling for Medea (Takaesu et al., 2006), it has been proposed that the activin pathway antagonizes BMP signaling (Sander, Eivers, Choi, & De Robertis, 2010).

Recent developments have also implicated electrochemical signaling pathways as growth regulators (Dahal et al., 2017; George et al., 2019). Ca^{2+} signaling is involved in the Hh, Dpp, and Wnt morphogen networks. For instance, it has been shown that calcium signaling is involved in Dpp secretion, (Dahal et al., 2012; Dahal et al., 2017), and to correlate with disk age and size (Brodskiy et al., 2019). In addition, spatial variations in intercellular Ca^{2+} waves are altered in knockdowns of Dpp and Hh signaling (Brodskiy et al., 2019), and under reduced (increased) Dpp signaling, Ca^{2+} signaling activity increased (decreased). In Dpp-secreting cells, calcium sensitive IrK activity is necessary for pulsatile Dpp secretion (Dahal et al., 2012; Dahal et al., 2017). In addition to its effects on the Dpp-pathway, Ca^{2+} levels in the endoplasmic reticulum (ER) affect the Wnt pathway (Suisse & Treisman, 2019). Mutations of SERCA reduce ER calcium

stores and inhibit Wnt-signaling via Arm by inhibiting its ability to translocate to the nucleus. Instead, Wnt remains bound to E-Cad until it is degraded. Interestingly, the Hippo pathway is activated when disruptions to ER calcium are strong enough (Suisse & Treisman, 2019).

Because several different pathways are affected by calcium signaling, it is challenging to construct a comprehensive theory to explain the known observations. Nonetheless, one mechanism, the *Calcium shunt hypothesis*, has been proposed to explain Ca^{2+}-dependent growth (Brodskiy et al., 2019; Paravitorghabeh et al., 2019). The theory is that the partition of PIP_2 between growth pathways and downstream calcium signaling modulates growth, but this is unproven, and understanding the effect of calcium in morphogenesis (George et al., 2019) is still an active area of research.

3 | THE ROLE OF MECHANICAL FORCES IN WING DISK MORPHOGENESIS

A growing wing disk is subject to a variety of mechanical forces occurring at different length scales that may influence its size and shape. Firstly, since the disk is growing in the intact larva, there are forces from tissue attachments acting on the disk. Figure 7a shows the shape of an early third instar in vivo disk when it is attached to a muscle that can exert force on it, and Figure 7b shows the shape after severing the muscle fiber. The large change in shape indicates that this force is significant, but what effects these forces have on the final disk shape are not known. However, since disks transplanted into mature adults grow to normal size, despite the fact that they are in a different mechanical environment, one may expect the effects to be minor (Bryant & Levinson, 1985). However, this remains to be clarified.

Second, the PE is attached to the periphery of the growing layer of columnar cells (Figure 1). It may exert restraining forces on the disk as it stretches due to the growth of the disk. When stretching is inhibited, hinge or notum cells are repositioned next to the peripodial membrane, and this leads to smaller disks (Fletcher et al., 2018). A mathematical model may shed light on this phenomenon, and on the fold formation described below.

At the level of the pouch proper, the compartment boundaries provide barriers that cells cannot cross, and the importance of local increases in bond tension along the AP and DV boundaries for separating cell lineages is well known (Aliee et al., 2012; Landsberg et al., 2009; Major & Irvine, 2005; Major & Irvine, 2006; Michel et al., 2016; Rudolf et al., 2015). The increased tension, induced by upregulation of actomyosin in boundary cells by signals that induce localized Hh (AP) and Notch (DV) activity (Klein, 2001), is sufficient to prevent intermixing of cells from different compartments and contributes to biased intercalations, division orientation anisotropy, and boundary smoothness. In addition, columnar disk cells are also attached to the basement membrane (Figure 5), which exerts additional restraining forces on the disk.

Growth leads to spatially variable mechanical properties of the disk, for as it grows, tangentially oriented actomyosin cables form in the disk periphery (LeGoff, Rouault, & Lecuit, 2013). Cells exert passive forces due to growth and active forces due to cell movement on neighboring cells, and tension changes between the center and periphery of the disks during growth. In younger third-instar disks, the center is under higher tension relative to the peripheral region compared with older disks (Pan et al., 2018). These forces lead to feedbacks that may regulate growth, size, and patterning (Buchmann, Alber, & Zartman, 2014; Heisenberg & Bellaïche, 2013; Irvine & Harvey, 2015; LeGoff & Lecuit, 2016; Mao & Baum, 2015) and are important in later pupal morphogenetic transformations (Classen, Anderson, Marois, & Eaton, 2005; Etournay et al., 2015; Li, Naveed, Kachalo, Xu, & Liang, 2014; Sugimura & Ishihara, 2013). Thus cell and tissue growth occurs on a complex, evolving landscape of local and global mechanical forces, and understanding the logic of how mechanics and growth are intertwined in the disk is a major open problem.

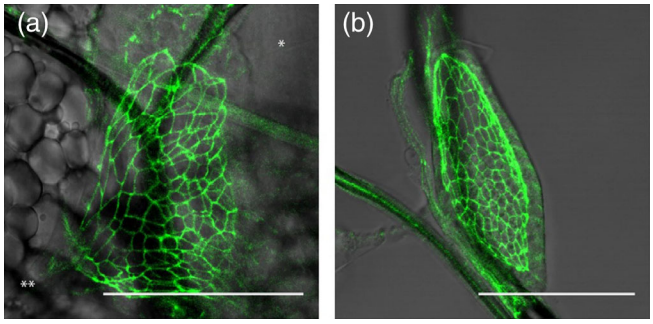

FIGURE 7 Images of a wing disk in which the apical cell outlines of the disk proper are marked by GFP fused to E-Cadherin. In (a) the large muscle fiber between the wing, leg, and haltere disks is exerts a substantial force on the disk at (**). (b) shows the same wing disk after dissection, which relaxes the external force and leads to a significant change in cell shape. The scale bar is 50 mm. From (Nienhaus et al., 2012)

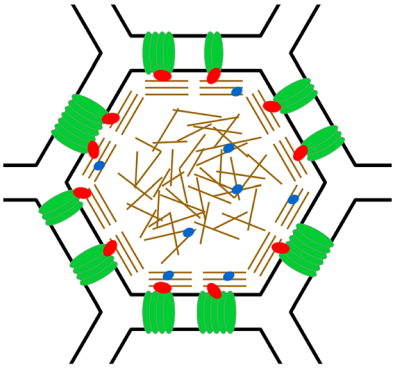

FIGURE 8 An apical section of a cell in a tissue, showing the E-Cad junctions (green), the cortex, Arm (red), myosin (blue) and the apical CSK

3.1 | The mechanical properties of the disk

Cell-level mechanical effects are mediated by the CSK and its interaction with AJs (Figure 8) (Leckband & De Rooij, 2014; Pinheiro & Bellaïche, 2018). E-Cads on opposing membranes form homophilic bonds via their extracellular domains, and their intracellular domains act as mechanotransducers via their linkage to the CSK through adapter proteins. These include α- and β-catenin (Armadillo [Arm] in *Drosophila*) and vinculin, which link the E-Cads to the cortical actomyosin network. This circumferential cortical bundle produces forces both normal and tangential to the membrane, which are transmitted across the AJs, and these cell-to-cell connections in turn mediate tissue-level contractile forces. During growth, many regulators of actomyosin are upregulated, and the process of cell division involves highly precise regulation of actomyosin contractility (Pinheiro et al., 2017). Actomyosin levels are spatially variable throughout the disk, being lower in the center and elevated in the periphery and in cells along compartment boundaries (Aliee et al., 2012; Major & Irvine, 2006; Pan et al., 2018). The role of actomyosin in exerting tensile force on adherens junctions is crucial in the activation of many of the pathways discussed later (Sun & Irvine, 2016).

The core AJ complex consists of E-Cad bound to p120-catenin, and Arm, which binds to α-catenin. The latter can bind directly to F-actin, (Leckband & De Rooij, 2014; Pinheiro & Bellaïche, 2018) forming a link to the CSK. p120-catenin also interacts with the microtubules (Singh et al., 2018), myosin VI, and Rho GTPase (Bryant & Stow, 2004). Thus, it is directly involved with the attachment of the CSK to AJs. The AJs respond anisotropically to applied forces (Kale et al., 2018): tangentially oriented forces stemming from the cortex tend to disrupt E-Cad binding, whereas normally oriented forces due to the apical CSK can strengthen cell–cell adhesion. Distinct networks of actomyosin are involved in these distinct responses to forces. The strengthening of junctions under normal force and weakening under tangential force is one mechanism that allows for epithelial integrity to be maintained in a tissue while permitting enough plasticity for division and cell rearrangements.

An important property of disk cells is their ability to modulate tissue-scale mechanical properties in response to stress through the reorganization of the actomyosin or other load-bearing proteins. Even without myosin contractility, cross-linked networks of actin can exhibit a range of stiffnesses and mechanical properties (Bieling et al., 2016). In the wing disk, normal stress leads to the reinforcement of AJs (Kale et al., 2018; Pinheiro & Bellaïche, 2018; Venkatesan Iyer, Piscitello-Gómez, Paijmans, Jülicher, & Eaton, 2019) and the reorientation of actomyosin to resist deformation (Acharya et al., 2018; Duda et al., 2019). Modulation of mechanical properties can thereby influence the stress and strain distributions and may thereby affect growth in the disk.

3.2 | The role of the basement membrane

Throughout development, interaction of columnar cells with the basement membrane (BM) is necessary to maintain their shape in the disk (Domínguez-Giménez, Brown, & Martín-Bermudo, 2007), and to guide apposition and folding along the DV axis (Domínguez-Giménez et al., 2007). In addition computational models show that the interaction is necessary to properly form folds in the third instar (Sui et al., 2018; Tozluoğlu et al., 2019). The connection of the BM to the peripheral portion of the wing disk also influences fold formation (Sui et al., 2018). Stiffening or degradation of the basement membrane in the disk appears to have strong effects on wing disk size, but not on Hippo signaling, thus questioning the role of Hippo signaling in growth (Ma, Cao, Dai, & Pastor-Pareja, 2017). However, it was later claimed

that BM degradation does not influence apical tension (Pan et al., 2018) that modulates key Hippo pathway components, even though it can affect cell shape and leads to smaller wings. On the other hand, the effect of elevated BM compression on apical tension remains untested, although recent evidence indicates no effect on Hippo signaling or the final disk size (Ma et al., 2017). Given the large aspect ratio of disk cell height to diameter—height up to 40 μm and disk diameter approximately 250 μm—even slight curvature, in conjunction with compressive forces due to collagen in the basement membrane and the tensile interaction of cytoskeletal elements with the AJs, may be sufficient to cause simultaneous basal compression and apical tension. 3D modeling of the disk may help sort out the answer to this question.

Three types of folds are formed in the disk: a fold between the notum and the hinge, the fold in the central hinge, and the fold separating the hinge and the wing pouch (Sui et al., 2018; Sui, Pflugfelder, & Shen, 2012) (Figure 1). These folds are caused by active processes in the disk when the growth rate is relatively high (Bryant & Levinson, 1985), possibly arising in the basement membrane, since it can exert compressive forces on the pouch cells (Pastor-Pareja & Xu, 2011). Evidence for the role of the BM in fold formation comes from recent laser-ablation experiments which indicate that basal edge tension of disk cells is three to five times as high as apical edge tension and can be regulated to promote hinge-pouch fold formation (Sui et al., 2018). In contrast, the hinge-hinge fold forms by an active apico-basal shortening process governed by actomyosin contractility, leading to shape changes that initiate folding (Sui et al., 2018). Furthermore, a finite deformation-based model that takes into account growth and the effect of apical stiffness due to actomyosin along with basement properties is able to reproduce the location of the disk-hinge fold (Tozluoğlu et al., 2019). Crucially, in order for realistic folding to occur in that model, an external resistance—such as BM interaction—to wing disk deformation was necessary.

Whether fold formation affects disk growth, size, or patterning is not known, but an accurate representation of the geometry of the folding disk requires a three-dimensional mechanical theory. Many current and previous wing disk growth and mechanics models assume a flat disk with relevant mechanics, biochemistry, and growth occurring in a planar apical layer. However, growth and mechanical properties of the wing disk are inherently three-dimensional given the large aspect ratio, which casts doubt on the validity of planar or shell approximations (Figure 9). Differences also occur along the apico-basal axis of cells in terms of protein localization, morphogen transport (Harmansa et al., 2017), and mechanics.

Further experiments are needed to define the mechanical interaction between the BM and columnar cells and to understand how feedback between the cells and the BM leads to coordinated growth. Several recent simulation studies have identified an important role for the BM and ECM in guiding fold formation in the growing wing disk (see Figure 9), and in the overall mechanical properties of the wing disk (Atzeni, Lanfranconi, & Aegerter, 2019; Keller, Lanfranconi, & Aegerter, 2018; Tozluoğlu et al., 2019). How the basement membrane and ECM are temporally regulated to tune stress in the wing disk may have a profound effect on wing disk growth (Pastor-Pareja & Xu, 2011).

4 | MECHANOTRANSDUCTION IN THE DISK

To date, all known mechanical effects on growth act via the Hippo pathway (K. D. Irvine, personal communication, 2019), primarily by regulating the localization of Wts at the AJs, and thereby controlling its effect on Yki. Increased tension, either in the cortex attached to the AJs or in the apical CSK, stimulates growth by increasing sequestration of Wts at the membrane (Aragona et al., 2013; Fa-Xing & Guan, 2013; Nelson et al., 2005; Rauskolb, Sun, Sun, Pan, & Irvine, 2014; Reddy & Irvine, 2013). At AJs experiencing tension in the normal direction, α-catenin unfurls and binds with the LIM domain protein Ajuba (Jub) and can then sequester Wts, preventing it from being activated (Alégot et al., 2019; Rauskolb et al., 2014). Most Wts is bound to Jub at the cell membrane in the disk (Sun, Reddy, & Irvine, 2015), and Wts

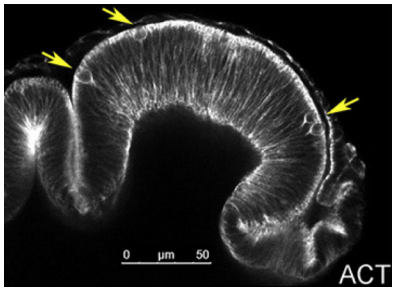

FIGURE 9 A 2D slice of the disk along its apicobasal axis (Meyer, Ikmi, & Gibson, 2011), yellow arrows are cells that are dividing. Note the large aspect ratio and curvature of the disk

expression tends to mirror Jub expression in much of the disk (Pan et al., 2018). Under low tension, Wts is released from Jub and can bind with Expanded-containing complexes to become activated (Sun et al., 2015), inhibiting Yki nuclear localization. The sensitivity of the wing disk to the control of Hippo signaling through Jub is dynamic and correlates with decreasing tension in the wing disk as it grows (Pan et al., 2018; Rauskolb et al., 2014). This mechanism introduces feedback that inhibits growth when CSK tension drops due to crowding (Pan, Heemskerk, Ibar, Shraiman, & Irvine, 2016). Aside from its role in the Hippo pathway, Jub also affects disk mechanics by modulating AJ stability during topological changes in cell contacts (Rauskolb, Cervantes, Madere, & Irvine, 2019; Razzell, Bustillo, & Zallen, 2018) and through its role in a pathway that relaxes actomyosin contractility (Rauskolb et al., 2019).

Since Hippo signaling depends on the mechanical state of the disk, the CSK plays a critical role throughout. Null mutants of the actin capping proteins Cp_α and Cp_β, or over-activity of the formin diaphanous leads to increased F-actin production, which down-regulates the Hippo pathway by increasing membrane localization of Wts and increases tissue overgrowth (Sansores-Garcia et al., 2011). Because OE of F-actin does not affect cell-polarity, and simultaneous OE of Wts offsets the effect of increased F-actin, it seems that the primary effect of F-actin OE is on localization of Wts (Sansores-Garcia et al., 2011). Conversely, Hippo has been shown to suppress F-actin polymerization, although the exact mechanism is unknown (Fernández et al., 2011; Sansores-Garcia et al., 2011; Sun & Irvine, 2016).

In addition to acting as an F-actin capping protein, Cp_α also regulates Yki nuclear translocation through an unresolved mechanism that likely involves Hippo signaling (Fernández et al., 2011; Janody & Treisman, 2006). In the wing disk periphery, the Wg/Vg feed-forward mechanism leads to increases in Cp_α and Cp_β levels. This may alter cell mechanical properties since Cp_α binds with F-actin, thereby altering the filament length distribution (Hu, Matzavinos, & Othmer, 2007).

Arm has dual roles in disk cells: transducing Wg signals (Hall, Hoesing, Sinkovics, & Verheyen, 2019; Leckband & De Rooij, 2014) and in cell adhesion via AJs, and thus there is a competition for it. Arm has binding sites for adhesion components such as α-catenin, and for transcription control regulators (Bejsovec, 2018). Arm changes conformation in response to tension, which allows for Src phosphorylation, and phosphorylation may regulate the switch between the cell adhesion and signaling roles of Arm (Röper et al., 2018). Altogether these results indicate an intricate balancing between the Hippo pathway and the cytoskeleton.

In addition to actin, the motor protein NMII also acts in the mechanotranduction pathways, NMII generates the contractile force in the CSK and cortex, and increased NMII leads to increased E-Cad activity at the AJs (Hall et al., 2019). The increased E-Cad binds more Arm and actin, which in turn reduces Wg signaling, increases Wts localization, and increases Yki activity. Reduction of NMII leads to an increased apical surface, decreased junctional tension, and

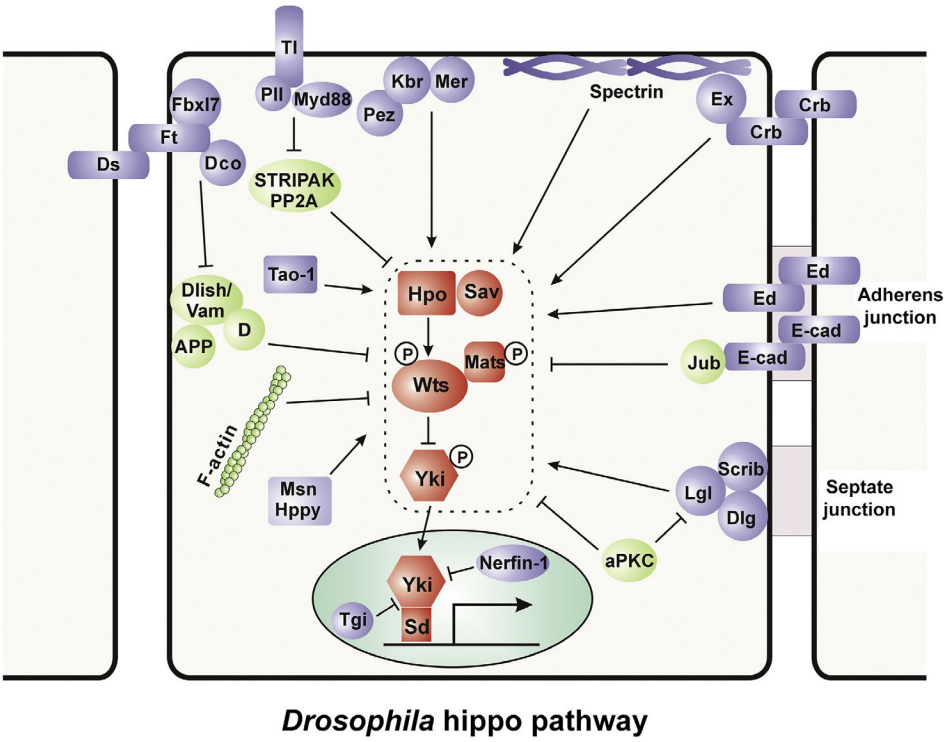

FIGURE 10 Other regulators of the Hippo pathway (Reprinted with permission from Zheng and Pan (2019))

smaller wings (Tsoumpekos, Nemetschke, & Knust, 2018). Cortical Yki also promotes NMII activation via activation of a myosin light chain kinase, which leads to a positive feedback loop (Xu et al., 2018). The role of actin in the tension-sensing is also complex: some results show that the binding rate of cofilin, which severs actin filaments, is reduced when actin bundle tension is high (Hayakawa, Tatsumi, & Sokabe, 2011), and this promotes Yki activity.

The junctional adhesion protein, Ed (Figure 10) also affects Yki phosphorylation (Yue, Tian, & Jiang, 2012). Ed homodimerizes with molecules on adjacent cells upon cell–cell contact, and complements E-Cad complexes in promoting cell–cell adhesion (Wei et al., 2005). Ed regulates the Hippo pathway upon cell–cell contact by binding with Sav, thereby inhibiting its degradation in the cytosol (Yue et al., 2012). Ed also regulates Wg levels away from the DV boundary (Yue et al., 2012).

Yet another mechanotransduction pathway involves α, β, and β_H-spectrins (Fletcher et al., 2015). In conjunction with other upstream Hippo regulators such as the Crumbs complex, spectrins regulate Yki localization in non-proliferative cells, such as those in the PE (Fletcher et al., 2018). It is believed that dilution due to areal expansion reduces Wts activity by inhibiting the Hippo dimerization needed for Wts activation, thus allowing for the nuclear accumulation of Yki (Fletcher et al., 2015; Fletcher et al., 2018). However, the dilution mechanism is probably less active in the disk proper where cells retain a columnar shape (Pan et al., 2018).

The complex interplays reflected in these and additional results suggest the need for a synthesis of known effects of the CSK on the Hippo pathway and its interactions with the biochemical pathways (Irvine & Harvey, 2015).

5 | A MODEL FOR THE CONTROL OF DISK SIZE

The foregoing shows that a great deal is known about many components involved in growth and patterning, and a number of cell-based models that treat the interior of each cell as a continuum have been proposed (Marciniak-Czochra & Ptashnyk, 2008; Sample & Shvartsman, 2010). One involves a cell-based model of the Hippo pathway which showed how cell–cell interactions via the Ft/Ds pathway can influence Yki levels and affect local growth (Gou et al., 2018). Furthermore, the preceding discussion of mechanotransduction suggests that a similar cell-based model can be constructed to explain how forces transmitted from cell-to-cell via AJs can affect Yki levels and growth, and as a next step the two models could be combined. Challenges associated with using such highly resolved models at the tissue level are (a) computational feasibility, and (b) upscaling: can the detailed model be approximated by a macroscale model? The second point is a crucial theoretical question for growing tissues, where local cell–cell differences and cell-competition play a role in how cells choose whether to grow and divide or not.

At present there is no general model that can integrate all the known facts and predict how the size of the disk is controlled. One step in that direction originated in a continuum-level mechanical description which postulates that faster-growing cells exert stress on their neighbors, whose reciprocal stress slows the growth rate of the faster-growing cells (Shraiman, 2005). This creates a stress gradient across the disk, which might then lead to a uniform growth rate over the disk, but there was no mechanism for terminating growth. A more recent cell-based model incorporates a simplified network for morphogen signaling and a mechanism for growth termination (Aegerter-Wilmsen et al., 2012), and we call the result the mechanical feedback model (MFM). The MFM is built on the following essential hypotheses—others are described in (Aegerter-Wilmsen et al., 2012).

1. Centrally secreted morphogens induce growth, which leads to compressive forces in the disk center due to growth incompatibilities and resistance from tissue surrounding the center. The Dpp gradient is assumed to scale with disk size, but the Wg gradient need not.
2. When compression exceeds a certain level, mechanosensitive growth regulatory pathways halt growth in the center of the disk.
3. In the periphery, stretching induced by the expansion of the disk center and morphogen signaling induces growth.
4. Cells can measure the tension gradient and growth stops when both the compression in the center and the tension gradient exceed thresholds.

Experimental observations have confirmed the increase of central compression and peripheral tension (LeGoff et al., 2013; Nienhaus, Aegerter-Wilmsen, & Aegerter, 2009; Pan et al., 2018; Schluck & Aegerter, 2010) and a computational implementation of the model that uses a cell-based descriptions of the tissue has been used to confirm that realistic wing sizes can be predicted under a number of additional assumptions (Aegerter-Wilmsen et al., 2012). Of course a

simple model of an isotropically growing disk shows that central compression and peripheral tension are inherent properties that will appear. In any case, the first three hypotheses are well-founded. At present there is no experimental evidence that cells can measure a stress gradient, but it is possible that a plausible theoretical model could be built on the observations in the previous section.

There are however a number of observations that the model cannot explain. Since Dpp plays an essential role in growth control, it is difficult to explain the fact that *dpp*/*brk* KOs exhibit enhanced lateral growth and reduced medial growth (Schwank, Tauriello, et al., 2011). In addition, the Wg (Alexandre et al., 2014) and Dpp (Schwank, Tauriello, et al., 2011) gradients may not be necessary for growth. Further, since the Dpp secreting stripe extends across the entire disk, portions of the disk boundary will experience high Dpp levels, and its effect on growth cannot be offset by compression of boundary cells, since they are in fact under tension. A similar difficulty applies to explaining the independence of growth control in compartments. If one compartment grows more rapidly, how is tension balanced along the compartment boundary? Also whatever mechanism halts growth must be able to deal with the fact that peripheral cells maintain high nuclear Yki levels and are under tension throughout the third instar (Pan et al., 2018).

Other aspects that are not addressed with the model are as follows. It is known that high nuclear Yki levels and Dpp signaling, but not Wg, are present in peripodial cells which stretch during growth, but do not proliferate (Baena-López et al., 2003; Fletcher et al., 2018; Manning et al., 2018; McClure & Schubiger, 2005). Understanding what downstream factors allow proliferation in the wing disk but not the peripodial membrane may shed light on the MFM. In addition, the Vg feed-forward recruitment pathway, which may contribute up to 20% of the final disk size (Muñoz-Nava, Alvarez, Chara, Flores-Flores, & Nahmad, 2019), must somehow be integrated into the MFM.

A major gap in our current knowledge of cell growth concerns how addition of mass is controlled and why compressive forces inhibit growth. Associations between the Hippo pathway and osmotic stress in mammals are known (Ma, Meng, Chen, & Guan, 2019), but what effect they have in the wing disk is unknown. At the molecular level stress can be transmitted to neighboring cells via the AJs, but how this is transduced by the Hippo pathway to signal mass uptake in preparation for division is not understood. Perhaps changes in the osmotic balances in cells under compression can inhibit nutrient uptake needed for division, or that the pressure gradient is important in cell growth. Very little is definitively known at this point. In other model organisms, calcium dynamics play an important role in cell shape changes in the context of cell competition. Differential osmotic regulation, regulated in part by Ca^{2+}, in adjacent winner and loser cells can facilitate the extrusion of loser cells from the tissue.

Whereas the MFM incorporates part of the Hippo pathway, a description of growth also requires the integration of extrinsic growth controls, transduced via the dTOR pathway (Vollmer et al., 2017). The disk also is mechanically connected to the notum, the basement membrane (Domínguez-Giménez et al., 2007; Pastor-Pareja & Xu, 2011), apical extracellular matrix (Heer & Martin, 2017), peripodial cells which are opposed to the disk cells (Baena-López et al., 2003), and muscles within the larva (Nienhaus et al., 2012). As described earlier, recent studies have shown that the basement membrane and the extracellular matrix have significant effects on the overall mechanical properties of the disk (Atzeni et al., 2019; Keller et al., 2018).

6 | EPILOG AND FUTURE DIRECTIONS

In the preceding we have discussed several aspects of a fundamental question in the development of organs and organisms, which is how local information and signals at the cell level are integrated with global signals at the tissue, organ or organism level to produce the appropriate response at the cell level, whether it be growth, homeostasis, or a decision to move. Extracellular signals activate downstream STNs which may lead to a decision to divide or not, but since a cell in a tissue receives many signals, they must be arbitrated in order to make a decision as to how to respond. In the case of mechanics, changes in stresses must generally lead to changes in STN output since there is at present no known mechanism by which stress can affect gene expression directly in Drosophila (K. D. Irvine, personal communication, 2019). We have illustrated these processes in the *Drosophila* wing disk, but the abstract structure is that of a complex network in which multiple signals are funneled into a few decision-making channels, and this structure applies throughout biology. In what follows, we address how tissue-level systems and local interactions combine to regulate growth in the wing disk.

Disk size is determined by disk-level and compartment-specific control, and while several theories have addressed the former, how compartment-specific control is achieved is an open question, but it cannot be achieved with many disk growth models for reasons given earlier. Extrinsic control also mediates tissue growth (Buchmann

et al., 2014), and since Yki-dependent growth requires dTOR activity, the extrinsic control structure may depend on a hypothetical mechanism in which dTOR affects the state of nuclear Yki (Parker & Struhl, 2015). Thus the dTOR and Hippo pathways may act in parallel, in that Hippo assesses local growth suitability, and dTOR ensures that organismal factors such as nutrition are sufficient to support the increased growth in the early stage of the cell cycle. Mechanical inputs may also be extrinsic. Dramatic evidence of muscle-induced deformation in Figure 7 contrasts with the often-assumed view of the wing disk as a suspended organ isolated from other larval tissues (Nienhaus et al., 2012).

The effects of these intrinsic and extrinsic controls affect proliferation through the integrated outputs of several STNs and mechanical interactions. Though there are models of STNs, few have integrated mechanics and signaling or extrinsic control. In the MFM, cellular-level growth rates are assumed to result from summing different STN outputs (Aegerter-Wilmsen et al., 2012). However, there are a number of observations that cannot be explained by the model, but many refinements are possible by using a more detailed network (Figure 2). An earlier model (Hufnagel et al., 2007) is also inadequate since it relies on a centrally secreted morphogen, whereas the AP and DV stripes of Dpp and Wg span the disk. Morphogen scaling is also problematic in these models. Various schemes of integrating active and passive processes in growing tissue have been introduced (Goriely, 2017; Tlili et al., 2015), and extending this paradigm to study interactions between multiple STNs and between STNs and mechanics may elucidate the roles of individual pathways in disk development.

A further complication is that disk cells exhibit heterogeneous gene expression across the disk, increased tension along compartment boundaries (Major & Irvine, 2005; Major & Irvine, 2006; Michel & Dahmann, 2016; Rudolf et al., 2015), and the organization of the CSK varies in space and time to adapt to growth-induced stress (LeGoff et al., 2013). As discussed in Section 3.2 disk growth is also coordinated with the surrounding PE and BM tissues. As the wing disk grows, the PE and BM must change shape to accommodate the growth. However, the role of the BM remains controversial. Increased BM compression does not affect disk size, but BM degradation leads to smaller wing disks (Ma et al., 2017). While BM degradation has been shown to not influence cytoskeletal tension (Pan et al., 2018), it remains to be determined whether this holds for disks with elevated BM compression.

Coordination of disk growth with that of the BM and PE may explain one role of the Zecca–Struhl feed-forward mechanism (Zecca & Struhl, 2010). Recruitment, unlike proliferation allows for the wing disk to increase in mass without altering force balances with neighboring tissues because nonwing cells become pouch cells when the PE becomes too stretched to permit further expansion due to growth. However, this would have to be reconciled with observations that Vg increases growth rates and upregulates C_{p_a}, altering cytoskeletal properties (Janody & Treisman, 2006). The Zecca–Struhl mechanism also coincides spatially and temporally with fold formation, but whether it plays a role in that process or whether fold formation enhances recruitment is unknown.

Although disk size is governed by large-scale growth control, disk development must ultimately be achieved through cellular level behaviors. Known facts about the regulation of Hippo through cell–cell interactions have been studied in a model known as the WAMND—"*what are my neighbors doing*"—model (Gou et al., 2018). In this model, the Hippo target gene activity of a cell is affected nonautonomously by the Ds and Fj levels of its neighbors. In the wing disk, the Ft–Ds–Fj pathway also plays an important role in PCP, thus this system couples tissue growth and PCP. In addition to Hippo signaling, a WAMND-like system transduces Dpp signals, and clonal expression of activated Tkv shows that juxtaposition of cells receiving different Dpp signals leads to nonautonomous proliferation on both the higher and lower Tkv sides (Rogulja & Irvine, 2005). Another WAMND system allows nearby cells to coordinate their cell-cycle progression. This may affect signaling as cells may be (un)responsive to different types of signals at different points in the cell-cycle.

Another aspect of WAMND arises in cell competition. Competitive cell–cell interactions occur when differences in context-dependent fitness levels between neighboring cells causes the elimination of less-fit "loser" cells, or the takeover of more-fit "winner" cells (Levayer & Moreno, 2013). Cell competition can be triggered by numerous factors (de la Cova, Abril, Bellosta, Gallant, & Johnston, 2004; Levayer & Moreno, 2013) including differences in proliferation rates between nearby cells. Although apoptosis is relatively rare during most of wing disk development, it occurs during the fast-growing early third instar (Milán, Campuzano, & García-Bellido, 1997) and was suggested to be important for the wing disk to abstain its normal size (de la Cova et al., 2004). On the other hand, blocking apoptosis has little effect on the final size of disk compartments (Martín et al., 2009). However, that result might reflect compensation by global control mechanisms rather than the effect of cell–cell competition.

Because copy numbers of signaling molecules and other key components are frequently low, stochastic effects should be considered with a view toward understanding if and how network structure plays a role in adapting to noisy

TABLE 2 Additional definitions of symbols

Term	Abbreviation
BM	Basement membrane
BMP	Bone morphogenetic protein
DSL	Delta-serrate-notch
dTOR	Drosophila target-of-Rapamycin
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
FRAP	Fluorescence recovery after photobleaching
GFP	Green fluorescent protein
HSPG	Heparan sulfate proteoglycan
KO	Knock-out
OE	Over-expression
PE	Peripodial epithelium
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase,

signals. Noise can affect the precision of morphogen-induced gene expression in simple networks (England & Cardy, 2005) and key components of the Dpp pathway, for example, are present at nanomolar concentrations (Shimmi & O'Connor, 2003) in the disk, yet disk patterning and size are remarkably reproducible. Though it has been argued that even such low concentrations are sufficient to mitigate stochastic noise (Kicheva et al., 2007), the network structure may play a role in mitigation. How the functions of STNs and mechanical regulation are maintained in the presence of fluctuations is still a major question in cellular biology.

Finally, studies of vertebrate systems show that signal transduction and growth control pathways are highly conserved across species, thus much of what is learned about *Drosophila* applies in higher organisms. Tissue overgrowth in *Drosophila* is similar to tumor growth in mammalian systems, and a better understanding of how morphogens and mechanics interact in development will shed light on how these signaling processes interact in the microenvironment of a tumor to affect its growth (Herranz, Weng, & Cohen, 2014; Milán, 2014). The ubiquity of the pathways across species and the balances of signals between them within an organ highlight the need for mathematical models to understand these complex systems. The inclusion of feedback loops in models may help illustrate how this dizzying array of pathways and interactions actually works. Cross-talk and feedback in reaction networks can yield surprising behavior that is not evident except by simulation.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Jia Gou: Writing-original draft-equal; writing-review and editing-equal. **Jay Stotsky**: Writing-original draft-equal; writing-review and editing-equal. **Hans Othmer**: Conceptualization-lead; funding acquisition-lead; supervision-lead; writing-original draft-equal; writing-review and editing-equal.

ENDNOTES

[1] Of course what are local factors, be they at cell, tissue, organ or organism level, and what are global factors is context-dependent, and these are frequently designated as intrinsic and extrinsic factors in a given context (Bryant & Simpson, 1984).

[2] For symbol definitions not in text see Table 2.

FURTHER READING

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