A *Drosophila* PIAS homologue negatively regulates *stat92E*

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Transcriptional activation by, and therefore the physiologic impact of, activated tyrosine-phosphorylated STATs (*s*ignal *t*ransducers and *a*ctivators of *t*ranscription) may be negatively regulated by proteins termed PIAS (*p*rotein *i*nhibitors of *a*ctivated *s*tats), as shown by previous experiments with mammalian cells in culture. Here, by using the genetic modifications in *Drosophila*, we demonstrate the *in vivo* functional interaction of the *Drosophila* homologues *stat92E* and a *Drosophila* PIAS gene (*dpias*). To this end we use a LOF allele and conditionally overexpressed *dpias* in JAK-STAT pathway mutant backgrounds. We conclude that the correct *dpias*/*stat92E* ratio is crucial for blood cell and eye development.

A variety of extracellular polypeptides cause tyrosine phosphorylation and activation of the mammalian STATs (*s*ignal *t*ransducers and *a*ctivators of *t*ranscription; refs. 1 and 2), which participate in regulating a wide variety of events in embryology, hematopoiesis, and growth control. Several mechanisms of negative regulation of the transiently activated STATs have been discovered. (*i*) Induced proteins, variously called SOCS, JABS, or SSI proteins (3–5), prevent cytoplasmic STAT activation. (*ii*) Cytoplasmic tyrosine phosphatases (SHP 1) deactivate the JAK receptors halting STAT activation (6). (*iii*) Tyrosine phosphatase-mediated inactivation of tyrosine phosphorylated STATs also occurs in the nucleus (7, 8).

Finally, Chung and coworkers (9, 10) reported another possible means of directly deactivating STAT molecules. Proteins that bind to and block the *in vitro* DNA binding of activated STATs were found, when overexpressed in transfected cells, to strongly inhibit STAT-driven transcription. Five such mammalian proteins have now been identified and named, PIAS (for *p*rotein *i*nhibitor of *a*ctivated *S*TAT) 1, 3, xα, xβ, and y (11). In transfected cells, overexpression of PIAS1 inhibited STAT1-induced transcription, overexpression of PIAS3 inhibited STAT3-induced transcription, and PIAS1 and PIAS3 coimmunoprecipitate with tyrosine-phosphorylated STAT1 and STAT3, respectively (9, 10). These results strongly implied a negative regulatory role of the PIAS proteins on activated STATs but did not prove a physiological relevance in animals.

The JAK-STAT pathway operates in *Drosophila* as well as in mammals (12). STAT92E (13, 14) is activated by hopscotch (HOP), the single *Drosophila* tyrosine kinase of the JAK family (15), after cells encounter *outstretched* (OS), a membrane-associated ligand, whose receptor is not yet identified (16). STAT92E then activates transcription, for example of the even skipped (*eve*) stripe 3 + 7 enhancer (13, 14). In addition to early lethality and segmentation phenotypes, LOF alleles of *os* and *hop* have uncovered widespread involvement of the pathway later in development, most well studied in eye ontogeny (17, 18).

Moreover, two hyperactive *hop* alleles, $hop^{Tum\text{-}l}$ and hop^{T42}, cause blood cell tumor formation, which can be suppressed by a LOF allele of *stat92E*, $stat^{HJ}$ (19, 20). In mammals a conserved STAT5-dependent pathway is likely to be involved in leukemias of functionally similar cell types of the myeloid lineage (21, 22).

We now report functional aspects of the single *Drosophila* PIAS gene first identified many years ago as a suppressor of position effect variegation, termed *Su(var)2–10*, and shown recently by Hari *et al.* (23) to be a gene required for normal chromosome function. This gene, also recently described as *zimp* by Mohr and Boswell (24), has strong homology to the mammalian PIAS genes. Because the PIAS proteins have been well described in mammalian cell literature as interacting with STAT proteins, we believe the name for the *Drosophila* gene that might best serve the wider scientific community is the simple term *dpias*. A lethal *P* element insertion in the *dpias* locus produces a LOF allele furnishing a genetic tool to explore the interaction of *dpias* with genes in the JAK-STAT pathway. For this study, we also generated transgenic fly stocks overexpressing dPIAS. Our experiments demonstrate that STAT92E and dPIAS have to be correctly balanced for normal blood cell and eye development to occur.

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Materials and Methods

Plasmid Constructs and Cultured Cells. For transgenic plasmid constructs, the coding sequences of expressed sequence tag (EST) clones AA803041 [*dpias*(537)] and AA390747 [*dpias*(522)] were sequenced and cloned into the pUAST vector (*Eco*RI/*Xba*I). For the pull-down assay, a *dpias* coding sequence corresponding to amino acids 270–409 was cloned into pGEX 5–1(*Eco*RI/*Sma*). The construct expressing FLAG epitope-tagged STAT92E in Schneider S2 cells under the control of the actin promoter and extract preparation for these cells was described earlier (14).

Reverse Transcription (RT)–PCR from Larval Extracts. $dpias^{03697}$/$dpias^{03697}$ were distinguished from $dpias^{03697}$/*CyO,actin-GFP,-dpias*$^{+}$ mutants by means of the larval marker actin-GFP. First instar larvae were chosen as the source of mRNA because the maternal contribution of *dpias* mRNA (determined by mRNA *in situ* assays) was presumed to be low at this stage, and viable homozygous $dpias^{03697}$ mutants could still be found. RNA was extracted from larvae of each genotype and semiquantitative RT-PCR assays were performed as described (25). Primer sequences used for dPIAS cDNA amplification were 5′-GCCG-TATACCTGGTAAAGAAGCTCACC-3′ and 5′-TGGTGTGC-TCCAAGATCCATCCTG-3′. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA amplification 5′-ACCGTC-GACGGTCCCTCT-3′ and 5′-GTGTAGCCCAGGATTCCCT-3′ were used.

Control Stocks and Methods. Control stocks and markers for Table 1: *CyO* was exchanged with *Adv* (as shown in Table 1); *Sp1* and *Bl.* $dpias^{03697}$ were exchanged with two deficiencies covering the *dpias* locus *Df(2R)NP3* (as shown in Table 1) and *Df(2Rw45-30n)* with similar results (Bloomington Stock Center, Bloomington, IN). In the cross shown in Table 2, *TM2* and *TM3* were used instead of *TM6b* with similar results. In Fig. 3, *TM2* was

Abbreviations: EST, expressed sequence tags; RT, reverse transcription; GST, glutathione *S*-transferase; WT, wild type; hs, heat shock.

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Table 1. Decreasing *dpias* gene dosage enhances the tumor frequency in $hop^{Tum\text{-}l}$ mutants

F1 Genotype	Total number	With tumor	Without tumor	% with tumor	Fold increase
Parental cross: $hop^{Tum\text{-}l} \times dpias^{03697}/CyO$					
$hop^{Tum\text{-}l}/+$; $dpias^{03697}/+$	323	257	66	80	2.2
$hop^{Tum\text{-}l}/+$; $CyO/+$	236	88	148	37	
Parental cross: $hop^{Tum\text{-}l} \times dpias^{03697}/Adv$					
$hop^{Tum\text{-}l}/+$; $dpias^{03697}/+$	351	226	125	64	2.0
$hop^{Tum\text{-}l}/+$; $Adv/+$	372	118	254	32	
Parental cross: $hop^{Tum\text{-}l} \times Df(2R)Np3bw/CyO$					
$hop^{Tum\text{-}l}/+$; $Df(2R)Np3bw/+$	256	241	15	94	2.4
$hop^{Tum\text{-}l}/+$; $CyO/+$	288	113	175	39	

The difference between the two genotypes was statistically significant with a P value of $P < 0.01$.

exchanged with *TM6b* and vice versa, and *CyO* was replaced with *Bl*, *Sco*, and *Adv*, which had no detectable influence on the eye phenotypes.

Two independent transgenic lines, UAS-*dpias*(537) and UAS-*dpias*(522), respectively, were used in this study with equal effects in the described experiments.

Alternative splicing at the C terminus of the *dpias* gene results in at least three protein isoforms that share the first 515 amino acids but differ at their C termini. *dpias* 522AA was derived from EST AA390747 [amino acids 515–522 = TLDPFLQ (see ref. 24)] (23). *dpias* (526) was derived from EST AA536416: amino acids 515–526 = AVSAMNTMRKAK. *dpias*(537) was derived from EST AA803041: amino acids 515–537 = EDNDENCMAKAKEDSVIDLLDSP [see also Hari *et al.* (23)]. These sequences are available from FlyBase (http://flybase.bio.indiana.edu/seqs/).

The frequency of the eye phenotype suppression in Fig. 3 *H* and *I* was calculated in the following way. Eye sizes of os^{1};$dpias^{03697}$/+;$stat^{06346}$/+, and os^{1};CyO/+;$stat^{06346}$/+ progeny segregating from the same parental cross were compared randomly in pairs, and flies were sorted into two pools (larger and smaller eyes), according their relative eye size. Of the flies of the larger-eyed pool, 85% were of the os^{1};$dpias^{03697}$/+;$stat^{06346}$/+ genotype. This difference was not caused by *CyO*, because in crosses with other dominant makers on chromosome 2, the result was similar: os^{1};$dpias^{03697}$/+;$stat^{06346}$/+ vs. os^{1};Adv/+;$stat^{06346}$/+ with 87%, os^{1};$dpias^{03697}$/+;$stat^{06346}$/+ vs. os^{1};Sco/+;$stat^{06346}$/+ with 89%, and os^{1};$dpias^{03697}$/+;$stat^{06346}$/+ and os^{1};Bl/+;$stat^{06346}$/+ with 85% (over 200 flies were tested in each cross).

Fly Stocks. *L(2)03697* (Bloomington Stock Center) is a lethal *P* element insertion stock with no detectable *dpias* expression. $stat^{06346}$ and $hop^{Tum\text{-}l}$ (gifts from C. Dearolf, Massachusetts General Hospital, Harvard Medical School, Boston) were used in genetic interaction experiments as well as os^{1} (Bloomington Stock Center). *ey*-Gal4, heat shock (*hs*)-Gal4, and GMR-Gal4 (Bloomington Stock Center) were used to overexpress UAS-*dpias*(522) and UAS-*dpias*(537). These transgenic strains carrying stable insertions on chromosomes 1 and 2, respectively, were generated by *P* element-mediated transformation. Eye clones were generated as described (26), except *P{ry+7.2 = ey-FLP.D}6, ry506* (Bloomington Stock center) was used as a flipase source; *y{1} w[*]; P{$w^{+mW.hs}$ =>w^{hs}>}G13 L ! FRT 42B* (Bloomington Stock Center) was recombined with *L(2) 03697* and crossed to *y{1} w[*]; P{$w^{+mW.hs}$ = >w^{hs}>}G13 P{w[+mC]-mCD*::GFP.L}LL5* (Bloomington Stock Center). $dstat^{-/-}$ eye clones were identified by the absence of *w* expression. [The *w* gene on FRT 42B is flanked by two FRT cassettes, which also causes intrachromosomal recombination removing *w* (27).] The *w* gene on *P{w[+mC]-mCD*::GFP.L}LL5* is strongly expressed. Therefore, complete removal of *w* by intra- and extrachromosomal recombination can be seen easily.

STAT92E-dPIAS Pull-Down Assay. It was performed as previously described (28), except that we established an S2 cell line expressing an epitope-tagged STAT92E (FLAG). Also, a glutathione *S*-transferase (GST) fusion protein (GST + dPIAS residues 270–409, which includes the zinc finger domain) was expressed in *Escherichia coli* and purified on GST beads. Nuclear extracts from untreated S2 cells or cells treated with peroxide vanadate (2 mM H_2O_2/1 mM for 15 min; refs. 8 and 14) were incubated with the GST-dPIAS fusion protein or GST protein alone, and then samples were exposed to FLAG Ab-conjugated beads. The beads were washed and the eluate was subjected to SDS/PAGE and Western blotting with an anti-GST Ab. The protein concentration of the nuclear extract of each sample was equalized before the addition of Flag-conjugated beads and before vanadate/H_2O_2 treatment by splitting the detached S2 cells into equal volumes.

Tumor-Suppression Experiments. Abdominal tumors were identified under a dissecting microscope (Zeiss) at ×30 magnification, and adult females were scored as positive if they had at least one abdominal tumor 0–12 h after eclosion. Flies were grown at 25°C under noncrowded conditions. Pilot experiments established that at this temperature, $hop^{Tum\text{-}l}$ female viability is comparable to wild-type (WT) siblings, and that 30–40% of $hop^{Tum\text{-}l}$ heterozygous females have tumors in several different genetic backgrounds.

Histological Analysis. Adult flies were prepared for scanning electron microscopy as described by Kimmel *et al.* (29). Eyes were sectioned and analyzed according to Tomlinson and Struhl (30). Pictures were taken by using a Zeiss Axiophot microscope.

Table 2. Overexpressing *dpias* suppresses the tumor frequency in $hop^{Tum\text{-}l}$ mutants

F1 Genotype	Total number	With tumor	Without tumor	% with tumors	Fold decrease
Parental cross: $hop^{Tum\text{-}l}$ × UAS-*dpias*(537); *hs*-Gal4/*TM6b*					
$hop^{Tum\text{-}l}/+$; UAS-*dpias*(537)/+; *hs*-Gal4/+	257	48	209	19	1.9
$hop^{Tum\text{-}l}/+$; UAS-*dpias*(537)/+; *TM6b*/+	385	141	244	37	

The difference between the two genotypes was statistically significant with a P value of $P < 0.01$.

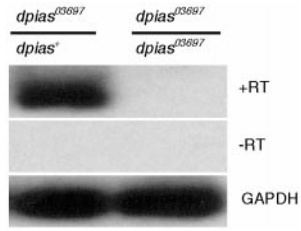

Fig. 1. Semiquantitative RT-PCR of $dpias^{03697}/CyO$;Actin-GFP,$dpias^{+}$ (*Left*) and homozygous $dpias^{03697}$ (*Right*) first instar larvae. +RT, reverse transcriptase added and primers amplifying *dpias* mRNA. −RT, no reverse transcriptase added and primers amplifying *dpias* mRNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), reverse transcriptase added and primers amplifying *gapdh* mRNA.

Results

***l(2)03697* Is a LOF Allele of *dpias*.** By matching the available flanking sequence of the *P* element insertion of the stock *l(2)03697* (BDGP; ref. 31) with the 5′ untranslated region (UTR) of a cDNA highly homologous to the mammalian PIAS genes, we identified a putative mutant allele of the *Drosophila* PIAS gene before the *Drosophila* genomic sequence was completed.

The *P* element insertion at the *dpias* locus (the $dpias^{03697}$ allele) blocked all mRNA formation. mRNA expression was assayed by semiquantitative RT-PCR of larvae homozygous and heterozygous for the insertion. Therefore, the $dpias^{03697}$ allele constitutes a strong LOF or a null allele of the *dpias* gene (Fig. 1; see *Materials and Methods* for details).

dPIAS Physically Interacts with STAT92E. Coimmunoprecipitation of mammalian PIAS and tyrosine-phosphorylated STATs (9) has been established, and the interacting region of PIAS3 with STAT3 lies in the center of the molecule embracing a portion of a putative zinc finger domain (11). By using an *in vitro* protein association assay, we found that a similar region of a dPIAS–GST fusion molecule bound to a FLAG-tagged STAT92E protein (Fig. 2). The interaction depended on prior activation of the STAT92E protein brought about by the inhibition of tyrosine dephosphorylation with vanadate/peroxide (32), which was used because natural activation of STAT92E has not been accomplished in cell culture.

Suppression of Melanotic Tumor Formation by *dpias*. Because the $dpias^{03697}$ allele is a homozygous lethal, we designed genetic interaction crosses in which flies heterozygous for the recessive $dpias^{03697}$ allele were scored for the possible enhancement or suppression of known phenotypes in JAK-STAT pathway mutants.

$hop^{Tum\text{-}l}$ is a dominant hyperactive allele (increased HOP activity at elevated temperature; ref. 33) that causes tumor formation (19). This tumor formation, which is suppressed by *stat92E* LOF mutants (32), results from excessive proliferation of blood cells (plasmatocytes) that form melanotic abdominal tumors in larvae and pupae that can be scored in adults (19). At 25°C, 37% of heterozygous $hop^{Tum\text{-}l}$ adult females had at least one abdominal tumor (Table 1 *Parental Cross:* $hop^{Tum\text{-}l} \times dpias^{003697}/CyO$, bottom row). (Hemizygous males have a much higher tumor rate and were not scored in this experiment.) Reduction of a negative activating regulator of this pathway should cause an increase in tumors. The percentage of flies with at least one tumor more than doubled in the $hop^{Tum\text{-}l}/+;dpias^{03697}/+$ genotype compared with the progeny with two WT *dpias* alleles ($hop^{Tum\text{-}l}/+;CyO/+$; Table 1 *Parental Cross:* $hop^{Tum\text{-}l} \times dpias^{003697}/CyO$, top row). Control crosses in which *CyO* was exchanged with other marked chromosomes, such as *Adv* (Table 1 *Parental Cross:* $hop^{Tum\text{-}l} \times dpias^{003697}/Adv$), and a chromosomal deletion that removes the *dpias* gene, *Df(2R)Np3*, is shown in Table 1 *Parental Cross:* $hop^{Tum\text{-}l} \times Df(2R)Np3bw/CyO$. Even though we found slight variations in the tumor frequency between these stocks (which could be a result of variations in population density), these controls produced a similar increase in tumor frequency compared with their siblings with two WT copies of *dpias*, minimizing the possibility of genetic background as a contributing factor to the elevation of tumor frequency.

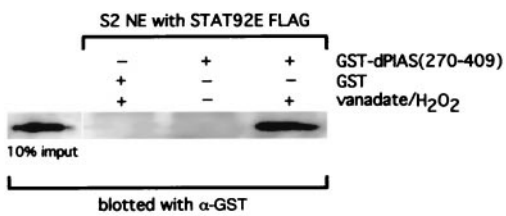

Fig. 2. The conserved central domain of dPIAS interacts directly with vanadate/H_2O_2-activated STAT92E (see text for details).

If the observed increase in tumor formation was truly caused by a reduction of *dpias* gene dosage, then an increase of dPIAS should decrease tumor formation. The binary UAS-Gal4 expression system (34) allows for tissue-specific and conditional overexpression of transgenes in *Drosophila*. Transgenic animals carrying UAS-*dpias* constructs of two of the three identified dPIAS isoforms of 537- and 522-aa in length (see *Materials and Methods* for details) were prepared and used in the following genetic interaction crosses. [No significant difference in effect between transgenic lines with these two isoforms could be detected in this study and for simplicity, from now on, we refer only to the 537-aa-containing construct as UAS-*dpias*(537)]. A stock containing UAS-*dpias*(537) was crossed to a driver stock carrying a transgene expressing the transcriptional activator Gal4 under the control of a heat-shock promoter (*hs*-Gal4). The double transgenic progeny was crossed into the $hop^{Tum\text{-}l}$ background and received a daily heat shock, 37°C for 30 min, from larval stages to eclosion. A significant decrease of tumor-bearing flies was observed in the hs-Gal4 genotype as compared with the control *TM6b* (Table 2). Other control crosses excluded *TM6b* as a contributing factor to this effect (see *Materials and Methods*). These experiments on tumor frequency support the conclusion that dPIAS interacts negatively with the JAK-STAT pathway made overactive by $hop^{Tum\text{-}l}$, which leads to tumor formation. We conclude that dPIAS decreases the transcriptional impact of the overactive STAT92E.

***dpias* and *stat92E* in Eye Development.** We next examined the role of *dpias* in eye development because hypomorphic mutants of *hop* and *os* have small eyes (17, ‖). We used two different lines, GMR-Gal4 and *ey*-Gal4, in which *dpias* overexpression depends on Gal4 activation at different times during eye development. When the GMR-Gal4 line was used to drive UAS-*dpias*(537), we observed no obvious effect on eye size or texture (not shown). Also, the *ey*-Gal4 transgene by itself had no effect on eyes (Fig. 3*A*). However, when we activated UAS-*dpias*(537) with the *ey*-Gal4 driver, eye size was severely reduced and the remaining small eye had a rough texture (Fig. 3*B*). A doubling of the transgene dosage further aggravated this phenotype and resulted in complete loss of the eyes in most of the surviving progeny (Fig. 3*C*). Because *ey*-Gal4 is active very early in eye development (before cellular differentiation) and GMR-Gal4 at later stages [during cellular differentiation (35)], we conclude that overexpression of *dpias*(537) has an effect primarily on cells in the early proliferating eye disk.

We further investigated whether this occurs because of a decreased activity of the JAK-STAT pathway. To this end, we crossed small-eyed UAS-*dpias*(537)/*CyO;ey*-Gal4 flies (as in Fig. 3*B*) to a stock carrying a heat shock-inducible *stat92E* gene (*hs-stat92E*) (32) and raised the progeny under mild heat-shock

‖Verderosa, F. J. & Muller, H. J. (1954) *Genetics* **39,** 999 (abstr.).

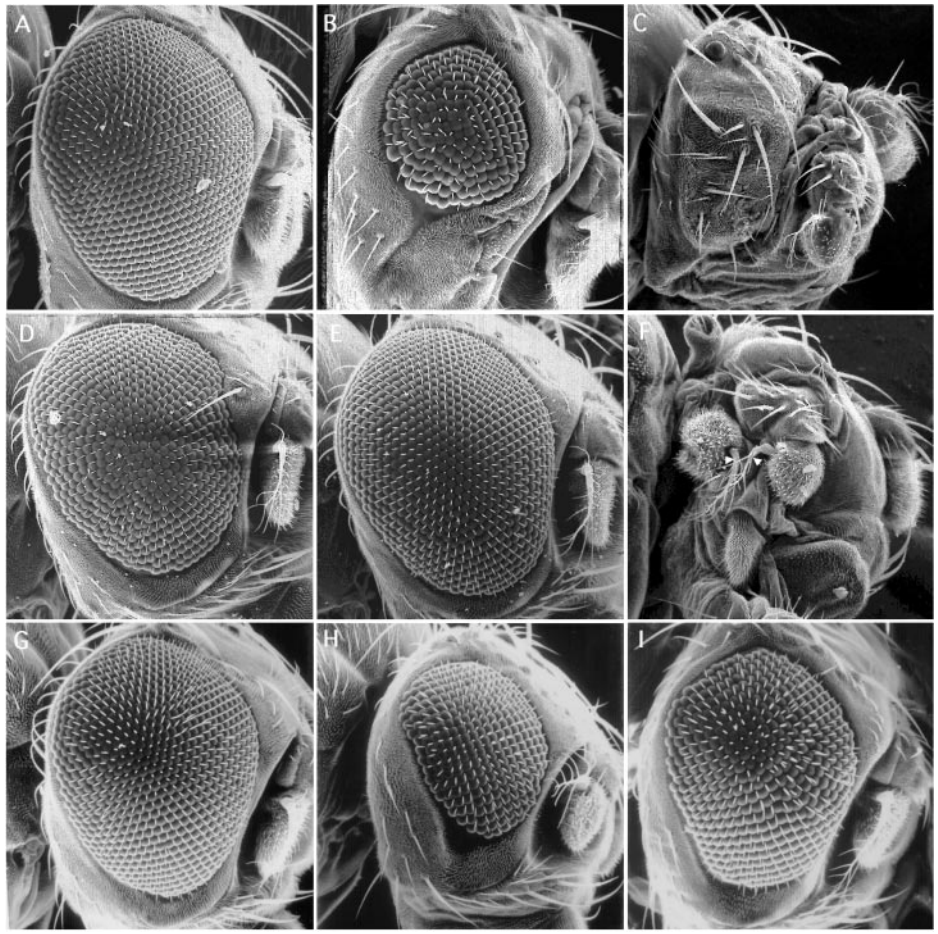

Fig. 3. Eye phenotypes and genetic interaction demonstrated by various gene dosages of *dpias*, *stat92E*, and *os*. (*A*) *ey*-Gal4/*TM2* flies appear WT. (*B*) Overexpression of *dpias*(537) by using one copy of UAS-*dpias*(537) driven by *ey*-Gal4 results in small and rough eyes (UAS-*dpias*(537)/*CyO;ey*-Gal4/*TM2*). (*C*) The majority of flies with two copies of UAS-*dpias*(537) have no eyes (UAS-*dpias*(537)/UAS-*dpias*(537);*ey*-Gal4/*TM2*). (*D*) Eye size and texture of eyes as in *B* can be rescued with simultaneous overexpression of STAT92E (UAS-*dpias*(537)/*hs-stat92E;ey*-Gal4/*TM2*). (*E*) Flies heterozygous for $stat^{06346}$ have phenotypically WT eyes ($stat^{06346}$/*TM2*). (*F*) When heterozygous $stat^{06346}$ flies are crossed to flies (as in *B*), an additional antennae can develop in place of the eye. The two resulting antennae (aristae) are marked by white arrows (UAS-*dpias*(537)/*CyO;*$stat^{06346}$/*ey*-Gal4). (*G*) $dpias^{03697}$/+ eyes have WT appearance. (*H*) The eyes of os^1;*CyO*/+;$stat^{06346}$/+ flies are small but can be partially rescued in size by reducing the gene dosage of *dpias* as shown in a sibling (*I*) with the genotype os^1;$dpias^{03697}$/+;$stat^{06346}$/+.

conditions (see *Materials and Methods*). A significant rescue of eye size and texture was observed only in progeny that carried the hs-*stat92E* transgene but not in genotypes without the hs-*stat92E* transgene segregating from the same cross (compare Fig. 3 *B–D*). Moreover, a similar eye-size rescue effect was achieved by crossing the $hop^{Tum\text{-}l}$ stock with small-eyed UAS-*dpias*(537)/*CyO;ey*-Gal4 flies (not shown), further bolstering the notion that activated STAT92E is required for eye development and that dPIAS counteracts the activated STAT92E.

We went on to investigate how the *stat92E* LOF allele, $stat^{06346}$, might affect small-eyed UAS-*dpias*/*CyO;ey*-Gal4 flies. (Heterozygous $stat^{06346}$/*TM2* was used because homozygotes of $stat^{06346}$ die as first instar larvae). Fig. 3*E* depicts an eye from a heterozygote for the $stat^{06346}$/*TM2* mutant that is phenotypically WT. When this stock was crossed to the small-eyed UAS-*dpias*(537)/*CyO;ey*-Gal4 stock, however, the small-eyed phenotype was clearly made more severe, resulting in the loss of eyes similar to those with two copies of the UAS-*dpias*(537) transgene (like Fig. 3*C* and therefore not shown). Yet, frequently (27 of 100) the enhancement of phenotype seemed to go even further, and the progeny grew antenna in place of eyes (see Fig. 3*F*). Thus, *stat92E* has a role in the early phase of eye development and determination.

We also tried to establish whether the dPIAS–STAT92E interaction occurs naturally during eye development. [Enhancer trap lac-Z stains of $dpias^{03697}$/+ mutants and detection of STAT92E by antiserum had indicated that both genes are coexpressed in developing third instar larval eye disks (data not shown and refs. 17 and 18).] To this end, we used the hypomorphic LOF mutant os^1, which reduces function of the only known ligand in the JAK-STAT pathway in flies resulting in a small-eye phenotype (16, ‖). When we introduced $dpias^{03697}$ into the os^1 background (os^1;$dpias^{03697}$/+), a subtle increase in eye size in os^1;$dpias^{03697}$/+ progeny was observed as compared with the siblings segregating from the same cross with WT *dpias* (os^1;*CyO*/+; not shown). This increase of eye size became more pronounced when JAK-STAT function of this genotype was further reduced in a background also heterozygous for the LOF $stat^{06346}$ allele (os^1;$dpias^{03697}$/+;$stat^{06346}$/+; compare Fig. 3 *H–I*). In contrast to the drastic eye-size differences described in Fig. 3 *A–F*, the increase in eye size observed in os^1;$dpias^{03697}$/+;$stat^{06346}$/+ vs. os^1;*CyO*/+;$stat^{06346}$/+ was more modest but still significant on average (see *Materials and Methods*). To exclude genetic background as a contributing factor, we used several control stocks in which *CyO* was replaced with *Adv*, *Sco*, and *Bl*, but none of these substitutions had any significant influence on the difference in eye size (not shown; see *Materials and Methods*). Also, *dpias*/+ flies are phenotypically WT as shown in Fig. 3*G*. We conclude that by removing one copy of the WT *dpias* gene, suboptimal JAK-STAT activity in eyes brought about by the os^1 allele can be partially

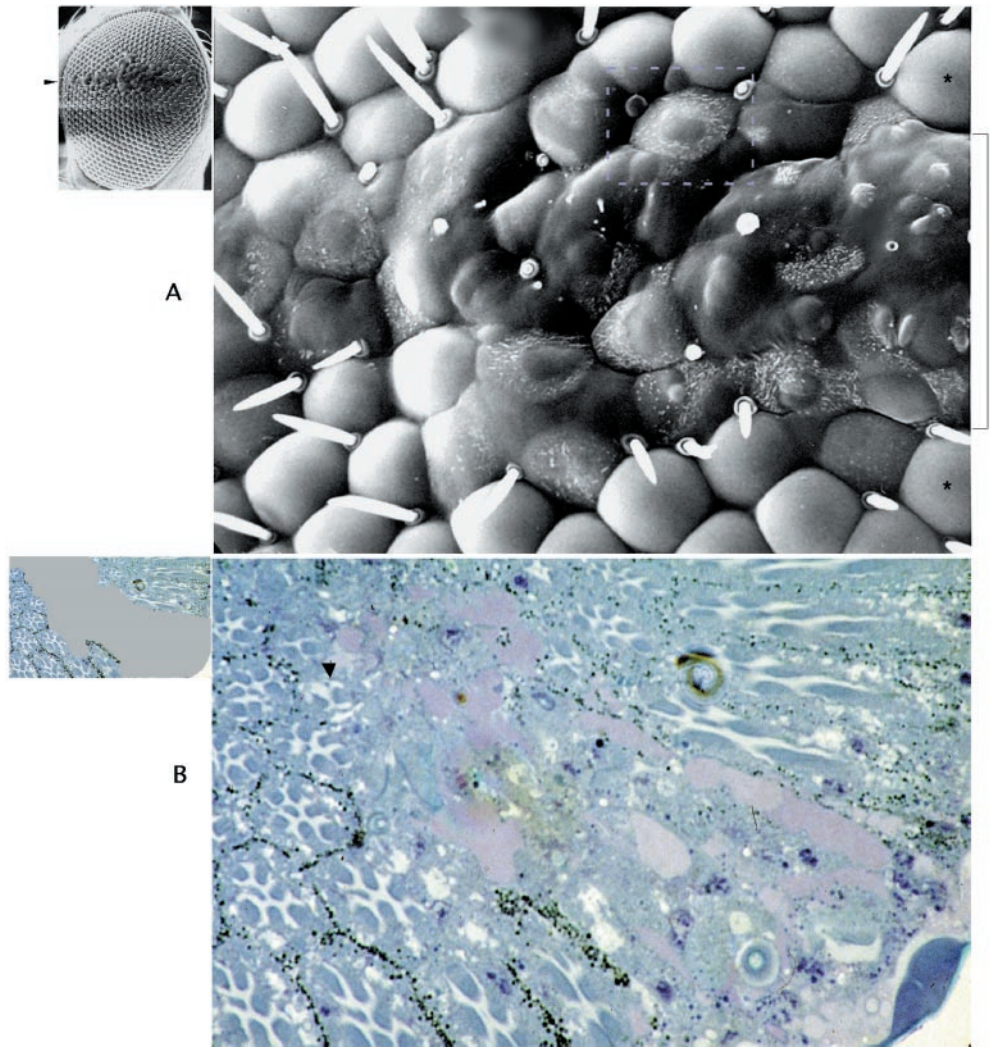

Fig. 4. (*A Left*) SEM of a larger equatorial *dpias*$^{-/-}$ eye clone extending along the equator (marked by an arrow), which can be seen to extend horizontally along the equator. In the blow up (*A Right*), normal lenses outside the clone, located dorsally and ventrally of the clone (two are marked with an asterisk), can be seen. In the center of the clone (marked by the bracket), the lens architecture is completely lost and the surface is bulged out. The *Inset* marks an example of a ''partial lens phenotype'' (37) with dotted lines, straddling the clonal border with ectopic hairs. (*B Left*) Diagram of a sectioned *dpias*$^{-/-}$ eye clone delineating the clonal area in gray. In the blow up (*B Right*), partial (arrow) or completely failed cellular differentiation (center) can be observed in the clonal area.

compensated. Furthermore, this effect occurs without ectopic expression of transgenes and partially depends on *stat92E* gene dosage.

Removal of *dpias* Activity in LOF Eye Clones. The effect of replacing both WT copies of the *dpias* gene with the mutant *dpias*^{03697} alleles in eyes was examined next. We used the yeast recombinase (flipase) system (26) to generate clonal patches of mutant homozygous *dpias*^{03697} cells (from now on called *dpias*$^{-/-}$) within heterozygous phenotypically WT flies. Fig. 4*A Left* shows an example of an SEM of an eye with a *dpias*$^{-/-}$ clone extending along the dorsoventral midline. Fig. 4*A Right*, an enlarged view of the center of this eye, shows that the lens structure completely failed to develop and was replaced by a heterogeneous bulged-out surface lacking bristles. Partially differentiated lenses surrounded the border of the clone.

Sections through *dpias*$^{-/-}$ clones (Fig. 4*B Right*) revealed that cellular differentiation into photoreceptors and other cell types had failed, especially in the center of these clones. Along the clonal borders, partially differentiated ommatidia could be seen with incomplete sets of photoreceptors. (Fig. 4*B Left* indicates schematically the area of the clone in gray.) Other *dpias*$^{-/-}$ clones (not shown) had apparently undergone necrotic and/or apoptotic cell death, because scars in the eyes were found frequently. These results indicate that *dpias* gene function plays a critical role in proper growth, differentiation, and survival of potentially all apparent cell types in the developing eye.

Discussion

From the recent completion of the genomic sequence and annotation of the *Drosophila* genome, there seems to be only a single STAT gene, *stat92E*, and now a single PIAS homologue, *dpias*. We show here that the tyrosine-phosphorylated STAT92E and dPIAS can interact directly and specifically, and that a *P* element inserted in the *dpias* gene suppresses *dpias* mRNA formation.

Tumor Formation in the Dysregulated JAK-STAT Pathway. The overgrowth of plasmatocytes (20) and melanotic abdominal tumor formation caused by the *hop*$^{Tum\text{-}l}$ allele presumably depends on too much activated STAT92E, because *stat92E* LOF mutants such as *stat*^{HJ} suppress tumor formation (32). By the same logic, we infer that dPIAS regulates the number of active STAT92E

molecules, because increased dPIAS decreases tumor formation and decreased dPIAS increases tumor formation, indicating that HOP, STAT92E, and dPIAS act together in this pathway. This type of behavior—genetic removal increasing tumorigenesis and overexpression reducing tumorigenesis—is characteristic of genes in mammals that are labeled tumor-suppressor genes. By this definition, *dpias* would be a tumor suppressor. Recent widespread reports of persistently active STAT3 in a variety of human tumors (22) and the demonstration of an engineered constitutively active STAT3 as an oncogene (36) coupled with the present results predict that mutations in human PIAS3 might very well allow for persistent activation of STAT3, resulting in tumor formation. This interpretation is further supported by recent findings of Hari *et al.* (23): Certain transheteroallelic *dpias* [*Su(var)2–10*] LOF alleles in otherwise genetically WT backgrounds caused melanotic tumors in third instar larvae.

***stat92E* and *dpias* Interact in Eye Development.** A dramatic developmental role of *dpias–stat92E* interaction was found in eye development. Overexpression of dPIAS early (driven by *ey*-Gal4) aborts eyes, but loss of *stat92E* function later (by overexpression of dPIAS under the control of GMR-Gal4) has no apparent detrimental effect on cell growth or survival. Similar observations were made by Papayannopoulos *et al.* (35), using the same Gal4 driver stocks in combination with other early and late eye genes. Therefore, factors controlling *stat92E* function must be normally balanced in a critical time window in early eye development. Further increased expression of *dpias* or coupling with heterozygosity for the *stat92E* LOF allele $stat^{06346}$ led to transformation events with antennae frequently replacing eyes. LOF alleles of the *Drosophila* JAK-kinase *hop* of increasing severity cause the same sequence of increasing phenotypic abnormalities (17). Moreover, os^{1}, a hypomorphic LOF allele of a JAK-STAT pathway ligand, results in small eyes (16, ‖). This phenotype could be partially suppressed and the eye size increased by reducing the *dpias* gene dosage, implying that with no transgenic intervention, dPIAS and STAT92E naturally interact in eye formation and eye determination.

In results to be described elsewhere, we have used a naturally occurring dominant negative *stat92E* to examine effects on eye development and find phenotypes similar to *dpias*(537) overexpression. The two sets of data substantiate a previously unrecognized role for STAT92E in growth, cell survival, and determination in early eye development.

The Consequences of Removal of dPIAS Activity on Eye Development. Through somatic recombination, patches of eye cells were created that presumably lack dPIAS, leaving any activated STAT92E unopposed. Under these conditions, none of the apparent retinal cell types differentiated normally.

The lack of, or abnormal differentiation of, lens structure observed on the surface of $dpias^{-/-}$ clones, in particular in the clonal border areas (see *Insert* in Fig. 4*A Right*), appears to be phenotypically similar to *Notch* GOF phenotypes as reported in an earlier study (37). Overexpression of activated *Notch* delayed the differentiation of cone cells, the cells that secrete the lens material. Therefore, we infer that cone cell differentiation in surviving $dpias^{-/-}$ clones might be similarly affected.

We also found that in sections through $dpias^{-/-}$ clones, retinal cellular differentiation failed and was replaced by a heterogeneous cell mass. Other clones had apparently undergone either apoptotic or necrotic cell death, as indicated by frequent scars. Which of theses diverse phenotypes might be caused by unopposed overactive STAT92E remains to be seen. It will be important to learn whether members of the mammalian PIAS genes are playing related roles in STAT-dependent tumor suppression, cell death, and differentiation.

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