Oncogene (2006) 25, 5933–5941

www.nature.com/onc

ORIGINAL ARTICLE

The *Drosophila* homolog of the human tumor suppressor gene *BHD* interacts with the JAK-STAT and Dpp signaling pathways in regulating male germline stem cell maintenance

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Birt–Hogg–Dubé syndrome (BHD) is a rare, inherited genodermatosis characterized by hair follicle hamartomas, kidney tumors and spontaneous pneumothorax. The *BHD* locus was mapped to chromosome 17p11.2 by linkage analysis, and germline mutations in a novel gene (*BHD*) were identified in a panel of BHD families. Using RNA interference to decrease expression of the *Drosophila* BHD homolog (*DBHD*), we have demonstrated that DBHD is required for male germline stem cell (GSC) maintenance in the fly testis. Reduction of *DBHD* gene activity suppresses the GSC overproliferation phenotype associated with overexpression of either *unpaired* (*upd*) or *decapentaplegic* (*dpp*). Further genetic interaction experiments suggest that DBHD regulates GSC maintenance downstream or in parallel of the JAK/STAT and Dpp signal-transduction pathways. These findings suggest that the BHD protein may regulate tumorigenesis through modulating stem cells in human.
Oncogene (2006) **25,** 5933–5941. doi:10.1038/sj.onc.1209593; published online 24 April 2006

Keywords: tumor suppressor; *Drosophila* BHD homolog; male germline stem cell; stem cell maintenance; JAK/STAT signaling pathway; Dpp signaling pathway

Introduction

Birt–Hogg–Dubé syndrome (BHD) is characterized by benign skin papules (fibrofolliculomas), lung cysts, spontaneous pneumothorax and an increased risk for developing kidney tumors (Birt *et al*., 1977; Schmidt *et al*., 2001; Zbar *et al*., 2002). The most common form of kidney tumor seen in BHD patients is an oncocytic hybrid tumor that comprises features of both chromophobe renal carcinoma and renal oncocytoma (Pavlovich *et al*., 2002). Germline mutations in a novel gene at chromosome 17p11.2 were identified in a panel of BHD families (Nickerson *et al*., 2002). The human *BHD* gene encodes a protein, folliculin, with an open-reading frame of 579 amino acids (AAs). Folliculin contains a glutamic acid-rich, coiled-coil domain with no significant homology to any known human protein. However, folliculin homologs were identified in many species, including *Drosophila*, *Caenorhabditis elegans*, mouse, dog and rat, implying a critical biological role for folliculin. Moreover, germline mutation in the rat and dog homologs of the *BHD* gene also resulted in inherited kidney tumors (Lingaas *et al*., 2003; Okimoto *et al*., 2004), suggesting that the *BHD* gene has a tumor suppressor function. Furthermore, recent evidence of somatic second-'hit' mutations in renal tumors from BHD patients (Vocke *et al*., 2005) strongly supports the Knudson 'two-hit' tumor suppressor model for *BHD*. To understand the biological function of the *BHD* tumor suppressor gene, we studied the biological effect of downregulation of the *Drosophila BHD* homolog (*DBHD*). By using RNA interference (RNAi) to decrease the expression of *DBHD* in *Drosophila*, we demonstrated that DBHD is required for germline stem cell maintenance in the fly testis and functions either downstream or in parallel of the JAK/STAT and decapentaplegic (Dpp) signal-transduction pathways.

Results

Blocking Drosophila BHD *homolog activity through dsRNA-mediated RNAi in flies*

The amino-acid sequence analysis identified a single protein encoded by the *CG8616* gene, which is the *Drosophila* homolog of the human tumor suppressor folliculin. *CG8616*/*DBHD* encodes a protein of 460 amino acids (AAs). Among the 460 amino acids (AAs), 138 amino acids (AAs) (138/460 = 30%) are identical and 211 amino acids (AAs) (211/460 = 46%) are similar

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Received 20 December 2005; revised 14 March 2006; accepted 14 March 2006; published online 24 April 2006

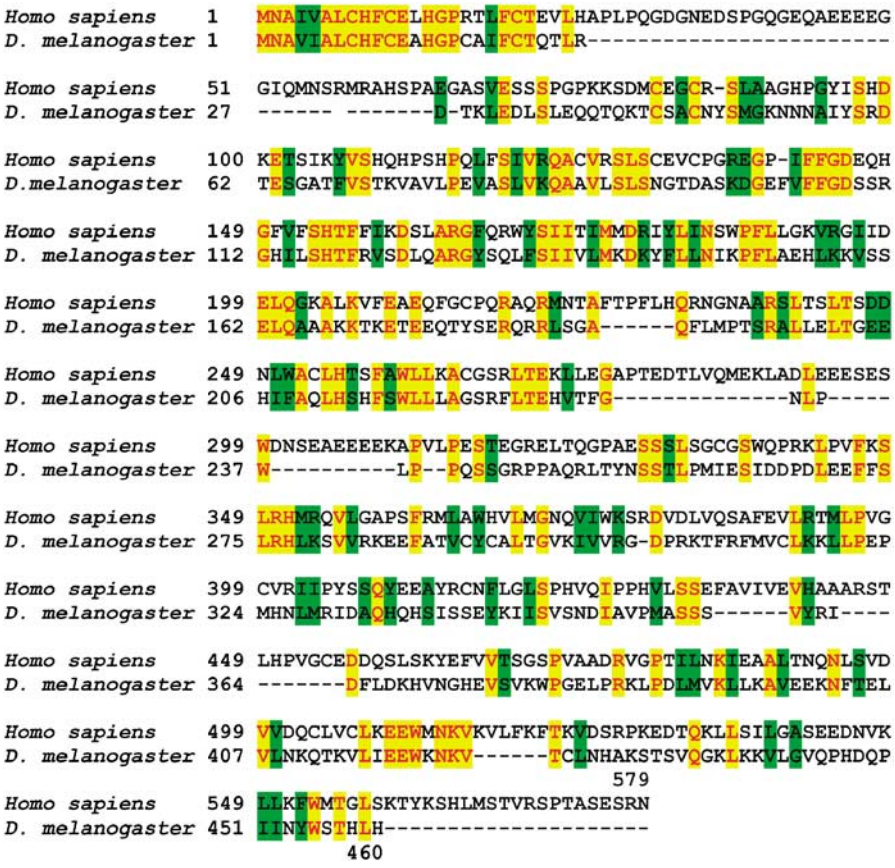

Figure 1 Sequence alignment of human and *Drosophila* Birt–Hogg–Dubé syndrome (BHD) proteins. Identical and similar residues are highlighted with red or green letters, respectively.

to their human counterpart (Figure 1). To study the subcellular localization of DBHD, we fused the coding sequence of DBHD to a V5 tag in a vector that can be expressed in *Drosophila* S2 cell cultures. As shown in Figure 2, V5-tagged DBHD was mainly expressed in the cytoplasm (Figure 2b).

There is no loss-of-function mutation available for *DBHD*. We employed double-stranded RNA (dsRNA)-mediated RNAi in flies to examine loss-of-function phenotypes for *DBHD*. dsRNA encompassing the coding region of *DBHD* was generated by using the UAS-Gal4 system to express sense–antisense transcription of the *DBHD* gene by two convergent arrays of Gal4-dependent *UAS* sequences (Giordano *et al*., 2002). To check the effectiveness of the RNAi technique, we monitored *DBHD* mRNA levels using quantitative reverse transcription–polymerase chain reaction (RT–PCR) (Figure 2a). *Drosophila* Birt–Hogg–Dubé syndrome mRNA levels were reduced to 25% of wild-type levels based on gel densitometry in embryos expressing *iDBHD* under a *V32-Gal4* driver.

Drosophila BHD *homolog is required for male germline stem cell maintenance*

The *Nos-Gal4/UAS-iDBHD* male flies have reduced fertility. We analysed DBHD function in male germline stem cells. *Drosophila* spermatogenesis takes place within the tubular testis (reviewed by Fuller, 1993, 1998). At the tip of the testis is a germinal proliferation center that is composed of a group of 12 non-dividing somatic cells, called the hub, and a small number of germline stem cells (GSCs; 16–18 in larvae, 5–9 in adult; Figure 2c). We first did *in situ* hybridization experiments using *DBHD* antisense RNA probe and found that *DBHD* mRNA is strongly expressed in early-stage germ cells, including GSCs (Figure 2i). We then stained the testes with anti-Fas III (to mark the hub cells), mAb1B1 (to mark the fusomes) and anti-Vasa (to mark the germ cells) antibodies. Expression of *UAS-iDBHD* using a *Nos*-Gal4 driver (Kiger *et al*., 2001; Tulina and Matunis, 2001) resulted in a significant reduction of GSCs (Figure 2e). Although an average of 7.91 GSCs ($n = 52$) could be clearly visualized in the wild-type testis (Figure 2d), an average of 3.70 GSCs ($n = 76$; Figure 2e) was detected in the *Nos-Gal4/UAS-iDBHD* testis. An average of 7.85 GSCs ($n = 60$) was detected in the *Nos-Gal4/UAS-DBHD* testis, indicating that expression of *UAS-DBHD* (*Nos-Gal4/UAS-DBHD*) does not significantly affect the number of GSCs (Figure 2f). In addition, there were holes at spermatogonial locations in the *Nos-Gal4/UAS-iDBHD* testes (Figure 2e–h, green arrows), indicating that *iDBHD* also caused spermatogonia loss.

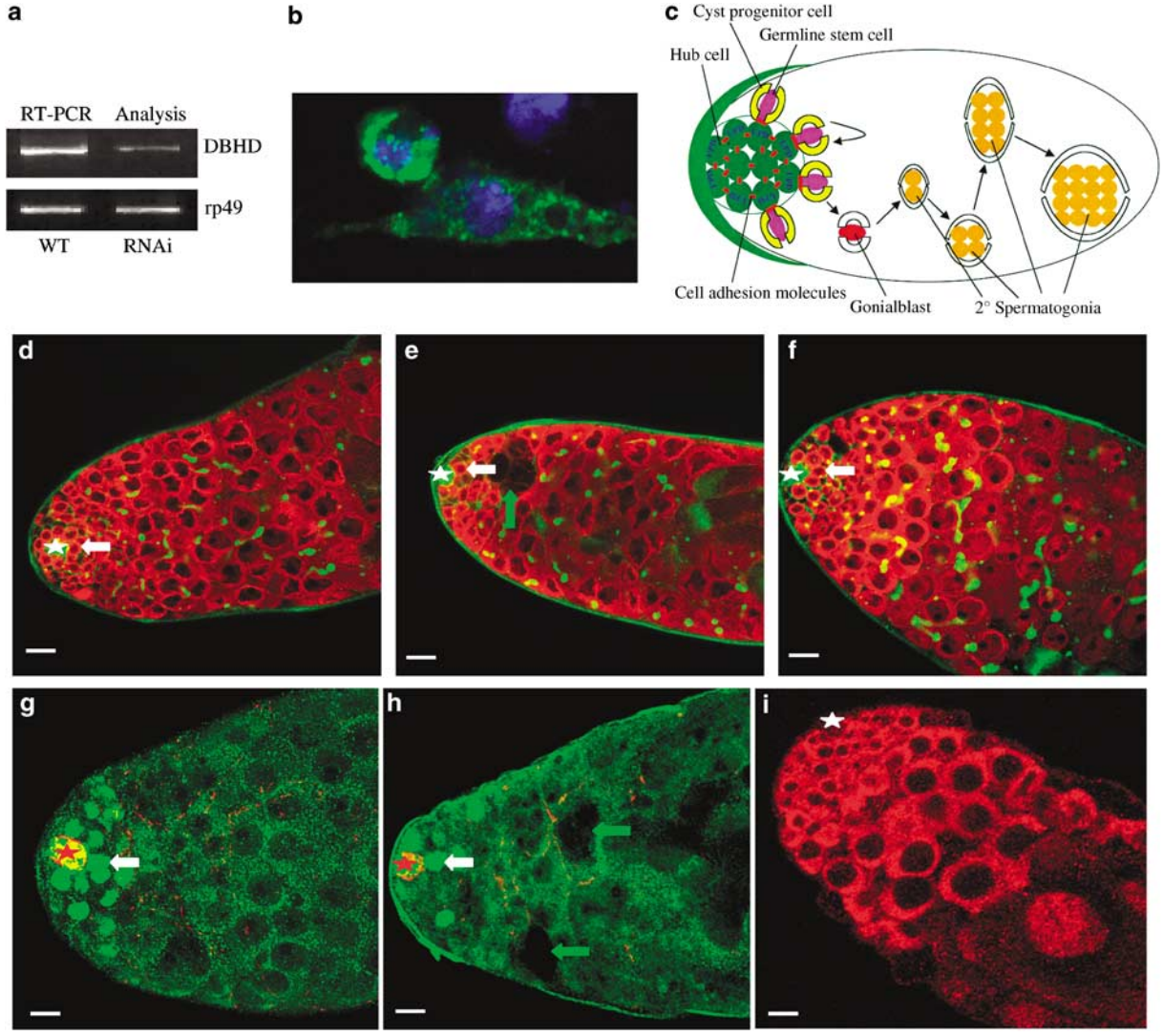

Figure 2 *Drosophila BHD* homolog (*DBHD*) regulates male germline stem cell (GSC) maintenance. (**a**) Reverse transcription–polymerase chain reaction (RT–PCR) analysis of *DBHD* mRNA in *V32-Gal4/+* and *V32-Gal4/UAS-iDBHD* embryos. rp49 mRNA was used as an internal control. In the *V32-Gal4/UAS-iDBHD* embryos, *DBHD* mRNA level is reduced to ~25% of the level observed in *V32-Gal4/+* embryos. (**b**) Expression of V5-tagged DBHD construct in S2 cells. The DBHD protein localizes to the cytoplasm. The cells were double-labeled with V5 antibody (green) to visualize the DBHD protein and 4′,6-diamidine-2′-phenylindole dihydrochloride (blue) to visualize the DNA. (**c**) A sagittal section of the *Drosophila* testis apex is drawn schematically and leaves out most of the cells for clarity. Both the GSCs (pink) and the somatic stem cells (yellow) are anchored around the hub (green). Asymmetric division of both stem cells results in spermatogenic cysts, in which each gonialblast is encased by two somatic cyst cells. Four more consecutive divisions produce a cyst of 16 spermatogonia. (**d**–**f**) Testes immunostained with anti-Vasa antibody to label germ cells (red), anti-Fas III antibody to label the hub (green, stars) and mAb1B1 (green) to label fusomes. In wild-type (**d**) and *Nos-Gal4/UAS-DBHD* (**f**) control testes, seven to nine GSCs (**d**, **f** arrows) contact the hub. In *Nos-Gal4/UAS-iDBHD* (**e**), only three to four GSCs (arrow) contact the hub. (**g**, **h**) Testes immunostained with anti-β-Galactosidase (green) from M5-4 marker and anti-Arm (red, stars). (**g**) Wild-type testis with M5-4 marker. β-Galactosidase (green) is expressed in the hub (red star), GSCs (arrow) and nearby gonialblasts. (**h**) In the M5-4/+; *Nos-Gal4/UAS-iDBHD* testis, the number of β-Galactosidase-positive GSCs and gonialblasts is significantly reduced, but the hub staining looks normal. The testis in (**e**) and (**h**) also has a big hole (green arrows) at the spermatogonia location. (**i**) is *in situ* hybridization of the wild-type testis using *DBHD* antisense RNA probe. Scale bars in (**d**)–(**i**) represent 10 μm.

To further verify the stem cell loss phenotype of *iDBHD* mutation, we examined the expression of an M5-4 marker. The enhancer trap line M5-4 drives β-Galactosidase expression in hub cells, GSCs and gonialblasts (Tran *et al*., 2000; Figure 2g) in wild-type testes. In all *DBHD* mutant testes examined, the number of β-Galactosidase-positive cells decreased dramatically (Figure 2h), whereas the expression of β-Galactosidase and Armadillo (Arm) (Figure 2h) in the hub looked normal in the *iDBHD* mutant testes.

We next examined *bam*-green fluorescent protein (*GFP*) transgene (a *bam* promoter fused to the GFP gene; Chen and McKearin, 2003; Kawase *et al*., 2003) expression. Consistent with earlier reports, we found that *bam* was expressed in two- to 16-cell spermatogonia but not in GSCs and gonialblasts in the wild-type testis

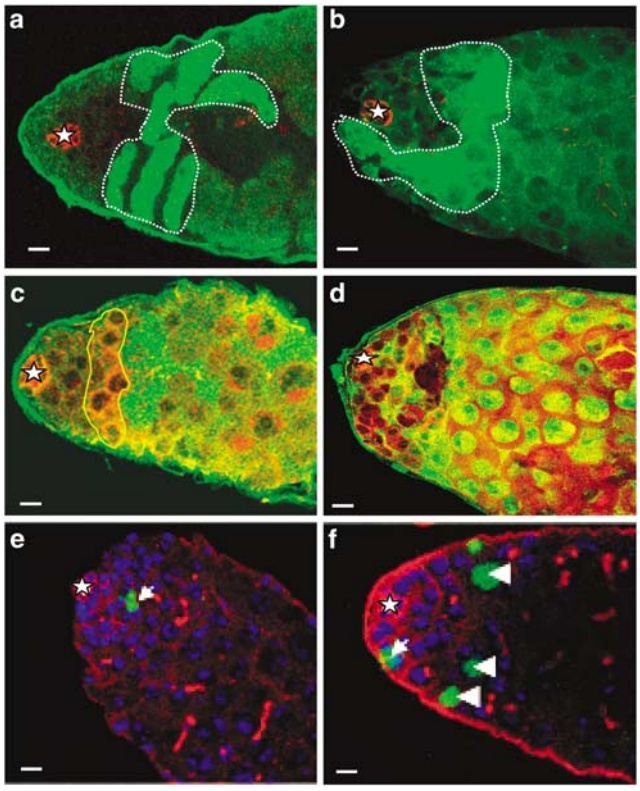

Figure 3 *Drosophila BHD* homolog (*DBHD*) regulates germline stem cell (GSC) differentiation. (**a**, **b**) Bam-GFP expression in wild-type (**a**) and *Nos-Gal4/UAS-iDBHD* (**b**) testes. GFP is expressed in the spermatogonia (green, outlined). White stars mark the Fas III-positive hub. In the *Nos-Gal4/UAS-iDBHD* testis, GSCs are reduced, and GFP-positive spermatogonia move toward the tip. (**c**, **d**) Clonal overexpression of *iDBHD* (GFP-positive cells) in GSCs causes ectopic Bam expression (red) in these cells (**d**). These testes were stained with anti-GFP (green) and anti-BamC (red). Genotypes: *hs-flp/Y; UAS-iDBHD/Act > CD2 > Gal4 UAS-GFP* (**c**) and *hs-flp/Y; UAS-iDBHD/tub > CD2 > Gal4 UAS-GFP* (**d**). (**e**, **f**) Testes stained to detect dying cells (green). White stars mark the Fas III-positive (red) hubs. In the wild-type testis, few dying spermatogonial cysts were detected (**e**, arrow). In the *Nos-Gal4/UAS-iDBHD* testis, increased dying cell clusters at the spermatogonial location (**f**, arrowheads) were detected. Scale bars in (**a**)–(**f**) represent 10 μm.

(Kiger *et al*., 2000; Tran *et al*., 2000; Kawase *et al*., 2003; Figure 3a). In the *Nos-Gal4/UAS-iDBHD* testis, GFP-positive spermatogonia moved forward, toward the tip, because of GSC loss (Figure 3b). As an alternative approach to express *iDBHD* in GSCs, we used the flip-out-Gal4/UAS method (Pignoni and Zipursky, 1997) to generate GFP-marked clones of cells expressing *iDBHD*. We used both *Act > CD2 > Gal4* and *tub > CD2 > Gal4* drivers. In *Act > CD2 > Gal4* testes, GFP-marked clones were only generated in late-stage germ cells and anti-BamC antibody staining was detected in normal spermatogonia (Figure 3c); in *tub > CD2 > Gal4* testes, GFP-marked clones were generated in GSC locations and anti-BamC antibody staining were also detected in these locations (Figure 3d), suggesting that these GFP-marked *iDBHD*-expressing cells have differentiated into spermatogonia.

To determine whether stem cell loss might be caused by cell death, we examined cell death in wild-type ($n=32$) and *Nos-Gal4/UAS-iDBHD* ($n=46$) testes by using an ApopTag Fluorescein Direct Detection Kit. No dying GSCs were detected in either wild-type (Figure 3e) or *Nos-gal4/UAS-iDBHD* (Figure 3f) testes. A few dying cysts (Figure 3e, arrowhead) were detected in the wild-type testis. Significant increases of dying cysts were detected at the spermatogonial location in the *Nos-gal4/UAS-iDBHD* testis (Figure 3f, arrowheads), which may explain the holes observed in the above experiments.

In summary, overexpression of *UAS-iDBHD* in the testis resulted in GSC differentiation rather than cell death.

Drosophila BHD *homolog may function downstream of* unpaired *in regulating germline stem cells*

Hub cells express the ligand *unpaired* (*upd*), which activates the JAK/STAT pathway in adjacent germ cells to regulate GSCs' self-renewal (Kiger *et al*., 2001; Tulina and Matunis, 2001). We examined the interaction between DBHD and the JAK/STAT signal-transduction pathway during male GSC fate determination. Ectopic expression of *UAS-upd* using the *Nos*-Gal4 driver resulted in ectopic cells with GSC and somatic stem cell features, and ballooning of the whole testis (Kiger *et al*., 2001; Tulina and Matunis, 2001; Figure 4a). Simultaneous expression of *UAS-iDBHD* with *UAS-upd* significantly suppressed the phenotypes of overexpressing *UAS-upd* in the testis; the numbers of GSCs were significantly reduced, and the shape of the testes became closer to that of the wild type (Figure 4b). Further, heterozygosity for a deficiency (*Df(3L)Exel6111/+*) that reduces by half the dosages of CG8616 (*DBHD*) and several other genes in chromosome regions 65E7-F4 also significantly suppressed the phenotypes of over-expressing *UAS-upd* in the testis (Figure 4c).

To verify whether *iDBHD* specifically suppresses the number of GSCs in *upd*-overexpressed testes, we examined the expression of the M5-4 marker. In all *Nos-Gal4/UAS-upd* testes examined, the number of β-Galactosidase-positive cells was expanded dramatically (Figure 4d). Simultaneously expressing *UAS-iDBHD* with *UAS-upd* (*UAS-upd + UAS-iDBHD/Nos-Gal4*) significantly suppressed the phenotype of GSC expansion, which is associated with the *upd* overexpression (Figure 4e). As a control, we also co-expressed *UAS-stat92E* with *UAS-upd* (*UAS-upd + UAS-stat92E/Nos-Gal4*; Figure 4f) and observed that simultaneous expression of *UAS-stat92E* with *UAS-upd* does not affect the GSC expansion phenotype caused by the *upd* overexpression. These results suggest that DBHD may function either downstream or in parallel of Upd and specifically regulates GSC proliferation.

Drosophila BHD *homolog may function downstream of STAT92E*

To determine whether DBHD operates upstream or downstream of STAT92E, we tested whether the GSC loss phenotype caused by a *stat92E* mutation could be

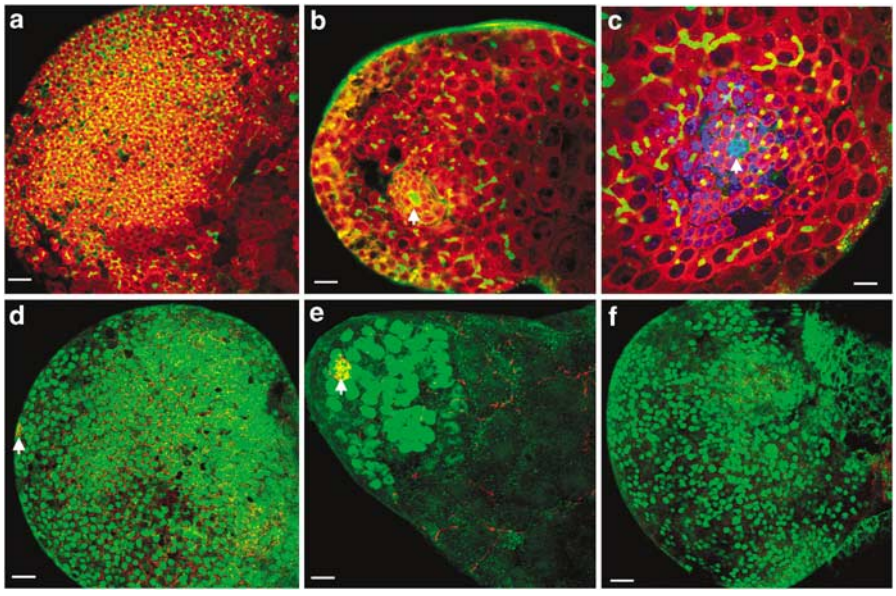

Figure 4 *Drosophila BHD* homolog (*DBHD*) functions downstream of *unpaired* (Upd). (**a**–**c**) Testes immunostained for germ cells (with α-Vasa, red), the hub (with α-Fas III, green) fusomes (with mAb1B1, green), and DNA (with 4′,6-diamidine-2′-phenylindole dihydrochloride, blue). Expressing *iDBHD* (**b**) or reducing *DBHD* dosage by half (**c**) suppresses *upd* overexpression phenotype (**a**). The flies' genotypes are: (**a**) *Nos-Gal4/UAS-upd*; (**b**) *Nos-Gal4/UAS-upd + UAS-iDBHD* and; (**c**)-*UAS-upd/+; Nos-Gal4/Df(3L)Exel6111*. Arrowheads in (**b**) and (**c**) mark the hubs; the hub in (**a**) cannot be clearly identified owing to deformation of the testis. (**d**–**f**) Testes immunostained with anti-β-Galactosidase (green) from M5-4 marker and anti-Arm (red, arrowheads). (**d**) In M5-4/*UAS-upd*; *Nos-Gal4/+* testis, β-Galactosidase-positive germline stem cells (GSCs) and gonialblasts (green) are everywhere. (**e**) In the M5-4/*UAS-upd*; *Nos-Gal4/UAS-iDBHD* testis, the numbers of β-Galactosidase-positive GSCs and gonialblasts are dramatically reduced. (**f**) In the M5-4/*UAS-upd UAS-stat92E*; *Nos-Gal4/+* testis, β-Galactosidase-positive GSCs and gonialblasts (green) are everywhere. The hub in (**f**) can not be clearly identified owing to deformation of the testis. Scale bars in (**a**), (**d**) and (**f**) represent 30 μm; scale bars in (**b**), (**c**) and (**e**) represent 15 μm.

negated by overexpression of DBHD in the testis. If DBHD is required downstream of STAT92E, overexpression of DBHD may suppress *stat92E* loss-of-function phenotypes. We utilized a temperature-sensitive *stat92E* allele (*stat92E*^{F}). *In trans* to a null allele, *stat92E*^{F} flies are normal at room temperature (RT, 22°C), but have little or no STAT92E activity at 29°C (Baksa *et al*., 2002; Brawley and Matunis, 2004; data not shown). We analysed testes from *stat92E*^{F}*/Nos-Gal4.stat92E*^{6C8} and *UAS-DBHD/+*; *stat92E*^{F}*/Nos-Gal4.stat92E*^{6C8} flies raised at RT, and then shifted to 29°C.

The GSCs, spermatogonia and spermatocytes can be distinguished by fusome morphology; GSCs contact the hub with round fusomes; spermatogonia with branching fusomes and spermatocytes are displaced away from the hub in wild-type (Figure 2c), *stat92E*^{F}*/Nos-Gal4.stat92E*^{6C8} (Figure 5a) and *UAS-DBHD*; *stat92E*^{F}*/Nos-Gal4.stat92E*^{6C8} (Figure 5b) flies at RT. After shifting the temperature to 29°C, GSCs, spermatogonia and spermatocytes were gradually lost over time in the *stat92E*^{F}*/Nos-Gal4.stat92E*^{6C8} testes. After 4 days at 29°C, GSCs were completely lost in all *stat92E*^{F}*/Nos-Gal4.stat92E*^{6C8} testes (57/57 = 100%), and only a fraction of the testes (10/57 = 18%) retained spermatogonia (Figure 5c). After 7 days at 29°C, GSCs, spermatogonia and spermatocytes were completely lost in all *stat92E*^{F}*/Nos-Gal4.stat92E*^{6C8} testes (Figure 5e; 48/48 = 100%). However, overexpression of *DBHD* in the *stat92E*^{F}*/stat92E*^{6C8} testes significantly slowed down the germ cell loss phenotypes. After 4 days at 29°C, 26% of the *UAS-DBHD; stat92E*^{F}*/Nos-Gal4.stat92E*^{6C8} testes (16/62) had one or more GSCs (Figure 5d), and most of the testes (51/62 = 82%) retained spermatogonia. After 7 days at 29°C, 8% of the *UAS-DBHD; stat92E*^{F}*/Nos-Gal4.stat92E*^{6C8} testes (4/53) had one or more GSCs, and 64% of the testes (34/53) retained spermatogonia (Figure 5f). These results indicate that overexpression of DBHD partially suppresses *stat92E* loss-of-function phenotypes, and DBHD may function either downstream or in parallel of STAT92E in regulating GSC maintenance.

Decapentaplegic may function downstream of STAT92E

Decapentaplegic, a transforming growth factor (TGF-β) family member, also regulates GSC self-renewal by activating its corresponding signal-transduction pathway in GSC (Kawase *et al*., 2003). To determine whether Dpp operates upstream or downstream of STAT92E, we tested whether the GSC loss phenotype caused by *stat92E* mutation could be negated by overexpression of Dpp in testis. In this experiment, we used *UAS-upd* as a control. We compared testes from *UAS-upd*; *stat92E*^{F}*/Nos-Gal4.stat92E*^{6C8} and *UAS-dpp*; *stat92E*^{F}*/Nos-Gal4.stat92E*^{6C8} flies raised at RT, and then shifted to 29°C. Upd functions upstream of STAT92E, and overexpression of *upd* should not suppress *stat92E* loss-of-function phenotypes. As expected, GSCs were nearly completely lost in the

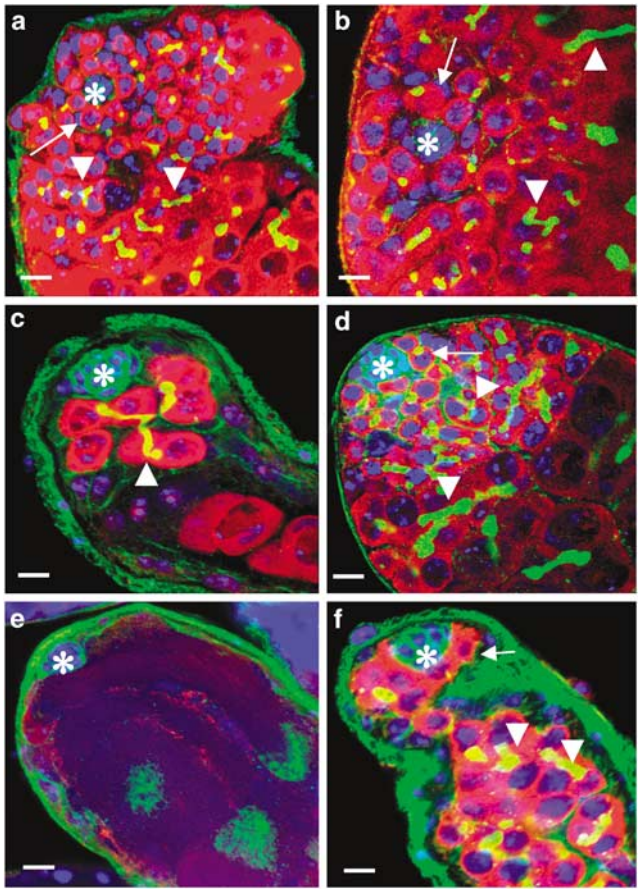

Figure 5 Overexpression of *Drosophila BHD* homolog (*DBHD*) partially blocks germline stem cell differentiation caused by conditional loss of *stat92E*. Testes immunostained for germ cells (with α-Vasa, red), the hub (with α-Fas III, green, asterisk), fusomes (with mAb1B1, green) and DNA (with 4′,6-diamidine-2′-phenylindole dihydrochloride, blue). (**a**, **c**, **e**) *stat92E*^{F}/*Nos-Gal4.-stat92E*^{6C8} testes shifted to 29°C at day 0, day 4 and day 7, respectively. (**b**, **d**, **f**) *UAS-DBHD*; *stat92E*^{F}/*Nos-Gal4.stat92E*^{6C8} testes shifted to 29°C at day 0, day 4 and day 7, respectively. Scale bars in (**a**)–(**f**) represent 10 μm.

UAS-upd; *stat92E*^{F}/*Nos-Gal4.stat92E*^{6C8} testes after 4 days at 29°C (68/69 = 99%), and only a fraction of the testes (9/69 = 13%) retained spermatogonia (Figure 6a). After 7 days at 29°C, GSCs, spermatogonia and spermatocytes were completely lost in all *UAS-upd*; *stat92E*^{F}/*Nos-Gal4.stat92E*^{6C8} testes (Figure 6b; 55/55 = 100%). However, overexpression of *dpp* in the *stat92E*^{F}/*stat92E*^{6C8} testes significantly slowed down the germ cell loss phenotypes. After 4 days at 29°C, 41% of the *UAS-dpp; stat92E*^{F}/*Nos-Gal4.stat92E*^{6C8} testes (24/59) had one or more GSCs (Figure 6c), and all of the testes (59/59 = 100%) retained spermatogonia. After 7 days at 29°C, 17% of the *UAS-dpp; stat92E*^{F}/*Nos-Gal4.stat92E*^{6C8} testes (7/41) had one or more GSCs, and 88% of the testes (36/41) retained spermatogonia (Figure 6d). Because overexpression of *dpp* partially suppresses *stat92E* loss-of-function phenotypes, the Dpp signal-transduction pathway may function either downstream or in parallel of the JAK/STAT signal transduction pathway.

We further investigated the relation between the JAK/STAT and the Dpp signal transduction pathways. Ectopic expression of *UAS-upd* using the *C587-Gal4* driver in the M5-4 testis resulted in moderate expansion of β-Galactosidase-positive cells (Figure 6e). Removing one copy of the Dpp receptor (*tkv*) significantly suppressed the phenotype of *C587-Gal/UAS-upd* in the testis (Figure 6f). These data further suggest that Dpp signal-transduction pathway functions either downstream or in parallel of the JAK/STAT signal-transduction pathway in regulating male GSC maintenance.

Drosophila *BHD may function downstream of the decapentaplegic signal-transduction pathway*

We further examined the relation between DBHD and the Dpp signal-transduction pathway. Ectopic expression of *UAS-dpp* using the *Nos*-Gal4 driver resulted in ectopic GSCs (Kawase *et al*., 2003; Figure 6g). We used the enhancer trap line M5-4 as a GSC marker in this experiment. In all *Nos-Gal4/UAS-dpp* testes examined, the number of β-Galactosidase-positive cells was expanded significantly (Figure 6g). Simultaneously expressing *UAS-iDBHD* with *UAS-dpp* (*UAS-dpp* + *UAS-iDBHD/Nos-Gal4*) significantly suppressed the GSC expansion phenotype associated with the *dpp* overexpression (Figure 6h). These results suggest that DBHD may also function either downstream or in parallel of Dpp in regulating male GSC proliferation.

Discussion

Possible roles of BHD in regulating germline stem cells in the fly testis

Our studies suggest that the Dpp signal-transduction pathway functions downstream of the JAK/STAT signal-transduction pathway, and that DBHD functions downstream of the Dpp in regulating male GSC maintenance. One possible explanation for this result is that the DBHD and the Dpp signal transduction pathway synergize on downstream targets. Although the BHD is a novel protein, some indirect evidence indicates that the BHD may be an RNA-binding protein. First, the DBHD was found to bind an mRNA-binding protein (RBP9) in a yeast two-hybrid screen (flybase, fly GRID). Second, the BHD protein has a weak Pumilio homolog domain (data not shown), and the Pumilio is an RNA-binding protein. In the fly ovary, the Pumilio/Nanos (another RNA-binding protein) and the Dpp/Mad signaling work synergistically to regulate GSC self-renewal and differentiation (Gilboa and Lehmann, 2004; Chen and McKearin, 2005; Szakmary *et al*., 2005). Similarly, the Dpp signaling and the DBHD may also synergistically regulate GSC self-renewal and differentiation in the fly testis.

Our current study demonstrates that the *Drosophila* ortholog of the human tumor suppressor gene *BHD* regulates male GSC maintenance and functions downstream of the JAK/STAT and Dpp signal-transduction pathways. The human BHD protein, folliculin, may

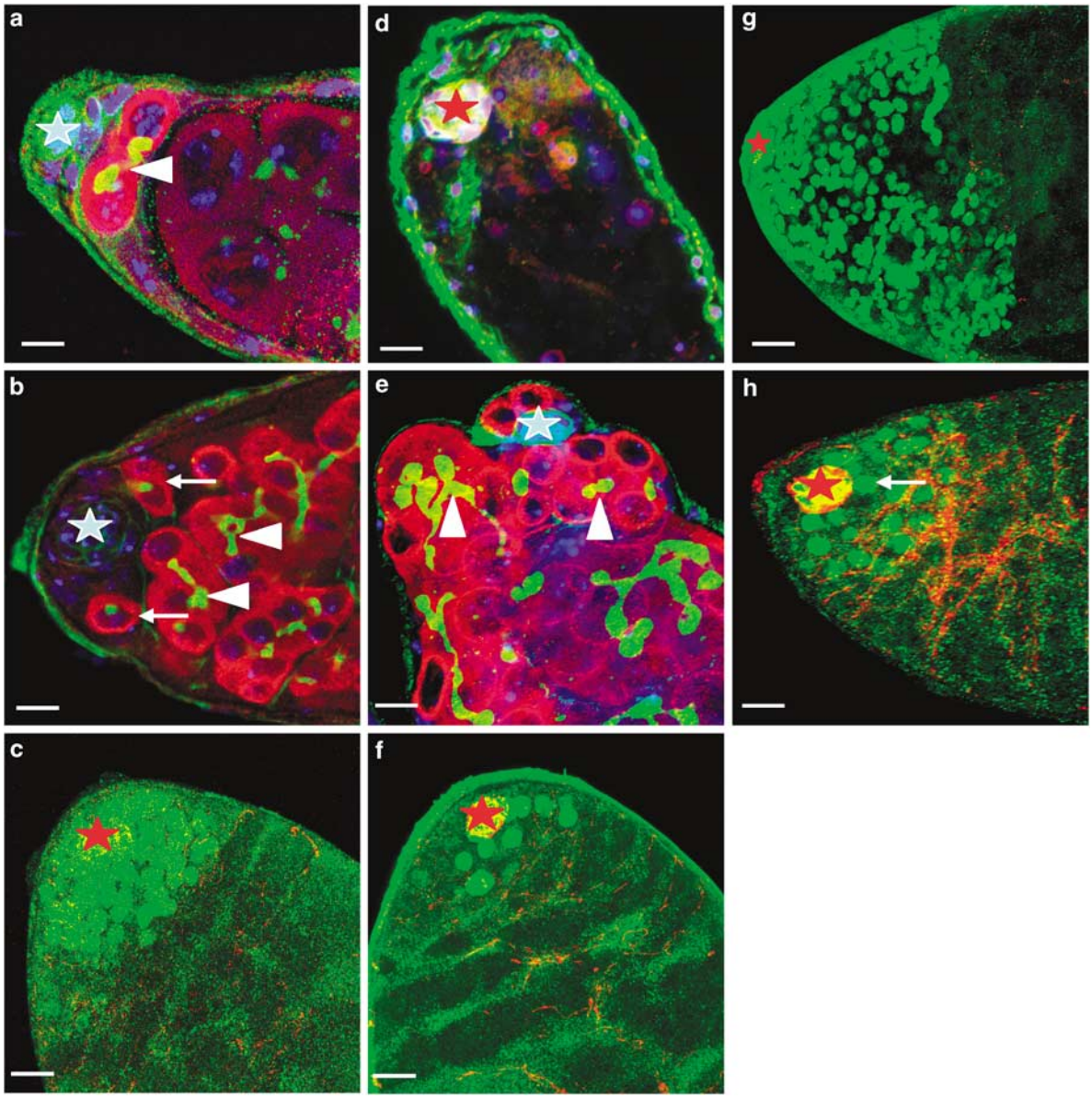

Figure 6 *Drosophila BHD* homolog (*DBHD*) functions downstream of decapentaplegic (Dpp). Testes immunostained for germ cells (with α-Vasa, red), the hub (with α-Fas III, green, asterisk), fusomes (with mAb1B1, green) and DNA (with 4′,6-diamidine-2′-phenylindole dihydrochloride, blue). (**a**, **b**) *UAS-unpaired (upd)*; $stat92E^{F}$/*Nos-Gal4*.$stat92E^{6C8}$ testes shifted to 29°C at day 4 and day 7, respectively. (**c**, **d**) *UAS-dpp*; $stat92E^{F}$/*Nos-Gal4*.$stat92E^{6C8}$ testes shifted to 29°C at day 4 and day 7, respectively. (**e**) *C587-Gal4*/*Y; M5-4 UAS-upd*/+ testis. β-Galactosidase-positive germline stem cells (GSCs) and gonialblasts (green) are expanded. (**f**) *Gal4*/*Y; M5-4 UAS-upd*/tkv^{str-II} testis. The numbers of β-Galactosidase-positive GSCs and gonialblasts are significantly reduced. (**g**) M5-4/*UAS-dpp*; *Nos-Gal4*/+ testis. β-Galactosidase-positive GSCs and gonialblasts (green) are expanded. (**h**) M5-4/*UAS-dpp*; *Nos-Gal4*/*UAS-iDBHD* testis. The numbers of β-Galactosidase-positive GSCs and gonialblasts are dramatically reduced. Stars mark the hub, arrows point to GSCs and arrowheads point to spermatogonia. Scale bars in (**a**)–(**f**) and (**h**) represent 10 μm; scale bar in (**g**) represents 20 μm.

control tumor development either through modulating stem cells or through regulating the JAK/STAT or TGF-β signal-transduction pathways. Reducing the DBHD activity in the fly testis causes a loss of GSCs. Mutations in certain human and mouse genes have been reported to cause germ cell loss and testicular tumors owing to the overproliferation of Sertoli cells or other somatic gonadal cells (Chomette *et al*., 1985; Youngren *et al*., 2005). Further experiments are underway to clarify these potential roles of *DBHD* in *Drosophila*; the results are expected to provide a greater understanding of how mutations in *BHD* lead to the BHD syndrome phenotype in humans.

Materials and methods

Drosophila *stocks*

Transgenic lines of *UAS-DBHD* and *UAS-iDBHD* were generated by inserting *DBHD* cDNA into the *pUAST* and

Sym-pUAST vectors, respectively (Brand and Perrimon, 1993; Giordano *et al*., 2002), and injecting the constructs into embryos. *Nos-Gal4* was from the Bloomington stock center. *UAS-upd*, *stat92E*^{6C8} and *V32-Gal4 (matα4-GAL-VP16)* were described previously (Hou *et al*., 1996; Chen *et al*., 2002); *stat92E*^{F}, a temperature-sensitive *stat92E* allele (Baksa *et al*., 2002; Brawley and Matunis, 2004), was a gift from C Dearolf through S DiNardo. The deficiency *Df(3L)Exel6111* removes CG8616 (*DBHD*) and several other genes in chromosome regions 65E7-F4 and was obtained from the Bloomington Stock Center. M5-4 is a P-element enhancer trap that expresses LacZ in the hub, GSCs and gonialblasts (provided by S DiNardo; Tran *et al*., 2000; Fabrizio *et al*., 2003); *bam-GFP* (provided by T Xie; Chen and McKearin, 2003). *UAS-dpp* and *tkv*$^{str\text{-}II}$ were from K Basler. *Act>CD2>Gal4 UAS-GFP* and *tub>CD2>Gal4 UAS-GFP* were from T Neufeld.

Flies were raised on standard *Drosophila* media at 25°C, unless otherwise indicated. Chromosomes and mutations that are not described in the text can be found in Flybase (http://flybase.bio.indiana.edu).

In situ *hybridization*

In situ hybridization to whole-mount testes by using a digoxigenin-labeled antisense *DBHD* RNA probe was performed as described previously (Hou *et al*., 1996).

Immunofluorescence staining and microscopy

Transfection and staining of S2 cells were performed as described previously (Chen *et al*., 2002). Fixing and staining of the testes were performed as described (Tulina and Matunis, 2001). The following antisera were used: rabbit polyclonal anti-Vasa antibody (1:5000; gift from R Lehmann); rabbit polyclonal anti-β-Galactosidase antibody (1:1000; Cappel, ICN Pharmaceuticals, Inc., Aurora, OH, USA); mouse monoclonal anti-Hts antibody 1B1 (1:4; Developmental Studies Hybridoma Bank (DSHB)); mouse monoclonal anti-Fas III antibody (1:10; DSHB); mouse monoclonal anti-Armadillo N7A1 (1:4; DSHB); rabbit polyclonal anti-GFP antibody (1:200; Molecular Probes, Eugene, OR, USA); rat anti-BamC antibody (1:500; gift from D. McKearin). Secondary antibodies were goat anti-mouse, goat anti-rat and goat anti-rabbit immunoglobulin G conjugated to Alexa 488 or Alexa 568 (1:400; Molecular Probes). 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) was used to stain DNA. Confocal images were obtained by using a Zeiss LSM510 system, and processed using Adobe Photoshop 7.0.

Generating GFP-marked germline stem cell clones

GFP-marked *iDBHD* GSC clones were generated using the flip-out-Gal4/UAS method (Pignoni and Zipursky, 1997). In brief, 2 days old *yw hs-flp/Y; UASiDBHD/Act>CD2>Gal4 UAS-GFP* or *yw hs-flp/Y; UASiDBHD/tub>CD2>Gal4 UAS-GFP* male flies were heat-shocked at 37°C for 1 h and then transferred to fresh food at 25°C. The testes were removed and processed for antibody staining 2 days after the heat-shock treatment.

Detection of apoptosis

We used an ApopTag Fluorescein Direct Detection Kit (Intergen, Purchase, NY, USA) to detect cell death in the testes. The testes were dissected and fixed in 4% formaldehyde in PBX (Phosphate Buffer saline with 0.1% Triton X-100), as described above. Fixed testes were washed in phosphate-buffered saline, and cell death was detected according to the manufacturer's instruction.

Quantitative RT–PCR assay for Drosophila BHD *homolog and RP49 mRNA levels*

Total RNAs were isolated from stages 6 to 12 of *V32-Gal4/+ or V32-Gal4/UAS-iDBHD* embryos using Trizol (Gibco BRL, Life technologies Inc., Grand Island, NY, USA), and then purified with Qiagen Rneasy kit. To avoid DNA contamination, the RNAs were first treated with 1 mu DNase I (2 μ/ml) for 30 min at 37°C. Total RNA (100 ng) was reverse transcribed (RT) using SuperScriptIII First-Strand Synthesis System for RT–PCR (Invitrogen, Carlsbad, CA, USA). The RT–PCR products were visualized in 1% agarose gel.

Acknowledgements

We thank C Dearolf, T Xie, S DiNardo, K Basler, T Neufeld, and the Bloomington Stock Center for fly stocks; R Lehmann and the Developmental Study Hybridoma Bank for antibodies; S Wincovith, S Garfield, E Cho, and S Lockett for help on the confocal microscope; H Shin for generating transgene lines; N Parrish for help on preparation of the manuscript. This research was supported by the Intramural Research Program of NIH, National Cancer Institute and by federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. N01-C0-12400.The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsement by the US government.

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