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The Fat/Hippo Signaling Pathway Links Within-Disc Morphogen Patterning to Whole-Animal Signals During Phenotypically Plastic Growth in Insects

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Background: Insects exhibit a diversity of environmentally sensitive phenotypes that allow them to be an extraordinarily successful group. For example, mandible size in male stag beetles is exquisitely sensitive to the larval nutritional environment and is a reliable signal of male condition. Results: To date, studies of how such phenotypically plastic traits develop have focused on two types of mechanistic processes. Local, tissue-specific genetic mechanisms specify the shape and approximate final size of structures, whereas whole-animal hormonal signaling mechanisms modulate trait growth in response to environmental circumstance, including the body size and nutritional state of each individual. Hormones such as juvenile hormone, ecdysteroids, and/or ligands of the insulin-signaling pathway specify whether traits grow and regulate how much growth occurs across a diversity of insect groups. What remains to be shown is how the local, tissue-specific developmental genetic pathways interact with these whole animal hormonal signaling pathways during development to yield phenotypically plastic patterns of trait growth. Conclusions: Because the Fat/Hippo signaling pathway coordinates trait growth and development through its interactions with morphogens and hormonal pathways, we propose that Fat/Hippo signaling is a missing mechanistic link coordinating environmentally sensitive trait development in insects. *Developmental Dynamics 244:1039–1045, 2015*.

Key words: Fat/Hippo Signaling; phenotypic plasticity; insect development; appendage patterning; polyphenism

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Introduction

From the weapons of sexual selection in horned beetles (Emlen et al., 2007, 2012) to the wing polymorphisms of aphids (Brisson, 2010) and crickets (Zera, 2003; Clark et al., 2015), and the caste polyphenism in social insects (Nijhout, 1994; Miura, 2005), environmentally sensitive or condition-dependent phenotypes are widespread in insects and are typically highly adaptive. While many studies have shown the ecological importance of phenotypically plastic traits, we are only just beginning to understand how trait growth responds to the environment at the physiological and genetic level (recently reviewed by Zera, 2003; Emlen and Allen, 2004; Brisson, 2010; Beldade et al., 2011; Hartfelder and Emlen, 2011; Emlen et al., 2012; Koyama et al., 2013; Lavine et al., 2015; Nijhout et al., 2014; Nijhout and Callier, 2014). For example, in the imaginal discs of holometabolous insects, which we focus on here, the final size of each structure depends both on a complex matrix of interactions between within-disc patterning morphogens such as Hedgehog (Hh), Wingless (Wg), Decapentaplegic (Dpp), and Ultrabithorax (Ubx), which specify the trait's shape and approximate final size, and also on whole-animal circulating signals such as insulin/insulin-like growth factors (ILS), juvenile hormone (JH), and ecdysteroids, which transduce environmental stimuli to the tissues and cells of the developing insect (Stern and Emlen 1999; Emlen and Allen 2004; Kojima, 2004; Emlen et al., 2007, 2012; Koyama et al., 2013; Lavine et al., 2015; Nijhout et al., 2014; Nijhout and Callier, 2014). The question we address in this commentary is how do local, within-trait morphogens interact with whole-animal endocrine signals to result in a trait that is exquisitely attuned to its environment? We propose the idea that the Fat/Hippo signaling pathway may be a major link between local patterning genes and circulating signals during the development and growth of phenotypically plastic traits in insects, and in animals generally. We briefly describe

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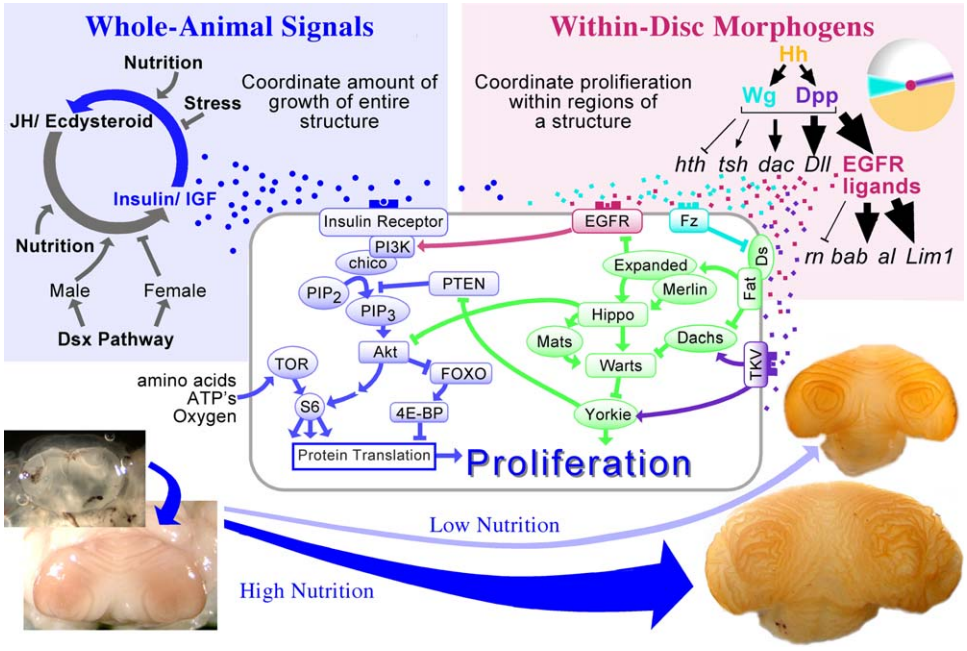

Fig. 1. Pathways known to be involved in condition-dependent trait growth. Morphogen signals specify the shape and approximate final size of developing insect appendages ("within-disc morphogens", top right). Overall amounts of growth are modulated in response to several whole-animal-circulating signals (e.g., insulin-like peptides, juvenile hormone [JH], whose levels are sensitive to the nutritional state of the animal. The Fat/Hippo pathway has been shown to integrate signaling pathways from within-disc morphogens to whole-animal signals. The sex-determination pathway (Dsx) is very important for regulating sex-specific trait expression and trait growth. Pathway interactions from Kojima (2004), Wu and Brown (2006), Zhou et al. (2010), Wartlick et al. (2011), Mirth et al. (2011), Nijhout et al. (2014). Akt, Protein Kinase B; Al, Aristaless; Bab, Bric-a-brac; Dac, Dachshund; Dll, Distal-less; Dpp, Decapentaplegic; Ds, Dachsous; EGFR, Epidermal Growth Factor Receptor; FOXO, Forkhead Box O; Fz, frizzled; Hh, Hedgehog; Hth, Homothorax; Mats, Mob as tumor suppressor; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; PIP2, phosphatidyl inositol bisphosphate; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PTEN, phosphatase and tensin homolog; Rn, Rotund; S6, S6 kinase; TOR, Target of Rapamycin; Tsh, Teashirt; Tkv, Thickveins; Wingless, Wg; 4E-BP, 4E Binding Protein. Modified from Lavine et al. (2014).

what is presently known about the functions of these pathways and their interactions, and argue that members of the Fat/Hippo signaling pathway include high priority candidate genes worthy of future study in the context of nutrition- and environment-sensitive growth of animal structures.

Within-Disc Morphogens

Insect appendage primordia are patterned by cascades of gene networks that unfold within the field of cells that will form the trait. Interactions among these patterning gene products delineate anterior-posterior, dorsal-ventral, and proximal-distal compartments within the developing imaginal disc cells. During larval development, the epithelial cells within each of the imaginal discs become subdivided by a hierarchical sequence of spatially-explicit signals that diffuse from cell to cell (e.g., Hedgehog, Hh; Wingless, Wg; and Decapentaplegic, Dpp; Fig. 1; Cohen, 1993; Serrano and O'Farrell, 1997; Teleman and Cohen, 2000). These signals act as local morphogens, directing the development of cells as they diffuse through the tissue (Lawrence and Struhl, 1996; Day and Lawrence, 2000). Partially overlapping gradients of these morphogens specify unique domains within the disc epithelium, and cells at the intersections of these domains take active roles in coordinating both patterning and growth of the resulting appendage. Importantly, interactions between positional signals stimulate and coordinate cell proliferation locally within the disc (Johnston and Gallant, 2002; Kojima, 2004).

Appendage size is roughly specified by homeotic genes acting together with other effectors. For example, during normal *Drosophila* wing and haltere development, the homeotic gene *Ubx* regulates appropriate haltere growth and size. When *Ubx* expression in primordial haltere cells is removed, the cells proliferate, resulting in abnormally large haltere sizes compared with cells expressing *Ubx* (Crickmore and Mann, 2006). Expression of *Ubx* appears to affect the growth of the haltere through its action on the TGF-β homolog Dpp (Crickmore and Mann, 2006), a morphogen that itself coordinates patterning and growth in animal cells during development (Restrepo et al., 2014). In *Drosophila*, *Ubx* reduces both Dpp production and mobility in haltere cells through enhanced expression of the Dpp receptor, Thick veins (Crickmore and Mann, 2006). What this suggests is that interactions between upstream genes, such as *Ubx*, and downstream regulators of cell growth and proliferation, such as Dpp, ultimately specify the approximate final size and shape of a structure.

Although details vary by appendage type and species, all traits examined thus far are formed via this same homeotic gene-appendage patterning network interaction. Specifically, interactions among these genes specify the identity of each structure and likely coordinate, to some extent, their final relative sizes. For example, forewings are typically larger than halteres in flies, and there are many species of insect such as water striders and crickets where one pair of legs differs markedly in size from the others. Species-typical trait differences in size such as these appear to be coordinated by this homeotic gene-appendage-patterning interaction (Mahfooz et al., 2004, 2007; Khila et al., 2009; Refki et al.,

2014). However, growth of tissues in insects and other animals is also often modulated in response to signaling from whole-animal physiological pathways, resulting in coordinated growth of the various body parts. Such mechanisms cause the relative sizes of traits to scale with among-individual variation in overall body size (i.e., allometry; Stern and Emlen, 1999; Shingleton et al., 2007; Nijhout et al., 2014). They can modify the growth of specific body parts dramatically as, for example, occurs in exaggerated sexually selected structures, which are unusually sensitive to nutrition (Emlen et al., 2012, Warren et al., 2013; .Lavine et al., 2015); or they may have little effect on growth at all, as occurs in *Drosophila* and rhino beetle male genitalia, which are less sensitive to nutrition than other body parts (Tang et al., 2011; Emlen et al., 2012; Johns et al., 2014). Finally, endocrine signals can couple facultative growth of body parts such as wings with season, crowding, or other aspects of environmental circumstance (e.g., polyphenism; Nijhout, 1994; Zera, 2003; Hartfelder and Emlen, 2011).

Endocrine Regulation of Growth

The insulin/insulin-like signaling (ILS) pathway is a highly conserved physiological pathway that transduces the nutritional status of an individual to its cells and functions in growth, metabolism, reproduction, and aging (Claeys et al., 2002; Tatar et al., 2003; Wu and Brown, 2006; Clemmons et al., 2010; Teleman, 2010). Warren et al. (2013) have reviewed the ILS pathway with regard to its role in the growth of condition-dependent sexually selected exaggerated traits, and Koyama et al. (2013) have reviewed ILS and Target of Rapamycin (TOR) signaling in the regulation of nutrition-dependent, developmentally plastic, organ-specific responses in insects. We briefly summarize these findings here.

The ILS pathway integrates physiological condition and metabolism with growth. Because of the action of this pathway, tissues grow faster in well-fed and unstressed individuals, who have increased levels of IGFs/insulin/ILPs, than they do in poorly fed, diseased, or stressed individuals (Broughton et al., 2005; Dionne et al., 2006; Tang et al., 2011). Downstream signaling cascades of the ILS pathway act in tissue- and cell-specific manners (Claeys et al., 2002; Clemmons et al., 2010), such that the nutrition- and condition-dependent plasticity of a trait is determined by its relative sensitivity to ILS signaling (Wu and Brown, 2006; Shingleton, 2010; Tang et al., 2011; Emlen et al., 2012). In fruit flies and rhinoceros beetles, for example, male genitalia are insensitive to the ILS pathway and grow to a specific size regardless of the animal's physiological condition (Tang et al., 2011; Emlen et al., 2012; Johns et al., 2014). In contrast, wings, which are moderately sensitive to ILS signaling, grow larger in large, well-fed individuals than in smaller, poorly fed individuals (Tang et al., 2011; Emlen et al., 2012; Johns et al., 2014). In the Asian rhinoceros beetle, *Trypoxylus dichotomus*, the male head horn is approximately eight times more sensitive to signaling through the ILS pathway than are other traits like wings, resulting in unusually rapid growth in the largest, best-fed males to produce extreme weapons (Emlen et al., 2012; Johns et al., 2014). Thus, trait differences in sensitivity to ILS signaling appear to underlie corresponding differences in the relative plasticity of their growth.

The ILS pathway has also been shown to contribute to socially mediated caste differentiation in social insects such as honeybees (Ament et al., 2008; de Azevedo and Hartfelder, 2008; Mutti et al., 2011) and termites (Hattori et al., 2013). Activation of the ILS pathway can stimulate cell growth and protein synthesis (Hara et al., 1998; Ruvinsky and Meyuhas 2006), and it modulates circulating JH, possibly by triggering the release of neuropeptides that influence JH production (Tu et al., 2005). As these examples suggest, in addition to their direct effects on growth, ILS and TOR signaling can control production of other hormones that also influence body size and shape (Koyama et al., 2013).

Two important effector hormones in insects are JH and ecdysteroids, which, through downstream actions, regulate growth, molting, metamorphosis, and reproduction (Nijhout 1994; Nijhout et al., 2014; Hartfelder and Emlen 2011; Jindra et al., 2013). Not surprisingly, these hormones and their receptors regulate trait expression and growth in at least some condition-dependent insect traits.

JH has diverse functions during insect development (Mutti et al., 2011; Jindra et al., 2013). Its classic roles are to ensure a stationary molt when titers are high (Mutti et al., 2011; Jindra et al., 2013; Restrepo et al., 2014) and to regulate developmental switches between alternative phenotypes (Nijhout, 1994). But JH can also stimulate cell proliferation (Truman et al., 2006; Mutti et al., 2011; Jindra et al., 2013; Restrepo et al., 2014) and link trait growth with nutrition (Emlen et al., 2005; Truman et al., 2006; Tang et al., 2011; Mirth and Shingleton 2012). Perturbations to JH affect the size of a number of condition-dependent insect traits including eyestalks of stalk-eyed flies (Cotton et al., 2004; Fry, 2006), mandibles of stag beetles (Gotoh et al., 2011) and broadhorned flour beetles (Okada et al., 2006), and horns of dung beetles (Emlen and Nijhout, 1999; Shelby et al., 2007). JH has also been shown to be critical to caste determination in social insects (Miura, 2005; Ament et al., 2008; de Azevedo and Hartfelder, 2008; Mutti et al., 2011).

Linking Within-Trait Patterning Mechanisms to Whole Animal Signals

Although it has been clear for decades that growth of the majority of insect appendages was phenotypically plastic and sensitive to the nutritional state of each animal, the precise molecular mechanisms responsible for nutrition-sensitive patterns of growth are only just now becoming clear. Somehow, the within-trait genetic regulatory networks responsible for delineating the formation of dorsal-ventral, anterior-posterior, and proximal-distal axes of tissue growth, which specify relative amounts of growth within specific regions of each developing structure, and, in so doing, determine the shape and approximate final size of the structure, must be modified in response to the particular environmental circumstances encountered by each individual. Therefore, the activities of genes in these networks must be modulated in response to ILS, JH, and/or ecdysteroid signals and their receptors so that the overall amounts of growth of each trait are adjusted in accordance with the stress and/or nutritional state of each individual. Indeed, this mechanistic link between global and local developmental processes likely occurs in the development of the overwhelming majority of animal appendages across a stunning diversity of species.

We suggest that the Fat/Hippo signaling pathway may provide this crucial missing link, based on its emerging role as a regulator of appendage growth, and also on preliminary results of ours, which implicate genes in this pathway in the regulation of growth of sexually selected beetle weapons (Fig. 2).

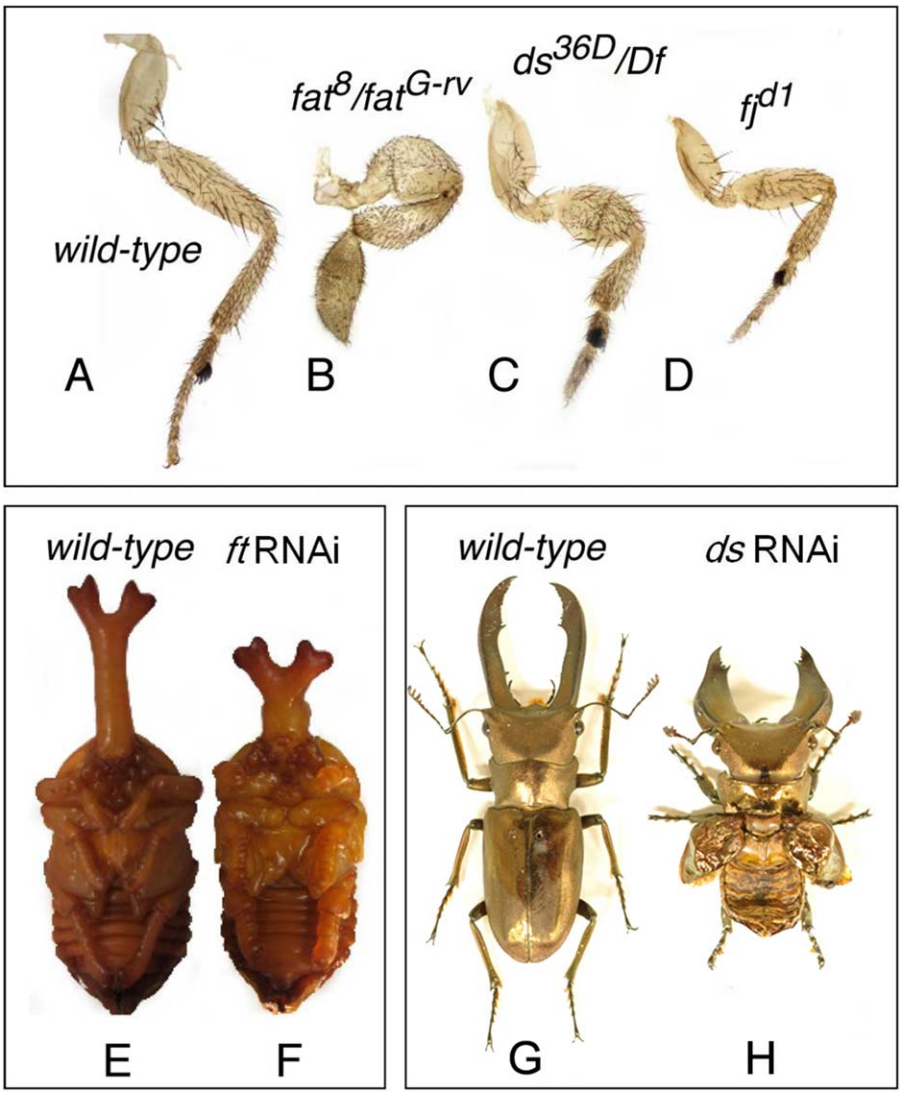

Fig. 2. Appendage phenotypes in *ft, ds*, and *fj* mutants [*Drosophila* legs from Supp. figure S1 in Mao et al. (2006); reproduced with permission of the publisher] and beetles (*Trypoxylus dichotomus* head horn and *Cyclommatus metallifer* mandibles from our unpublished work). Adult legs, all at the same magnification, from (**A**) Wildtype, (**B**) $ft^{8}/ft^{\text{G-rv}}$, (**C**) $ds^{36D}/Df^{(2L)ED94}$, (**D**) fj^{dl}. Mutations in each of these Fat pathway components result in shortened legs, but their phenotypes are distinct. *fj* legs are simply shortened. *ds* mutant legs are shortened but also thicker. *ft* mutant legs are shorter and sometimes much thicker. Pupal *T. dichotomus* shown on their ventral side. **E** is the wild-type control male of the same body size as the knockdown male (**F**). Adult male *C. metallifer* mandibles are shortened in response to *ds* RNAi (**G**) compared to control males (**H**) of the same body size. The *ft* and *ds* knockdown resulted in shortened legs and wings in addition to a much reduced weapon (head horn or mandibles) relative to body size. Males were injected with 1 μg of dsRNA against the *T. dichotomus ft* gene and *C. metallifer ds* gene in nuclease free water, injected into the T2 segment of 15 male larvae at the end of the gut purge stage.

The FAT/HIPPO Signaling Pathway

This pathway was first identified in *Drosophila* as an intracellular signaling pathway but has since been found to be evolutionarily conserved across taxa (Irvine, 2012; Matis and Axelrod, 2013). The Fat/Hippo pathway is interconnected with other signaling pathways in intriguing ways that suggest it may enable cells to integrate many different types of information such as relative position, developmental stage, and nutritional state. Upstream members of the pathway include *fat* (*ft*), *dachsous* (*ds*), and *four-jointed* (*fj*) (Pan, 2010; Bando et al., 2009, 2011; Matis and Axelrod, 2013, Figs. 1, 2). Both Ft and Ds are large, atypical cadherin molecules that act as ligand-receptor pairs (Pan 2010, Bando et al., 2009, 2011, Matis and Axelrod, 2013). The golgi-localized protein kinase Fj regulates the activity of Ft and Ds by phosphorylation (Pan, 2010; Brittle et al., 2010; Bando et al., 2009, 2011), and these proteins have been shown to be necessary for the establishment of planar cell polarity (PCP) during imaginal disc growth (Matis and Axelrod, 2013). Planar cell polarity is established by the unequal distribution of Ft and Ds protein heterodimers within the cell (Lawrence et al., 2008; Yoshida et al., 2014).

In addition to the establishment of PCP, members of the Fat/Hippo signaling pathway are also critical for trait growth. *fat* and *ds* were first identified as tumor suppressor genes (Matis and Axelrod, 2013). In *Drosophila* and *Gryllus* (crickets), *fj, ft*, and *ds* mutants have short leg (*fj*) or short and thick leg (*ft* and *ds*) phenotypes (Mao et al., 2006; Bando et al., 2009, 2011). In these mutants, although each appendage segment retains its original

identity (i.e., tarsus, tibia, and femur are recognizable), their size and shape are largely disrupted and have overgrowth as well as cell disorganization phenotypes (Fig. 2). This disorganization in cellular proliferation is also seen in the *ft* knockdown, RNAi phenotype of appendages of the Asian rhinoceros beetle *Trypoxylus dichotomus* and, most interestingly, in the large, nutrition-sensitive head horn of males (Fig. 1). Similarly dramatic effects are observed for RNAi knockdown of *Ds* in the nutrition-sensitive male mandibles of the stag beetle *Cyclommatus metallifer* (Fig. 1). Because this is preliminary data, we do not show the distribution of phenotypes across a large range of male body sizes, but the clear trend is for cellular disorganization and stunted trait sizes across all male body sizes in both species (data not shown). The mechanism by which this occurs in *Drosophila* is through the effects of *fat, fj,* and *ds* on the Wart-Hippo signaling cascade, which directly regulates cell proliferation (Lawrence et al., 2008; Straßburger et al., 2012; Matis and Axelrod, 2013; Fig. 1).

The Hippo signaling pathway coordinates cell density-dependent mechanisms of cellular proliferation with traditional growth factor signaling pathways to mediate tissue and organ growth (Gumbiner and Kim, 2014). It does this by sensing and responding to the physical organization of cells in tissues, through cell-cell adhesion, cell junctions, cell polarity mechanisms, cell shape, tension, and actin organization (reviewed in Gumbiner and Kim, 2014). The Hippo pathway is a highly conserved serine kinase cascade that mediates tissue growth and organ size; in *Drosophila*, mutations in this pathway result in organized overgrowth of imaginal discs resulting in normal, but enlarged organs (Halder and Johnson, 2011; Sebé-Pedrós et al., 2011; Tumaneng et al., 2012; Bossuyt et al., 2014).

Hippo is a serine-threonine kinase that, with two additional proteins, Mob as tumor suppressor (Mats) and Salvador (Sav), phosphorylates the kinase Warts (Wts). The phosphorylated and activated Wts kinase then phosphorylates the terminal target transcriptional co-activator Yorkie (Yki) to prevent Yki from entering the nucleus (Huang et al., 2005; Lawrence et al., 2008; Matis and Axelrod, 201; Fig. 1). Yorkie is a transcriptional co-activator required for the transcription factor Scalloped to bind DNA and induce cellular proliferation.

Activated Hippo signaling thus results in inhibition of cellular growth by keeping Yorkie in the cytoplasm; loss of any of those four genes (*hpo, mats, sav, wts*) or overexpression of *yki* results in overgrowth of the tissue (Pan, 2010; Grusche et al., 2011; Irvine, 2012). But what makes the Hippo pathway a likely candidate for mechanisms of plasticity is that it has also been found to integrate signals from a diversity of upstream regulators shared from mammals to *Drosophila* (Fig. 1; Gumbiner and Kim, 2014). These upstream inputs include Kibra, Expanded (Ex), Merlin (Mer), the Fat signaling pathway, Crumbs (Crb), atypical Protein Kinase C (aPKC), and Lethal giant larvae (Lgl), as well as morphogens and growth factors such as Wingless (Wg), Epidermal Growth Factor, (EGF), and ILPs/IGFs (Harvey and Tapon, 2007; Reddy and Irvine, 2008; Zeng and Hong, 2008; Badouel et al., 2009; Zhao et al., 2010; Pan, 2010; Halder and Johnson, 2011). By responding to secreted growth factors from a diversity of other pathways that promote and regulate growth, the Fat/Hippo signaling cascade integrates a diversity of environmental and intrinsic signals. As such, the Wts-Hpo signaling factors are now considered to be general regulators of organ size in both *Drosophila* and mammals (Reddy and Irvine, 2008; Kango-Singh and Singh, 2009; Grusche et al., 2010; Halder and Johnson, 2010; Oh and Irvine, 2010; Pan, 2010; Yi and Kissil, 2010; Zhao et al., 2010; Irvine, 2012).

The ability of the Fat/Hippo signaling pathway to act as a nexus coordinating a diversity of inputs makes it an important candidate pathway for linking the activity of within-disc morphogens with circulating levels of whole animal signals of nutritional state, body size, and physiological condition, as well as signals sensitive to environmental factors such as crowding and photoperiod.

Future Directions

For decades it has been clear that insect hormones play critical roles in the control of plastic trait growth in insects (e.g., Rembold et al., 1974; Lenz, 1976; Hardie, 1980; Hardie and Lees, 1985; Rachinsky and Hartfelder, 1990) and, for just as long, it has also been clear that insect appendages are patterned by largely trait-autonomous cascades of genetic interactions that unfold within each developing trait (e.g., Garcia-Bellido 1965; Lawrence and Morata, 1976; Campbell et al., 1993; Cohen, 1993; Day and Lawrence, 2000; Parker, 2011). As molecular technologies have improved, so, too, has our understanding of the molecular details of both types of mechanism (e.g., Kojima 2004; Weihe et al., 2005; Koyama et al., 2013; Nijhout and Callier, 2014). It has also become increasingly apparent that these processes must interact; somehow, signals from the former must affect the activities of the latter. We suggest that the molecular links between whole-animal physiological signals and within-trait patterning comprise a significant outstanding problem in developmental biology, lying at the heart of most mechanisms of developmental plasticity.

The Fat/Hippo signaling cascade (Fig. 1) may provide this missing link. Recent studies hint at the crosstalk between Fat/Hippo signaling and other pathways in the regulation of both of planar cell polarity and organ growth (Rogulja et al., 2008, Wartlick et al., 2011, Irvine, 2012). For example, Rogulja et al. (2008) provide evidence that the Fat/Hippo signaling pathway is regulated by a differential Dpp gradient in the developing wing disc in *Drosophila* (Rogulja et al., 2008). And the Fat/Hippo downstream transcription factor Yki is regulated in part by the ILS pathway (Straßburger et al., 2012) and by EGFR signaling (Reddy and Irvine, 2013). Thus, elements of the Fat/Hippo pathway respond to whole-animal circulating signals crucial to the plastic regulation of trait growth, and they also interact with local morphogens of the appendage patterning cascade, and the feedback this pathway provides is known to affect the final sizes of traits. Although the nuances of these interactions have yet to be fully explored, and, in particular, the putative role of this pathway in nutrition-sensitive, or other forms of plastic growth, has yet to be directly tested, the Fat/Hippo signaling cascade comprises a promising candidate for the molecular link between local patterning networks and the whole-animal circulating signals critical to phenotypically plastic trait growth. Future experiments will be needed to investigate the role of Fat/Hippo signaling in coordinating growth of plastic traits such as the exaggerated, exquisitely nutrition-sensitive weapons and ornaments of sexual selection and soldier castes of social insects, as well as in the seasonally polyphenic traits of a diversity of insect species.

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