RESEARCH ARTICLE

The JAK/STAT Pathway Regulates Proximo-Distal Patterning in Drosophila

Aidee Ayala-Camargo, Laura A. Ekas, Maria Sol Flaherty, Gyeong-Hun Baeg, and Erika A. Bach

JAK/STAT signaling is thought to control growth and proliferation. However, here we show a novel role for this pathway in the patterning of Drosophila appendages. Loss of Stat92E function results mainly in ventralizations and multiplications of the proximo-distal axis in leg and antenna, primarily through the ectopic misexpression of wingless. We also show that the pathway ligand Unpaired is expressed in two domains in leg and antenna that abuts those of wingless and decapentaplegic. We report that JAK/STAT signaling represses both wingless and decapentaplegic, restricting them to their respective domains in leg and antenna. In a reciprocal manner, we show that wingless and decapentaplegic restrict unpaired to its two domains. Thus, a main function of the JAK/STAT pathway in leg and antennal development is to promote the formation of a single proximo-distal axis per disc by constraining the intersection of wingless and decapentaplegic to the center of the disc. Developmental Dynamics 236:2721–2730, 2007. © 2007 Wiley-Liss, Inc.

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INTRODUCTION

In mammals and Drosophila, the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway is a critical regulator of proliferation and has been shown to promote the formation of tumors (Levy and Darnell, 2002; Arbouzova and Zeidler, 2006). For instance, an oncogenic allele of jak2 causes hematopoietic cancers and a dominant active stat3 allele causes tumors in nude mice (Bromberg et al., 1999; Bowman et al., 2000; Darnell, 2005). Similarly, hyper-activation of the Drosophila JAK/STAT pathway leads to dramatic tissue overgrowths that resemble human tumors (Harrison et al., 1995; Kiger et al., 2001; Tulina and Matunis, 2001; Bach et al., 2003). However, little is known about the role of this pathway in pattern formation. The presence of only one JAK and one STAT in Drosophila, compared to four JAKs and seven STATs in mammals, make the fruit fly an ideal system to study the role of this pathway during development. Three Unpaired ligands (Upd, Upd2, and Upd3) are thought to bind to the receptor Domeless (Dome), resulting in its activation and subsequent trans-phosphorylation and activation of the associated JAK kinase Hopscotch (Hop) (reviewed in Arbouzova and Zeidler, 2006). The activated Hop proteins phosphorylate and activate the transcription factor Stat92E, which then migrates to the nucleus and acts as a transcription factor.

Development of the proximo-distal (P-D) axis is essential to the formation of mammalian limbs and arthropod appendages. Significant insights into P-D axis formation have been made in Drosophila imaginal discs, groups of larval epithelia that give rise to adult structures (Cohen, 1993). The Drosophila leg and antenna are homologous structures based on homeotic mutations, and both have been used successfully to study patterning of the P-D axis (Postlethwait and Schneider-
The leg consists of five segments: the proximal coxa, followed by the trochanter, femur, tibia, and tarsus, and the distal-most structure, the claw. The adult antenna consists of six segments: from the proximal a1 to the distal a6, also called the arista. Like the vertebrate limb, the *Drosophila* leg and antenna arise from the differentiation of three different axes: antero-posterior (A-P), dorso-ventral...
(D-V), and P-D. The secreted factor Hedgehog (Hh) is produced by cells in the posterior compartment of the leg and induces the expression of two secreted factors, the Bone Morphogenetic Protein (BMP) Decapentaplegic (Dpp) and the Wnt protein Wingless (Wg), in anterior cells adjacent to the compartment boundary (Basler and Struhl, 1994; Tabata and Kornberg, 1994). The mutually antagonistic relationship between Dpp and Wg establishes the D-V axis by creating spatially restricted domains, in which each gene maintains its own expression pattern (Brook and Cohen, 1996; Jiang and Struhl, 1996; Johnston and Schubiger, 1996; Penton and Hoffmann, 1996; Theisen et al., 1996; Heslop et al., 1997). In addition to their roles in patterning the D-V axis, Dpp and Wg determine the P-D axis of the leg and antenna. The juxtaposition of dpp- and wg-expressing cells in the central region of both discs activates Distal-less (Dll), which encodes a homedomain protein that is required for the formation of distal leg and antennal structures (Cohen et al., 1989; Diaz-Benjumea et al., 1994; Campbell and Tomlinson, 1995). Ectopic expression of wg on the dorsal side, or dpp on the ventral side, of the leg disc results in ectopic expression of Dll and a secondary P-D axis (Campbell et al., 1993; Struhl and Basler, 1993; Diaz-Benjumea et al., 1994). Lower levels of Wg and Dpp initiate the transcription of dachshund (dac), which encodes a nuclear factor required for development of the femur and tibia (Mardon et al., 1994; Lecuit and Cohen, 1997). In addition to Dll and Dac, the TALE class homeodomain protein Homothorax (Hth) is essential for patterning of the proximal structures in the leg and antenna (Casares and Mann, 2001; Dong et al., 2001).

In this study, we address the role of the JAK/STAT pathway in patterning by addressing its role in P-D axis formation. Specifically, we find that the JAK/STAT pathway can repress both wg and dpp in an autonomous manner, although Stat92E represses wg more robustly than it does dpp. Furthermore, we show that both wg and dpp restrict upd to its anterior and posterior domains in leg and antennal discs. Through these reciprocal inhibitory interactions, the expression domains of upd, wg, and dpp become mutually exclusive. Thus, we show that JAK/STAT signaling regulates the development of one central P-D axis per leg or antennal disc by constraining the expression domains of dpp and wg.

**RESULTS**

**Upd Expression and JAK/STAT Pathway Activity Are Detected Throughout Leg and Antennal Imaginal Disc Development**

We recently reported that upd is expressed in two distinct domains in leg and antennal discs, one in the anterior and the other in the posterior compartment (Bach et al., 2007). In both discs, upd-expressing cells abut the Wg and Dpp domains (Fig. 1A–D; see also Fig. 6G). An upd2 null mutant is viable and fertile and has no antennal or leg defects, while upd3 is not expressed in either disc (data not shown). These data suggest that these factors do not act in these discs or have redundant roles with Upd. To assess the range of Upd activity, we used a 10XSTAT92E-GFP reporter that specifically reflects JAK/STAT pathway activity in vivo (Bach et al., 2007). This reporter is activated throughout larval development in leg and antennal discs (Bach et al., 2007).

Interestingly, its expression is lowest in the Wg-expressing domains (Fig. 1E–H). These data indicate that in both discs the JAK/STAT pathway is activated by Upd, which acts over a long range. Additionally, the mutual spatial and temporal exclusion of cells with JAK/STAT activity from those that express Wg suggests that a negative regulatory interaction exists between these pathways.

**Loss of JAK/STAT Pathway Activity Leads to Defective Patterning of the P-D Axis**

To assess the role of stat92E in the developing leg and antenna, stat92E clones were randomly induced and marked by the absence of the yellow (y) gene product in the adult. In the leg, y stat92E clones are associated with supernumerary appendages and incomplete duplications (Fig. 2A,D, arrowhead and inset, and data not shown). Furthermore, stat92E clones promote the formation of ventro-lateral structures like ectopic transverse bristles of the prothoracic legs next to the normally positioned ventral ones (Fig. 2B,C yellow arrowhead). In addition, other ventro-lateral structures, such as the sex combs in the male prothoracic leg, are increased in number when they reside within y stat92E clones (Fig. 2E,F). The formation of supernumerary limbs and of both ectopic transverse bristles and sex combs has been observed when ectopic wg expression is present on the dorsal side of the leg disc (Campbell et al., 1993; Struhl and Basler, 1993).

In the antenna, y stat92E clones exhibit supernumerary structures, including the duplication or triplication of segments a2-a6 (Fig. 2G,H arrowheads and data not shown). Other phenotypes, such as reduced or absent medial and distal segments (a3–a6), as well as antenna-to-leg transformations, are also observed (Fig. 2K, arrowheads). These defects are fully rescued by co-expression of a full-length stat92E transgene (Stat92E FL) (Ekas et al., 2006), indicating that they were specifically due to the loss of JAK/STAT signaling (Fig. 2L). Lastly, we de-
terminated that mutant clones of dome or hop, genes that act upstream of Stat92E, result in antennal phenotypes similar to those observed in stat92E clones, including the formation of supernumerary segments (Fig. 2I,J, arrowheads). Taken together, these data indicate that the JAK/STAT pathway regulates P-D patterning in both the leg and antenna.

Stat92E Is Required for the Proper Expression of Major P-D Patterning Genes

To determine if the stat92E adult phenotypes arise during larval development, we examined the effect of loss of stat92E activity in leg and antennal imaginal discs. First, we examined the expression of major P-D patterning genes. In the leg, DIl and Dac are expressed in a central and medial domain, respectively, while Hth is expressed in the proximal domain (Fig. 3A; See Supplemental Fig. 1A, which can be viewed at www.interscience.wiley.com/pages/1058-8388/suppmat). Third instar leg discs carrying stat92E clones often contain at least one secondary P-D axis (Fig. 3B, arrowhead). These ectopic P-D axes are only found on the dorsal side of the leg disc but can form in either the anterior or posterior compartment (Fig. 3B,D, arrowheads). Bric-a-brac (Bab), a BTB/POZ-domain protein required for the development of distal joints in the leg and antenna (Godt et al., 1993; Coudere et al., 2002), is also observed in these ectopic P-D axes (Fig. 3C,D, arrowhead). Consistent with a lack of defects in the coxa and trochanter in adult legs containing stat92E clones, we found that Hth expression is not altered in stat92E clones in the leg disc (Supplemental Fig. 1A,B).

Similarly, additional P-D axes are observed in antennal discs containing stat92E clones. In the third instar antenna, Hth is expressed in a1, a2, and faintly in a3, while DIl is expressed in a2–a6 (Supplemental Fig. 1C) (Dong et al., 2000). We show one disc that contains three P-D axes, as assessed by Dll expression (Fig. 3E, asterisks). One of these ectopic axes occurs within the stat92E clone, while the bifurcation that generated the other two axes follows the stat92E clone boundary (Fig. 3E,E'). We presume that comparable antennal discs would give rise to the duplicated or triplicated antennal structures similar to those shown in Figure 2H–J. In addition, we also observed absent or reduced Dll expression in large stat92E clones, and a concomitant expansion in Hth expression throughout the proximal and distal antenna (Supplemental Fig. 1C,D). These results are consistent with the adult phenotypes where a3–a6 segments are most frequently lost (Fig. 2K).

JAK/STAT Signaling

Autonomously Represses wg Expression

The defects observed in stat92E clones in leg and antennal discs and their corresponding adult structures suggested that wg was ectopically expressed. In wild type leg discs, Wg protein is expressed in a wedge in ventral anterior cells close to the A-P boundary, while dpp, as assessed by expression of the dpp-LacZ reporter (Blackman et al., 1991), is synthesized by dorsal anterior cells adjacent to this boundary and at lower levels by ventral anterior cells (Fig. 4A). In wild type antennal discs, Wg and dpp domains are reversed relative to the leg (Fig. 4C). As expected, ectopic Wg is expressed within stat92E clones on the dorsal side of the leg disc. Moreover, we observe a secondary P-D axis adjacent to the ectopic Wg domain, as assessed by ectopic non-autonomous dpp expression and local outgrowths (Fig. 4B, arrowhead). Similarly, ectopic Wg is also seen in stat92E antennal clones, as is an additional P-D axis, and non-autonomous ectopic expression of dpp (Fig. 4D, arrowhead). Furthermore, the ectopic Wg protein observed in stat92E clones reflects autonomous ectopic transcription of the wg gene, as monitored by the enhancer trap wgx (Kassis et al., 1992) (Fig. 4E,E' arrowhead). We note that the lateral posterior domain in the antennal disc appears to be the most sensitive to loss of JAK/STAT signaling, as ectopic wg is always observed in stat92E clones in this region (Fig. 4E).

We next attempted to identify the region of the wg gene regulated by Stat92E. The 3' cis genomic region of the wg gene regulates wg expression in many imaginal discs (Pereira et al., 2006). These authors found that a ~2-kb enhancer called wg2.3Z was the smallest enhancer from this 3' cis genomic region that could partially recapitulate wg expression in leg and other ventral discs (Supplemental Fig. 2A). We were unable to detect wg2.3Z expression in leg discs by immuno-fluorescence using antibodies specific for β-galactosidase (data not shown). However, we could detect its expression in wild type discs by X-Gal staining (Supplemental Fig. 2B,D). This enabled us to ask whether wg2.3Z is ectopically expressed in discs containing stat92E clones. Indeed, this reporter is ectopically expressed in these discs and in regions where we observe re-patterning and overgrowth (Supplemental Fig. 2C,E, yellow arrowheads). However, we were unable to determine if Stat92E regulates this wg enhancer autonomously.

The data presented to this point suggest that Stat92E represses wg. Therefore, we reasoned that ectopic activation of the JAK/STAT pathway should lead to wg repression. To address this issue, we assessed whether hop-expressing clones could autonomously repress expression of wg, as monitored by the wgx' enhancer trap. As expected, in both leg and antennal discs, ectopic activation of JAK/STAT signaling represses wg in an autonomous manner (Fig. 5A,A' and data not shown). These results indicate that JAK/STAT signaling represses wg.

JAK/STAT Signaling Also Represses dpp Expression

We also find that dpp can be autonomously expressed in stat92E clones (4/24 discs), although at a lower penetrance than ectopic expression of wg. We next specifically examined leg
Fig. 3. Expression of proximal and distal markers in \textit{stat92E} clones in leg and antennal discs. \textit{stat92E} clones were generated in a \textit{Minute} (B, D) or non-\textit{Minute} (E) background and lack GFP (green). All discs are third instar. In a wild type leg disc, Dac (blue) is expressed in the femur, tibia, and first tarsal segment, while Dll (red) is expressed in all tarsal segments and distal tibia (A). In a leg disc that contains large \textit{stat92E} clones, a secondary P-D axis is observed in the posterior compartment (B, arrowhead). In a wild type leg disc, Bab (red) is expressed in 4 concentric rings within the Dll domain (C). A \textit{stat92E} clone in the anterior compartment of the leg disc is associated with a secondary P-D axis, in which Bab is ectopically expressed (D, arrowhead). A \textit{stat92E} clone that runs through the D-V axis in an antennal disc is associated with a triplicated P-D axis, as assessed by Dll (red) staining (E, asterisks). The expression of Hth follows the edge of the clone ventro-laterally and marks the proximal edge of the ectopic axes (E'). hop-expressing clones (abbreviated hop FO and marked by GFP) do not alter the expression of Dll (red) (F,F'). D, dorsal; V, ventral; A, anterior; P, posterior.

Fig. 4. \textit{wg} and \textit{dpp} are ectopically expressed in \textit{stat92E} clones. All discs are third instar. \textit{stat92E} clones were generated in a \textit{Minute} (B,F) or non-\textit{Minute} (D,E) background and lack GFP (green). Wild type discs (A,C). A–E: Wg is ectopically expressed in \textit{stat92E} clones in leg and antennal discs. In a wild type leg disc, \textit{dpp} (blue) is expressed in the dorsal domain, while \textit{Wg} protein (red) is expressed ventrally (A). In a leg disc containing a dorsal anterior \textit{stat92E} clone, \textit{Wg} is ectopically expressed (B, arrowhead), which leads to a duplicated axis, as assessed by the local outgrowth and ectopic, non-autonomous \textit{dpp} expression adjacent to the ectopic \textit{Wg} (B). In a wild type antennal disc, \textit{dpp} is expressed ventrally, while \textit{Wg} is expressed dorsally (C). In an antennal disc containing \textit{stat92E} clones, \textit{Wg} expression expands into the ventral domain and is associated with a secondary P-D axis, in which \textit{Wg} is ectopically expressed (D). In an antennal disc containing an anterior \textit{stat92E} clone, the \textit{wg} gene (\textit{wgP} in red), is ectopically expressed within the clone (E,E' arrowhead). F: \textit{dpp} is ectopically expressed within \textit{stat92E} clones. A \textit{stat92E} clone in the dorsal lateral domain of the posterior compartment of a leg disc leads to ectopic expression of \textit{dpp} (blue) in an autonomous manner (F,F' arrowhead). \textit{wgP} (\textit{wg}-lacZ (anti-\textit{\beta}-Gal) is red in E and \textit{dpp-lacZ}, (anti-\textit{\beta}-Gal) is blue in A–D and F. Magenta lines mark one side of the clone boundary in F,F'.
discs in which \textit{dpp} was ectopically expressed in an autonomous manner (\(n = 13\) discs) and we made the following observations: (1) in all cases, \textit{dpp} is ectopically expressed only in the dorsal domain and never in the ventral domain (13/13 discs); and (2) small patches of ectopic \textit{dpp} are observed at the dorsal lateral margin of the disc, close to the boundary with the proximal region that gives rise to the trocanter and coxa (10/13) (Fig. 4F,F' arrowhead).

Our results indicate that the loss of Stat92E function can result in ectopic expression of \textit{dpp} within the \textit{stat92E} clone boundary. To assess if JAK/STAT signaling leads to autonomous repression of \textit{dpp}, we examined \textit{dpp} expression in \textit{hop}-expressing clones at various locations in the leg disc. We find that \textit{dpp} can be repressed in dorsal \textit{hop} flip-out clones located at the lateral edge of the \textit{dpp} expression domain but not in those located close to the A-P boundary (Fig. 5B,B' arrowhead, and data not shown). Previous work has shown that loss of \textit{wg} expression or signaling in the leg disc leads to ectopic expression of \textit{dpp} in the ventral domain (Brook and Cohen, 1996; Jiang and Struhl, 1996). However, we find that \textit{dpp} is never expressed within ventral \textit{hop}-expressing clones located in or adjacent to the \textit{wg} expression domain (21 discs examined) (Fig. 5C,C' arrowheads, and data not shown). Lastly, we find that the effects of Stat92E on \textit{wg} and \textit{dpp} are not due to alteration in Hh expression in the absence of Stat92E (Supplemental Fig. 1F,G). Taken together these results suggest that JAK/STAT signaling represses \textit{dpp}. However, the fact that supernumerary P-D axes and local repatterning are always associated with \textit{stat92E} clones on the dorsal side of the leg disc suggests that the repressive effects of Stat92E are greater on the \textit{wg} gene than on the \textit{dpp} gene.

\textbf{\textit{wg} and \textit{dpp} Restrict \textit{upd} Expression}

It is not known how \textit{upd} becomes restricted to two domains as in leg and antennal discs (Fig. 6A, arrowheads). We observed that the cells producing \textit{upd}, \textit{dpp}, and \textit{wg} do not overlap in either leg or antennal discs (Fig. 6G and data not shown). These mutually exclusive expression domains, together with our results that Stat92E represses \textit{wg} and \textit{dpp}, raise the possibility that there are multiple negative regulatory interactions between these factors. To investigate this issue, we examined \textit{upd} mRNA in leg discs that had temperature-sensitive mutations in key P-D axis patterning genes. Notch (N) signaling is necessary and sufficient to induce transcription of the \textit{upd} gene in the eye disc (Chao et al., 2004; Reynolds-Kenneally and Mlodzik, 2005). Consistent with these published reports, we observed loss of \textit{upd} at the posterior margin of \(N^{ad}\) eye discs at the restrictive temperature (data not shown). In contrast, \textit{upd} remains in its two domains in leg and
antennal discs when N signaling is reduced, thus ruling out the hypothesis that N regulates upd in these tissues (Fig. 6C, arrowheads, and data not shown). However, we observed that upd is negatively regulated by Hh signaling, as upd mRNA is found throughout hhTs2 leg discs at the restrictive temperature (Fig. 6B). To investigate whether the repressive effects of Hh were due to Wg and/or Dpp, we examined upd expression in hetero-allelic combinations that behave as wgts or dppTs alleles (van den Heuvel et al., 1993; Kenyon et al., 2003). When wg signaling is reduced, we find that upd expression collapses into a single domain that is aberrantly localized to the ventral leg disc (Fig. 6D, arrowhead). Similarly, a single upd domain that localized to the dorsal leg disc is observed when dpp signaling is reduced (Fig. 6E, arrowhead). Consistently, we also find that Upd activity is significantly altered in the absence of dpp signaling. In a dppTs leg disc, Wg is ectopically expressed in the dorsal domain, which is consistent with previous reports (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996). Moreover, the 10XSTAT92E-GFP reporter is ectopically expressed at the dorsal-most region of these discs, consistent with the dorsal expression of upd in these discs, but is lost from the central region of the disc (compare Fig. 1E to 6F). Taken together, our results indicate that both wg and dpp repress upd and hence restrict its expression to two domains in the leg disc (Fig. 6H).

DISCUSSION

Numerous studies have documented the function of the JAK/STAT pathway in proliferation and growth control. However, we report here for the first time that these three major patterning factors (Upd, Wg, and Dpp) cross-regulate one another and that this regulation is crucial for proper P-D patterning of leg and antennal discs.

Although we show that Stat92E autonomously represses both wg and dpp, it must be stressed that Stat92E appears to more strongly repress wg than it does dpp. This conclusion is supported by the following observations. First, ectopic wg is observed more frequently than ectopic dpp in stat92E clones. Second, stat92E clones only give rise to P-D axis duplication and overgrowth on the dorsal side of
the leg disc. We presume that Stat92E repression of dpp may be cell-type specific, as we have previously shown that Stat92E represses wg but not dpp in the eye disc (Ekas et al., 2006). In contrast, our work demonstrates that the repression of wg by activated Stat92E is a broad mechanism utilized during pattern formation in imaginal discs and one that has recently been highlighted (Tolwinski, 2007). We have demonstrated the repression of wg by Stat92E in the eye and notal region of the wing disc (Ekas et al., 2006), as well as antennal and leg discs (this study). In the eye disc, we have identified a small (263 bp) enhancer that is negatively regulated by Stat92E in an autonomous manner (wg 2.11Z) (Ekas et al., 2006). The inability to look at the wg2.3Z enhancer that is negatively regulated by Stat92E in leg and antennal discs leaves open the possibility that Stat92E regulates these two enhancers by the same molecular mechanism (Ekas et al., 2006; Pereira et al., 2006). Canonical Stat92E binding sites are not present in either wg enhancer, which suggests that Stat92E does not directly repress wg but rather acts through another factor. In the simplest scenario, activated Stat92E induces the expression of a repressor that acts directly on wg in eye, wing (notal region), antennal, and leg discs. However, we cannot rule out the possibility that Stat92E regulates wg by distinct mechanisms in different discs. Moreover, there may be cryptic Stat92E binding sites in these wg enhancers, through which Stat92E may directly repress wg. Future work will be needed to address these issues.

**Do Mammalian STATs Repress BMP and Wnt Genes in Limb Formation?**

Our study raises the possibility that the JAK/STAT pathway also plays an important role during mammalian development by negatively regulating the expression of vertebrate Wnts and BMPs. Furthermore, our study also highlights the question of whether Wnts and BMPs regulate expression of cytokines/growth factors that activate JAK/STAT signaling during development. While it is not currently known whether the JAK/STAT pathway functions during mammalian limb development, BMP and Wnt genes do play fundamental roles in this process (Capdevila and Izpisua Belmonte, 2001). The elucidation of the roles of the JAK/STAT pathway during development have been hampered by the early embryonic lethality of stat3-deficient mice, and likely genetic redundancy between Stat3 and Stat5, which are most similar to Stat92E (Takeda et al., 1997; Levy and Darnell, 2002). The effects of a stat3/stat5 double knockout mouse have not been reported. The generation of mice with conditional single stat3 or with double stat3 and stat5 deficiency only in the limb bud will be required to address this issue. Nevertheless, the homology between molecules involved in limb development in Drosophila and mammals and the critical role of Stat92E in Drosophila appendage development presented here, our observations are likely to provide new insights into the role of STAT proteins in limb development in higher organisms.

**Experimental Procedures**

**D. melanogaster Stocks**

These stocks are described in FlyBase: y; stat92E^{E55C9}, stat92E^{E397}; dome^{E468}; hop^{C111}; dpp-lacZ; wg-lacZ (wg^E); UAS-hop; UAS-gfp; P{AyGAL4}25 P{UAS-GFP.S65T}/T2; hs-flp MKRS/ TM6B; hs-flp; ey-flp; FRT^{82B} ubi-GFP(S65T)_ub/TM6B, Tb^1, FRT^{82B} M(3)96C, Ubi-GFP, N^p; h^L1{25}/TM6B, Tb; a wg temperature sensitive heteroallelic combination ug^D_{114/CX3}, referred to as wg^T in the text; a dpp temperature-sensitive heteroallelic combination dpp^{h^24/h^25}, referred to as dpp^T in the text. We also used y, hs-flp; FRT^{82B} hsCD2, y^+TM2 and y, hs-flp; FRT^{82B} hsCD2, y^- Minute/ TM2 (Casares and Mann, 2001), upd2 null allele (Hombria et al., 2005); upd3-Gal4, UAS-GFP (Agaigre et al., 2003); 10XSTAT92E-GFP (Bach et al., 2007); UAS-3HA-stat92E, referred to as “Stat92E FLX” in text and figures (Ekas et al., 2006), and wg2.3Z (Pereira et al., 2006).

**stat92E Clones**

stat92E^{E55C9} and stat92E^{E397} were used interchangeably as they produce identical phenotypes (Silver and Montell, 2001; Ekas et al., 2006). We induced stat92E clones randomly using hs-flp or in the eye-antennal disc using ey-flp. Collections of larvae of the genotype y, hs-FLP; FRT^{82B} stat92E/FRT^{82B} hsCD2, y^- were heat shocked for 2 hr at 37°C during first or second instar. The adult legs and antennae were dissected and mounted in a 2:1 mixture of Canada balsam and methyl salicylate. To provide stat92E cells with a growth advantage, we employed the Minute technique, which allows mutant cells to grow faster than surrounding heterozygous cells and to encompass the majority of the compartment in which they arise (Morata and Ripoll, 1975). To rescue stat92E antennal phenotypes, we used the MARCM technique (Lee and Luo, 1999) to drive expression of the UAS-3HA-stat92E transgene only in stat92E clones.

**hop and dome Clones**

We used dome^{E48}, a strong hypomorphic allele, and hop^{C111}, an amorphic allele, and generated clones in the eye-antennal disc using the ey-Gal4 UAS-flp, GMR-hid technique (Stowers and Schwarz, 1999). This system relies upon the presence of a recessive cell lethal mutation on the chromosome arm of interest to generate large clones in the antenna.

**Temperature-Sensitive Experiments**

Flies of the appropriate genotype were allowed to lay eggs at the permissive temperature 18°C for 2 days. Their offspring were then shifted to the restrictive temperature 29°C for 2 days, and then they were returned to 18°C. For the N^p experiments, we isolated male larvae for the dissection of discs. For h^L1{25} experiments, Tb^- larvae were dissected. The wg and dpp alleles were maintained over a compound chromosome SM6~/TM6B and Tb^- larvae were isolated for dissec-
tion. Discs were fixed and sense and anti-sense upd ribo-probes were generated according to the protocol in Bach et al. (2003).

Antibody Staining and Microscopy

Antibody stainings were performed as described (Bach et al., 2003). We used mouse anti-Wg (1:15), mouse anti-β-galactosidase (1:20), mouse anti-Dac (1:20), mouse anti-Dacil (1:500) (Duncan et al., 1998); rabbit anti-β-galactosidase (1:2,000) (Cappel); rabbit anti-Hh (1:1,000) (Pai et al., 1998); mouse anti-sense ribo-probes were generated (Bach et al., 2003). We used fluorescent secondary antibodies at 1:250 (Jackson Laboratories). We collected fluorescent images (at 25×) using a Zeiss LSM 510 confocal microscope, and bright field pictures of adult legs or antennae (at 10, 20, or 40×) using a Zeiss Axiosplan microscope with a Spot camera.

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