A Sensitized Genetic Screen to Identify Novel Regulators and Components of the Drosophila Janus Kinase/Signal Transducer and Activator of Transcription Pathway

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ABSTRACT

The JAK/STAT pathway exerts pleiotropic effects on a wide range of developmental processes in Drosophila. Four key components have been identified: Unpaired, a secreted ligand; Domeless, a cytokine-like receptor; Hopscotch, a JAK kinase; and Stat92E, a STAT transcription factor. The identification of additional components and regulators of this pathway remains an important issue. To this end, we have generated a transgenic line where we misexpress the *upd* ligand in the developing Drosophila eye. GMR-upd transgenic animals have dramatically enlarged eye-imaginal discs and compound eyes that are normally patterned. We demonstrate that the enlarged-eye phenotype is a result of an increase in cell number, and not cell volume, and arises from additional mitoses in larval eye discs. Thus, the GMR-upd line represents a system in which the proliferation and differentiation of eye precursor cells are separable. Removal of one copy of *stat92E* substantially reduces the enlarged-eye phenotype. We performed an F_1 deficiency screen to identify dominant modifiers of the GMR-upd phenotype. We have identified 9 regions that enhance this eye phenotype and two specific enhancers: *C-terminal binding protein* and *Daughters against dpp*. We also identified 20 regions that suppress GMR-upd and 13 specific suppressors: *zeste-white 13*, *pineapple eye*, *Dichaete*, *histone 2A variant*, *headcase*, *plexus*, *kohtalo*, *crumbs*, *hedgehog*, *decapentaplegic*, *thickveins*, *saxophone*, and *Mothers against dpp*.

THE *Ja*nus *k*inase (JAK)/*s*ignal *t*ransducer and *a*ctivator of *t*ranscription (STAT) pathway is a phosphotyrosine-driven signaling system that responds to extracellular cues and triggers specific responses in the nucleus within minutes of activation (Levy and Darnell 2002). Extracellular ligands bind to and induce multimerization of cell-surface cytokine receptors, which constitutively associate with nonreceptor protein tyrosine kinase JAKs. Upon receptor activation, the JAKs are activated by auto- or transphosphorylation, and they in turn phosphorylate and activate a class of latent cytosolic transcription factors, STATs, at the plasma membrane. Activated STATs translocate to the nucleus and induce transcription of target genes. The JAK/STAT pathway is evolutionarily conserved and plays important roles in many biological processes in both vertebrates and invertebrates (Zeidler *et al.* 2000; Levy and Darnell 2002). Moreover, mutations in *JAK* and *STAT* genes cause cancer and immune deficiency in humans (Russell *et al.* 1995; Lacronique *et al.* 1997). Discovered as a key signaling pathway of cytokine receptors, the JAK/STAT pathway has been extensively characterized biochemically in mammalian tissue culture systems (Bach *et al.* 1997; Levy and Darnell 2002; O'Shea *et al.* 2002). However, a systematic genetic approach to identify new components and regulators of the JAK/STAT pathway has lagged behind biochemical ones. The redundancy of this pathway in mammals, which have four *JAK* and seven *STAT* genes, makes a genetic approach difficult in this system (Levy and Darnell 2002). However, in the fruit fly Drosophila, which has only one *JAK* and one *STAT* gene, a genetic approach is feasible (Zeidler *et al.* 2000).

There are currently four key members of the Drosophila JAK/STAT pathway: a secreted ligand, Unpaired (Upd), also called Outstretched (Os; Harrison *et al.* 1998; Sefton *et al.* 2000); a cytokine-like receptor, Domeless (Dome; Brown *et al.* 2001), also called Master of marelle (Mom; Chen *et al.* 2002); a nonreceptor, cytosolic tyrosine Janus kinase Hopscotch (Hop; Binari and Perrimon 1994); and a STAT Stat92E (formerly known as Marelle; Hou *et al.* 1996; Yan *et al.* 1996). Upd biochemically activates and genetically interacts with Hop (Harrison *et al.* 1998). Dome has similar overall structure and low but significant homology to gp-130 and leukemic inhibitory factor receptor (Hombria and Brown 2002). Dome interacts genetically with *stat92E*

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and has been shown to associate with Upd when both are expressed in mammalian cells (Brown *et al.* 2001; Chen *et al.* 2002). In mammals, *p*rotein *i*nhibitors of *a*ctivated *S*TATs (PIAS) and *s*uppressor *o*f *c*ytokine *s*ignaling (SOCS) proteins negatively regulate the JAK/STAT pathway (Levy and Darnell 2002). Drosophila possess one *PIAS* homolog, DPIAS [also called *Suppressor of variegation 2-10* (*Su(var)2-10*) and *zimp*], that interacts genetically and biochemically with the JAK/STAT pathway (Chung *et al.* 1997; Mohr and Boswell 1999; Betz *et al.* 2001; Hari *et al.* 2001). Drosophila also have three *SOCS* genes, but no mutations in any of them have been reported (Hombria and Brown 2002; Hou *et al.* 2002). The expression of one of them, *SOCS36E*, depends on the activity of the JAK/STAT pathway, thus making it a reporter for activation of the pathway (Callus and Mathey-Prevot 2002; Karsten *et al.* 2002).

In Drosophila, the JAK/STAT pathway is involved in sex determination, stem cell renewal in the male germline, border cell migration and stalk cell development in oogenesis, embryonic segmentation, tracheal development, larval hematopoiesis, and ommatidial rotation (Binari and Perrimon 1994; Harrison *et al.* 1995; Hou *et al.* 1996; Yan *et al.* 1996; Luo *et al.* 1999; Zeidler *et al.* 1999; Sefton *et al.* 2000; Brown *et al.* 2001; Kiger *et al.* 2001; Silver and Montell 2001; Tulina and Matunis 2001; Beccari *et al.* 2002; Chen *et al.* 2002; McGregor *et al.* 2002).This plethora of biological outcomes is mirrored in the mammalian system, where biochemistry and gene targeting experiments have demonstrated a role for this pathway in numerous processes, including embryonic development, neuronal survival, and development of the immune system and immune responses (reviewed in Levy and Darnell 2002; O'Shea *et al.* 2002).

To identify regulators and components of the Drosophila JAK/STAT pathway, we have generated a transgenic Drosophila line (GMR-upd) that ectopically overexpresses the ligand Upd in the developing eye-imaginal disc. Overexpression of Upd in the developing eye results in an enlarged eye, which is a phenotype that is easy to score visually and that can be used to screen enhancers and suppressors of the activation of the JAK/STAT pathway. To verify this, we found that the hyperactive JAK/STAT pathway in GMR-upd can be modulated by changes in the genetic dose of other known components of the pathway, making GMR-upd a sensitized genetic background for this pathway. The methodology we have used has proven highly successful in the dissection of signal transduction pathways, for example, the *sevenless* and the *ras* pathways (Simon *et al.* 1991; Therrien *et al.* 2000). We performed a sensitized screen to identify dominant modifiers of the GMR-upd, enlarged-eye phenotype using a set of overlapping deficiencies of the Drosophila genome. We found 20 regions that suppress and 9 regions that enhance the enlarged-eye phenotype. Within these deficiencies, we identified 10 suppressors and two enhancers. We also found 3 suppressors of GMR-upd not covered by these deficiencies. In addition, we characterized the enlarged-eye phenotype to aid in understanding the mechanism of the interactions. Interestingly, we found that the GMR-upd phenotype is due to an increase in cell number and not cell size and can be modulated by the *dpp* pathway.

MATERIALS AND METHODS

Stocks: The deficiency kit, a set of overlapping deletions of the Drosophila genome, was obtained from the Bloomington Stock Center and has been estimated to cover 70–80% of the euchromatin of the Drosophila genome. Flies were grown on standard food at 25° unless mentioned otherwise. GMR-upd/Balancer flies were crossed to flies carrying a specific deficiency or mutation. The parents were allowed to lay eggs for 4 days and then were transferred to a new vial. In general, at least 15 progeny of the correct genotype were scored, and an interaction was significant only if most of the progeny exhibited the same phenotype (*i.e.*, suppression or enhancement of the enlarged-eye phenotype). All stocks were crossed to GMR-upd three independent times.

Constructs: The GMR-upd transgene was made by ligating a PCR fragment of the entire coding region of *upd* with *Eco*RI (5′) and *Stu*I (3′) ends into BSKS at the *Eco*RI and *Hinc*II sites to generate BSKSupdΔ3′. The lack of mutations in the updΔ3′ insert was verified by sequencing the entire region amplified by PCR. The updΔ3′ insert was excised from BSKS by digestion with *Bss*HII. The 3′ recessed termini were filled in with Klenow and then the blunted insert was digested with *Eco*RI to generate a updΔ3′ insert with *Eco*RI (5′) and blunt (3′) ends. This fragment was ligated into pGMR at the *Eco*RI and *Stu*I sites (Hay *et al.* 1994). The resulting pGMR-updΔ3′ plasmid was verified by restriction digest and sequencing. To obtain the GMR-upd transgenic line, the pGMR-updΔ3′ plasmid, together with a plasmid encoding the Δ2-3 transposase, was coinjected into w^{1118} embryos by standard protocol (Rubin and Spradling 1983). The G_0 generation was crossed to w^{1118} flies and grown at 16° until eclosion. The resulting transgenic lines, yw P[w* GMR-updΔ3′]^{19}/FM7 and w; P[w* GMR-updΔ3′]^{28}/TM3, Sb^1, resulted from an insertion of the transgene into the X and third chromosomes, respectively. We utilized the yw P[w* GMR-updΔ3′]^{19}/FM7, hereafter called GMR-upd19, most extensively. However, to examine genetic interactions between GMR-upd and alleles on the X chromosome, we utilized the w; P[w* GMR-updΔ3′]^{28}/TM3, Sb^1 transgene, hereafter called GMR-upd28.

Flip-out clones: y w UAS-upd52/y w UAS upd52; hh^{P30}/hh^{P30} were crossed to w; flipout actin Gal4, UAS-eGFP/CyO; hs-flp, MKRS/TM6B, Tb (Basler and Struhl 1994). Larvae were subjected to heat shock for 1 hr at 37° during first or second instar, and green fluorescent protein (GFP)-positive larvae were dissected 24 or 48 hr after heat shock and stained with an anti-β-galactosidase antibody to mark hh-LacZ.

Stainings: Dissections were performed in 1× PBS and tissues were stained with rabbit anti-β-galactosidase (ICN; 1:200, preadsorbed), rat anti-Elav (1:50), mouse anti-Prospero (1:4), rabbit anti-phospho-histone3 (1:200; Upstate Biotechnology, Lake Placid, NY) or Alexa Fluor 568-conjugated phalloidin (1:100; Molecular Probes, Eugene, OR). Elav and Prospero antibodies were obtained from the Developmental Studies Hybridoma Bank. Secondary antibodies (1:200) were obtained from the Jackson lab. Stained tissues were mounted by the SLOWFADE light antifade kit (Molecular Probes) and analyzed on a Leica LSM NT confocal microscope (Department

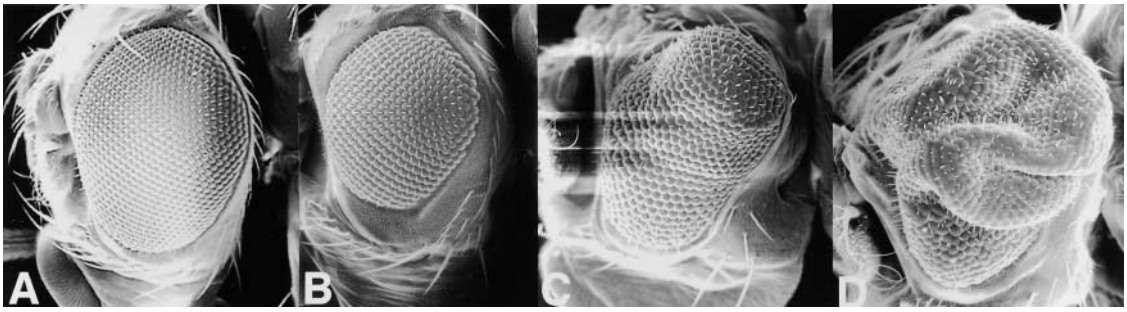


FIGURE 1.—The JAK/STAT pathway controls size of the adult eye. Scanning electron micrographs of a WT eye (A). Heteroallelic combinations of *upd* (*w os/y w os1A*) result in a small eye (B). Ectopic misexpression of Upd using ey-Gal4II (C) or directly using a transgene GMR-Upd (D) results in an enlarged eye. In A–D, anterior is to the left and posterior to the right; dorsal is up and ventral is down. Scanning electron micrographs taken at ×100 magnification.

of Genetics, Harvard Medical School) or an LSM510 Zeiss confocal microscope (Pharmacology Department, NYU School of Medicine). *In situ* hybridization was performed as described in HAUPTMANN and GERSTER (2000). X-gal staining was performed as described in HAZELRIGG (2000). Samples for *in situ* and X-gal stainings were developed on the same day, using the same probe and for the same length of time and were analyzed on an Axiophot 2 compound microscope.

Adult sections: Newly eclosed flies were fixed in osmium tetroxide as described in WOLFF (2000). Sections of 1 μm were cut and mounted on microscope slides. The sections were analyzed using a phase 3 condenser on an Axiophot compound microscope at ×63 under immersion oil.

Scanning electron microscopy: Adult flies were dehydrated in ethanol, subjected to drying and sputter coating, and analyzed on an Amray 1000a SEM (Cambridge Instruments) or a Leo SEM (Zeiss), both at the Harvard School of Public Health, or a JEOL 840 model (Department of Cell Biology, NYU School of Medicine).

Inverse PCR: Inverse PCR was performed as described in HUANG *et al.* (2000). PCR products were sequenced by the Biopolymer Facility at the Howard Hughes Medical Institute, Harvard Medical School, and aligned with Drosophila genomic sequences using BLAST.

Flow cytometry: Collections of embryos and staining and flow cytometric analysis of the cell cycle were performed as described in NEUFELD *et al.* (1998) using a Becton Dickinson FACSvantage. We isolated GFP-positive larvae, dissected the eye-antennal discs, removed the antennal discs, and dissociated and stained only eye-imaginal disc cells. The statistics for each fluorescence-activated cell sorter (FACS) experiment are independent (see NEUFELD *et al.* 1998) and hence are presented separately, rather than as a meta-analysis. The results in Figure 6 are representative of three individual experiments.

RESULTS

The JAK/STAT pathway is involved in the establishment of eye size: A hetero-allelic combination (*w os/y w os1A*, hereafter called *os/os1A*) of a viable *upd* allele (*os*) and a small deletion that removes the *upd* locus (*os1A*) results in a normally patterned eye that is considerably smaller than that of wild type (WT; Figure 1, A and B). In contrast, increased expression in the eye of an *upd* ortholog, the *Om1E* gene, in the closely related species *D. anannasae*, leads to an enlarged-eye phenotype (JUNI *et al.* 1996). Thus, we reasoned that ectopic misexpression of *upd* in the developing eye in *D. melanogaster* would also result in an enlarged eye. We used the Gal4-UAS system to ectopically misexpress *upd* in the developing eye-imaginal disc (BRAND and PERRIMON 1993). We employed four Gal4 drivers: eyeless-Gal4 (ey-Gal4), elav-Gal4, GMR-Gal4, and dpp-Gal4. ey-Gal4 is expressed throughout the eye disc very early in larval development and, in third instar, at high levels in cells posterior to the morphogenetic furrow and in a faint and fading pattern anterior to the furrow (HALDER *et al.* 1995; HAUCK *et al.* 1999; see also Figure 6D). elav-Gal4 and GMR-Gal4 are both expressed in cells posterior to the morphogenetic furrow (HAY *et al.* 1994; JONES *et al.* 1995). dpp-Gal4 is expressed only in the cells in the morphogenetic furrow (STAEHLING-HAMPTON *et al.* 1995). We observed enlarged eyes in flies expressing UAS-upd under the control of all four Gal4 driver lines (Figure 1C and Table 1). In all cases, the enlarged eyes have prominent outgrowths, primarily in the dorsal portion of the eye.

We also compared Gal4-mediated *upd* misexpression with that of *upd* directly under the control of the GMR promoter, since GMR has been used in many modifier screens (HARIHARAN *et al.* 1995). We therefore generated a transgene in which the coding region of *upd* was placed directly under the control of the GMR promoter, which contains multiple tandem binding sites for the eye-specific transcription factor Glass and which is expressed in cells posterior to the morphogenetic furrow (HAY *et al.* 1994). Animals expressing one copy of the GMR-upd transgene have greatly enlarged adult compound eyes, with dramatic dorsal outgrowths (Figure 1D). In addition, the eyes of GMR-upd flies do not appear rough, and the external morphology of the eye and the position of interommatidial bristles is relatively normal. Taken together, these data indicate that ectopic expression of Upd in the developing eye leads to a substantial increase in the size of the eye. Since we observe the same enlarged-eye phenotype using either the Gal4-UAS system or the GMR promoter, we used these two systems interchangeably in the characteriza-

TABLE 1

The JAK-STAT pathway can control the size of the eye

Gal4 line	UAS line	Enlarged eye
ey	upd	Y
ey	dome	N (small)
ey	hop	Y
ey	hop^{Tum-l}	Y
ey	stat92E	N
ey	SOCS36	N
elav	upd	Y
elav	dome	ND
elav	hop	Y
elav	hop^{Tum-l}	Y
elav	stat92E	N
GMR	upd	Y
GMR	dome	N
GMR	hop	Y
GMR	hop^{Tum-l}	Y
GMR	stat92E	N
dpp	upd	Y
dpp	dome	N (small)
dpp	hop	Y
dpp	hop^{Tum-l}	Lethal
dpp	stat92E	N
dpp	SOCS	N

Ectopic expression of Upd or Hop or Hop^{Tum-l} in the developing eye results in an enlarged-eye phenotype in the adult. However, when full-length Dome is misexpressed in the developing eye, it acts as a dominant-negative receptor and results in a small adult eye. Ectopic misexpression of Stat92E or SOCS36E did not result in a visible phenotype. We used eyGal4 insertions on both the second and third chromosomes and got identical results using either driver. elav-Gal4 is an insertion of the third chromosome. GMR-Gal4 and dpp-Gal4 are insertions on the second chromosome. We used two independent UAS-upd lines (UAS-upd4 and UAS-upd30, both insertions on the second chromosome) and obtained the same results from both. UAS-dome, UAS-hop, UAS-hop^{Tum-l}, UAS-stat92E, and UAS-socs36E are all insertions on the second chromosome. ey, eyGal4; elav, elav-Gal4; GMR, GMR-Gal4; dpp, dpp-Gal4; Y, Yes; N, No; ND, not determined.

tion of the enlarged-eye phenotype described below, depending on which line was most convenient.

We next asked whether ectopic expression of *upd* in the developing eye could rescue the small-eye phenotype of *os/os1A* using the ey-Gal4 driver. Importantly, we rescued the small-eye phenotype in *os/os1A* animals using UAS-upd (Figure 2C) but not using UAS-GFP (Figure 2B). These results demonstrate that *upd* regulates the size of the developing eye.

Upd is a secreted molecule that can act in a cell-nonautonomous manner (Harrison *et al.* 1998; Zeidler *et al.* 1999). Therefore, we wanted to determine if ectopic misexpression of cytosolic components of the JAK/STAT pathway, which presumably act cell autonomously, could also rescue the small eye in *os/os1A* and could generate a phenotype when expressed in wild-type flies. Using the Gal4-UAS system, we expressed UAS-dome, UAS-domeΔCyt, UAS-hop, UAS-hop^{Tum-l}, UAS-stat92E, and UAS-SOCS36E using the Gal4 drivers mentioned above. Misexpression of full-length Dome using ey-Gal4 in an *os/os1A* mutant does not rescue the small-eye phenotype (Figure 2E). In fact, *os/os1A; ey-Gal4/UAS-dome* flies actually have smaller eyes than *os/os1A* flies do. Expression of a full-length Dome or a cytoplasmically truncated and presumably inactive Dome (DomeΔCyt) in the wild-type eye discs resulted in a small-eye phenotype that looked similar to the small eye observed in *os/os1A* flies (Figure 2F; Table 1; data not shown). This result indicates that full-length Dome can act as a dominant-negative molecule, an observation that has been made after expressing UAS-dome in other tissues (E. A. Bach, unpublished data; S. Brown and J. C.-G. Hombria, personal communication). However, after coexpression of Upd and full-length Dome together in the developing eye, we still observed an enlarged eye (data not shown). Presumably, full-length Dome does not act as a dominant-negative when Upd is also misexpressed in the eye disc. Expression of wild-type Hop in *os/os1A* partially rescued the small-eye phenotype (Figure 2G), although not as well as Upd (Figure 2C). Expression of the wild-type Hop or the activated Hop^{Tum-l} resulted in an enlarged eye in all combinations (Figure 2H and Table 1; Harrison *et al.* 1995; Luo *et al.* 1995). These data indicate that the growth observed by misexpression of Upd to the developing eye results from signals downstream of Hop. Ectopic misexpression of the negative regulator SOCS36E exacerbated the small-eye phenotype in *os/os1A* animals (Figure 2K). However, when misexpressed in wild-type animals, SOCS36E does not lead to a small-eye phenotype, which has been observed previously (Figure 2L; Callus and Mathey-Prevot 2002). In contrast, ectopic expression of *stat92E* does not rescue the small-eye phenotype in *os/os1A* flies (Figure 2I). In fact, ectopic misexpression of *stat92E* to the developing eye, using any of the Gal4 drivers, failed to produce a phenotype (Figure 2J and Table 1). This is presumably due to the misexpression of *stat92E* not leading to the activation of this transcription factor. This has also been observed in mammalian tissue culture experiments where overexpression of wild-type full-length STATs do not result in their activation without the addition of a stimulating ligand (Bach *et al.* 1997; Darnell 1997). Nonetheless, these data indicate that the JAK/STAT pathway can control the size of the developing eye.

Similarly, we addressed whether the GMR-upd phenotype was dependent on activation of the JAK/STAT pathway. We established two independent transgenic lines, GMR-upd19/FM7 and GMR-upd28/TM3, Sb. In either line, the expression of the GMR-upd transgene does not result in embryonic lethality, and homozygous animals exhibit pupal lethality (data not shown). Animals expressing one copy of the GMR-upd transgene have a greatly enlarged adult compound eye, with sig-

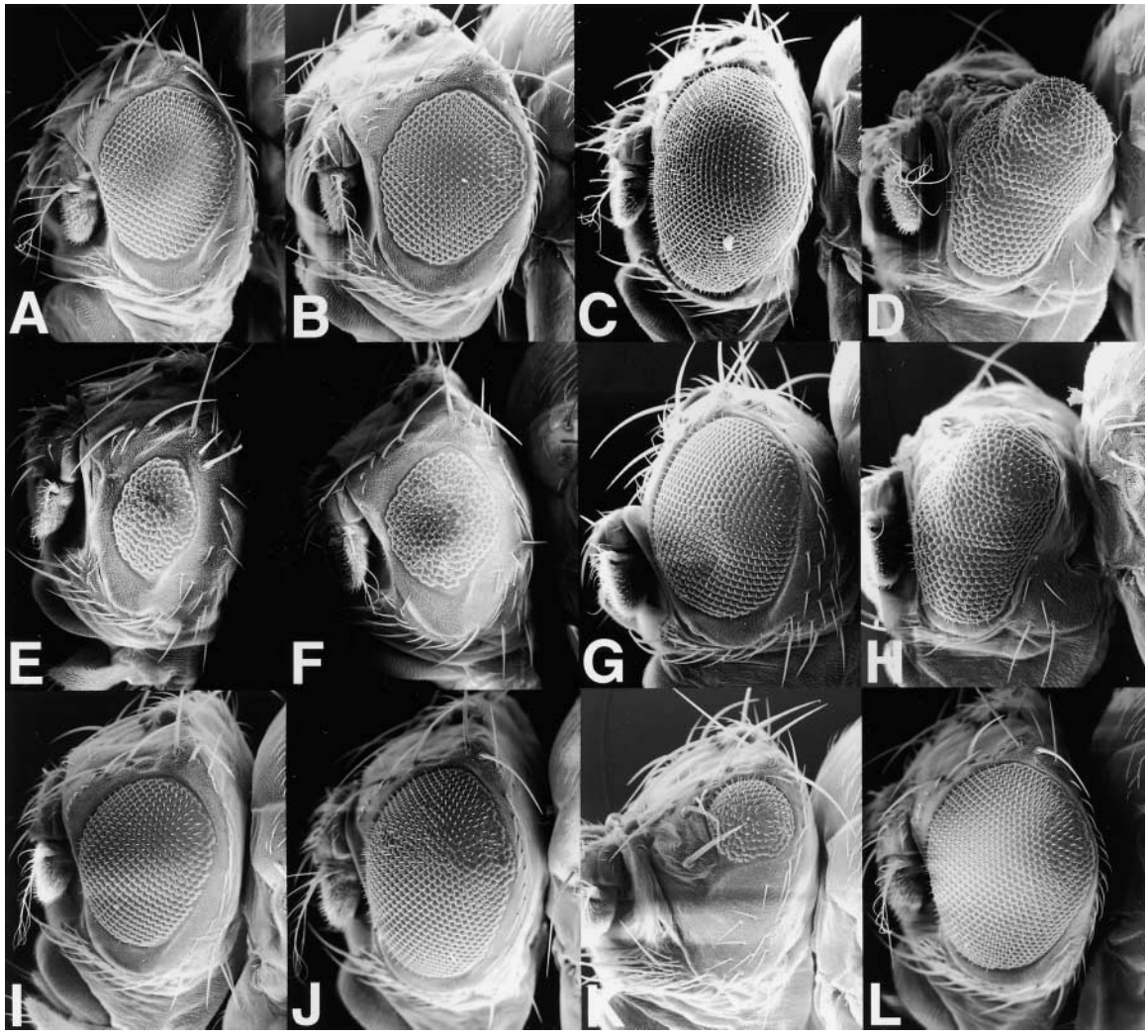


FIGURE 2.—The *upd* small eye is rescued by ectopic expression of Upd. Genotypes: *w os/y w os1A* (A); *w os/y w os1A; ey-Gal4/UAS-GFP* (B); *w os/y w os1A; ey-Gal4/UAS-upd* (C); *ey-Gal4/UAS-upd* (D); *w os/y w os1A; ey-Gal4/UAS-dome* (E); *ey-Gal4/UAS-dome* (F); *w os/y w os1A; ey-Gal4/UAS-hop* (G); *ey-Gal4/UAS-hop* (H); *w os/y w os1A; ey-Gal4/UAS-stat92E* (I); *ey-Gal4/UAS-stat92E* (J); *w os/y w os1A; ey-Gal4/UAS-SOCS36* (K); and *ey-Gal4/UAS-SOCS36* (L). This small-eye phenotype associated with *w os/y w os1A* (A) can be rescued by ectopic misexpression of *upd* (C) to the developing eye disc but not by ectopic misexpression of GFP (B). The small eye is partially rescued by ectopic misexpression of Hop (G) but not of Stat92E (I). The small eye is exacerbated by ectopic misexpression of Dome (E) and Socs36 (K) . Ectopic misexpression of Upd (D) and Hop (H) in wild type using the ey-Gal4 II driver results in enlarged eyes, while Dome (F) generated a small eye and Stat92E (J) and Socs36 (L) had no effect. All crosses were performed at 25°, except B, which was done at 16° and H, which was performed at 20°. In A–L, anterior is to the left and posterior to the right; dorsal is up and ventral is down. Scanning electron micrographs were taken at 100×.

nificant dorsal outgrowths in GMR-upd19 and GMR-upd28 (Figure 3, B and C, respectively). We predicted that reduction in the dose of *stat92E* would modify (*i.e.*, suppress) the GMR-upd phenotype. When we reduce by 50% the dose of *stat92E*, using the hypomorphic alleles $stat92E^{06346}$ or $stat92E^{fC68}$, there is a dramatic suppression of the enlarged-eye phenotype in both GMR-upd19 and GMR-upd28 (Figure 3, D and F, and data not shown). In addition, when we reduce the dose of *glass*, which drives the GMR promoter, using the viable $glass^{3}$ allele, we also suppress the phenotype (Figure 3E and data not shown). We reduced the dose of *hop, dome,* and *upd* to assess if this would modify the enlarged-eye phenotype. The GMR-upd phenotype is moderately suppressed when we remove a copy of *hop*, using the null allele hop^{C111}, or *dome*, using the hypomorphic alleles $dome^{217}$ or $dome^{A68}$, although not to the same extent as when the dose of *stat92E* is reduced (Figure 3, G and H, respectively, and data not shown). However, a weak allele of *hop*, hop^{msv1}, does not modify the phenotype (data not shown). Reduction in the dose of *upd*, using the null allele upd^{yc43}, the strong hypomorph upd^{ym55}, or the *os1A* deficiency, does not modify the phenotype (data not shown). This is presumably because Upd is so highly expressed in GMR-upd that a reduction in the amount of endogenous *upd* does not modify the

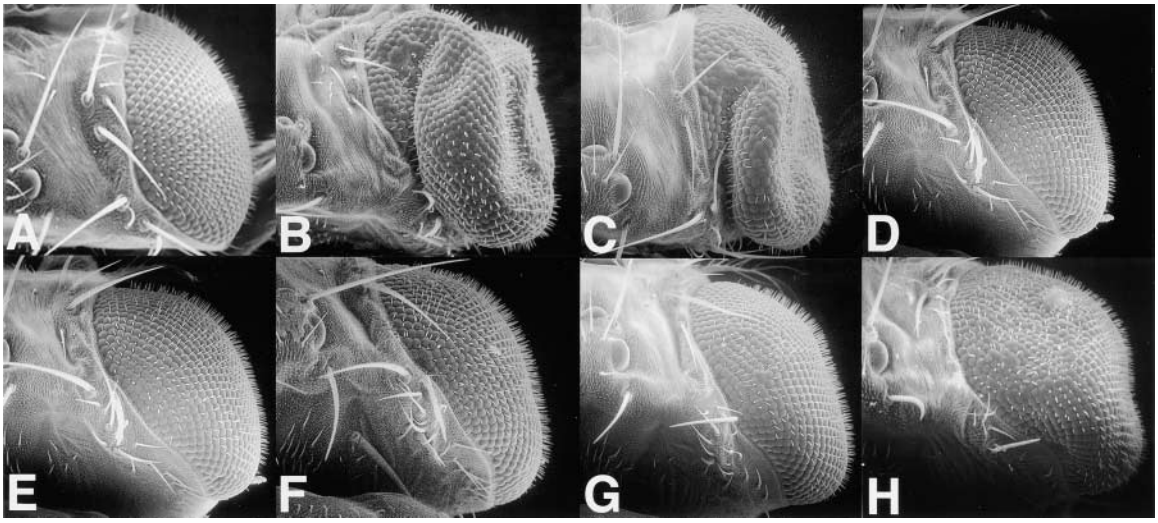


Figure 3.—GMR-upd is a sensitized genetic background. Genotypes: WT (A); *GMR-upd19/+* (B); *GMR-upd28/+* (C); *GMR-upd19/+; stat92E^{06346}/+* (D); *GMR-upd19/+; glass^{3}/+* (E); *GMR-upd28/stat92E^{06346}* (F); *hop^{C111}/+; GMR-upd28/+* (G); and *dome^{217}/+; GMR-upd28/+* (H). One copy of the GMR-upd transgene inserted on the first chromosome *GMR-upd19* (B) or on the third *GMR-upd28* (C) results in an enlarged eye. Removal of one copy of *stat92E* (D and F) or *glass* (E) suppresses the enlarged-eye phenotype. Removal of one copy of *hop* (G) or *dome* (H) moderately suppresses the enlarged-eye phenotype. Scanning electron micrographs, dorsal view, taken at ×200.

phenotype. Therefore, the GMR-upd phenotype is specific to activation of the JAK/STAT pathway in the developing eye.

Characterization of GMR-upd transgenic line: In wild-type eye discs, *upd* is expressed in first and second instar at the posterior margin (Figure 4A). By third instar, endogenous *upd* expression has largely disappeared, and the observed staining in the furrow indicates macrophages (Figure 4B). In contrast, in third instar eye discs from GMR-upd19 animals, *upd* is expressed in all cells posterior to the morphogenetic furrow (Figure 4C). Importantly, third instar GMR-upd eye discs are larger than those of wild type (compare Figure 4C with 4B). However, first and second instar eye discs from GMR-upd are the same size as wild type (data not shown). These data demonstrate that the overgrowth observed in GMR-upd begins in third instar. Interestingly, *dome* is strikingly upregulated in cells anterior to the furrow

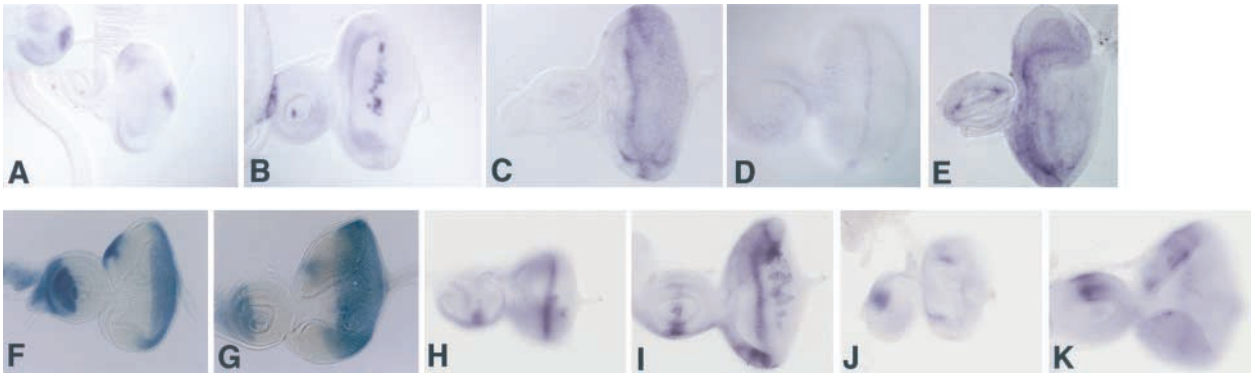


Figure 4.—Expression of *upd*, *dome*, *hh*, *dpp*, *wg* in WT and GMR-upd eye discs. Expression patterns of *upd* (A–C), *dome* (D and E), *dpp* (H and I), and *wg* (J and K) were examined by *in situ* hybridization using RNA probes. *hh* (F and G) expression was monitored by X-gal staining using an enhancer trap *hh^{P30}* (*hh-LacZ*). WT discs (A, B, D, F, H, J) and GMR-upd19 discs (C, E, G, I, K). In WT larvae, *upd* is expressed at the posterior margin in first instar (disc, top left) and second instar eye discs (disc, center) (A), but not highly expressed in third instar eye discs (B). In third instar GMR-upd eye discs, *upd* is expressed in all cells posterior to the morphogenetic furrow (C). Dome expression is barely detectable in WT third instar eye discs (D), but is greatly upregulated in all cells anterior to the morphogenetic furrow in GMR-upd (E). We observed normal expression of *hh* in both WT (F) and GMR-upd (G). *dpp* is expressed in cells of the furrow in WT third instar eye discs (H), and its expression is slightly enhanced in GMR-upd (I). *wg* is expressed at the lateral margins in WT third instar eye discs (J) and is still expressed there in GMR-upd (K); however, the staining pattern is slightly enhanced. Note that in A–K, GMR-upd third instar discs are larger than WT. The positive staining observed posteriorly in B and I indicates macrophages. In A–K, anterior is to the left and posterior to the right; dorsal is up and ventral is down.

in third instar GMR-upd discs (Figure 4E). In wild-type third instar eye discs, *dome* expression is not observed or is barely detectable (Figure 4D). These data suggest that *dome* is a target of the JAK/STAT pathway in the eye.

Secreted factors Hedgehog (Hh), Decapentaplegic (Dpp), and Wingless (Wg) have been shown to induce proper morphogenesis and to influence proliferation in the eye-imaginal disc (Heberlein and Treisman 2000). Therefore, we wanted to investigate whether these molecules are expressed normally in third instar GMR-upd discs. In wild-type eye discs, Hh is produced by differentiated photoreceptors posterior to the furrow (Heberlein *et al.* 1993; Ma *et al.* 1993). We analyzed *hh* expression using an enhancer trap (hh^{P30}) and found that its expression in differentiating photoreceptors is normal in both wild-type and GMR-upd discs (Figure 4, F and G). In third instar, *dpp* is expressed in the cells of the furrow (Heberlein *et al.* 1993; Ma *et al.* 1993; Heberlein and Treisman 2000). *dpp* is expressed at the correct place in GMR-upd but at slightly elevated levels compared to wild type (Figure 4, H and I; data not shown). The observed staining in the posterior part of GMR-upd disc is not *dpp* but rather macrophages (Figure 4I). In wild-type third instar eye disc, *wg* is expressed at the dorsal and ventral margins (Heberlein and Treisman 2000). In both wild-type and GMR-upd discs, *wg* is expressed in its normal pattern. However, there appears to be more *wg* in the GMR-upd discs compared to wild type (Figure 4, J and K). The increased *dpp* and *wg* expression may be the by-product of a greater number of cells in GMR-upd discs. However, our previous work has shown that *upd* does not regulate *wg* expression and vice versa (Zeidler *et al.* 1999).

GMR-upd eyes have more cells due to increased mitoses: We reasoned that the increased size of GMR-upd eyes could be due to an increase in cell number. This is supported by the observation that *GMR-Gal4, UAS-upd/+* animals exhibit more facets than wild type exhibit (Chen *et al.* 2002). In addition, we stained third instar eye discs from wild-type and GMR-upd animals with an antibody to Elav to mark neuronal cell fate and with phalloidin to mark filamentous actin. GMR-upd discs have more Elav-positive clusters than wild type (compare Figure 5, A and B). These data support the hypothesis of an increase in cell number in GMR-upd discs.

The increased numbers of cells in GMR-upd discs could arise from a decrease in apoptosis or an increase in cell division. To investigate the former, we removed one copy each of *hid*, *reaper*, and *grim* using the H99 deficiency (White *et al.* 1994). If Upd prevents apoptosis, then removal of these apoptotic genes should result in an enhancement of the GMR-upd phenotype. However, we observed no modification of the GMR-upd phenotype when the dose of *hid*, *reaper*, and *grim* is reduced by 50% (data not shown). Similarly, when we ectopically misexpressed the baculovirus p35 or the caspase inhibitor DIAP1, using GMR-p35 or GMR-DIAP1, the GMR-upd phenotype was not modified (Davidson and Steller 1998; Goyal *et al.* 2000; data not shown). These data suggest that a reduction in apoptosis does not account for the enlarged-eye phenotype.

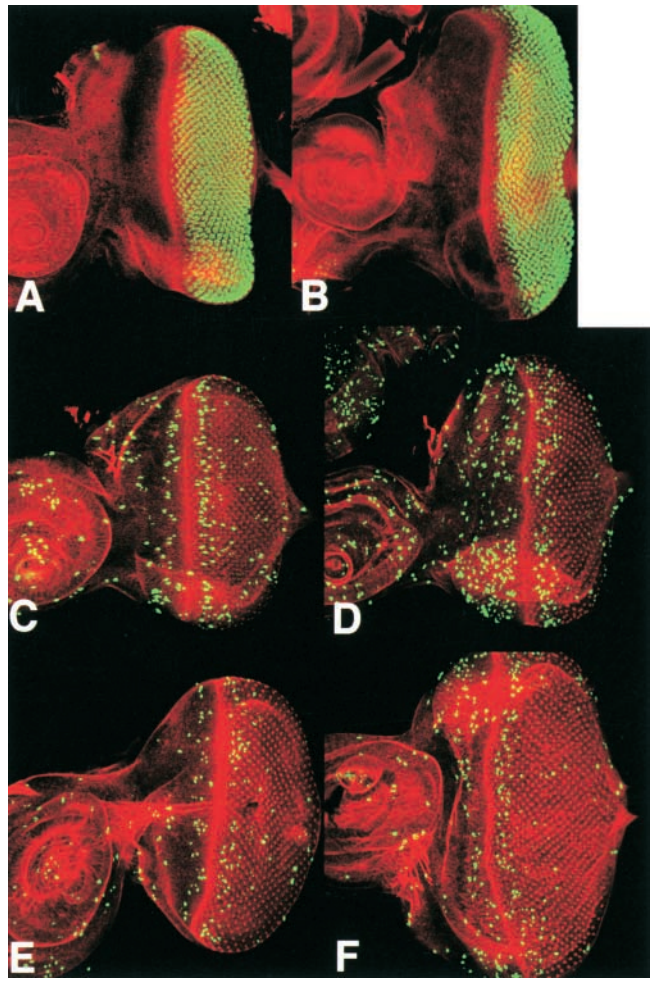


Figure 5.—GMR-upd eye discs have more cells than WT (A and B). Third instar eye-antennal discs were stained with an antibody to Elav (in green), which marks photoreceptors, and rhodamine-conjugated phalloidin (in red), which marks filamentous actin and hence the morphogenetic furrow. There are more Elav-positive clusters in GMR-upd (B) compared to WT (A). (C–F) Third instar eye-antennal discs were stained with an antibody to PH3 (in green), which marks cells in mitosis, and with rhodamine-conjugated phalloidin (in red) at 96 hr (C and D) and at 110 hr (E and F) AED. (C–F) Misexpression of Upd does not lead to extra rounds of cell divisions in the second mitotic wave, *i.e.*, posterior to the furrow. However, GMR-upd discs (D) contain more mitotic cells in the region anterior to the furrow compared to WT (C). Older GMR-upd discs (F) are substantially larger than WT (E). Images of third instar eye-antennal discs taken on a confocal microscope at ×20 magnification of WT (A, C, and E) and GMR-upd19/+ (B, D, and F) discs. In A–F, anterior is to the left and posterior to the right; dorsal is up and ventral is down.

We next investigated whether the enlarged-eye phenotype could be due to increased mitoses induced by Upd. In eye-imaginal disc development, there are two

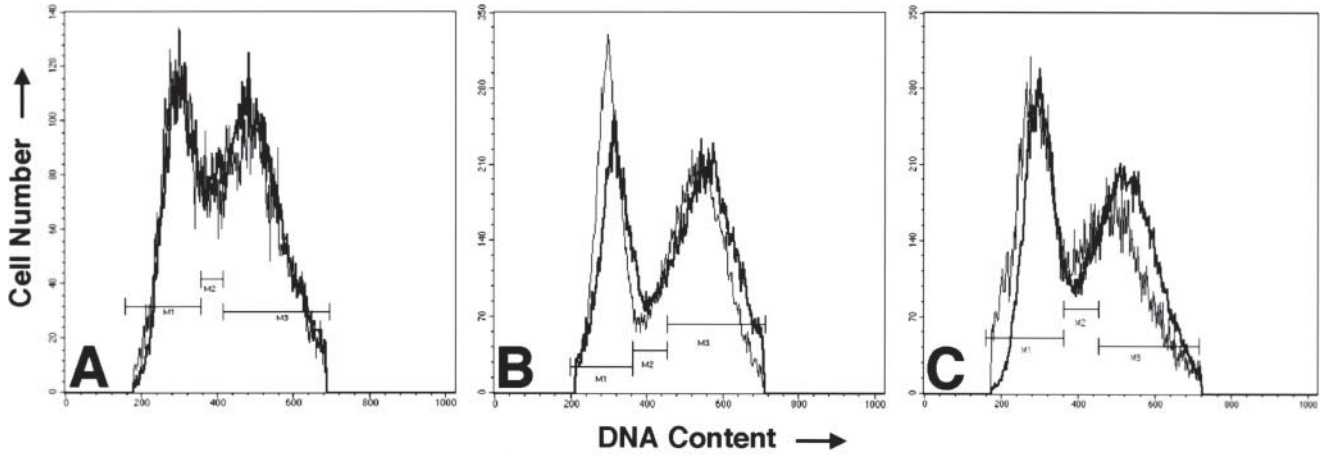
waves of mitosis (Wolff and Ready 1993). In the first mitotic wave, cells anterior to the morphogenetic furrow undergo asynchronous rounds of cell division. In the second mitotic wave, cells immediately posterior to the furrow undergo one more round of mitosis as they adopt specific cell fates. To investigate if the first or second wave of mitosis in the eye was affected by ectopic expression of *upd*, we stained GMR-upd or wild-type third instar eye discs with an antibody to phospho-histone 3 (PH3), which marks cells in mitosis. We examined PH3 expression at 96 and 110 hr after egg deposition (AED), which under our culture conditions corresponds roughly to middle and late third instar as assessed by the position of the furrow. At both time points, wild-type and GMR-upd discs had similar numbers of mitotic cells posterior to the furrow (Figure 5, C–F). These data indicate that the second mitotic wave is not affected by ectopic misexpression of *upd* to the developing eye. However, at 96 hr AED, there are more total cells in GMR-upd discs than in wild-type discs, and, importantly, there are more mitotic cells anterior to the furrow in GMR-upd discs compared to wild type (data not shown and Figure 5D). Thus, there are more undifferentiated cells to be patterned by the morphogenetic furrow in GMR-upd eye discs. At 110 hr AED, GMR-upd discs contain two to four times more cells and have more PH3-positive than wild-type cells (Figure 5F). These data suggest that in GMR-upd eye discs, Upd produced by cells posterior to the furrow can diffuse away from its production site and induce proliferation in the Dome-expressing, unpatterned cells anterior to the furrow.

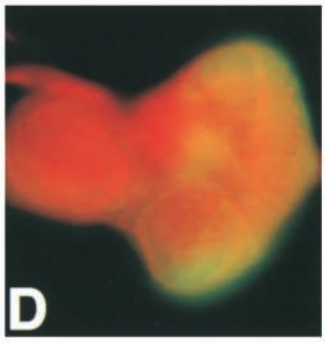
We performed cell-cycle analysis by flow cytometry on live eye-imaginal disc cells (Neufeld *et al.* 1998). We expressed *upd* in the developing eye disc using an ey-Gal4, UAS-GFP recombinant that we made. In this line, cells posterior to the furrow are strongly GFP-positive cells, and cells anterior to the furrow, which correspond to the more mitotic population mentioned above, are largely GFP negative and are referred to as GFP^{lo} (Figure 6D; Halder *et al.* 1995; Hauck *et al.* 1999). Thus, in discs from *ey-Gal4, UAS-GFP/UAS-upd* animals, the GFP-positive cells posterior to the furrow produce Upd, and we assume that Upd induces proliferation of the GFP^{lo}, Dome-expressing cells anterior to the furrow. We examined the cell-cycle distribution at 90, 96, and 110 hr AED. At 90 hr AED, histograms of GFP^{lo} cells showed similar cell-cycle distribution in *ey-Gal4, UAS-GFP/+* and *ey-Gal4, UAS-GFP/UAS-upd* (Figure 6, A and E). At 90 hr AED, there are similar numbers of total eye disc cells in both genotypes (data not shown). At 96 hr AED, cell-cycle profiles of GFP^{lo} cells still appear similar between the two genotypes; however, there is a reproducible increase in the number of cells in G_2/M in *ey-Gal4, UAS-GFP/UAS-upd* compared to *ey-Gal4, UAS-GFP/+*: 50 *vs.* 55%, respectively (Figure 6, B and E). By 110 hr AED, GFP^{lo} cells from *ey-Gal4, UAS-GFP/UAS-upd* eye discs have more cells in G_2/M than do those from *ey-Gal4, UAS-GFP/+*: 46 *vs.* 34%, respectively (Figure 6, C and E). Therefore, we conclude that Upd increases the number of cycling cells in the eye disc.

GMR-upd larval eye discs and adult eyes are patterned normally: When cells "exit" the morphogenetic furrow in wild-type third instar larvae, they receive specific signals to assume cell fates and positions within the ommatidia (Wolff and Ready 1993). The differentiating photoreceptors rotate 90° toward the equator, and eventually the dorsal and ventral halves of the eye form mirror images relative to the equator (Figure 7C). We used the position of the R7 cell, which expresses both Propero and Elav, within the ommatidium to assay ommatidial rotation. In wild-type and GMR-upd genotypes, the yellow R7 cell is in its expected position within the ommatidium, indicating normal rotation (Figure 7, A and B). We also examined adult sections to look at ommatidial rotation and photoreceptor differentiation. In wild-type discs, we observed the expected complement of photoreceptors and normal rotation of ommatidial clusters toward the equator (Figure 7, D and F). In GMR-upd adult sections, photoreceptor differentiation appears to be normal, although occasionally we observed the loss or gain of a photoreceptor within an ommatidium (Figure 7E). However, we did not observe a consistent loss or gain of any particular photoreceptor or support cell after analyzing eye sections of several GMR-upd animals (data not shown). We did observe abnormal ommatidial rotation in both dorsal and ventral halves of the adult eye in GMR-upd (Figure 7G), which is consistent with a previously observed role of the JAK/STAT pathway in ommatidial rotation (Luo *et al.* 1999; Zeidler *et al.* 1999). In addition, the adult sections have allowed us to examine the contribution, if any, of changes in cell volume to the GMR-upd phenotype. We observed no increase in cell volume of photoreceptors or their support cells in eyes from GMR-upd animals (Figure 7E). In fact, there appears to be a slight decrease in their cell volume compared to wild type, perhaps due to competition among cells for nutrients and space.

Taken together, these data indicate that Upd acts as a growth factor in the developing Drosophila eye. Loss-of-function mutations in *upd* are associated with a small eye. Misexpression of *upd* to the developing eye results in a greatly enlarged eye-imaginal disc and compound eye. The enlargement is a result of an increase in the number of cells within the eye and not an increase in their volume. Moreover, although there are more cells in GMR-upd eyes, these cells appear to be patterned normally.

A deficiency screen to identify dominant modifiers of GMR-upd: To determine how many loci in the Drosophila genome contain modifiers of the GMR-upd phenotype, we used a set of deficiency stocks from the Bloomington Stock Center that contain overlapping deletions in the Drosophila genome and crossed them to GMR-upd. Although initially we used the GMR-upd28/TM3, Sb line for our screen, the majority of the screen





E

Hours AED	Genotype	G1%	S%	G2/M%
90	wt	40	21	40
	ey-upd	37	23	39
96	wt	39	11	50
	ey-upd	32	13	55
110	wt	49	17	34
	ey-upd	38	15	46

FIGURE 6.—Ectopic misexpression of Upd leads to more cells in G_2/M. Cell-cycle analysis by FACS on live GFP^{lo} eye-imaginal disc cells from WT (*ey-Gal4, UAS-GFP/+*; thin line) or ey-upd (*ey-Gal4, UAS-GFP/UAS-upd*; thick line). (A) At 90 hr the cell-cycle profile and total number of cells are roughly the same in WT and ey-upd. (B and E) At 96 hr AED eye discs from ey-upd have more cells (1.5-fold more) but no distinct increase in a particular portion of the cell-cycle profile in GFP^{lo} cells. However, there is a small but reproducible increase in the number of cells in G_2/M in ey-upd discs compared to WT. (C and E) At 110 hr AED, ey-upd discs have more GFP^{lo} cells in G_2/M and have 4-fold more cells than WT (E). All FACS profiles contained at least 20,000 events. M_1 represents cells in G_1 phase, M_2 in S, and M_3 in G_2/M. (D) eyGal4, UAS-GFP early third instar eye disc stained with phalloidin (red). GFP is strongly expressed in cells posterior to the furrow and faintly and in a fading pattern in cells anterior to the furrow. (E) Numeric representation of FACS profiles, percentage of GFP^{lo} cells in G_1, S, and G_2/M from WT and ey-upd discs at the indicated time AED. These data were obtained from experiments repeated three independent times with similar results. In D, anterior is to the left and posterior to the right; dorsal is up and ventral is down.

was conducted using the GMR-upd19/FM7. GMR-upd 19/Y are observed at a low frequency, and they are sterile as they are defective in the proper development/morphogenesis of the male reproductive tract, preventing release of motile sperm (E. A. BACH and A. A. KIGER, unpublished observations). Because we used the GMR-upd19/FM7 line for most of this study, we have screened only those deficiencies on the X chromosome that are covered by a duplication on the Y (*e.g.*, Df/DpY). To date, we have tested 166 deficiencies that together uncover 60% of the genome, almost all of the euchromatin on the autosomes, and a small portion of that on the X. We have identified 20 regions that suppress and 9 regions that enhance the GMR-upd phenotype (Tables 2 and 3). We have also identified 21 regions that, when heterozygous in the GMR-upd background, result in lethality (synthetic lethals) prior to adult stages (data not shown). Importantly, the deficiency Df(3R)H-B79 (92B3; 92F13) that uncovers *stat92E* (92E11-12) behaved as a suppressor of GMR-upd, thus validating the screen (Table 2). One prediction from these results is that reduction in the genetic dose of the negative regulators *DPIAS* or SOCS would enhance the GMR-upd phenotype. However, a DPIAS allele $Su(var)2\text{-}10^{03697}$ does not interact in our screen and there are no mutations in SOCS genes (HARI *et al.* 2001; data not shown). There may be buffering of the GMR-upd phenotype at the level of feedback loops, and thus it is possible that a 50% reduction in the dose of *DPIAS* does not modify the enlarged-eye phenotype.

Testing candidate genes: We tested mutations of several genes uncovered by deficiencies that control growth or survival in the imaginal eye, including *ras85D*, *epidermal growth factor receptor*, *raf*, *corkscrew*, *chico*, *Pten*, *Insulin Receptor* (*InR*), *frizzled*, *wg*, *Toll*, and *spaeztle*. However, mutations in these genes did not modify the GMR-upd phenotype (Table 4).

We then tested whether other genes uncovered by the interacting deficiencies could modify the GMR-upd phenotype. To date, we have tested >500 mutations that

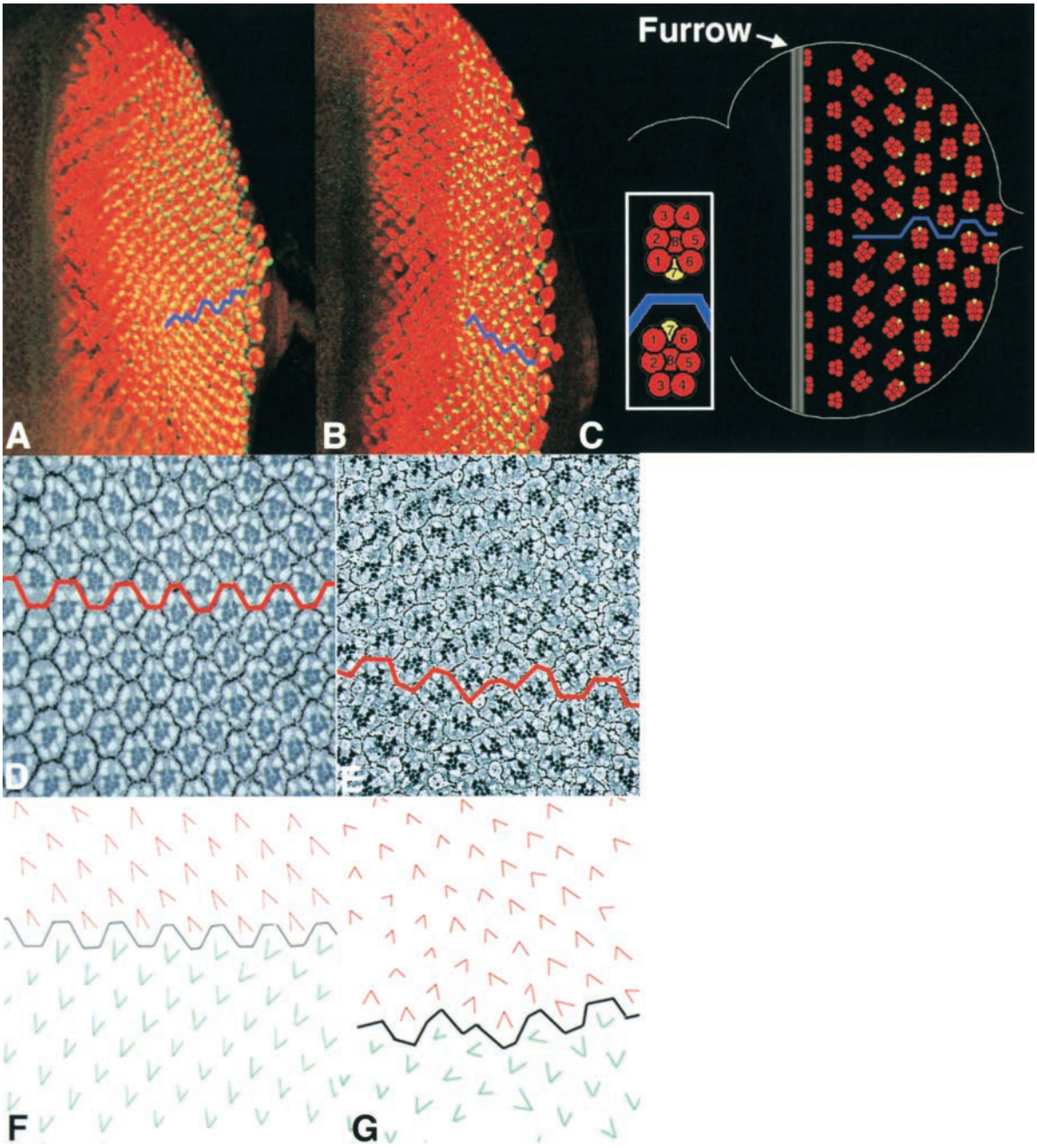


Figure 7.—Larval discs and adult eyes in GMR-upd animals are patterned normally. Positioning of the R7 photoreceptor in GMR-upd19 third instar eye discs occurs normally. WT (A) and GMR-upd (B) third instar eye discs were stained with antibodies to Prospero in green and Elav in red. The cells in yellow are R7 cells and cone cells and the equator has been marked manually in blue. In WT (A) and GMR-upd (B), rotation of the R7 cells occurs normally. (C) Schematic representation of larval ommatidial rotation. Sections of adult WT (D) and GMR-upd (E) animals reveal that misexpression of Upd in the developing eye does not perturb photoreceptor and secondary cell fates. Importantly, cell volume is not increased in GMR-upd (E) compared to WT (D). However, ommatidial rotation is abnormal in GMR-upd compared to wild type, which is best assessed in the schematics of the WT (F) and GMR-upd (G) adult sections. Dorsal ommatidia are represented by red and ventral by green in E and F. The equator is red in D and E and black in F and G.

map to the interacting deficiencies. *Df(1)64c18* (2E1-2; 3C2) uncovers *l(1)3Ag*, a mutation in *zeste-white 13* (*zw13*), which also strongly suppressed GMR-upd (Table 2). *Tp(3;Y)ry*^{506}*-85C* (87D1-2; 88E5-6; Y) acts as an enhancer in the screen and uncovers the *C-terminal Binding Protein* (*CtBP*) gene, which encodes a transcriptional corepressor. We tested two hypomorphic mutations in *CtBP*, one from the Bloomington Stock Center, *CtBP*^{03463}, and the other identified in a screen for epithelial morphogenesis that will be described elsewhere (M. Schober and N. Perrimon, unpublished observations). Interestingly, both mutations enhance the GMR-upd phenotype.

TABLE 2

A deficiency screen to identify suppressors of GMR-upd

Deficiency	Cytology	Interaction	Strength	Candidate gene	Interaction	Strength
Df(1)64c18	2E1-2; 3C2	Su	4	*zeste-white* 13	Su	4
Df(1)BK10	16A2; 16C7-10	Su	2			
Df(2L)cl-h3	25D2-4; 26B2-5	Su	2	*thickveins*	Su	2
Df(2L)J2	31B; 32A	Su	3	*pineapple eye*	Su	2
Df(2L)r10	35E1-2; 36A6-7	Su	2			
Df(2L)TW1	38A7-B1; 39C2-3	Su	2			
Df(2R)cn9	42E; 44C	Su	1	*saxophone*	Su	1
Df(2R)Pcl11B	54F6-55A1; 55C1-3	Su	2			
Df(2R)Egfr5	57D2-8; 58D1	Su	2	*plexus*	Su	2
Df(3L)66C-G28	66B8-9; 66C9-10	Su	2			
Df(3L)vin2	67F2-3; 68D6	Su	3			
Df(3L)vin5	68A2-3; 69A1	Su	3			
Df(3L)fz-M21	70D2-3; 71E4-5	Su	3	*Dichaete*	Su	2
Df(3L)W10	75A6-7; 75C1-2	Su	3			
Df(3L)kto2	76B1-2; 76D5	Su	3	*kohtalo*	Su	3
Df(3L)Pc-MK	78A2; 78C9	Su	2			
Df(3L)Ten-m-AL29	79C1-3; 79E3-8	Su	2			
Df(3R)p712	84D4-6; 85B6	Su	3	*ras85D*	NE	
Df(3R)H-B79	92B3; 92F13	Su	2	*stat92E*	Su	4
Df(3R)23D1	94A3-4; 94D1-4	Su	2	*hedgehog*	Su	2
Df(3R)crb-F89-4	95D7-D11; 95F15	Su	3	*crumbs*	Su	2
Df(3R)crb87-5	095F7; 96A17-18	Su	2	*crumbs*	Su	2

The deficiency kit was crossed to the GMR-upd19 and GMR-upd28 lines and eye phenotype in the progeny (Df/GMR-upd) was scored. We identified 20 regions that suppress the GMR-upd phenotype. We identified 10 genes within these deficiencies that similarly modified the enlarged-eye phenotype. The interactions were classified under the "modifier" category as suppressor (Su) and also by strength, in ascending order, with 4 indicating a strong suppression and 1 a mild suppression. Suppression obtained by removing a copy of *stat92E* and was assigned a score of 4. NE, no effect.

Df(2L)J2 (31B-32A) acts as a suppressor in our screen and uncovers the *pineapple eye* (*pie*). A viable allele, pie^{EB3}, also suppresses the GMR-upd phenotype (Table 3). *Df(3L)fz-M21* (70D2-3; 71E4-5) acts as a suppressor of GMR-upd and uncovers *Dichaete* (*D*), also called *fish hook* (*fish*; Table 2). Hypomorphic mutations in *D*, $fish^{87}$, and $fish^{96}$ suppress the GMR-upd phenotype. In addition, D^1, a dominant mutation, enhances the phenotype (Table 2).

In the course of trying to identify the gene(s) responsible for the enhancer activity of *Df(3R)Tl-P* (97A; 98A1-2), we identified a mutation, $His2Av^{05146}$, in the *Histone 2A variant* gene at 97D2 that suppresses the enlarged-eye phenotype (Tables 2 and 3). Therefore, we assume that *Df(3R)Tl-P* contains both an enhancer and suppressor of GMR-upd. We also identified a novel *P*-element insertion $l(3)B4\text{-}3\text{-}20^1$ that suppressed GMR-upd. Inverse PCR showed that this *P*-element was inserted in the *headcase* (*hdc*) gene at 99E. *hdc* is a nuclear factor required for imaginal cell development, and its expression is regulated by the transcription factor *escargot* (*esg*; Steneberg *et al.* 1998). Interestingly, an *esg* allele, esg^{k00606}, also suppressed GMR-upd (data not shown). *Df(3L)kto2* (76B1-2; 76D5) acts as a suppressor in the screen and uncovers the *kohtalo* (*kto*) gene. A hypomorphic mutation, kto^1, acts as suppressor in the screen (Table 2). *Df(2R)Egfr5* (57D2-8; 58D1) suppresses the GMR-upd phenotype, and we identified two hypomorphic mutations in *plexus* (*px*), px^1 and px^{k08613}, which strongly suppressed the GMR-upd phenotype (Table 2). *Df(3R)crb-F89-4* and *Df(3R)crb87-5* act as suppressors in the screen and uncover 95D7-D11; 95F7 and the *crumbs* (*crb*) gene (Table 2). Mutations in *crb*, crb^1, and crb^{j1B5} act as suppressors of the GMR-upd phenotype (Table 2).

Dpp pathway genes modulate GMR-upd: *Df(2L)cl-h3* (25D2-4; 26B2-5) and *Df(2R)cn9* (42E; 44C) suppress the GMR-upd phenotype and uncover type I Dpp receptors *thickveins* (*tkv*) and *saxophone* (*sax*; Table 2) (Brummel *et al.* 1994). Notably, hypomorphic *tkv* (tkv^{k16713}, tkv^1, tkv^{04535a}) and *sax* (sax^1, sax^2, sax^4) alleles also suppressed the GMR-upd phenotype (Figure 8, F and G, and Table 5). Given these data, we tested other alleles in *dpp* pathway genes. Seven hypomorphic *dpp* alleles suppressed the enlarged-eye phenotype, as did a hypomorphic mutation in a type II Dpp receptor *punt* (*put*), put^{135} (Figure 8E; Table 5; Letsou *et al.* 1995; data not shown). Importantly, a null mutation in *Mothers against dpp* (*Mad*), Mad^{k00237}, the Co-Smad in Drosophila that transduces *dpp* signals, strongly suppresses the enlarged-eye phenotype to the level observed with *stat92E* (Figure 8D; Table 5; Wiersdorff *et al.* 1996). However, *Df(2L)JS17* (23C1-2; 23E1-2), which removes the *Mad* gene, did not interact in our screen and may also contain an enhancer. Impor-

TABLE 3

A deficiency screen to identify enhancers of GMR-upd

Deficiency	Cytology	Interaction	Strength	Candidate gene	Interaction	Strength
Df(2L)sc19-4	25A5; 25E5	En	2			
Df(2L)J-H	27C2-9; 28B3-4	En	1	*wingless*	NE	
Df(2L)spd	27D-E; 28C	En	2			
Df(2R)X1	46C; 47A1	En	1			
Df(2R)en-B	47E3; 48A	En	1			
Df(2R)Chi	60A3-7; 60B4-7	En	1			
Tp(3;Y)ry506-85C	87D1-2; 88E5-6;Y	En	1	*C-terminal binding protein*	En	1
Df(3R)DG2	89E1-F4; 91B1-B2	En	2	*Daughters against dpp*	En	1
Df(3R)Tl-P	97A; 98A1-2	En	1	*Toll, spaetzle*	NE, NE	
Df(3R)Tl-P	97A; 98A1-2	En	1	*Histone 2A variant*	Su	2

The deficiency kit was crossed to the GMR-upd19 and GMR-upd28 lines and eye phenotype in the progeny (Df/GMR-upd) was scored. We identified nine regions that enhance the GMR-upd phenotype. We identified two genes uncovered by these interacting deficiencies that also modified the enlarged-eye phenotype. The interactions were classified under the "modifier" category as enhancer (En) and also by strength, in ascending order, with 2 indicating a strong enhancement and 1 a mild enhancement.

tantly, another interacting deficiency, *Df(3R)DG2* (89E1-F4; 91B1-B2), acts as an enhancer in our screen and uncovers the *Daughters against dpp* (*Dad*) gene (Tsuneizumi *et al.* 1997). Dad is a negative regulatory SMAD in Dpp signal transduction, and mutations in *Dad* should enhance the GMR-upd phenotype (Table 2). As expected, a hypomorphic allele of *Dad*, Dad^{1}, enhanced the enlarged-eye phenotype (Figure 8H and Table 5). Since Hh induces *dpp* expression in third instar eye discs, it was interesting to observe that *Df(3R)23D1* (93F; 94F), which uncovers *hh*, acts as a suppressor in the screen (Table 2). Hypomorphic alleles of *hh*, hh^{IJ35}, and hh^{G31} moderately suppressed the GMR-upd phenotype (Table 2). We also noted that *Df(2R)en-B* (47E3; 48A) enhances the GMR-upd phenotype and uncovers the *en* gene (Table 3). However, an overlapping deficiency *Df(2R)en-A* (47D3; 48B2) that also removes the *en* gene does not modify the GMR-upd phenotype (data not shown). Therefore, we assume that the enhancer uncovered by *Df(2R)en-B* is not *en*.

These data raise the possibility that Upd induces the *hh* gene. We tested this hypothesis directly by making flip-out clones of UAS-upd in a *hh-lacZ* genetic background. Ectopic expression of *upd* did not induce *hh* in any region of the eye disc or in the wing disc (supplemental Figure 1 available at http://www.genetics.org/supplemental/; data not shown). These data indicate that *hh* is not a direct target of the JAK/STAT pathway.

The GMR-upd modifiers do not alter Glass-mediated phenotypes: We performed a secondary screen to determine whether the modifiers of *GMR-upd* also affected Glass-mediated transcription (supplemental Figure 2 available at http://www.genetics.org/supplemental/). *GMR-hid 1M/+* flies have a small eye that is two-thirds the size of wild type and is rough and glassy in the posterior half (supplemental Figure 2A available at http://www.genetics.org/supplemental/). This phenotype is strongly suppressed by reduction in the dose of *glass* (supplemental Figure 2B available at http://www.genetics.org/supplemental/). Importantly, neither *stat92E* allele modified *GMR-hid* (supplemental Figure 2, C and D, available at http://www.genetics.org/supplemental/). Moreover, none of the enhancers and suppressors of GMR-upd behaved in a similar manner with *GMR-hid*. For example, *mad* strongly suppresses GMR-upd; however, it did not modify *GMR-hid*. In addition, *fish* alleles, which both suppress *GMR-upd*, actually enhance *GMR-hid* (supplemental Figure 2 available at http://www.genetics.org/supplemental/). The same results were obtained using another Glass-dependent eye phenotype (*i.e.*, GMR-Gal4). Taken together, these data indicate that the modifiers identified in our screen are likely to modify JAK/STAT-dependent phenotypes rather than Glass-dependent ones.

Ectopic expression of Dpp does not rescue the *upd* small-eye phenotype: We observed a consistent genetic interaction between GMR-upd and *dpp* pathway genes. Since *dpp* is slightly increased in GMR-upd discs (Figure 4I), we reasoned that Upd may directly induce expression of *dpp*. We found one consensus optimal Stat92E binding site in the *dpp* locus; however, the functional significance of this site is unknown (Yan *et al.* 1996; data not shown). We attempted to rescue the *os/os1A* small-eye phenotype by ectopically misexpressing *dpp* using UAS-dpp, an activated form of its receptor *tkv* using UAS-tkv^{QD}, or activated *hh* using UAS-hh-N driven by ey-Gal4 (Wiersdorff *et al.* 1996). Ectopic misexpression of *dpp* or tkv^{QD} resulted in more eye tissue in *os/os1A* when compared to GFP. However, neither rescued to the extent observed with UAS-upd or UAS-hop (compare Figure 8L with Figure 2, C or G, and data not shown). In contrast, UAS-hh-N resulted in a smaller eye

TABLE 4

Mutations that do not modify the GMR-upd phenotype

Gene	Allele	Uncovered by	Cytology of gene	Gene function
ras85D	ras^{C40B}	*Df(3R)p712*	(84D4-6; 85B6)	Serine/threonine kinase
epidermal growth factor receptor (egfr)	flb^{CO}	*Df(2R)Egfr5*	(57DD2-8; 58D1)	Receptor tyrosine kinase (RTK)
	Ellipse			
raf	raf^{11-29}	*Df(1)64c18*	(2E1-2; 3C2)	Serine/threonine kinase
corkscrew (*csw*)	csw^{LE120}	*Df(1)64c18*	(2E1-2; 3C2)	Protein tyrosine phosphatase
	csw^{VA199}			
chico	$chico^{1}$	*Df(2L)J2*	(31B-32A)	IGF receptor binding protein
	$chico^{2}$			
pten	$Pten^{mgh3}$	*Df(2L)J2*	(31B-32A)	Dual specificity protein phosphatase
	$Pten^{mgh1}$			
Insulin receptor (*InR*)	InR^{217}			RTK
	InR^{327}			
	InR^{31}			
frizzled (*fz*)	fz^{J22}	*Df(3L)fz-M21*	(70D2-3; 71E4-5)	Wingless (Wg) receptor
	fz^{K21}			
	fz^{H51}			
wg	wg^{1}	*Df(2L)J-H*	(27C2-9; 28B3-4)	Secreted morphogen
	wg^{IG22}			
Toll (*Tl*)	Tl^{9QRE}	*Df(3R)Tl-P*	(97A; 98A1-2)	Transmembrane receptor
	Tl^{RxA}			
	Tl^{9Q}			
	Tl^{10B}			
spaeztle (*spz*)	spz^{2}	*Df(3R)Tl-P*	(97A; 98A1-2)	Secreted ligand
	spz^{rm7}			
Hairless (*H*)	H^{1}	*Df(3R)H-B79*	(92B3; 92F13)	Antagonist of the Notch pathway
	H^{2}			
	H^{3}			
	H^{25}			
lethal(2) giant larvae (*lgl*)	lgl^{4}			Epithelial polarity
scribble (*scrib*)	$scrib^{j7B4}$			Epithelial polarity

We tested candidate genes that had been previously shown to be involved in cell proliferation and/or survival in imaginal tissue. The alleles listed are hypomorphs, except ras^{C40B}, csw^{LE120}, $Pten^{mgh3}$, and flb^{CO}, which are amorphs.

than did *os/os1A* with extra bristles (data not shown). Although it is possible that we did not express *dpp*, tkv^{QD}, or *hh-N* at the appropriate time to engender rescue of the small-eye phenotype, these results demonstrated that neither *dpp* nor *hh-N* can substitute for *upd* in the developing eye.

We assessed whether mutations in JAK/STAT pathway genes can modify an eye phenotype dependent on hyperactivation of the Dpp pathway. *GMR-Gal4/+; UAS-*tkv^{QD}*/+* flies have rough, glassy eyes (supplemental Table 1 available at http://www.genetics.org/supplemental/). Reducing the dose of *glass* strongly suppressed the roughness in the eye, while reduction in the dose of *mad* partially modified the eye phenotype. The *GMR-Gal4/+; UAS-*tkv^{QD}*/+* phenotype was not modified by reduction in the dose of *stat92E*, *hop*, *upd*, or *dome* (supplemental Table 1 available at http://www.genetics.org/supplemental/). These data indicate that the JAK/STAT pathway is not a direct target of the *dpp* pathway.

We also assessed whether visible *dpp* and *upd* mutants interacted genetically. Homozygous dpp^{blk} flies have small eyes (Staehling-Hampton *et al.* 1995). We compared the eye size in the following genotypes: *os/os*, *os*/Y, *os/+*, dpp^{blk}/dpp^{blk}, $dpp^{blk}/+$, *os/+*; $dpp^{blk}/+$, and *os*/Y; $dpp^{blk}/+$ (supplemental Table 1 available at http://www.genetics.org/supplemental/). As expected, *os/os*, *os*/Y, and dpp^{blk}/dpp^{blk} flies had a small-eye phenotype, while $dpp^{blk}/+$, *os/+*; $dpp^{blk}/+$ flies had wild-type eyes. *os*/Y; $dpp^{blk}/+$ flies have a small-eye phenotype identical to that observed in *os*/Y flies, indicating that the reduction in dose of *dpp* does not modify the *os* phenotype (supplemental Table 1 available at http://www.genetics.org/supplemental/). Taken together, these data indicate that the JAK/STAT and Dpp pathways do not directly regulate each other.

DISCUSSION

The JAK/STAT pathway controls eye size: Our results indicate that Upd and the JAK/STAT pathway control

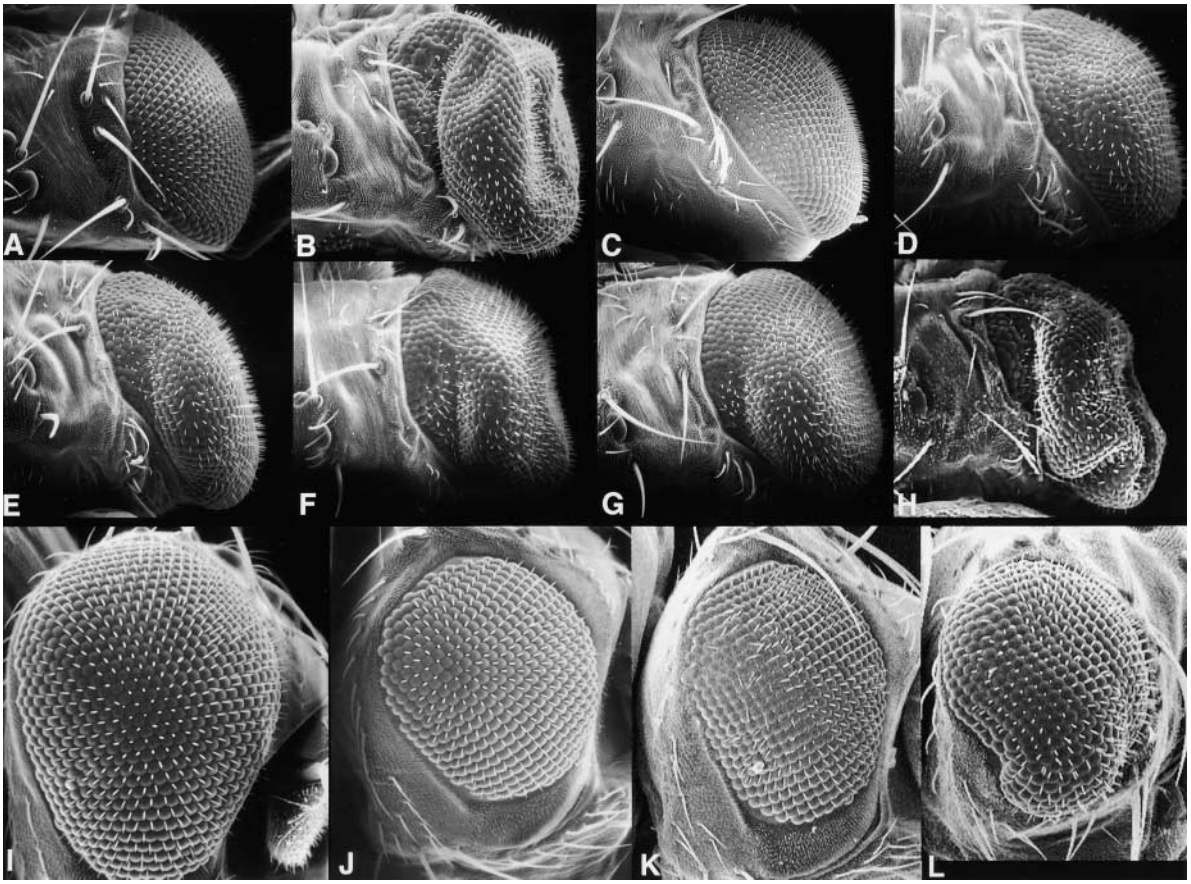


Figure 8.—*dpp* pathway genes modify the GMR-upd phenotype. Genotypes: WT (A); *GMR-upd19/+* (B); *GMR-upd19/+; stat92E^{06346}/+* (C); *GMR-upd19/+; Mad$^{l(2)k00237}$/+* (D); *GMR-upd19/+; dpp^{10638}/+* (E); *GMR-upd19/+; tkv$^{l(2)k16173}$/+* (F); *GMR-upd19/+; sax^{1}/+* (G); *GMRupd19/+; Dad^{1}/+* (H); WT (I); *w os/y w os1A* (J); *w os/y w os1A; ey-Gal4/UAS-GFP* (K); *w os/y w os1A; ey-Gal4/UAS-dpp* (L). Mutations in *dpp* (E), its receptors *tkv* (F) and *sax* (G), or the Dpp pathway positive signal transducer *Mad* (D) all suppress the GMR-upd phenotype. (C). Removing a copy of the Dpp pathway negative regulator *Dad* enhances the GMR-upd phenotype (H). The small eye in *os/os1A* (J) can be rescued by ectopic misexpression of *upd* (see Figure 2C) to the developing eye disc but not by ectopic misexpression of GFP (K) and can be only slightly rescued by ectopic misexpression of *dpp* (L). In A–H, scanning electron micrographs were taken at ×200 magnification; in I–L, at ×100. See Table 5 for more details on the genes in the *dpp* pathway that modify GMR-upd.

the size of the Drosophila eye. Heteroallelic hypomorphic combinations of *upd* result in a small adult eye, while ectopic misexpression of *upd* in the developing fly eye results in a greatly enlarged eye. This phenotype is specific to activation of the JAK/STAT pathway in the developing eye because reduction in the dose of *stat92E* or the eye-specific transcription factor *glass* results in suppression of the enlarged eye. Our results suggest that ectopic misexpression of *upd* in the developing eye results in additional mitoses of precursor cells in the region of the eye disc anterior to the furrow. These additional cells are patterned normally by the morphogenetic furrow, resulting in increased numbers of ommatidia in GMR-upd discs.

The GMR-upd phenotype is distinct from other enlarged-eye phenotypes: The enlarged-eye phenotype observed by ectopic misexpression of an activated form of *ras85D* using the *ey* enhancer, *ey-ras^{V12}*, is the result of ectopic R7 cells and also appears very rough (Karim and Rubin 1998). Our results indicate that the GMR-upd phenotype is distinct from the *ey-ras^{V12}* because GMR-upd eyes are patterned normally, are not rough, and are not modified by *ras85D* mutations. The enlarged eyes observed with misexpression of the Drosophila *InR* using GMR-Gal4 results primarily from increased cell volume (Brogiolo *et al.* 2001; Britton *et al.* 2002). Our results indicate that in the Drosophila eye the JAK/STAT and InR pathways do not interact, at least when ectopically misexpressed. Reduction in doses in *InR* pathway genes, such as *InR*, *Pten*, and *chico*, do not modify the GMR-upd phenotype. Moreover, the GMR-upd phenotype results from increased cell numbers, not from increased cell volume. In fact, cells in GMR-upd adult eyes actually exhibit decreased cell volumes when compared to wild type. Interestingly, the enlarged-eye phenotype in GMR-upd shares similarities with that produced as a nonautonomous effect of expression of an activated form of Notch (N^{intra}) in the eye, with promi-

TABLE 5

Dpp pathway genes modify GMR-upd

Gene	Allele	Interaction	Strength
decapentaplegic	dpp^{10638}	Su	2
decapentaplegic	dpp^{d12}	Su	2
decapentaplegic	dpp^{d6}	Su	2
decapentaplegic	$dpp^{d\text{-}ho}$	Su	2
decapentaplegic	dpp^{S11}	Su	2
decapentaplegic	dpp^{d5}	Su	2
decapentaplegic	dpp^{s1}	Su	2
thickveins	tkv^{16173}	Su	2
thickveins	tkv^{1}	Su	2
thickveins	tkv^{04535a}	Su	1
saxophone	sax^{4}	Su	2
saxophone	sax^{2}	Su	2
saxophone	sax^{1}	Su	2
Mothers against dpp	Mad^{k00237}	Su	4
Daughters against dpp	Dad^{1}	En	1

Mutations in *dpp*, its receptors *tkv*, *sax*, and *put*, and the signal transducer *mad* all suppress the GMR-upd phenotype, while a mutation in the negative regulator of this pathway *dad* enhances GMR-upd. The *dpp* locus (22F1-4) is haploinsufficient. *tkv* (25D1-2) is located in the interacting Df(2L) sc19-4, *sax* (42B) is located in the interacting deficiency Df(2R) cn9, *dad* (89E6-7) is located in the interacting Df(3R)DG2, and *mad* (at 23D3) is located in noninteracting Df(2L)JS17. Su, suppressor; En, Enhancer; 4, strong modification, for example, that observed with stat92E; 1, mild modification.

nent dorsal outgrowths (Go *et al.* 1998; Kurata *et al.* 2000). This observation is also interesting in light of the fact that we identify *CtBP*, which represses N pathway activity, as an enhancer of GMR-upd. It is possible that CtBP represses Stat92E itself or negatively regulates transcriptional coactivation by Stat92E.

Identification of modifiers of GMR-upd: We established that the GMR-upd line is a sensitized genetic background and performed an F_1 screen for dominant modifiers of the GMR-upd phenotype using a set of overlapping deletions of the Drosophila genome. We identified 20 loci that suppress and 9 that enhance the enlarged-eye phenotype. The gene(s) in these deficiencies that are responsible for the modification of the phenotype may represent new components of or new interactors with the JAK/STAT pathway. We identified 13 mutations as Su(GMR-upd): *zw13*, *crb*, *pie*, *D*, *His-2Av*, *kto*, *hdc*, *px*, *hh*, *dpp*, *tkv*, *sax*, and *Mad*. In addition, we identified two mutations as En(GMR-upd): *CtBP* and *Dad*.

Identification of suppressors of GMR-upd: *zw13* interacts genetically with the meiotic kinesin-like genes *nod* and *ncd* and encodes a poorly characterized protein with RNA-recognition motifs. Therefore, Zw13 may be important in regulating *upd* expression. We also identified *crb* as a suppressor of GMR-upd. Crb is a PDZ-containing protein involved in the establishment and maintenance of apical-basal polarity in epithelia (Pellikka *et al.* 2002). *crb* may suppress the GMR-upd phenotype by altering the localization of Dome and/or Upd or the signaling output of the JAK/STAT pathway in the eye.

We identified several transcription factors as suppressors of GMR-upd: *pie*, *D*, *His2Av*, *kto*, *px*, and *hdc*. Pie is a nuclear protein that contains a PHD finger, which is a C4HC3 zinc-finger-like motif thought to facilitate chromatin-mediated transcriptional regulation (Aasland *et al.* 1995). Eyes from *pie* homozygotes show irregular spacing of ommatidia, although the ommatidia have the normal array of photoreceptors (Baker *et al.* 1992). Notably, *pie* homozygous flies also have held-out wings, a phenotype shared by *os* flies and flies that overexpress full-length Dome (Lindsley and Grell 1968; E. A. Bach, unpublished observation). In embryonic segmentation, D directly regulates the expression of the pair-rule gene, *even-skipped* (*eve*), by binding to multiple sites located in downstream regulatory regions that direct formation of *eve* stripes 1, 4, 5, and 6 (Ma *et al.* 1998). This overlaps with the function at Stat92E, which is needed for proper expression of *eve* stripes 3 and 5 (Hou *et al.* 1996; Yan *et al.* 1996). Interestingly, *fish* and *upd* share related expression patterns and phenotypes. The early expression pattern of *fish* is almost identical to that of *upd* (Nambu and Nambu 1996). Like *upd*, *fish* is also required in the hindgut, and the *D* held-out wing phenotype is very similar to that of *os* (Lengyel and Iwaki 2002). *His2Av* belongs to the H2AZ variant subclass, which is involved in chromatin stability, chromatin remodeling, and transcriptional control (Redon *et al.* 2002). Given that mammalian STATs have been shown to mediate transcriptional changes within seconds of activation, it is possible that histone modification must be coordinated with transcriptional coactivation. Kto is the homolog of thyroid-hormone receptor associated protein (TRAP230), which was originally identified as part of the *trithorax* group, a large transcriptional coactivation complex (Kennison and Tamkun 1988). *kto* is involved in photoreceptor differentiation because homozygous mutant clones in the eye disc fail to develop into photoreceptors, although mutant cells can respond to Hh by expressing *dpp* (Treisman 2001). *hdc* encodes a nuclear factor involved in tracheal development, where it acts nonautonomously in an inhibitory signaling mechanism to determine the number of cells that will form unicellular sprouts in the trachea (Steneberg *et al.* 1998). Interestingly, it has been recently noted that *stat92E* is also required in tracheal development (Brown *et al.* 2001; Chen *et al.* 2002). However, whether *hdc* and *stat92E* interact, if at all, in this tissue is not known, nor is it understood whether any interaction exists in the eye disc. Px is a nuclear protein that, like Pie, contains a PHD zinc finger and is involved in venation in the wing (Matakatsu *et al.* 1999). It is not known if *px* mutants exhibit an eye phenotype. Clearly, future work must focus on the elucidation of any biochemical inter-

action between Stat92E and these transcription/nuclear factors and also whether they regulate the transcription of a common set of genes required for growth of the eye disc.

The Dpp pathway genes modify GMR-upd: The other modifiers identified in our modifier screen are genes in the Dpp pathway, specifically *dpp*, *tkv*, *sax*, *mad*, *hh*, and *Dad*. We initially reasoned that *upd* may exerts its proliferative effects through *hh* or *dpp*. However, we show that *hh* and *dpp* are expressed normally in GMR-upd. In addition, we demonstrate that ectopic misexpression of *hh* or *dpp* in the *os/os1A* flies does not rescue the small-eye phenotype whereas *upd* does and that ectopic expression of *upd* in flip-out clones does not induce *hh*. These results suggest that *upd* may not directly regulate *dpp* or *hh* expression. These data also suggest that Upd and Dpp and/or Hh may coregulate genes involved in the proliferation of eye precursor cells. This hypothesis is supported by observations in mammalian systems. The cytokines leukemic inhibitory factor and bone morphogenic protein 2 activate Stat3 and Smad1, respectively, and act synergistically in fetal neuroepithelial cultures to promote the differentiation of astrocytes from progenitor cells. The synergism requires functional Stat3 and Smad1. However, these proteins do not physically interact; rather, they both bind to p300/CBP to promote transactivation of target genes, such as glial fibrillary acidic protein, a marker of astrocyte differentiation (Nakashima *et al.* 1999).

The role of the JAK/STAT pathway in proliferation and growth control: In both mammals and flies, the JAK/STAT pathway plays an important role in the control of organ/tissue size. *Stat5* knock-out mice are runted due to impaired growth-hormone signaling (Levy and Darnell 2002). Similarly, *Socs-2* knock-out mice are significantly larger than their wild-type littermates, due to a lack of negative regulation of the growth-hormone pathway *in vivo* in the absence of the *Socs-2* gene (Metcalf *et al.* 2000). Overexpression of an activated, constitutively dimerized STAT, c-Stat3, results in the formation of tumors in mice (Bromberg *et al.* 1999). Importantly, the only gain-of-function mutations in any JAK are found in Drosophila *hop*. $hop^{Tum\text{-}l}$ and hop^{T42} are independent point mutations that give rise to hyperactive Hop proteins, overproliferation and premature differentiation of Drosophila larval blood cells (a so-called fly "leukemia"), melanotic tumors, and lethality (Harrison *et al.* 1995; Luo *et al.* 1995). Overexpression of *upd* or *hop* in the developing Drosophila eye leads to a greatly enlarged eye due to an increase in the number of cells in the eye disc. In contrast, hypomorphic mutations in *upd*, for example, *os* or *os/os1A*, lead to a small adult eye.

Although proliferation is clearly a result of activation of the JAK/STAT pathway in mammals and Drosophila, we know very little about how this pathway regulates the increase in cell number or the cell cycle. Our data suggest that activation of the JAK/STAT pathway in the eye disc increases the number of cycling cells, possibly by shortening the G_1 phase or by regulating the G_2/M transition of the cell cycle. As a secreted molecule, Upd presumably acts in a cell-nonautonomous manner and may promote proliferation directly through activation of Hop and Stat92E. However, the observed proliferation in GMR-upd may in fact be due to the ability of Upd to induce another molecule that can also act cell nonautonomously. At the moment we cannot differentiate between these two possibilities. Nonetheless, the fact that we observe more cells in GMR-upd indicates that Upd may regulate genes involved in proliferation in the eye disc. In addition to the 15 modifiers of GMR-upd described here, we have also identified several uncharacterized mutations that modify GMR-upd and may encode potentially novel molecules and uncover new functions of the JAK/STAT pathway. Given the high conservation between the Drosophila and mammalian JAK/STAT pathways, it is likely that the genes and functions we uncover in this screen will also be relevant to higher organisms.

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