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Utilization of *Drosophila* Eye to Probe the Functions of Two Mammalian Serine/Threonine Kinases, Snk and HsHPK

Mei-Ann Liu[a] A-Mei Huang[c] Chen-Kung Chou[b,c] Gwo-Jen Liaw[c,d] Cheng-Wen Wu[a]

[a]National Health Research Institutes, [b]Department of Medical Research, Veterans General Hospital, [c]Institute of Genetics, [d]Department of Life Science, National Yang-Ming University, Taipei, Taiwan, ROC

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Abstract
Here we report a quick functional analysis of two mammalian serine/threonine kinases, a serum inducible kinase (Snk) and *Homo sapiens* hepatoma protein kinase (HsHPK), using *Drosophila* eye as a model system. We generated transgenic fly lines carrying constructs of both kinases under control of the *GAL* upstream activating sequence (UAS). Each UAS line was then crossed to a line in which GAL4 expression was driven by one of the following promoters, *eyeless (ey), glass* or *decapentaplegic*. Thus, different kinase mutants can be ectopically expressed in a promoter-dependent manner. We observed that the ectopic expression of either the wild-type or active form of Snk driven by the *glass* promoter resulted in a rough-eye phenotype. Nevertheless, the ectopic expression of HsHPK under the control of the *ey* promoter resulted in a small-eye phenotype. The results of this study demonstrated that ectopic expression of these two mammalian genes could be achieved by the regulation of *Drosophila* promoters. In addition, the effects of these ectopically expressed genes on eye development could be an implication of their functions with respect to cell proliferation and differentiation. Thus, *Drosophila* eye, with the powerful genetic tools and vast information on eye development available, can be a useful system to probe the functions of mammalian genes in the postgenome era.

Introduction

As the postgenome era approaches, analyzing the massive amount of sequence information obtained (bioinformatics) and studying the functions of the genes identified (functional genomics) are becoming critical. Here we report an approach to expressing two mammalian serine/threonine kinases, a serum inducible kinase (Snk) and *Homo sapiens* hepatoma protein kinase (HsHPK), in the *Drosophila* eye as a way to study the in vivo functions of these genes. The adult *Drosophila* compound eye is the result of a series of precise developmental events [33, 35]. The highly regular and reiterative nature of the compound eye structure makes it easy to identify any disruption of the otherwise perfect lattice structure. Thus, even

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G.-J. Liaw
Department of Life Science, National Yang-Ming University
155, Li-Nong street, Sec. 2, Peitou
Taipei (Taiwan)
Tel. +886 2 2826 7232, Fax +886 2 2820 2449, E-Mail gjliaw@ym.edu.tw

subtle defects within a single ommatidium would commonly result in easily recognizable phenotypes such as a rough or small eye. In addition, the viability and fertility of the fly are usually not severely affected by any defects in their eyes, which makes it possible to maintain and analyze flies even when the eye development of those flies is severely interfered with. Furthermore, a wealth of information on the development of fly compound eyes has been accumulated [32]. The adult compound eye consists of approximately 750 unit eyes, or ommatidia, with 8 photoreceptors and 11 accessory cells in each unit, which is derived from the eye imaginal disc in larvae. Many genes and signaling pathways have been identified to be important for eye development. During embryonic development, eye master genes such as *eyeless (ey)*, *eye gone*, *sine oculis*, *eye absent* and *dachshund* specify eye disc formation. Starting from the third larval stage, the originally proliferative and unpatterned cells of the eye disc gradually differentiate to generate neuronal photoreceptor cells as well as other cell types. These cells are eventually assembled into patterned ommatidia and in turn are organized into a lattice compound eye. The differentiation and pattern formation of the ommatidium are synchronously initiated in the morphogenetic furrow (MF) as a wave moves from the posterior end toward the anterior of the disc [35]. The initiation of ommatidia differentiation requires the activities of *hedgehog*, *decapentaplegic* (*dpp*) and *wingless* signaling. During ommatidia differentiation, many transcription factors, such as *glass*, *atonal*, *hairy*, *extradenticle*, *Yan*, *pointed* and *Seven in absentia*, and two protein tyrosine kinase signaling pathways, *sevenless* and *Drosophila* EGF receptor, are involved in the formation of photoreceptor and border cells [for reviews, see ref. 5, 33]. Although loss-of-function mutations often reveal the function of a gene, many of them do not give any obvious phenotype, presumably due to gene redundancy. On the other hand, gain-of-function mutations have been quite helpful in suggesting functions in vivo. Numerous examples, including the expression of constitutively activated oncogenes and the misexpression of homeotic genes, show abnormal eye formation. Here, we ectopically expressed two mammalian genes in the *Drosophila* eye, in the hope that the ectopic expression of these genes would interfere with eye development, since many of the regulatory genes are evolutionarily conserved. Once a phenotype is detected, we can then analyze the mechanism by which these misexpressed genes interfere.

Snk is transcriptionally activated within 1 h of mitogenic stimulation without requiring de novo protein synthesis [30]. Sequence analysis indicates that Snk is closely related to a family of protein kinases, known as the polo-like kinase family, which have been implicated in cell cycle regulation. Members of this kinase family include Fnk/Prk in mice and humans [6, 18], polo in *Drosophila melanogaster* [19], CDC5 in *Saccharomyces cerevisiae* [15], plo1 in *Schizosaccharomyces pombe* [22], Plk/STPK13 in mice and humans [4, 9, 11, 12, 17], Plx1 in *Xenopus* [16], tbplk in *Trypanosoma brucei* [10] and Plc1/2 (plk-1) in *Caenorhabditis elegans* [2, 23]. Mouse Snk and *Drosophila* polo share 49% homology at the protein level. Genetic and biochemical evidence indicates that many of the polo-like kinases play important roles during mitosis. These include centrosome maturation, bipolar spindle formation, cdc25 activation, cyclin-dependent kinase-cyclin complex inactivation and cytokinesis [7, 8, 20, 21]. Snk appears to be distinct from other members of the polo family, largely because of its mitogen-inducible expression, and Snk is temporally and spatially expressed in developing mouse embryos, especially in the nervous system [Liu, unpubl. data]. The pattern of Snk expression suggests that Snk may play a role in regulating cell growth and differentiation. However, at present, the role of Snk in cellular proliferation is largely unclear.

HsHPK, also known as protein kinase U-alpha (PKU-α) and *Homo sapiens* Tousled-like kinase-2, is a member of an evolutionarily conserved protein kinase subfamily. Closely related homologs can be found in species ranging from the nematode [34], mosquito [3], fruit fly (GeneBank AF181637), zebrafish [Huang, pers. commun.], plant [25] and mouse [28], to human [14, 29, 36]. The most studied member of this subfamily is the *Arabidopsis thaliana* Tousled gene, which is essential for the normal development of leaves and flowers [24, 25]. In humans, HsHPK mRNA is most abundant in the testes, and is moderately expressed in many other tissues, but there is very little in the liver, brain, kidney or lung [14, 29, 36]. Huang et al. [14] reported that the expression of HsHPK is elevated in many hepatoma tissues, implying that this protein may play a role in the development of human hepatoma. Overexpression of HsHPK causes G2/M arrest in both human 293T cells and fission yeast [13]. These studies suggest that HsHPK/PKU-α might play a role in the regulation of cell growth. Yet the mechanism by which HsHPK/PKU-α functions in cell cycle regulation is unclear.

To further elucidate the function and regulation of Snk and HsHPK, we introduced them into *Drosophila* and induced their expression in the developing compound eye using three different promoters, *ey*, *dpp* and *glass*. The *ey* promoter would drive the introduced gene to be expressed

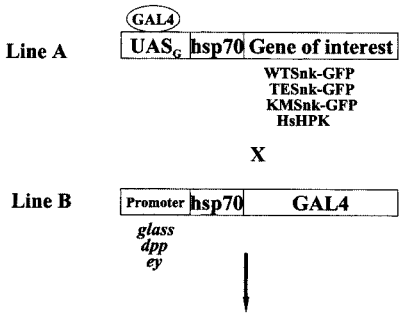

Fig. 1. Schematic diagram showing the generation of transgenic fly lines using the *GAL4-UAS* system [1]. Independent transgenic fly lines (Line A) carrying genes of interest downstream of the *Gal4* UAS were generated. Virgin females from Line A were mated with males from GAL4 lines (Line B) expressing the yeast transcriptional activator GAL4 under the control of the *glass* (GMR24), *dpp* or *ey* promoter. Thus, genes of interest can be ectopically expressed in a promoter-dependent manner. WT = Wild type; TE = active; KM = kinase dead.

in cells before the MF where the cells are actively proliferating. The *dpp* promoter would drive the gene to be expressed in the MF where the differentiation initiated. The *glass* promoter drives the gene to be expressed in cells behind the MF, where they undergo further differentiation and maturation. When *snk* and *hshpk* were ectopically expressed, driven by the *glass* and *ey* promoters, rough- and small-eye phenotypes were observed, respectively. These results, along with data obtained from previous studies, suggest that their functions are conserved between mammals and fly.

Materials and Methods

Green fluorescent protein (GFP) was fused to the C terminus of wild-type, activated and kinase-dead mutants of mouse Snk to form Snk-GFP fusion genes [Liu, unpubl. data]. Two oligonucleotides, CTCGAGAATTCaagtaggcagtgtcgggtg and CGGGATCCtgtaagagcatgttcaggc (letters in lower case represent the sequence of the *snk* coding region), were used to amplify the *snk* coding sequence, which was cloned into *Eco*RI and *Bam*HI sites of the pEGFP-N1 vector (Clontech Laboratories Inc., Palo Alto, Calif., USA). Different Snk-GFP mutants were then cloned downstream of the *Gal4* upstream activating sequence (UAS) into the *Eco*RI and *Not*I sites of a P element vector, pUAST [1]. At least six independent transgenic fly lines for each construct were generated using P element-mediated germ line transformation [27, 31]. Virgin females from each UAS line were mated with males from one of the three different GAL4 lines expressing the yeast transcriptional activator GAL4 under the control of the *glass* (GMR24), *dpp* or *ey* promoter. Similarly, a 2.1-kb *hshpk* cDNA [14], an *Eco*RI-*Xho*I DNA fragment which lacks the N-terminal 188 amino acids, was cloned into the *Eco*RI and *Xho*I sites of the pUAST vector, and the resulting transgenic fly lines were also crossed with the three different GAL4 lines. Thus, different Snk as well as HsHPK mutants can be ectopically expressed in a promoter-dependent manner (fig. 1). The morphology and histology of the affected compound eyes were examined using scanning electron microscopic and histological techniques [26].

Results and Discussion

Ectopic Expression of Snk Induces a Rough-Eye Phenotype

For all three promoters used to drive ectopic expression of Snk-GFP, only *glass*-driven expression gave an obvious eye phenotype. Since Snk was expressed as an Snk-GFP fusion protein, its expression level could be easily examined and quantified using confocal microscopy. Figure 2 shows that Snk-GFP was indeed expressed in a pattern similar to that of *glass*, posterior to the MF in the eye imaginal disc of the third-instar larvae. We observed that the ectopic expression of either the wild-type or active form of Snk resulted in a rough-eye phenotype (fig. 3B, C compared with A and F, G compared with E). Flies grown at 29°C had a higher level of expression of the transgene than those grown at 25°C (data not shown). The severity of rough eye was correlated with the amount of Snk-GFP protein detected in the third-instar eye imaginal disc (fig. 3F, G compared to B, C and data not shown). Upon examination of histological sections of the adult compound eyes, we observed that the lattice array became somewhat irregular with a reduced number of photoreceptor cells in 30–50% of ommatidia (fig. 4, arrowheads in B and C compared with arrows). Again, the reduction in photoreceptor cells is correlated with the amount of Snk-GFP protein detected in the third-instar eye imaginal disc (data not shown). The ectopic expression of kinase-dead Snk-GFP failed to show the strong rough-eye phenotype (fig. 3D, H, 4D), suggesting that the induction of this phenotype required its kinase activity. Our results indicate that Snk may disrupt the differentiation of neuronal cells, thus affecting the formation of photoreceptors. They are also consistent with the observation that Snk is highly expressed in the developing murine nervous system, suggesting a role in neuronal differentiation [Liu, unpubl. data]. The mechanism by which Snk interferes with eye development is under investigation.

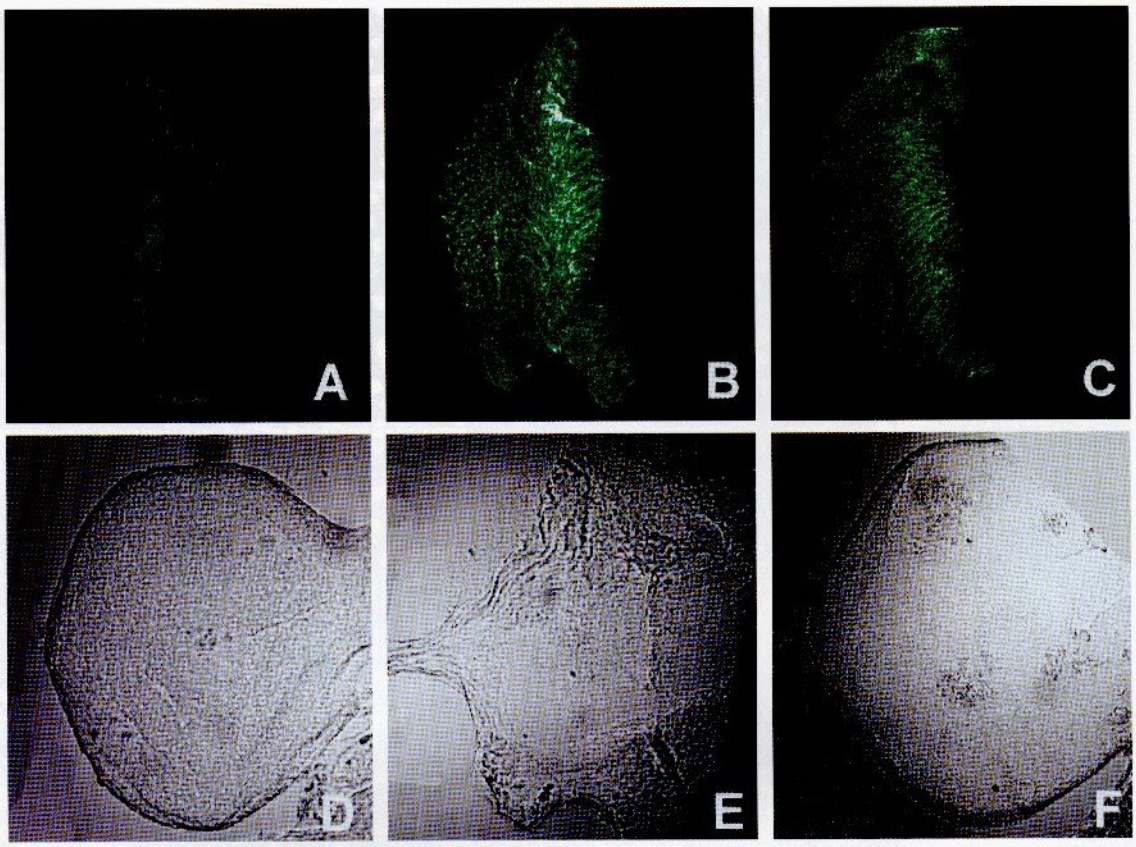

Fig. 2. Ectopic expression of Snk-GFP in the third-instar eye imaginal disc. **A–C** Expression of *UAS-Snk-GFP* transgenes driven by the *glass* promoter. Fluorescent micrographs show that wild-type (**A**), activated (**B**) and kinase-dead (**C**) Snk-GFP are expressed in cells posterior to the MF in the third-instar eye imaginal disc. **D–F** Differential interference contrast photomicrographs showing the same eye discs as in **A–C**. Anterior in all panels is to the right.

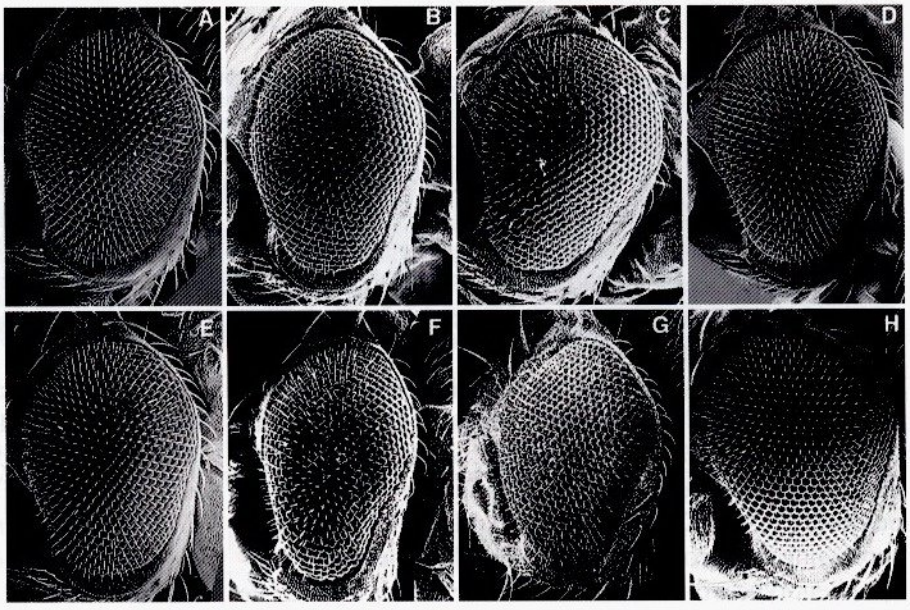

Fig. 3. Misexpression of both wild-type and active Snk-GFP causes rough-eye phenotypes in *Drosophila*. Scanning electron micrographs of the eyes of transgenic flies grown either at 25 °C (**A–D**) or at 29 °C (**E–H**). **A**, **E** Control flies (GMR24) expressing only GAL4 driven by the *glass* promoter. **B**, **F** Transgenic flies expressing wild-type Snk-GFP driven by *GMR-GAL4*. **C**, **G** Transgenic flies expressing constitutively activated Snk-GFP driven by *GMR-GAL4*. **D**, **H** Transgenic flies expressing kinase-dead Snk-GFP driven by *GMR-GAL4*. Posterior is to the right. Dorsal side is to the top.

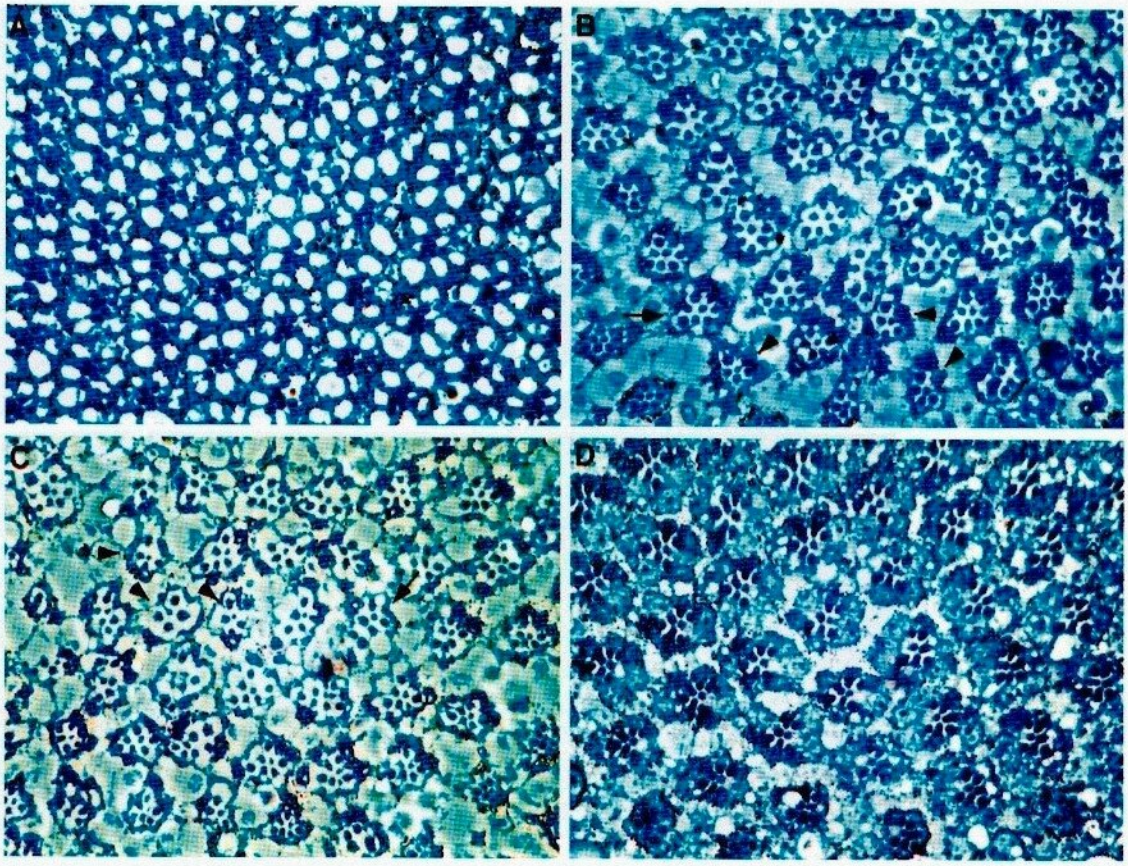

Fig. 4. Misexpression of both wild-type and active Snk-GFP reduces photoreceptor cells in ommatidia. Phase-contrast photomicrographs of tangential sections through the adult retina of flies grown at 25°C. **A** GMR24. **B** GMR-driven wild type. **C** GMR-driven active form. **D** GMR-driven kinase-dead form. Sections were stained with toluidine blue. Arrowheads indicate ommatidia with fewer photoreceptors. Arrows indicate ommatidia with the normal number of photoreceptors.

Ectopic Expression of HsHPK Induces a Small-Eye Phenotype

Although the HsHPK construct used in this study lacks its N-terminal 188 amino acids, it still contains the coiled-coil and the complete kinase domains, and the enzymatic activity of this protein remains, as demonstrated by in vitro kinase assays [14]. When HsHPK was ectopically expressed under the control of the *glass* or *dpp* promoter, no obvious phenotype was observed. However, a small-eye phenotype was induced when HsHPK was expressed under the control of the *ey* promoter (fig. 5B–D). The severity of the small-eye phenotype was correlated with increasing GAL4 activity by raising flies at a higher temperature (fig. 5C, D compared to B). Most of the HsHPK-expressing flies grown at 29°C showed a moderate small-eye phenotype with about 50 fewer ommatidia on average than that of the wild type (fig. 5C: 630 ± 17, compared to fig. 5A: 679 ± 23) and of control flies carrying either *ey-GAL4* or *UAS-HsHPK* (data not shown). However, about 10% of the HsHPK-expressing flies grown at 29°C showed a more severe small-eye phenotype with approximately 130 fewer ommatidia (fig. 5D: 547 ± 66, compared to fig. 5A). The reason for the more severe small-eye phenotype is unclear. Interestingly, the compound eyes of these flies always collapsed when samples were processed for scanning electron microscopy (as seen in fig. 5D). Analyses of histological sections of adult compound eyes showed that the photoreceptors are larger, with a smaller interommatidial space, in the HsHPK-expressing flies (fig. 6B, C compared to A). The number of photoreceptors in each ommatidium appeared to be normal. These results indicate that HsHPK is not involved in photore-

ceptor differentiation during eye development. Instead, HsHPK may be involved in the process which determines the overall ommatidial numbers during eye development. The mechanism by which HsHPK affects eye development is also under investigation.

Comments

The observation that ectopic expression of these two mammalian genes can elicit eye phenotypes indicates that they can interact and interfere with components in pathways regulating eye development in the fly. The observed interfering effect is specific, since only expression in specific cells can lead to an abnormal phenotype. Snk has no detectable effect when expressed in early, undifferentiated eye disc cells (driven by *ey-GAL4*) or in cells in the MF (driven by *dpp-GAL4*). However, Snk causes a loss of some photoreceptors when expressed in differentiating

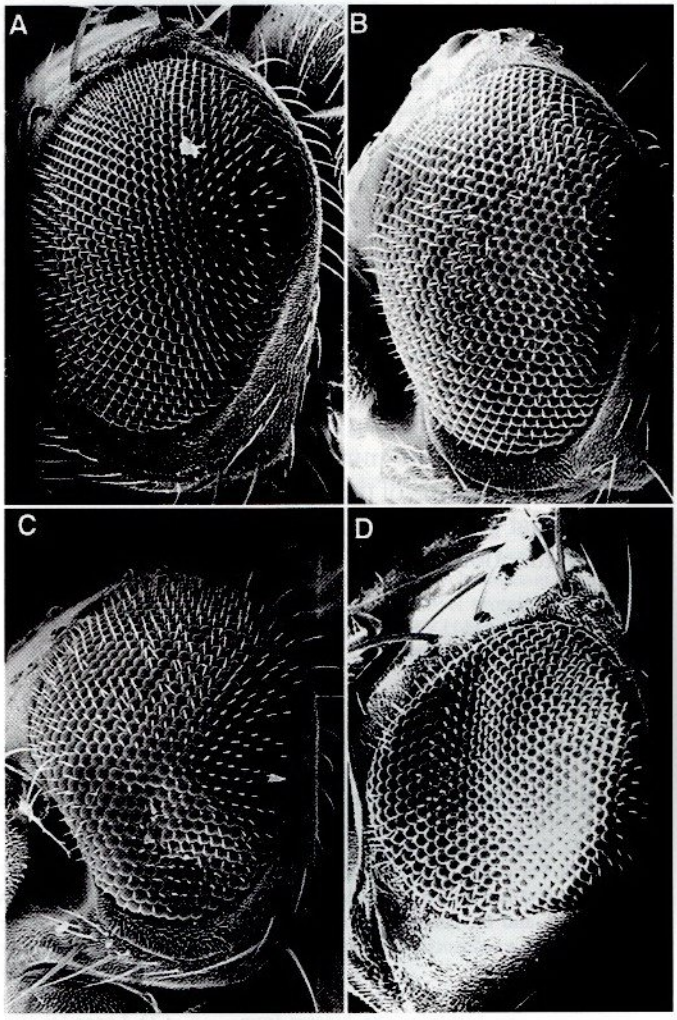

Fig. 5. Misexpression of HsHPK results in a small-eye phenotype. Scanning electron micrographs of the eyes of the wild-type as well as transgenic flies grown either at 25°C (**A**, **B**) or at 29°C (**C**, **D**). **A** Wild-type (Oregon R) fly with 679 ± 23 ommatidia in each compound eye. **B–D** Transgenic flies expressing HsHPK driven by *GMR-GAL4*. **C** Ninety percent of HsHPK-expressing flies show a moderate small-eye phenotype with 630 ± 17 ommatidia in each compound eye. **D** Ten percent of HsHPK-expressing flies show a more severe small-eye phenotype with only 547 ± 66 ommatidia in each compound eye. Posterior is to the right. Dorsal side is to the top.

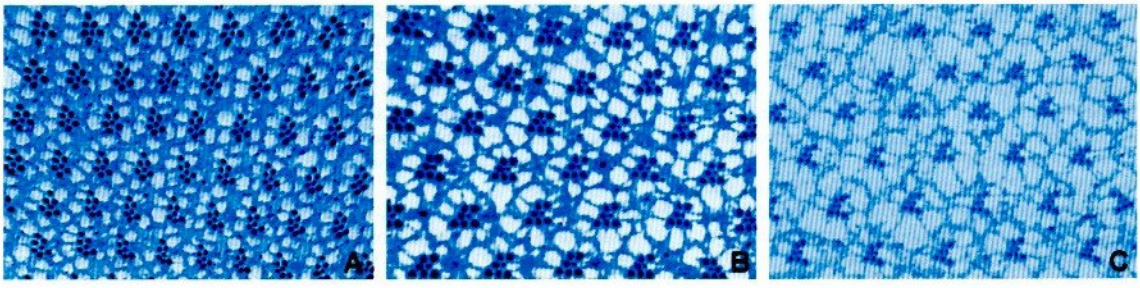

Fig. 6. Cellular disorganization in each ommatidium may contribute to a more severe small-eye phenotype. Phase-contrast photomicrographs of tangential sections through the adult retina of flies grown at 29°C. **A** Control flies ectopically expressing only the *GAL4* gene driven by the *ey* promoter *(ey-GAL4)*. **B**, **C** Transgenic flies expressing HsHPK driven by *ey-GAL4*. **B** Flies with a moderate small-eye phenotype. **C** Flies with a more severe small-eye phenotype.

eye disc cells (driven by *GMR-GAL4*). These results, consistent with those obtained from previous studies [29, Liu, unpubl. data], imply that the function of these two mammalian genes is conserved between mammals and fly. Currently, we are in the process of determining which photoreceptors are specifically affected. In contrast, expression of HsHPK has no effect on later stages of eye development, but causes an effect when expressed in undifferentiated eye cells (driven by *ey-GAL4*). To further determine the process in eye development with which these genes interfere, genetic interaction with genes known to affect the early (for HsHPK) or late (for Snk) developmental process can be examined. Furthermore, isolation of second-site mutations that act as dominant enhancers/suppressors of the elicited phenotype can identify genes that genetically interact with these two kinases. Thus, the functions of these two serine/threonine kinases can first be analyzed in flies and then tested in vertebrate systems. In summary, we report here two examples of misexpressing genes in *Drosophila* eye as an efficient method to probe the functions of mammalian genes. The effects of these ectopically expressed genes on eye development can be an implication of their functions with respect to cell proliferation and differentiation.

Acknowledgments

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