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Upd/Jak/STAT signaling represses *wg* transcription to allow initiation of morphogenetic furrow in *Drosophila* eye development

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Abstract

The initiation of retinal development in *Drosophila* begins at the posterior center (PC) of the eye disc margin. The front of the differentiation wave, recognized as a morphogentic furrow (MF), moves from posterior to anterior. What determines MF initiates from the specific PC site is still unclear. The *unpaired* (*upd*) gene is expressed at PC at early third instar, just before the time of MF initiation. Therefore, *upd* is expressed at the appropriate time and location for a specific role in defining the site of MF initiation. *upd* encodes a ligand for the Jak/STAT signaling pathway. In this report, we showed that the Upd/Jak/STAT signaling is required and sufficient to determine MF initiation. This is primarily achieved by repressing the transcription of *wingless* (*wg*), which is known to block MF initiation.

Keywords: Upd/Jak/STAT signaling; *Drosophila*; Eye; Morphogenetic furrow initiation; *wg* repression

Introduction

The *Drosophila* compound eye develops from the larval eye imaginal disc. At third instar, photoreceptor differentiation begins from the central point of the posterior margin and gradually progresses toward the anterior direction. The front of the differentiation wave is marked by a dorsoventral (D/V) line of apically constricted cells, called the morphogenetic furrow (MF), which sweeps over the eye disc in a posterior-to-anterior direction. The site of MF initiation is at the intersection of the D/V midline and the posterior margin of the eye disc. This is also the site where the eye disc connects via the optic stalk to the brain. We will call this spot the posterior center (PC).

The movement of MF can be divided into two phases: initiation and progression. MF progression requires the interplay among Decapentaplegic (Dpp), Hedgehog (Hh) and Notch (N) signaling pathways (Baonza and Freeman, 2001; Chanut and Heberlein, 1997b; Curtiss and Mlodzik, 2000; Dominguez and Hafen, 1997; Fu and Baker, 2003; Greenwood and Struhl, 1999; Heberlein et al., 1993; Ma et al., 1993; Pappu et al., 2003; Strutt and Mlodzik, 1997). MF progression is a reiterative process. Differentiating photoreceptors behind the MF express *hh*. The Hh then signals to the cells at the anterior edge of MF to express *dpp*. Dpp, with the help of N signaling, induces the cells to express the proneural gene *atonal* (*ato*). Hh also induces *ato* expression. Hh and N also independently repress the expression of the negative factor *hairy*. The upregulation of *ato* and downregulation of *hairy* allows these cells to enter into photoreceptor neural fate and later express *hh*. Together these events repeatedly drive the cycle of MF progression.

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MF initiation can be further divided into birth and reincarnation, two temporally and spatially separable phases (Kumar and Moses, 2001). Birth is the very first event that occurs at PC, and results in the formation of the first row of Ato-positive cells (Dominguez and Hafen, 1997). Once the first row of retinal cells is formed, they can then induce the progression of the MF. However, since the eye disc has a curvature, the D/V length of the MF gradually increases toward the middle of the eye disc. Therefore, the MF will have to be reinitiated repeatedly from the lateral margin as the MF progresses forward. This process is termed the reincarnation of MF. Whether these two stages are mechanistically different is not clear.

MF reincarnation requires Hh (Curtiss and Mlodzik, 2000; Dominguez and Hafen, 1997; Pappu et al., 2003), Dpp (Baonza et al., 2001; Burke and Basler, 1996; Chanut and Heberlein, 1997b; Curtiss and Mlodzik, 2000; Dominguez and Hafen, 1997; Kumar and Moses, 2001; Wiersdorff et al., 1996), Egfr (Kumar and Moses, 2001) and N (Kumar and Moses, 2001) signalings. Blocking or reduction of these signaling resulted in a block of MF initiation from the lateral margins.

MF birth is more difficult to study. Kumar and Moses (2001) used *dpp^{blk}-GAL4* to drive various constructs to block the signaling in the posterior and lateral margins of the eye disc and examined the effects on MF birth and reincarnation. However, since the *dpp^{blk}-GAL4* showed no expression at PC (Chanut and Heberlein, 1997b; Kumar and Moses, 2001), these experiments did not convincingly test the role of these signaling pathways in MF birth. Regulation of MF birth had been addressed in previous studies. Temperature shift experiments on temperature-sensitive (ts) allele of *dpp* (Chanut and Heberlein, 1997b), *hh* (Borod and Heberlein, 1998) and *Egfr* (Kumar and Moses, 2001) can cause the complete absence of retinal development. For *hh* and *Egfr*, the critical period has been defined to be around the time of MF birth (Borod and Heberlein, 1998; Kumar and Moses, 2001). *Mad* mutant clones located at PC also can cause the complete absence of retinal development (Wiersdorff et al., 1996). These results suggest that the Dpp, Hh and Egfr signalings are required for MF birth. The involvement for N signaling has not been conclusively determined. Although blocking of N signaling can result in the total absence of retinal development (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998), this effect may be due to its role in cell proliferation in early eye disc (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998).

Ectopic activation of Hh, Dpp, Egfr and N signaling can induce ectopic MF. However, there are some spatial differences. Hh signaling induces ectopic MF only from the internal region (Heberlein et al., 1993; Pan and Rubin, 1995; Strutt and Mlodzik, 1997), while Dpp signaling only induces ectopic MF from the anterior margin (Chanut and Heberlein, 1997a; Pignoni and Zipursky, 1997). Egfr and N have only been found to induce MF initiation from the lateral margins (Kumar and Moses, 2001). These spatial constraints suggest that additional factors are required. For example, Dpp plus Dl can induce ectopic MF in the internal region (Baonza and Freeman, 2001).

Whereas multiple signaling pathways promote MF initiation, only one signaling pathway, the Wingless (Wg), is known to play a negative role. *wg* is expressed broadly in the early second instar eye disc, and become restricted to the lateral margins beginning from late second instar (Baker, 1988; Cavodeassi et al., 1999; Cho et al., 2000). Reduction of Wg signaling can cause MF initiation from the lateral margins, predominantly from the dorsal side (Blackman et al., 1991; Heslip et al., 1997; Treisman and Rubin, 1995). Ectopic activation of Wg signaling blocked both MF initiation and progression (Heslip et al., 1997; Treisman and Rubin, 1995).

The *unpaired* (*upd*) gene is expressed at PC at second and early third instar (Halder et al., 1995; Pignoni and Zipursky, 1997; Tsai and Sun, 2004; Zeidler et al., 1999). The timing and location of *upd* expression suggested that it may have a specific role in MF initiation. *upd* encodes the ligand for the Jak/STAT signaling pathway (Harrison et al., 1998). In this study, we demonstrate that the Upd/Jak/STAT signaling is necessary and sufficient for MF initiation, at both the birth and reincarnation stages. The primary function of this signaling appears to be the suppression of *wg* expression.

Materials and methods

Fly stocks

Fly culture and crosses were performed according to standard procedure at 25 °C unless otherwise noted. In these experiments, *UAS-dome$^{\Delta cyt}$* (Brown et al., 2001) was from J. Castelli-Gail Hombria, *UAS-hop^{Tum-l}* (Luo et al., 1999) and *w hop^{m13} FRT18A/ FM6k* (Luo et al., 1999) were from C. R. Dearolf, and *STAT^{F} mwh e/TM3,Ser,Sb* (Baksa et al., 2002) was from Margaret Fuller, and *w E132-GAL4* (Halder et al., 1995) was from W. Gehring. *UAS-upd* (Harrison et al., 1998; Zeidler et al., 1999) was from N. Perrimon, *wg-lacZ/SM6-TM6B* (Kassis et al., 1992) was from C. T. Chien. *hsFLP^{22};Act5C>y^{+}>GAL4,UAS-GFP^{S65T}* (Ito et al., 1997) was from D. Yamamoto. *w FRT18A ubi-GFP^{S65T}* (from B. Edgar), *wg^{cx4}/SM6-TM6B* (Baker, 1987), *w;UAS-wg/CyO* (Azpiazu and Morata, 2000), *dpp-lacZ(BS3.0)* (Blackman et al., 1991), *os^{1}* and *STAT92^{06346}/TM3, ry^{RK} Sb^{1} Ser^{1}* were from the Bloomington Stock Center. The *grh-STAT-lacZ* reporter contains three Grainyhead (Grh) binding sites and four STAT consensus binding sites (S.B. and J.W.P., submitted for publication).

Clonal induction

Positively labeled flp-out expression clones were generated by crossing *UAS*-lines to *hs-FLP^{22};Act5C>y^{+}>GAL4 UAS-GFP^{S65T}* (Ito et al., 1997). Heat-shock induction of *hs-FLP^{22}* was at 37 °C for 30 min at 24–48 h after egg laying. Mutant clones were induced by the FLP–FRT method (Xin et al., 2002). For *hop^{m13}* mutant clones, *hs-FLP^{22}; P[ubi-nls-GFP]FRT18A* males were crossed to *w hop^{m13} FRT18A/ FM6k* virgins. Heat shock induction of *hs-FLP^{22}* was at 37 °C for 90 min at 24–48 h after egg laying.

STAT92E^{ts} and wg genetic interaction

wg^{CX4};STAT92E^{F}/SM6-TM6B and *STAT92E^{F}/TM6B* virgins were crossed with *STAT92E^{06346}/TM6B* males and their F1 progenies were raised at room temperature. The two crosses were set up under the same condition. F1 progeny with no balancer markers were scored for viability and adult eye phenotype.

Immunohistochemistry

Antibody staining for imaginal discs was done as previously described (Pai et al., 1998). Early and late third instar larval imaginal discs were dissected and stained. Primary antibodies were rat anti-Elav (1:500), mouse anti-Wg 4D4

(1:200, Developmental Studies Hybridoma Bank, University of Iowa) and rabbit anti-β-galactosidase (1:2000, Cappel). Secondary antibodies (Jackson ImmunoResearch) were Cy3 anti-rabbit, Cy5 anti-rabbit, Cy3 anti-rat, Cy5 anti-rat, FITC anti-mouse and Cy5 anti-mouse. Fluorescent images were obtained using a Zeiss LSM 510 confocal microscope.

wg enhancer dissection

Various fragments of the *wg* 1.7-kb enhancer were either PCR-amplified or cut by specific restriction enzymes from the genomic DNA and cloned into the *PH-stinger GFP* reporter vector (Barolo et al., 2000). The relative position and length of each construct were shown in Fig. 5. Germline transformants of each construct were generated as described previously (Jang et al., 2003). More than two independent transgenic lines for each construct were generated and tested.

Results

STAT signaling is active at the site of MF initiation

If the Upd/Jak/STAT signaling plays a role in MF initiation, then the signaling should be active at the right time and right location. We have previously shown that Upd-GFP can be distributed over a long range (Tsai and Sun, 2004), but it was not clear how far the endogenous Upd can reach and exert its effect on the Jak/STAT pathway. We used a *grh-STAT-lacZ* (S.B. and J.W.P., submitted for publication) as a reporter for Upd/Jak/STAT signaling activity. The reporter contains three Grainyhead (Grh) binding sites and four STAT consensus binding sites (Fig. 1A), so the *lacZ* expression is expected to be sensitive to STAT binding and reflects STAT signaling. Expression of the reporter in the embryo is consistent with it being induced by endogenous Upd signal (data not shown; S.B. and J.W.P., submitted for publication). In wild type eye disc, *upd* mRNA is detected at PC only before MF initiation (Tsai and Sun, 2004), although the Upd protein can persist into late third instar (Zeidler et al., 1999). The *grh-STAT-lacZ* expression is high in the posterior region in early third instar (Fig. 1B) and mid-third instar (Fig. 1C). The high expression region is broader than the domain of *upd* expression at PC (Tsai and Sun, 2004; Zeidler et al., 1999). Weak signal can be detected within most of the eye disc, consistent with it reflecting the long range of secreted Upd. At late-third instar, the *grh-STAT-lacZ* expression became weaker and more restricted to the PC (Fig. 1D), presumably reflecting the reduction of *upd* expression at this stage (Tsai and Sun, 2004). Weak signal can be detected anterior to MF. When *upd* expression was clonally induced (abbreviated as *Act>upd*), the *grh-STAT-lacZ* reporter expression was non-autonomously induced surrounding the clone in regions anterior to MF in late third instar eye disc (not

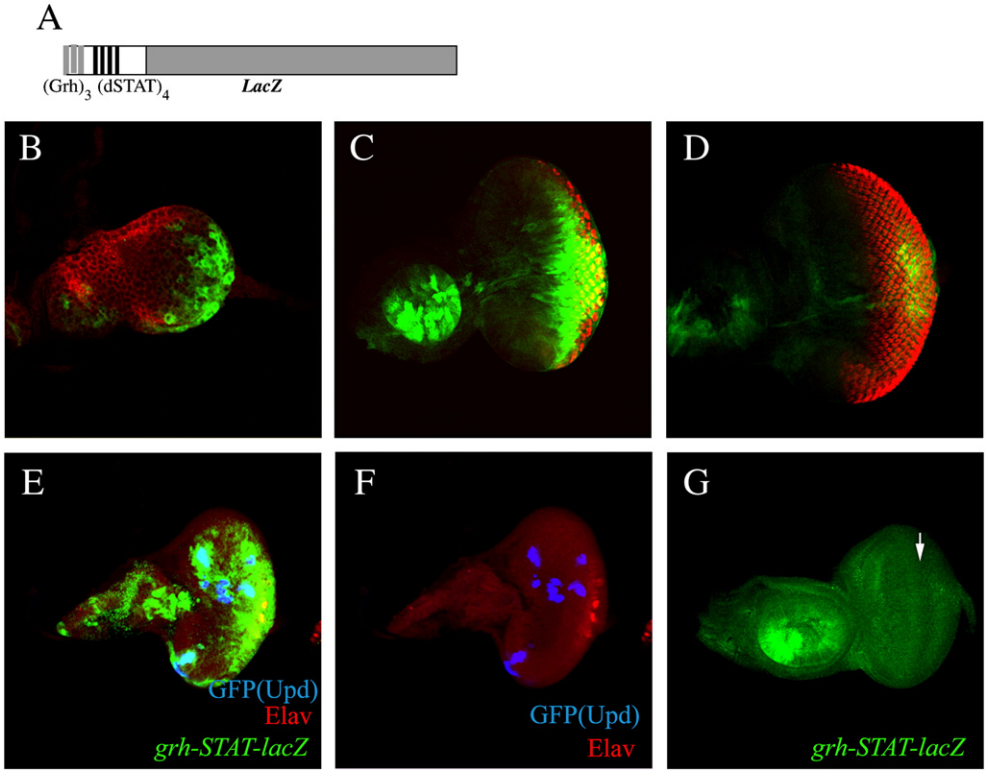


Fig. 1. STAT signaling is active at the site of MF initiation during early eye development. *grh-STAT-lacZ* is a reporter for STAT signaling. (A) The reporter contains three Grainyhead (Grh) binding sites and four STAT consensus binding sites. (B–D) The *grh-STAT-lacZ* expression was detected by anti-β-gal immunostaining (green). The *grh-STAT-lacZ* expression is high in the posterior region in (B) early third and (C) mid third instar eye disc. In late third instar eye disc (D), the expression becomes reduced and restricted to the posterior-most region. Weak expression anterior to MF can also be detected. At a different focal plane, strong expression was seen associated with the Bolwig nerve (not shown). In (B) the *grh-STAT-lacZ* expression abuts the Wg domain (anti-Wg, in red). (E, F) Several *Act>upd* clones (marked by coexpression of GFP, in blue) caused non-autonomous expression of *grh-STAT-lacZ* and slight overgrowth. The disc has just initiated the MF and has only one row of photoreceptors. (B–F) Photoreceptors were labeled by anti-ELAV (in red). (G) In the *upd* mutant os^{1}, the late third instar eye disc is smaller and the *grh-STAT-lacZ* expression is barely detectable. In contrast, the *grh-STAT-lacZ* expression in the antennal disc is still strong. The position of MF is indicated by an arrow. In this and subsequent figures, the discs and adult heads are positioned with anterior to the left, and dorsal to the top.

shown) and in mid-third instar eye disc at the time of MF initiation (Figs. 1E, F). These results suggested that the reporter is responsive to Upd signal. In late third instar os^{1} eye disc, the eye disc is smaller and the *grh-STAT-lacZ* expression is very weak, in contrast to the strong expression in the antennal disc (Fig. 1G). This result indicates that the reporter expression requires Upd signaling. Together these results validated the *grh-STAT-lacZ* as a reporter for STAT signaling and showed that the STAT signaling is active at the appropriate time and location for MF initiation. Similar conclusion was recently reported using an independently designed STAT92E reporter (Bach et al., 2007; Ekas et al., 2006).

Ectopic Upd can induce MF initiation from lateral margin and suppress wg transcription

We then tested whether Upd has the ability to induce MF initiation by ectopically expressing *upd* in the eye disc. We reported previously ectopic *upd*-expressing clones within the eye disc induced non-autonomous overgrowth (Tsai and Sun, 2004). When the clone occurred at the dorsal margin, it non-autonomously induced MF initiation and photoreceptor differentiation (Fig. 2A), as well as non-autonomous overgrowth (as seen by BrdU incorporation and anti-phospho-histone-3 staining, data not shown). *Act>upd* clones in the ventral margin did not induce ectopic MF initiation and photoreceptor differentiation (Fig. 2B).

Since *wg* is known to be expressed at the lateral margins and blocks MF initiation from the lateral margin, we then checked to see if *upd* affected *wg* expression. Indeed *upd*-expressing clones non-autonomously repressed Wg protein level when the clone is located in dorsal (Fig. 2C) or ventral (Fig. 2D) margins. The weak Wg signal (Fig. 2D) probably reflects the Wg protein produced by neighboring Wg-producing cells. The repression of *wg* is at the transcriptional level, since *wg-lacZ* is also repressed at both dorsal (Fig. 2E) and ventral margins (Fig. 2F). These findings suggest that *upd* may induce MF initiation by

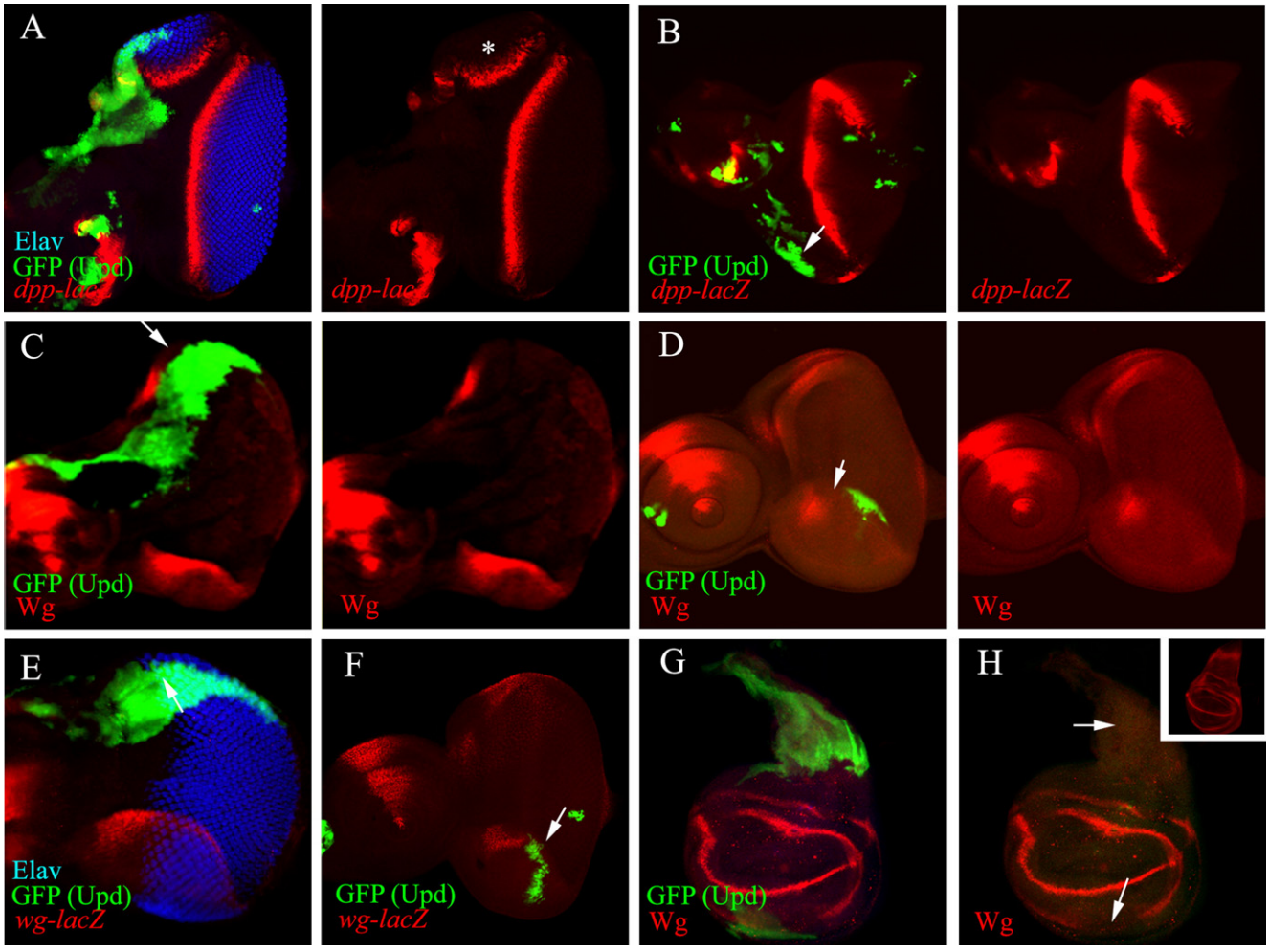


Fig. 2. Ectopic Upd/Jak/STAT signaling can repress *wg* expression and induce ectopic MF at lateral margins. (A–H) *Act>upd* clones (marked by coexpressing GFP, in green) in eye–antennal disc (A–F) and in wing disc (G, H). (A) An *Act>upd* clone at the dorsal margin non-autonomously induced (marked by an asterisk) ectopic MF and photoreceptor differentiation, indicated by *dpp-lacZ* (red) and ELAV (blue). (B) An *Act>upd* clone (marked by an arrow) in the ventral margin did not cause ectopic MF, indicated by *dpp-lacZ* (red). (C, D) An *Act>upd* clone (arrow) non-autonomously suppressed Wg level (red) in dorsal margin (C) and in ventral margin (D). In (A, B), the ectopic ommatidial clusters are revealed by anti-Elav staining (blue). (E, F) *wg-lacZ* (red) is non-autonomously repressed (arrow) by *Act>upd* clones in the dorsal margin (E) and ventral margin (F). (G, H) Wg level (red) is suppressed by *Act>upd* clones (arrows) in wing disc. Inset in (H) showed Wg (red) expression in wild-type wing disc.

repressing *wg* transcription. Upd-expressing clones in the wing disc also repressed Wg expression (Figs. 2G, H, compare with Wg expression in wild-type wing disc in Fig. 2H inset).

In contrast to the non-autonomous action of Upd, clonal expression of activated Hop (*Act>Hop^{Tum1}*) caused an autonomous repression of *wg-lacZ* expression in both dorsal and ventral margins (Supplementary Fig. 1). This result suggests that Upd acts through its downstream Jak/STAT signaling pathway to repress *wg* transcription.

Ectopic MF initiation by Upd depends on repression of wg

Since *wg* can block MF initiation, it is possible that the ectopic MF initiation by Upd is due to its repression of *wg* at the lateral margins. When *wg* and *upd* were coexpressed (*Act>wg+upd*) at the lateral margins, the ectopic MF was blocked (Fig. 3A). This result suggested that *wg* suppression is responsible for the induction of MF initiation by Upd. When *Act>wg+upd* clones were located within the eye disc, they caused phenotypes (blocking of MF progression and photoreceptor differentiation) (Fig. 3B) similar to those caused by *Act>wg* alone. These results suggest that Wg acts downstream of Upd signaling, and that the effect of Upd/Jak/STAT signaling on *wg* is primarily on *wg* transcription and not on blocking Wg function.

One *Act>wg+upd* clone located behind the MF and at the lateral margin showed both the induction of ectopic MF and the suppression of photoreceptor differentiation (Fig. 3A). Presumably, the former is due to the long range action of Upd to repress endogenous *wg*, and the latter is due to the action of Wg. Both Wg and Upd action are non-autonomous. This result suggested that the range of Upd action is longer than that of Wg.

Endogenous Upd/Jak/STAT signaling is required to repress wg expression

The above experiments were done with ectopic induction of the Upd/Jak/STAT signaling. We next checked whether the endogenous Upd/Jak/STAT signaling plays any role in the regulation of *wg* expression during eye development. In *os^{1}hop^{m13}/Y* mutant eye disc, *wg-lacZ* expression expanded posteriorly (Fig.

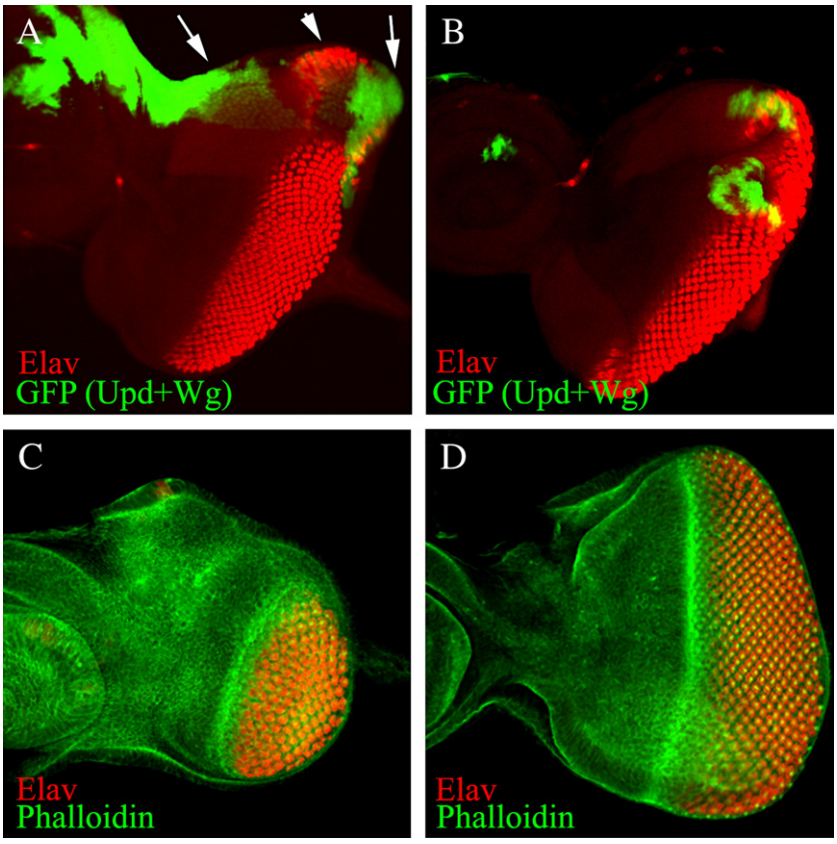


Fig. 3. Induction of MF by Upd/Jak/STAT signaling depends on repression of *wg*. (A–C) Co-expression of Upd and Wg (*Act>upd+wg*; marked by coexpressing GFP, in green) in eye discs. Photoreceptors were labeled by anti-Elav (red). (A) The ectopic MF induced by Upd was blocked (arrow). A posterior–dorsal clone blocked MF initiation (arrow) but also induced an ectopic MF more anteriorly (arrowhead). (B) *Act>upd+wg* clones blocked MF progression and photoreceptor differentiation. (D) In *os^{1}hop^{m13}/Y;wg^{cx4}/+* eye disc, the small eye field in *os^{1}hop^{m13}/Y* (C) can be significantly rescued. The *os^{1}hop^{m13}/Y;wg^{cx4}/+* were derived from the cross between *os^{1}hop^{m13}/FM7* and *w/Y;wg^{CX4}/CyO*. Male larvae were dissected and their eye discs stained by anti-ELAV to identify the *os^{1}hop^{m13}/Y* (non-FM7) eye discs. Because the *wg^{cx4}* chromosome was not balanced, the *wg^{cx4}/+* genotype cannot be positively scored. However, many of the *os^{1}hop^{m13}/Y* eye discs were nearly wild-type size, whereas the *os^{1}hop^{m13}/Y* eye discs were uniformly highly reduced (Figs. 4B, 5D), thus suggesting they are *os^{1}hop^{m13}/Y;wg^{cx4}/+*. Some eye discs of intermediate size were also observed, suggesting that the rescue is not complete. MF is marked by phalloidin staining (green). The reason that the *STAT92E^{ts}* eye disc phenotype can be stronger than those of the null *hop^{m13}/Y* may be there is some maternal contribution in *hop^{m13}/Y*.

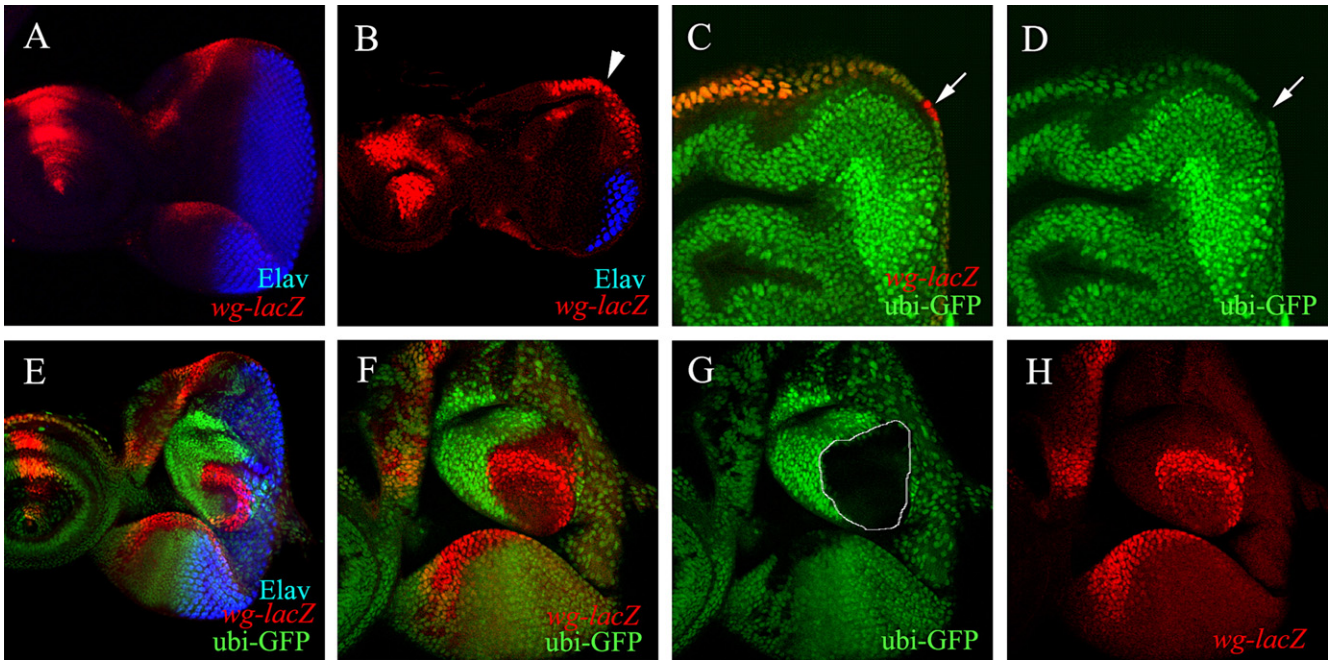


Fig. 4. Endogenous Upd/Jak/STAT signaling is required to repress *wg* expression. (A) *wg-lacZ* (A–F, H; red) is expressed in the dorsal and ventral margins in wild-type eye disc. (B) In the $os^{1}hop^{m13}$/Y mutant eye disc, *wg-lacZ* expression expanded posteriorly. The expansion is more pronounced on the dorsal side (arrowhead), but also evident on the ventral side in some other samples. (C–H) *wg-lacZ* is derepressed in the hop^{m13} mutant clones (marked by the absence of ubi-GFP marker, in green). (C, D) A small clone (arrow) at the dorsal posterior margin showed derepression of *wg-lacZ*. The internal region of the disc has some overgrowth. (E–H) A clone (outlined in white in G) in the interior region of the eye disc showed upregulated *wg-lacZ* expression and overgrowth. The block of photoreceptor differentiation (marked by anti-Elav, in blue) is apparent in different optical planes.

4B, compare with Fig. 4A). The expansion is more pronounced at the dorsal side, but also occurs at ventral in other samples (not shown). The mutant eye disc is small, due to the defect in cell proliferation. hop^{m13} is a null allele (Perrimon and Mahowald, 1986) and is a point mutation that results in a premature stop codon in the kinase-like domain (Luo et al., 1999). hop^{m13} mutant clones at the posterior–lateral margin (Figs. 4C, D) and within the interior of the eye disc (Figs. 4E–H) caused autonomous *wg-lacZ* expression. Within these clones, MF progression and photoreceptor differentiation were blocked (not shown). These effects were the expected consequences of *wg* expression. The hop^{m13} mutant clones also have overgrowth (Figs. 4E–H), presumably due to the proliferative effect of *wg* in early eye development (Blackman et al., 1991). These results suggested that the endogenous Upd/Jak/STAT signaling is required to restrict *wg* expression to the lateral margins. Although the *grh-STAT-lacZ* expression anterior to MF is barely detectable (Fig. 1C), the STAT signaling is active and required for *wg* repression, as shown by the upregulation of *wg-lacZ* in hop^{m13} mutant clones.

We then checked to see the relationship between the *grh-STAT-lacZ* reporter and *wg* expression. *wg-lacZ* is expressed in the dorsal half of the eye disc in early second instar and becomes restricted to the dorsal and ventral margins in late second and early third instar (Cavodeassi et al., 1999; Cho et al., 2000; Hazelett et al., 1998; Treisman and Rubin, 1995). If Upd/Jak/STAT signaling acts to repress *wg* expression, then the *grh-STAT-lacZ* and *wg-lacZ* expression domains should not overlap. Indeed, in second instar eye disc, Wg expression at the lateral margins abuts the *grh-STAT-lacZ* expression domain (Fig. 1A). These results suggest that the endogenous Upd/Jak/STAT signaling is active at the time of MF initiation and also showed that the range of endogenous Upd/Jak/STAT signaling is consistent with a function in preventing *wg* expression from the posterior margin of the eye disc.

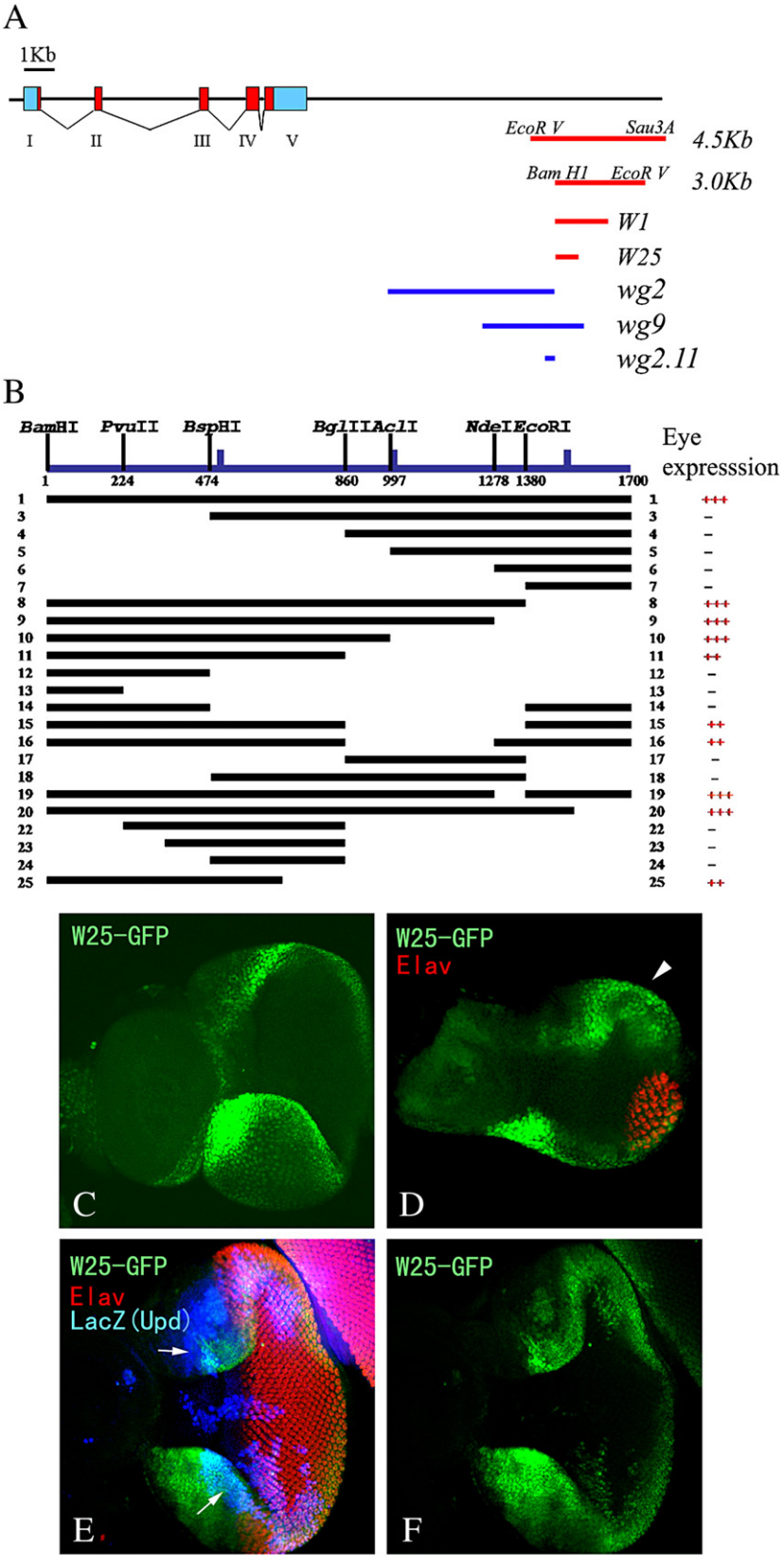
In $os^{1}hop^{m13}/Y$ mutant, the larval eye disc is highly reduced (Figs. 3C–5D; Tsai and Sun, 2004). When *wg* dosage was reduced (in wg^{cx4}/+), the small eye disc phenotype can be significantly rescued (Fig. 3D). This result suggests that the derepression of *wg* in the $os^{1}hop^{m13}/Y$ mutants is responsible for the block of eye development.

We also tested this relationship by reducing the *wg* dosage in the $STAT92E^{F}/STAT92E^{06346}$ temperature-sensitive mutant. In $STAT92E^{F}/STAT92E^{06346}$ mutants (N=115) raised at room temperature, 49% was lethal. Of the viable ones, 27% has small eye or eye defects. When the *wg* dosage was reduced to half in wg^{CX4}/+;$STAT92E^{F}/STAT92E^{06346}$ (N=87); the lethality dropped to 25%. Of the viable ones, all except one has normal eyes. Thus the $STAT92E^{ts}$ eye phenotype is significantly suppressed when the *wg* dosage is reduced. This result again supports the interpretation that the derepression of *wg* is responsible for the block of eye development.

Identification of a wg eye enhancer that responds to Upd signaling

The *wg* locus has been dissected and analyzed for enhancer activities and a 4.5-kb *Eco*RV–*Sau3*A fragment from the *wg*

locus was found to drive expression in the eye, wing and leg discs (J. Kim, unpublished observations). A 3.0-kb *Bam*HI–*Eco*RV subfragment specified strong expression in the eye and wing discs (Fig. 5A). Upon further dissection of this fragment, we found that a 1.7-kb subfragment (W1) specifies the expression in the eye disc, in a pattern that mimics the *wg* expression pattern (Fig. 5A). This 1.7-kb fragment was further dissected for analysis, and we identified a 680-bp subfragment



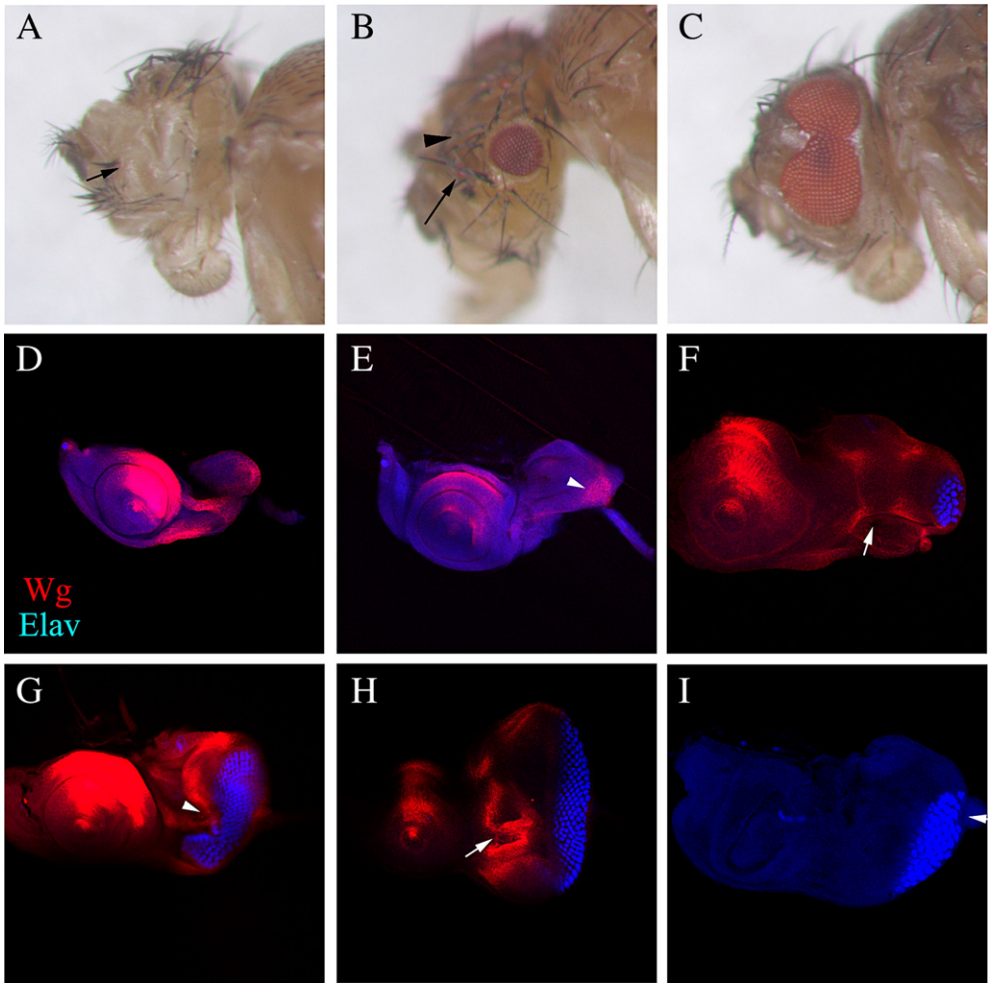


Fig. 6. MF initiation requires Upd/Jak/STAT signaling. *STAT92E^{F}/STAT92E^{06346}* is a temperature-sensitive mutant. Flies were raised at room temperature (24–28 °C). The *STAT92E^{ts}* adult flies showed a range of eye phenotypes that includes (A) no eye, (B) small eye and ectopic ocellus (arrow) and (C) an anterior nick. In the *STAT92E^{ts}* eye discs, Wg (red) is induced in the entire eye disc (D) or strongly at the PC region (E, arrowhead). There is no photoreceptor differentiation (Elav, blue) in these discs (D, E), suggesting a complete block of MF initiation. In (F–H) the eye field showed different degree of reduction. (F) The Wg in the ventral margin (arrow) is upregulated to become a pattern similar to that of the dorsal side. In (G, H), the Wg is upregulated in the interior region of eye disc, accompanied by tissue overgrowth and a block of MF progression and photoreceptor differentiation (G). (I) The *E132-GAL4* was used to drive a dominant-negative Upd receptor *dome$^{\Delta cyt}$* (*E132> dome$^{\Delta cyt}$*), and dorsal furrow initiation and retinal development were blocked. *E132>dome$^{\Delta cyt}$* flies were raised at 29 °C. Arrowheads indicate the position of the optic stalk.

(W25, Figs. 5A, B) that drove expression in the lateral margins of the eye disc (Fig. 5C). In *os^{1}hop^{m13}/Y* mutant eye disc, *W25-GFP* expression expanded posteriorly (Fig. 5D), suggesting that this *wg* eye enhancer is also repressed by the Upd/Jak/STAT signaling. Expansion of *W25-GFP* occurs at both dorsal and ventral side, although dorsal expansion is more pronounced. This expansion of *wg* expression is correlated with the lack of MF initiation from the dorsal half of the eye disc (Fig. 5D). The ventral eye is also reduced (Fig. 5D), suggesting that the expansion of *wg* blocked MF reincarnation from the lateral margin. However, *Act>upd* clones did not suppress *W25-GFP* expression in either dorsal or ventral margin (Figs. 5E, F), suggesting that the suppression of this enhancer requires additional factor.

Fig. 5. Upd/Jak/STAT signaling is necessary but not sufficiently to repress an eye-specific *wg* enhancer. (A) A 4.5-kb genomic *Eco*RV–*Sau*3A fragment downstream of *wg* transcript was found to specify the *wg* expression pattern in eye, wing and leg discs (J. Kim, unpublished). Further dissection found that a 3.0-kb *Bam*HI–*Eco*RV subfragment retained the expression in eye and wing disc, but only showed weak expression in leg discs. A 1.7-kb *Bam*HI–*Xho*I subfragment (W1) retained the eye expression pattern. (B) Serial dissection of the 1.7-kb fragment for enhancer activity. A minimal 680-bp fragment, *W25*, reflects *wg* expression in the eye disc (C). (D) *W25-GFP* expression (green) expanded posteriorly in *os^{1}hop^{m13}/Y* mutant. The expansion is more pronounced in dorsal than in the ventral side. The MF initiation is completely blocked from the dorsal side. The eye field in the ventral side is also reduced. (E, F) *Act>upd* clones (blue) in the dorsal and ventral margin did not repress *W25-GFP* expression (green). In (D, E), the photoreceptors were marked by anti-Elav (red). The relative location of the two distinct *wg* enhancers identified in this study and in Pereira et al. (2006) is shown in (A). Pereira et al. (2006) focused on the left side of *wg9* and defined the *wg2.11* enhancer fragment. The 3-kb fragment and its subfragments *W1* and *W25* that we analyzed lie immediately downstream of *wg2.11*. *W25* and *wg2.11* are separated by a *Bam*HI site.

Upd/Jak/STAT signaling is required for the birth of MF

Because *upd* is expressed at the PC, where MF birth occurs, we speculate it is involved in the birth stage of MF initiation. To test whether Upd/Jak/STAT signaling is required for the birth of MF, we first examined a *Stat92E* mutant. $Stat92E^{06436}/Stat92E^{F}$ is a temperature-sensitive combination (Baksa et al., 2002). The mutant flies showed a range of eye reduction phenotypes. The most severe is an absence of adult eye (Fig. 6A). Some eye discs lack MF initiation, accompanied by the expansion of *wg* expression into the posterior margin and the PC (Figs. 6D, E). The less severe class has a very small eye (Fig. 6B). Some eye discs showed posterior expansion of *wg* expression in both dorsal and ventral margins, and a block of MF initiation from the lateral margins (Fig. 6F). The weakest phenotype is an anterior indentation of the adult eye (Fig. 6C). Some eye discs showed internal *wg* induction, which blocked MF progression and photoreceptor differentiation (Figs. 6G, H). These results clearly showed that *Stat92E* is required for MF initiation, by suppressing *wg* expression, at both the birth and the reincarnation stages.

We also drove the expression of a dominant-negative Dome receptor ($Dome^{\Delta cyt}$) (Brown et al., 2001) using the *E132-GAL4* (*E132>*$Dome^{\Delta cyt}$). *E132-GAL4* (Halder et al., 1995) is a P[*GawB*] insertion about 2.8 kb upstream of the *upd* transcription start site, and drove expression in patterns similar to *upd* expression (Pignoni and Zipursky, 1997; Tsai and Sun, 2004). This way, we can block Upd signaling at the site of *upd* expression. *E132>*$Dome^{\Delta cyt}$ are pupal lethal. Interestingly, *E132>*$Dome^{\Delta cyt}$ eye discs showed a block of MF initiation only in the dorsal half (Fig. 6I). As the block involved the entire dorsal half, we interpret this as a block of the birth of MF.

Discussion

MF initiation: a new role for the Upd/Jak/STAT signaling

In this study, we report a new role for the Upd/Jak/STAT signaling in eye development. It is involved in MF initiation at both the birth and the reincarnation stages. This was demonstrated by both gain-of-function and loss-of-function experiments. Ectopic expression of *upd* was sufficient to induce ectopic MF initiation from the dorsal margins (Fig. 2A), while blocking of the signaling ($Stat92E^{ts}$*;E132>*$dome^{\Delta cyt}$) resulted in the block of MF initiation (Fig. 6). Four signaling pathways (Dpp, Hh, Egfr and N) are known to act in promoting MF initiation. Our finding adds Upd/Jak/STAT signaling to this group of positive signals to promote MF initiation. *upd* expression at PC is under the regulation of N signaling (Chao et al., 2004). The relationship between Upd/Jak/STAT signaling with Dpp, Hh and Egfr signaling awaits further study.

The Jak/STAT signaling pathway has been reported to be involved in many developmental events (for review, see Arbouzova and Zeidler, 2006; Hombria and Brown, 2002; Hou et al., 2002). For eye development, it has been shown to determine planar cell polarity (Zeidler et al., 1999) and cell proliferation (Bach et al., 2003; Chao et al., 2004; Reynolds-Kenneally and Mlodzik, 2005; Tsai and Sun, 2004). Our finding adds a new function to this versatile signaling pathway.

Upd/Jak/STAT signaling represses wg to allow MF initiation

Wg is the only negative signaling that blocks MF initiation. A main role for Dpp signaling is the repression of *wg* expression (Chanut and Heberlein, 1997b; Dominguez and Hafen, 1997), although this is not the only function of Dpp in MF initiation (Hazelett et al., 1998). Here we report Upd/Jak/STAT as the second signaling pathway that represses *wg* expression. As we were writing this report, Ekas et al. (2006) reported similar findings, demonstrating that the Upd/Jak/STAT signaling represses *wg* transcription and affects eye development. We further demonstrated that the suppression of *wg* appears to be the primary effect of Upd signaling on MF initiation. This is based on: (1) coexpression of *upd* and *wg* abolished its ability to induce ectopic MF initiation (Fig. 3); and (2) the no eye phenotype of $os^{1}hop^{m13}/Y$ mutants is recovered when the dosage of *wg* is reduced (Fig. 3). Further study should reveal the molecular mechanism of *wg* repression by Upd/Jak/STAT signaling. This relationship between these two signaling pathways is not restricted to the eye disc, since Upd signaling can also repress Wg expression in the wing disc (Fig. 2H). However, there is certain tissue specificity, since activated Jak did not repress *wg-lacZ* in the antennal disc (Supplementary Fig. 1). The relationship between Dpp and Upd signaling awaits further study.

Wg blocks eye development at several steps: MF initiation, MF progression and photoreceptor differentiation. In early eye disc, *wg* is expressed in a broad domain and later becomes restricted to the anterior lateral margins. The pushing back of *wg* expression is therefore critical for eye development. Our results show that the Upd signaling represses *wg* expression from the posterior margin, thereby allowing MF to initiate. In addition, the Upd signaling also represses *wg* expression from the internal region of the eye disc, as shown by *wg* derepression in hop^{m13} mutant clones (Fig. 4). Therefore Upd signaling is important also indirectly for MF progression and retinal differentiation.

Pereira et al. (2006) recently reported another *wg* eye enhancer. This 263-bp enhancer (*wg2.11*) is distinct from the *W25* enhancer we described here. Interestingly, *W25* lies immediately downstream of *wg2.11*. *W25* is expressed in both dorsal and ventral margins of the eye disc, while *wg2.11* is expressed in the dorsal margin and only very weakly in the ventral margin (Pereira et al., 2006). Whereas Upd signaling is required, but not sufficient, to repress *W25* expression, it is both required and sufficient to repress *wg2.11* expression (Ekas et al., 2006). *wg2.11* does not contain any STAT92E biding sites, suggesting that STAT92E may not directly regulate *wg* (Ekas et al., 2006). *W25* also does not contain any STAT92E binding sites (TTCNNNGAA) (Hou et al., 1996). There are four sites that each differ from the suggested STAT binding sequence by one bp. However, these were not conserved among *Drosophila melanogaster*, *Drosophila pseudobscura* and *Drosophila virilis*, so are probably not real STAT92E binding sites. Thus Upd

represses *wg* transcription probably indirectly and through at least two distinct, but very closely linked, elements. The mechanism for repression on these two elements may be distinct. The two *wg* enhancers will be valuable in the identification of what factor(s) mediates the repression effect by STAT signaling. The two elements may provide a tight and fail-safe regulation on *wg* expression, to make sure that MF is properly initiated.

What determines the site of MF initiation?

MF initiates from the PC, which coincides with the point at which the eye disc connects to the optic stalk. However, ectopic MF can initiate from the lateral margins, so the optic stalk is not the factor specifying the site of MF initiation. Several signaling pathways are involved in MF initiation. Around the time of MF initiation, Egfr is not activated specifically at PC (Y. C. Tsai, unpublished observations), Notch is activated along the D/V midline (Cho et al., 2000; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998), and *dpp* is expressed along the posterior and lateral margins (Dominguez and Hafen, 1997; Ma et al., 1993; Treisman and Rubin, 1995). Therefore none of these determines the site of MF initiation. *hh* is expressed at the PC in late second and early third instar (Blackman et al., 1991; Borod and Heberlein, 1998; Cavodeassi et al., 1999; Cho et al., 2000) but becomes expressed along the posterior margin just prior to MF initiation (Dominguez and Hafen, 1997). *upd* transcript and protein is detected at the PC at second and early third instar, but disappears after MF initiation (Tsai and Sun, 2004; Zeidler et al., 1999). The expression patterns suggest that *hh* and/or *upd* may be determining the site of MF initiation.

hh expression in the eye disc has been shown to be regulated by *sine oculis* (*so*), *pointed* (*pnt*) and *odd-skipped* and its family members (Bras-Pereira et al., 2006; Pauli et al., 2005; Rogers et al., 2005). The *hh* eye enhancer that is regulated by *so* and *pnt* specifies *hh* expression behind MF (Pauli et al., 2005; Rogers et al., 2005). *pnt* and *so* also express behind the MF (Cheyette et al., 1994; Rogers et al., 2005; Serikaku and O'Tousa, 1994). *so* is expressed in early eye disc, but the expression is uniform (Halder et al., 1995; Kumar and Moses, 2001). Therefore, the regulation by *so* and *pnt* probably does not determine the spatial pattern of *hh* expression at the posterior margin before MF initiation. The *odd* family members *odd* and *drumstick* (*drm*) are expressed at the posterior margin and in second and late third instar eye disc (Bras-Pereira et al., 2006). *odd* and *drm* probably act redundantly through *bowl*, another *odd* family member, to induce *hh* expression along the posterior margin (Bras-Pereira et al., 2006). However, this regulation appears to be along the entire posterior margin, and does not specify the PC.

Does *hh* regulate *upd* expression, or vice versa? Both are expressed at PC from second instar. Whether one is expressed earlier than the other has not been clearly determined. It had been reported that *ptc* mutant clones at the lateral margins or at the D/V midline can induce ectopic MF initiation and also induce *upd-lacZ* expression, even before MF initiation (Reifegerste et al., 1997). This result suggests that Hh signaling may act upstream to induce *upd* expression.

It was also found that *ptc* mutant clones not located at the lateral margins or midline do not induce *upd* expression (Reifegerste et al., 1997). This suggests that Hh signaling may not be sufficient to induce *upd* expression. It had been reported that *upd* expression is induced by *N* and *eyg* (Chao et al., 2004; Reynolds-Kenneally and Mlodzik, 2005). N signaling is activated at the D/V midline, and turns on *eyg* expression. Clonal induction of N^{act} or *eyg* can induce *upd* expression only when located near the posterior margin (Chao et al., 2004; Reynolds-Kenneally and Mlodzik, 2005) had found that the induction by N^{act} can occur also in the internal region of the eye disc. This discrepancy is perhaps due to the different level of induction achieved by different flp-out methods (Ito et al., 1997; Pignoni and Zipursky, 1997). Since *upd* is normally expressed at the intersection of the D/V midline with the posterior margin, we speculate that the normal level of N signaling at the D/V midline collaborates with another factor only present at the posterior margin (Chao et al., 2004). Together these results suggest that it is the synergistic action of N and Hh signaling that turns on *upd* expression at PC, which then induce MF birth.

In addition to the spatial control, there is probably a temporal control for MF initiation. While Upd is expressed at PC from second instar (Ekas et al., 2006; Halder et al., 1995; Pignoni and Zipursky, 1997; Tsai and Sun, 2004; Zeidler et al., 1999) and STAT signaling is active in this region from second instar (Fig. 1; Bach et al., 2007), MF initiation does not begin until early third instar. Additional factor(s) that is temporally regulated is likely required.

Dorsoventral bias

Ectopic Upd can repress *wg* expression in both dorsal and ventral margins (Fig. 2). However, the ectopic MF was induced only from the dorsal margin (Fig. 2). In $E132>Dome^{\Delta cyt}$, MF initiation was also blocked only on the dorsal side (Fig. 6I). This D/V bias is consistent with previous finding that in *wg* mutants the ectopic MF predominately originates from the dorsal margin (Blackman et al., 1991; Treisman and Rubin, 1995), also correlating with the stronger expression of *wg* in the dorsal margin than in the ventral margin (Blackman et al., 1991; Cavodeassi et al., 1999; Treisman and Rubin, 1995). However, the D/V bias is not complete, as Wg still functions in the ventral margin (Treisman and Rubin, 1995). Some $STAT92E^{ts}$ mutants also had complete loss of MF initiation (Figs. 6A, D, E). So the dorsal margin is only more sensitive to Wg signaling.

In $STAT92E^{ts}$ mutants, some eye discs showed enhanced *wg* expression in the ventral margin, in a pattern similar to the *wg* expression pattern at the dorsal margin (Fig. 6F). At the dorsal margin, *wg* expression is interrupted in a small region, which correlates with the site of ocellus formation (Royet and Finkelstein, 1997). Some $STAT92E^{ts}$ mutant adults also have ectopic ocellus located in anterior–ventral region of the head (Fig. 6B). These results suggest that in some $STAT92E^{ts}$ mutant, the upregulation of *wg* is accompanied with a ventral-to-dorsal transformation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.04.011.

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