The position and function of the Notch-mediated eye growth organizer: the roles of JAK/STAT and four-jointed

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In many animal systems, the local activation of patterning signals in spatially confined regions (organizers) is crucial for promoting the growth of developing organs. Nevertheless, how organizers are set up and how their activity influences global organ growth remains poorly understood. In the *Drosophila* eye, local Notch activation establishes a conserved dorsal–ventral organizer that promotes growth. The dorsal selector *Iroquois* complex defines the position of the organizer at the mid-first instar, and through its ligand, unpaired, the Janus kinase (JAK)/signal transducers and activator of transcription (STAT) pathway is thought to mediate global growth downstream of the organizer. However, here we show that the unpaired/JAK/STAT pathway is actually a fundamental element in the spatial control of the organizer, upstream from Notch activation. Furthermore, we identify *four-jointed*, a target of the Fat and Hippo tumour-suppressor pathways, as a mediator of the growth controlled by the organizer. These findings redefine the process of organizer formation and function, and they identify *four-jointed* as a regulatory node, integrating multiple growth-control pathways.

Keywords: Notch; growth; organizer; JAK/STAT; four-jointed

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INTRODUCTION

The control of organ size and shape remains a mystery although, in recent decades, this control has often been linked to the activity of spatially confined domains known as organizers (conserved signalling centres). However, how organizers instruct global tissue growth over long distances remains poorly understood (Affolter & Basler, 2007). Indeed, such issues are difficult to define as the control of growth by organizers almost always occurs as an integral part of pattern formation and genes that distinguish

between these two roles have remained elusive. However, clinical evidence implicating various organizer-forming genes in human carcinogenesis—for example, *Notch*, *Wnt* or *Hedgehog* (*hh*)—has revived interest in the connections between growth control and organizers.

The eye disc (adult-eye precursor) of *Drosophila melanogaster* provides a useful conceptual framework to study how organizers form and function in growth control. Growth and morphogenesis in the eye discs depend on a conserved Notch-mediated dorsal–ventral (DV) organizer. This organizer is initially established early in the second larval instar by the asymmetric distribution of the dorsal-compartment selector genes of the *Iroquois* complex (*Iro-C*), which is comprised of three related members: *mirror* (*mirr*), *araucan* and *caupolican* (McNeill *et al*, 1997; Cavodeassi *et al*, 1999). Downstream from the organizer, the *eyegone* (*eyg*) gene that is specific for the organizer cells controls eye size (Chao *et al*, 2004; Dominguez *et al*, 2004). Indeed, loss of *eyg* completely blocks growth without affecting eye specification or patterning, whereas gain of *eyg* fully reverts growth arrest in Notch mutants. As *eyg* encodes a transcription factor, its effects on growth might be mediated indirectly by regulating the expression of diffusible factors.

Activation of the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signalling pathway by constitutively activated JAK protein (*hop^{Tum-I}*) or overexpression of the *unpaired* (*upd*) ligand produces eye overgrowth (Bach *et al*, 2003; Chao *et al*, 2004; Tsai & Sun, 2004; Reynolds-Kenneally & Mlodzik, 2005), suggesting a role for the *upd*/JAK/STAT pathway in the control of eye size.

By activating the transcription factor *eyg*, Notch signalling is thought to be required for *upd* expression in the posterior disc margin—the so-called ‘firing point’—leading to diffusion of the *upd* protein from these posterior cells and organization of global eye growth (Chao *et al*, 2004; Reynolds-Kenneally & Mlodzik, 2005). The ‘firing point’ is the exact point where retinal differentiation starts in early third instar discs, and a point of integration of Notch and hedgehog (*hh*) signals (Dominguez & Casares, 2005). Therefore, it remains unclear whether *upd* is expressed in appropriate cells at the correct time to fulfil this role.

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Fig 1 | General *upd*/JAK/STAT activity drives dorsal overgrowth that is linked to ectopic organizer formation. (A) Control wild-type (wt) adult eye. (B) Ubiquitous overexpression of *upd* drives a dorsal protrusion (arrow). (C–H) Eye discs stained for β -galactosidase (*lacZ*) and *wg*, or the neuronal marker *Elav* as indicated. Expression of the *mirr-lacZ*, *Ser-lacZ*, and *slp-lacZ* enhancer trap lines in wild type discs (C,E,G) and *ey-Gal4>upd* discs (D,F,H). (I,J) Schematics of the changes in gene expression. Double arrows represent signalling from the organizer to the adjacent cells. Red arrowhead points to the organizer position (C,D); the small red arrowhead points to the dorsal-ventral axis in the head, and the white arrowhead points to the eye (E). an, anterior; D, dorsal; ey, eyeless; Iro-C, Iroquois complex; JAK, Janus kinase; mirr, mirror; Ser, Serrate; slp, sloppy pair; STAT, signal transducers and activator of transcription; upd, unpaired; V, ventral; wg, wingless.

Here, we show that activation of the JAK/STAT pathway by *upd* promotes eye growth before activation of the Notch pathway and *eyg*, and that by repressing *Iro-C* expression, *upd* proves to be instrumental in the positioning of the organizer. Furthermore, we identify *four-jointed* (*fj*) as a mediator of the JAK/STAT pathway and of Notch-dependent eye growth, a gene negatively regulated by the Hippo and Fat tumour-suppressor pathways. Together, these data provide a new framework for the formation and function of organizers in growth control.

RESULTS AND DISCUSSION

JAK/STAT pathway activation drives dorsal overgrowth

In various studies, the *upd* gene has been proposed as a promising downstream effector of the Notch-dependent DV organizer (Chao et al, 2004; Reynolds-Kenneally & Mlodzik, 2005), acting as a long-range diffusible signal (Zeidler et al, 1999; Tsai & Sun, 2004) to mediate global eye growth. Although this model is attractive for explaining how signals from the organizer reach the surrounding cells and promote global eye growth, it is yet to be shown. Furthermore, there are observations that do not concord with this model. First and foremost, ubiquitous expression of *upd* or of *hop^{Tum-1}* does not induce overgrowth across the entire eye but, rather, overgrowth is limited to the dorsal eye alone (Fig 1).

Dorsal overgrowth by *upd* is linked to ectopic organizer

To define the molecular basis underlying this asymmetric overgrowth, we specifically activated the JAK/STAT pathway in all eye cells during the proliferative phase of the disc using the regulatory sequences of the *eyeless* (*ey*) gene and a *UAS-upd* transgene (hereafter *ey-Gal4>upd*). We examined the expression and function of the dorsal selector *Iro-C* in these mutant discs (Fig 1C–J), *P{lacW}mirr^{P69D17}* (McNeill et al, 1997), and precisely defined the dorsal region of the early eye disc and, hence, the position of the organizer (Fig 1C).

Dorsal outgrowth caused by ubiquitous *upd* expression was always associated with a new *mirr⁺/mirr⁻* border owing to the repression of *mirr* in a dorsal-anterior domain of the eye disc (Fig 1D; supplementary Fig S1B online: 100% penetrance, *n* = 83). Several Notch target genes are prominently expressed on one or both sides of the DV boundary—for example, *Serrate* (*Ser*; Papayannopoulos et al, 1998), *eyg* (Chao et al, 2004; Dominguez et al, 2004), *fj* (Papayannopoulos et al, 1998; this study) and *Enhancer of split* (*E(spl)mβ*; Dominguez & de Celis, 1998)—and

Furthermore, we await verification that early *upd* expression can reverse the growth arrest in mutants of Notch signalling and *eyg*, and that the loss of core components of the JAK/STAT pathway severely reduces cell proliferation after the organizer is formed.

Fig 2 | Transient ventral expression and the activity of the upd/JAK/STAT pathway. (A–H) Late first-instar eye discs stained for wg protein (blue), in which GFP represents the JAK/STAT reporter (10XSTAT92E–GFP) and β -galactosidase (*lacZ*, red) monitors *upd-lacZ* (A–D) or *mirr-lacZ* (E–H) expression. (I) Control GFP⁺ clones and (J–L) *upd*⁺ clones induced at 0–24 h AEL. Arrow in the inset (J) indicates the repressed *mirr-lacZ*. (K) Histogram of the area of *upd*⁺ clones in D and V regions. Only discs with no *mirr* repression ($n = 18$) were scored. Data are means $\pm$ s.e.m. P -value of 0.0348 was derived from Student's t -test. (L) Mosaic *upd*⁺ eye discs stained for pH3 (purple) and cut (red). AEL, after egg laying; D, dorsal; GFP, green fluorescent protein; JAK, Janus kinase; mirr, mirror; pH3, phosphorylated histone H3; STAT, signal transducers and activator of transcription; upd, unpaired; V, ventral; wg, wingless.

act as molecular markers of organizer cell fate. In particular, *Ser* is expressed in a ventral stripe along the organizer complementary to the expression of *Iro-C*, specifically defining ventral organizer cell fate. The formation of a secondary organizer and the transformation from a dorsal-to-ventral cell fate was further shown by the ectopic expression of *Ser* in the dorsal region of discs overexpressing *upd* (Fig 1E,F; supplementary Fig S1C online). The repression of *mirr* and other *Iro-C* genes (data not shown) by *upd* is independent of Sloppy paired 1 and 2 (Fig 1G–J; see also supplementary information online), two members of the Forkhead family of transcription factors expressed in the ventral eye (Sato & Tomlinson, 2007). These findings might explain the puzzling asymmetric overgrowth following activation of the JAK/STAT pathway.

upd represses *Iro-C* by antagonizing hh and wg

Models of *Iro-C* regulation propose that *hh* expression in dorsal cells induces *wingless* (*wg*) in dorsal–anterior cells, and that both *wg* and *hh* are required for *Iro-C* expression (reviewed in Dominguez & Casares, 2005). This model is incomplete because both *wg* and *hh* signalling pathways have long-range activity in the early eye disc, as seen by the ubiquitous expression of target genes such as *homothorax* (Dominguez & Casares, 2005). Thus, the dorsal-restricted *Iro-C* domain might only be explained if *wg*

and/or *hh* signalling was counteracted by a ventral signal, factor ‘X’ in the early eye disc. Our findings suggest that *upd* could act as this inhibitory signal and, indeed, *upd* overexpression driven by *ey-Gal4* can repress *wg* or *wg* signalling in the dorsal margin. Consequently, some discs from these flies showed a typical *wg*-like phenotype ($\leq 35\%$, $n = 100$ larval discs scored; supplementary Fig S1 online), whereas higher levels of *upd* expression induced by the ‘flip-out’ *AyGal4* method produced the *wg*-like phenotype with a 100% penetrance (Fig 2J).

To establish a functional link between *upd* and the *wg* and/or *hh* cascades, we monitored the expression of the *mirr-lacZ* gene when *upd* was co-expressed with *wg* or *hh*. Neither the *wg* nor *hh* transgene was sufficient to activate *mirr* in the dorsal–anterior and ventral cells in the presence of *upd* (100% penetrance, $n = 22$). Furthermore, although *wg* could rescue growth, *hh* could not (supplementary Fig S1D–F online). Thus, we conclude that *upd* might repress *Iro-C* largely by preventing eye cells from responding to both the *hh* and *wg* cascades.

Iro-C DV expression occurs during the first instar (Fig 2) and *upd* is transiently expressed in ventral cells during the first (Fig 2A–D) and early second instar (supplementary Figs S2 and S3 online). A 10XSTAT92E–GFP reporter that accurately reflects JAK/STAT pathway activity (Ekas et al, 2006) further showed that JAK/STAT signalling is active at this early stage (Fig 2A–H).

Fig 3 | Inhibition of the JAK/STAT pathway alters the DV *Iro-C* expression. (A, G–I) *ey-FLP*-induced clones of JAK/STAT pathway genes marked by loss of *GFP* (green), with *mirr-lacZ* expression (red) and *wg* protein (blue). (B,F) Expression of *mirr-lacZ* in the larval and adult eye in wt (B,D) and *ey-Gal4>PIAS* (C,E,F) grown at 26.5 °C (B–E) or 25 °C (F). (G–I) Examples of eye discs with mutant clones running along the DV boundary and marked by loss of *GFP* (green). Note the altered border of *mirr-lacZ* expression (red). an, anterior; DV, dorsal-ventral; ey, eyeless; flp, flip; GFP, green fluorescent protein; *Iro-C*, Iroquois complex; JAK, Janus kinase; mirr, mirror; PIAS, protein inhibitor of activated STAT; STAT, signal transducers and activator of transcription; upd, unpaired; wg, wingless; wt, wild type.

Importantly, *wg* is expressed in a dorsal–anterior sector during this stage complementary to the activity of the JAK/STAT pathway (Fig 2A–H; supplementary Fig S2 online). Note that *GFP* is activated in a broader domain than *upd-lacZ*, overlapping 1–2 cell rows with *mirr-lacZ* (supplementary Fig S2 online), which is consistent with the secretion of the *upd* protein. This overlapping expression fits the observation that levels of mirr decrease near the DV boundary in wild-type discs.

Early and transient *upd* activity represses *Iro-C*

Next, we investigated whether the ability of *upd* to repress *Iro-C* is limited to a specific temporal window, consistent with its transient ventral expression. The *ey-Gal4* line drives expression from as early as the 6–23-cell eye-disc primordium in stage-15 embryos, persisting throughout the larval stages until retinal differentiation starts in the third instar. Therefore, we induced flp-out *AyGal4* clones of *upd*-expressing cells during the first, second and third

Fig 4 | *upd* drives overgrowth upstream from the Notch pathway. (A) There is no eye overgrowth induced by *upd* when the *mirr* border is eliminated by ubiquitous expression of *mirr* (*ey-Gal4>upd>mirr*). (B) Small eye size owing to ubiquitous *fng* expression is not altered by removing *upd* (*os¹*; C) or by adding *hop^{Tum-1}* (D) or a *upd*⁺ transgene (E; 100% penetrance). (F) Small eye size in ubiquitous *PIAS* (51 out of 63 flies with small eyes) or the eye loss (12 out of 63) is rescued by (G) *N¹CD* (100%, *n* = 32). (H) Gain of *upd* in *eyg*[−] (*eyg^{XE119}/eyg^{M3-12}*), a pharate adult is shown. *ey*, eyeless; *eyg*, eyegone; *fng*, fringe; *mirr*, mirror; *N¹CD*, Notch intracellular domain; *PIAS*, protein inhibitor of activated STAT; *upd*, unpaired.

instar, and then analysed the discs from the wandering third instar larvae (Fig 2I–L).

The *upd*⁺ clones that made up the dorsal margin were associated with premature retinal differentiation (*wg*-like phenotype) and *wg* repression (data not shown), which is consistent with recent studies (Ekas et al, 2006; Tsai et al, 2007). However, repression of the *mirr-lacZ* gene only occurred in clones induced 0–24 h after egg laying (AEL; Fig 2J; and detail in supplementary Fig S4 online) and not thereafter (see Fig 3). These observations are consistent with *upd* being necessary to initiate but not to maintain *Iro-C* repression, although the initial repression by *upd* might be maintained by polycomb-mediated epigenetic silencing (Netter et al, 1998) and by the *Sloppy pair* genes (Sato & Tomlinson, 2007; see model presented later).

We also found that mosaic larvae having clones of *upd*-expressing cells generated at 0–24 h AEL rarely survived to the third instar (<2%). In addition, control *GFP*⁺ clones grew to encompass most of the eye disc (74.8%, *n* = 10 mosaic discs scored; Fig 2I), whereas *upd*⁺ clones were smaller (26.32%, *n* = 18). Furthermore, *upd*⁺ clones were significantly smaller in the dorsal (*Iro-C*) region (Fig 2K, *P* = 0.0348), whereas the distribution and size of clones in the dorsal and ventral regions did not differ significantly in control discs (data not shown). Staining the mosaic discs with phosphorylated histone H3 (pH3), a marker for mitosis, indicated that clones of *upd*⁺ cells and adjacent wild-type cells do not overproliferate (Fig 2L).

We then generated clones of *JAK/STAT* pathway genes by using the *ey-FLP* technique (supplementary Table S2 online) to assess their consequences on growth and *mirr-lacZ* expression. Clones of homozygous mutant cells for the JAK protein *hop*, the receptor *dome* or the nuclear transducer *STAT92E* grew, suggesting the pathway was not essential cell-autonomously for disc growth

(Fig 3; Zeidler et al, 1999; Ekas et al, 2006). As none of these clones de-repressed *mirr-lacZ* (Fig 3A,G–I), we suspected that clones originated after the first instar could grow, but clones induced before first instar—the crucial period—could not survive or proliferate. This would be consistent with the large twin-spot areas adjacent to *hop* null mutant clones in the ventral eye region (Fig 3A). Indeed, when we assayed *hs-FLP*-induced clones of the receptor (*dome^{PG14}*; supplementary Table S2 online), clones induced at 0–24 h AEL were rarely recovered (1 clone in 109 eye discs), unlike those induced at 24–48 h AEL (8 out of 84). Thus, homozygous *JAK/STAT* clones induced after the first instar can grow and survive, and *Iro-C* expression is not altered.

By using the protein inhibitor of activated STAT (*PIAS*) to block the *JAK/STAT* pathway (Betz et al, 2001) and by growing flies at 26.5 °C we were able to recover adult flies severely deficient for *JAK/STAT* activity in early eye development, in which the *mirr-lacZ* is de-repressed ventrally (Fig 3C,E). The *mirr-lacZ* is mutant for the *mirr* gene and hence the reduction in eye size is ameliorated in the *mirr-lacZ*/+ background (compare fly eyes in Fig 3F with Fig 4G). In addition, the straight and smooth DV *Iro-C* border was altered severely in many *ey-Gal4>PIAS* eyes grown at 25 and 26.5 °C (Fig 3F; supplementary Table S1 online). Similarly, clones of cells mutant for *hop*, *dome* or *upd* mildly disturbed the DV *mirr* border (Fig 3G–I). We conclude that *JAK/STAT* pathway activity helps to generate or maintain the DV *Iro-C* boundary or its differential affinities.

JAK/STAT induces growth upstream from Notch pathway

To verify that *upd* lies upstream from the growth organizer, we revisited and extended the epistatic analyses of *JAK/STAT* and Notch (Chao et al, 2004; Reynolds-Kenneally & Mlodzik, 2005). The overgrowth induced by *upd* was reverted to that of the

Fig 5 | Fj mediates growth by the organizer. (A) The expression of *fj* (visualized by *fj-lacZ*, white arrows in A–C) diminishes when Notch activity is compromised (B). Atonal (red) marks the position of the morphogenetic furrow (A,B). (C) Ectopic dorsal *fj* expression (arrowhead) in discs overexpressing *upd*. *wg* (pink) unambiguously defines the DV axis in the discs. (D) The growth defect associated with diminished Notch activity is partly rescued by (E) the gain of *fj* expression. (F) Map of *GS-58F* and *GS-D67* within the *fj* locus. (G) New model of the sequential roles of *upd*/JAK/STAT, Notch and *fj* in normal eye growth. DI, Delta; DV, dorsal–ventral; ey, eyeless; eyg, eyegone; fj, four-jointed; fng, fringe; GS, Gene Search; hh, hedgehog; Iro-C, Iroquois complex; JAK, Janus kinase; N, Notch; Ser, Serrate; slp, sloppy pair; STAT, signal transducers and activator of transcription; upd, unpaired; wg, wingless; wt, wild type.

wild type, or beyond, by blocking the formation of the *Iro-C* border by ubiquitously expressing *mirr* or any of the *Iro-C* genes (Fig 4A; data not shown; 86% of flies reverted *upd*-mediated overgrowth, $n = 108$). *Iro-C* represses the *fringe* (*fng*) gene and the *fng*⁺/*fng*[−] border establishes the site of Notch activation. Hence, ubiquitous expression of *fng* by *ey-Gal4* produces a reproducibly reduced eye defect owing to the failure to activate fully the Notch receptor in the organizer (Dominguez et al, 2004). In this context, halving the dosage of *upd* did not enhance eye loss (Fig 4B,C). Similarly, constitutive activation of the JAK protein (*hop*^{Tum-1}) or generalized expression of *upd* did not rescue growth (Fig 4D,E; 100%, $n = 270$).

Halving the *upd*, *dome*, *hop* or *STAT92E* gene dosage, or downregulating any of the three *upd*-like genes by RNA-mediated interference, or the receptor *dome*, did not block the overgrowth induced by *Delta* (*DI*; supplementary Figs S3, S5 and S6 online;

supplementary Table S2 online). Conversely, expression of a constitutively active form of the Notch receptor N^{ICD} restored the growth impairment caused by *PIAS* (Fig 4F,G; see legend for quantification of the phenotypes) and the small eye size associated with diminished *upd* activity (*upd*^{OSIS}; Bach et al, 2003). Suppression of the overgrowth phenotypes is not caused by titration of the Gal4 protein by the addition of *UAS* transgenes as co-expression of one or two copies of *UAS-GFP* did not alter growth.

Finally, a gain of *upd* by *ey-Gal4* did not rescue the growth defect caused by loss of the endogenous Notch effector gene *eyg* (Fig 4H; 100%, $n = 30$). However, it was notable that *upd* overexpression reverted the failure to initiate retinogenesis in the *eyg* mutants without rescuing the growth defect (supplementary Fig S7 online). Perhaps the role of *upd* in retinogenesis downstream from *eyg* or experimental differences accounts for the apparent discrepancy between our results and the reported rescue

of *Notch* and *eyg* eye size by gain of *upd* at a later stage in development (Chao *et al*, 2004). Together these results suggest that an early phase of JAK/STAT signalling controls growth upstream from the organizer, whereas its late phase in retinogenesis—the ‘firing point’—lies downstream from the Notch–*eyg* (model in Fig 5).

JAK/STAT and Notch induce growth through Four-jointed

Our observations re-open the question of how signals from a local organizer reach the surrounding cells to promote global eye growth. Therefore, we searched our collection of Gene Search (GS) *P*-element lines for candidates to mediate JAK/STAT and Notch-induced growth. These GS lines were isolated in a forward genetic screen for their capacity to interact with the Notch signalling pathway in eye growth (D.F.-M., F.J.G.-A. & M.D., unpublished data). Interestingly, we identified two GS *P*-elements within the *fj* locus (*GS-58F* and *GS-D67*; Fig 5D–F), a Golgi transmembrane type II glycoprotein (Ishikawa *et al*, 2008 and citations therein).

The *fj* gene is expressed at the DV organizer and in a graded manner dependent on Notch and JAK/STAT pathway activation (Zeidler *et al*, 1999 and citations therein; Fig 5B,C). Initially *fj* was linked to DV ommatidial polarity determination through the Notch, wg and JAK/STAT pathways (Zeidler *et al*, 1999), and isolating *fj* in an unbiased forward genetic screen reveals it to be a functionally relevant target of these pathways in eye growth (Fig 5B–E). The gain of expression of *fj* in the GS *P*-element line *GS-D67* or with a *UAS-fj* transgene (data not shown) rescued the growth defect caused by reduced Notch signalling to some extent (Fig 5E; eye size 60–65% of the wild type, $n=371$). Thus, *fj* mediates growth through the JAK/STAT and Notch-induced organizer, although additional effectors might be needed to ensure the correct eye size. Remarkably, *fj*-related proteins—for example Fjx-1—have also been identified as targets of Notch in mammals (Rock *et al*, 2005), suggesting that similar events might also occur in other Notch-induced organizers.

fj was recently shown to be negatively regulated by the Fat and Hippo tumour-suppressor pathways during imaginal-disc growth control (Pan, 2007; Rogulja *et al*, 2008 and references therein). The Fat and Hippo tumour-suppressor pathways and the JAK/STAT and Notch-induced organizer seem to operate independently in growth control, as shown by dosage-sensitive genetic interactions (data not shown), although their control of growth seems to converge at *fj*. *fj* is a promising candidate to mediate the spread of organizer activity as its expression at the DV organizer is self-stimulated (Zeidler *et al*, 1999) and, hence, the *fj* domain is broader than that of *eyg*, the expression of which coincides precisely with the domain of Notch activation (Fig 5G). Furthermore, *fj* can influence Fat tumour-suppressor activity by directly phosphorylating Fat (Ishikawa *et al*, 2008).

Collectively, these findings re-define the model of organizer formation and function, in which an initial phase of JAK/STAT signalling regulates the position and shape of the DV organizer by repressing *Iro-C*. The local activation of Notch at the DV boundary then establishes the organizer, and *eyg* and *fj* activation by Notch executes the growth-promoting activity of the organizer (model in Fig 5G) and *fj*, as a target also of the Fat and Hippo tumour-suppressor pathways, acts as a regulatory node to integrate and

implement the signals acting cell autonomously and the organizer growth signal that acts globally.

METHODS

Drosophila strains. The mutant stocks and genotypes of larvae used for genetic mosaic analyses are described at <http://flybase.org/> and in supplementary Tables S2 and S3 online, except *fj GS-58F* and *GS-D67* that are described in this study. Clones of *upd*-expressing cells were induced by an 8 min 36 °C heat shock at 0–24 h AEL or 10 min at 37 °C at 24–48 h or 48–72 h AEL in the $y w hs-FLP; AyGal4 UAS-GFP_{nl}/UAS-upd; mirr-lacZ/+$ genotype. Clone size was quantified with ImageJ.

Antibody staining. Eye imaginal discs were stained as described by Dominguez *et al* (2004). The following antibodies were used: monoclonal rat anti-Elav anti-serum (1:100); mouse anti-wg (1:100) and mouse anti-cut (1:50) were obtained from Developmental Studies Hybridoma Bank at the University of Iowa; rabbit anti-pH3 (1:5,000, Upstate, Billerica, MA, USA); rabbit anti- β -galactosidase (1:2,000, Cappel, Solon, OH, USA); mouse anti- β -galactosidase (1:200, Promega, Maddison, WI, USA) and rabbit anti-atonal (1; 2,000, a gift of A. Jarman). Alexa Fluor-596- and -488 (Molecular Probes, Carlsbad, CA, USA) and Cy5-conjugated (Jackson ImmunoResearch, West Grove, PA, USA) secondary antibodies were used at 1:400. Discs were mounted in Fluoromount G (Southern Biotechnology, Birmingham, AL, USA). Images were acquired using a Leica TCS-NT Confocal microscope.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- Affolter M, Basler K (2007) The Decapentaplegic morphogen gradient: from pattern formation to growth regulation. *Nat Rev Genet* **8**: 663–674
- Bach EA, Vincent S, Zeidler MP, Perrimon N (2003) A sensitized genetic screen to identify novel regulators and components of the *Drosophila* janus kinase/signal transducer and activator of transcription pathway. *Genetics* **165**: 1149–1166
- Betz A, Lampen N, Martinek S, Young MW, Darnell JE Jr (2001) A *Drosophila* PIAS homologue negatively regulates stat92E. *Proc Natl Acad Sci USA* **98**: 9563–9568
- Cavodeassi F, Diez Del Corral R, Campuzano S, Dominguez M (1999) Compartments and organising boundaries in the *Drosophila* eye: the role of the homeodomain Iroquois proteins. *Development* **126**: 4933–4942
- Chao JL, Tsai YC, Chiu SJ, Sun YH (2004) Localized Notch signal acts through *eyg* and *upd* to promote global growth in *Drosophila* eye. *Development* **131**: 3839–3847
- Dominguez M, de Celis JF (1998) A dorsal/ventral boundary established by Notch controls growth and polarity in the *Drosophila* eye. *Nature* **396**: 276–278

- Dominguez M, Casares F (2005) Organ specification-growth control connection: new in-sights from the *Drosophila* eye-antennal disc. *Dev Dyn* **232**: 673–684
- Dominguez M, Ferres-Marco D, Gutierrez-Aviño FJ, Speicher SA, Beneyto M (2004) Growth and specification of the eye are controlled independently by Eyegone and Eyeless in *Drosophila melanogaster*. *Nat Genet* **36**: 31–39
- Ekas LA, Baeg GH, Flaherty MS, Ayala-Camargo A, Bach EA (2006) JAK/STAT signaling promotes regional specification by negatively regulating wingless expression in *Drosophila*. *Development* **133**: 4721–4729
- Ishikawa HO, Takeuchi H, Haltiwanger RS, Irvine KD (2008) Four-jointed is a Golgi kinase that phosphorylates a subset of cadherin domains. *Science* **321**: 401–404
- McNeill H, Yang CH, Brodsky M, Ungos J, Simon MA (1997) *mirror* encodes a novel PBX-class homeoprotein that functions in the definition of the dorsal-ventral border in the *Drosophila* eye. *Genes Dev* **11**: 1073–1082
- Netter S, Fauvarque MO, Diez del Corral R, Dura JM, Coen D (1998) *white+* transgene insertions presenting a dorsal/ventral pattern define a single cluster of homeobox genes that is silenced by the polycomb-group proteins in *Drosophila melanogaster*. *Genetics* **149**: 257–275
- Pan D (2007) Hippo signaling in organ size control. *Genes Dev* **21**: 886–897
- Papayannopoulos V, Tomlinson A, Panin VM, Rauskolb C, Irvine KD (1998) Dorsal-ventral signaling in the *Drosophila* eye. *Science* **281**: 2031–2034
- Reynolds-Kenneally J, Mlodzik M (2005) Notch signaling controls proliferation through cell-autonomous and non-autonomous mechanisms in the *Drosophila* eye. *Dev Biol* **285**: 38–48
- Rock R, Heinrich AC, Schumacher N, Gessler M (2005) Fjx1: a notch-inducible secreted ligand with specific binding sites in developing mouse embryos and adult brain. *Dev Dyn* **234**: 602–612
- Rogulja D, Rauskolb C, Irvine KD (2008) Morphogen control of wing growth through the Fat signaling pathway. *Dev Cell* **15**: 309–321
- Sato A, Tomlinson A (2007) Dorsal-ventral midline signaling in the developing *Drosophila* eye. *Development* **134**: 659–667
- Tsai YC, Sun YH (2004) Long-range effect of upd, a ligand for Jak/STAT pathway, on cell cycle in *Drosophila* eye development. *Genesis* **39**: 141–153
- Tsai YC, Yao JG, Chen PH, Posakony JW, Barolo S, Kim J, Sun YH (2007) Upd/Jak/STAT signaling represses wg transcription to allow initiation of morphogenetic furrow in *Drosophila* eye development. *Dev Biol* **306**: 760–771
- Zeidler MP, Perrimon N, Strutt DI (1999) Polarity determination in the *Drosophila* eye: a novel role for unpaired and JAK/STAT signaling. *Genes Dev* **13**: 1342–1353