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# *Drosophila happyhour* modulates JNK-dependent apoptosis

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**Mitogen-activated protein kinase kinase kinase kinase-3 (MAP4K3) is a Ste20 kinase family member that modulates multiple signal transduction pathways. We recently identified MAP4K3 as proapoptotic kinase using an RNA interference screening approach. In mammalian cells, MAP4K3 enhances the mitochondrial apoptosis pathway through the post-transcriptional modulation of selected proapoptotic Bcl-2 homology domain 3-only proteins. Recent data suggest that MAP4K3 mutations contribute to pancreatic cancer, which highlights the importance of studying the *in vivo* function of this kinase. To determine whether the cell death function is conserved *in vivo* and which downstream signalling pathways are involved, we generated transgenic flies expressing *happyhour* (*hppy*), the *Drosophila MAP4K3* orthologue. Here, we show that the overexpression of *hppy* promotes caspase-dependent apoptosis and that the hypothetical kinase domain is essential for inducing cell death. In addition, we show that *hppy* expression triggers the activation of both the c-Jun N-terminal kinase (JNK) and target of rapamycin (TOR) signalling pathways; however, only JNK signalling is required for apoptosis. Together, our results show that *hppy* has a JNK-dependent proapoptotic function in *Drosophila*, which reinforces the hypothesis that MAP4K3 might act as tumour suppressor by regulating apoptosis in higher eukaryotes.**
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Mitogen-activated protein kinases (MAPKs) are highly conserved in all eukaryotes and control various cellular processes, such as cell growth, differentiation and cell death. The three MAPK pathways, that is, those mediated by extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK), have been found to be deregulated in cancers.[1] Therefore, a better understanding of the molecular mechanisms controlling MAPK signalling pathways is crucial for designing effective cancer therapies. However, the analysis of these pathways in mammalian models is complicated by the multiple forms of crosstalk between the MAPK signalling pathways and the functional redundancy of various MAPK isoforms.

Mitogen-activated protein kinase kinase kinase kinase-3 (MAP4K3), a Ste20 family member, was identified as a new component of the target of rapamycin (TOR) signalling pathway with an RNA interference (RNAi) screen in *Drosophila* S2 cells.[2] In response to amino acids in cell culture, MAP4K3 modulates the phosphorylation of S6 protein kinase and the translation inhibitor, eukaryotic translation initiation factor 4E-binding protein (4E-BP), which are direct targets of TOR activity. Recently, the involvement of MAP4K3 in the regulation of TOR activity was confirmed *in vivo* with the characterisation of *Drosophila MAP4K3* mutant flies.[3] In addition, MAP4K3 activates the JNK pathway in mammalian cell lines upon stimulation with ultraviolet radiation and tumour necrosis factor-$\alpha$.[4] More recently, a genetic screen designed to identify novel modulators of ethanol sedation in flies identified loss-of-function mutations in *Drosophila MAP4K3* that result in increased resistance to the sedative effects of ethanol. This result inspired the naming of the MAP4K3-coding gene as *happyhour* (*hppy*).[5] Importantly, it was determined that Hppy regulates ethanol sensitivity by inhibiting epidermal growth factor receptor (EGFR)/ERK signalling *in vivo*. Taken together, these observations indicate that this kinase modulates multiple signal transduction pathways that affect cell survival.

We recently identified MAP4K3 as a proapoptotic kinase in an RNAi screen.[6] Our data indicate that MAP4K3 regulates apoptosis by modulating selected proapoptotic Bcl-2 homology domain 3 (BH3)-only proteins.[6,7] This modulation seems to occur at the post-transcriptional level through both the mammalian TOR complex 1 (mTORC1) and JNK signalling pathways. Interestingly, our previous work showed a significant decrease in MAP4K3 protein levels in human pancreatic tumour samples,[6] which is consistent with the previous finding that a somatic mutation in the *MAP4K3* gene is associated with pancreatic cancer.[8] This result strongly suggests that

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**Keywords:** *Drosophila*; apoptosis; *hppy*; MAP4K3; GLK; JNK
**Abbreviations:** ATP, adenosine triphosphate; Bcl-2, B-cell lymphoma protein-2; Bcl-XL, B-cell lymphoma-extra large; BH3, Bcl-2 homology domain 3; *da*, *daughterless*; 4E-BP, eukaryotic translation initiation factor 4E-binding protein; EGFR, epidermal growth factor receptor; *en*, *engrailed*; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; *hppy* KD, happyhour kinase dead; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAP4K3, mitogen-activated protein kinase kinase kinase kinase-3; mTORC1, mammalian TOR complex 1; *puc*, *puckered*; RGH, reaper, grim, hid; RNAi, RNA interference; TOR, target of rapamycin; TSC, tuberous sclerosis complex; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; UAS, upstream activation sequence
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MAP4K3 has a tumour suppressor role. Together, these observations highlight the need for more information on the role of MAP4K3 in cell death *in vivo*.

We characterised the role of the *Drosophila* orthologue of human *MAP4K3*, *hppy*, using a gain-of-function approach. Here, we show that *hppy* overexpression results in partial lethality during development and that the surviving flies have smaller wings due to the loss of epithelial cells. The ectopic expression of *hppy* in the larval wing disc induces cell death in a caspase-dependent manner. Furthermore, mutation of the putative adenosine triphosphate (ATP)-binding domain by the substitution of lysine 45 blocks apoptosis in *hppy*-overexpressing cells, indicating that the kinase activity of Hppy is required for cell death. Also, we demonstrate that Hppy induces the activation of TOR and JNK signalling pathways but only JNK signalling is required for its cell death function. Thus, we confirm that *hppy*, the *Drosophila MAP4K3* orthologue, has a JNK signalling-dependent proapoptotic function *in vivo*.

## Results

***hppy* expression induces defective wing phenotype in *Drosophila*.** We previously identified human MAP4K3 as a potent cell death inducer.[6] To determine the *in vivo* consequences of MAP4K3 expression, we cloned the *Drosophila* orthologue of human *MAP4K3*, *hppy*. The *Drosophila* genome encodes two *hppy* variant transcripts, $hppy^{RA}$ and $hppy^{RB}$. We employed the *UAS-Gal4* system to generate two independent fly lines expressing wild-type $hppy^{RB}$ and a kinase-dead version of this kinase (happyhour kinase dead (*hppy KD*)) and used real-time quantitative reverse transcription PCR (qRT-PCR) to assay transcript levels (Figure 1a). First, we noted that ubiquitous expression of *hppy* results in significant pupal lethality (Figure 1b) and that this lethality correlates with *hppy* expression levels (Figures 1a and b). Importantly, the surviving adult flies are smaller than the controls (data not shown), and have a reduced wing area (Figures 1c and d). As we previously showed that expression of human *MAP4K3* in cultured cells results in apoptotic cell death,[6] we tested whether the decrease in wing size upon *hppy* expression is related to cell loss during development by analysing the total number of bristle cells per wing in control flies and in flies expressing *hppy* and *hppy KD* (Figure 1e). We found a clear decrease in the total number of bristle cells in *hppy*-expressing flies, but not in flies expressing *hppy KD*. This result strongly suggests that the decrease in wing size results from significant cell loss caused by the kinase activity of Hppy.

***hppy* expression induces apoptosis in *Drosophila*.** To directly test whether the decrease in wing size observed in *hppy*-expressing flies is a consequence of cell loss,

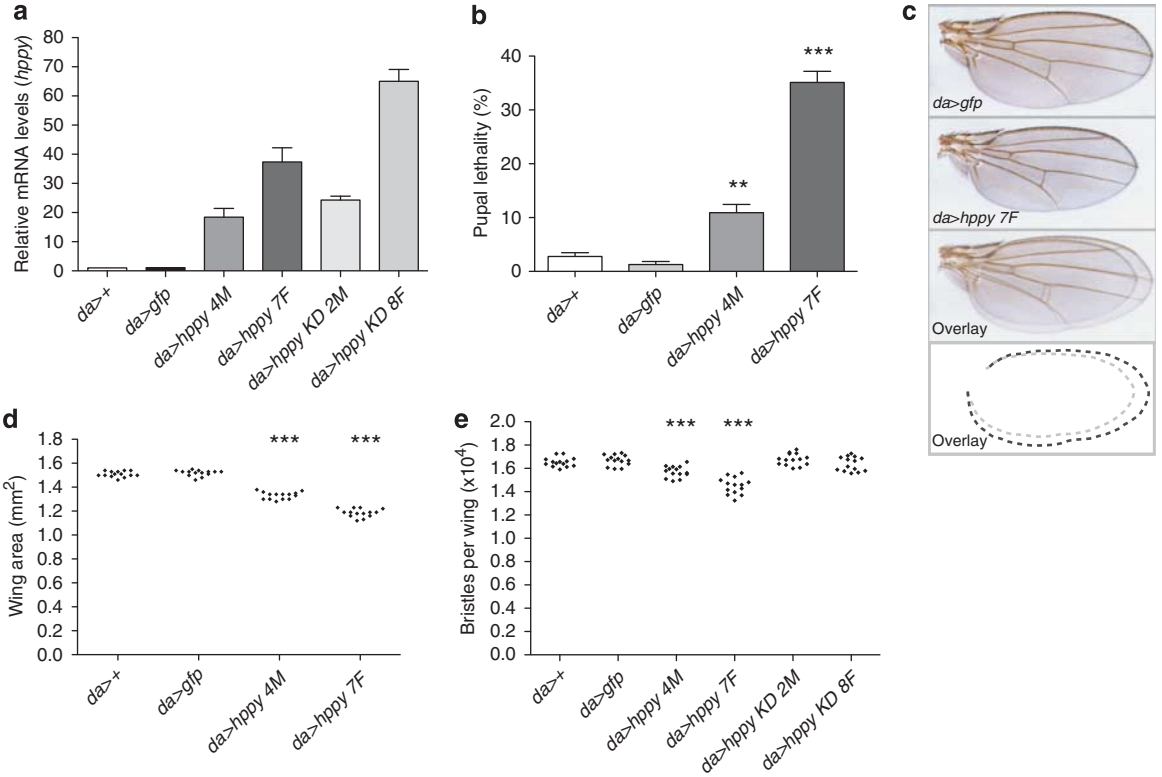


**Figure 1** Developmental consequences of *hppy* overexpression. (**a**) Analysis of *hppy* mRNA levels in transgenic flies. RNA was isolated from a single larva and analysed by qRT-PCR. The notations 4M, 7F, 2M and 8F refer to the independent transgenic lines employed in this study. The fold change in transcript levels is shown relative to controls (*da*$>+$). Mean values $\pm$S.E.M. are shown. (**b**) Analysis of pupal lethality in *hppy*-expressing flies. Percentages correspond to the number of dead pupae relative to the total number of pupae. Values represent the average of three independent experiments. Mean values $\pm$S.E.M. are shown. (**c**) Decreased wing area in adult *hppy*-expressing flies. Representative wings are shown. (**d**) Quantification of total wing area in *hppy*-expressing flies. (**e**) Analysis of epithelial cell number in *hppy*-expressing flies. Statistically significant values relative to controls (*da*$>+$) are indicated with asterisks (one-way ANOVA with Dunnett's post-test)

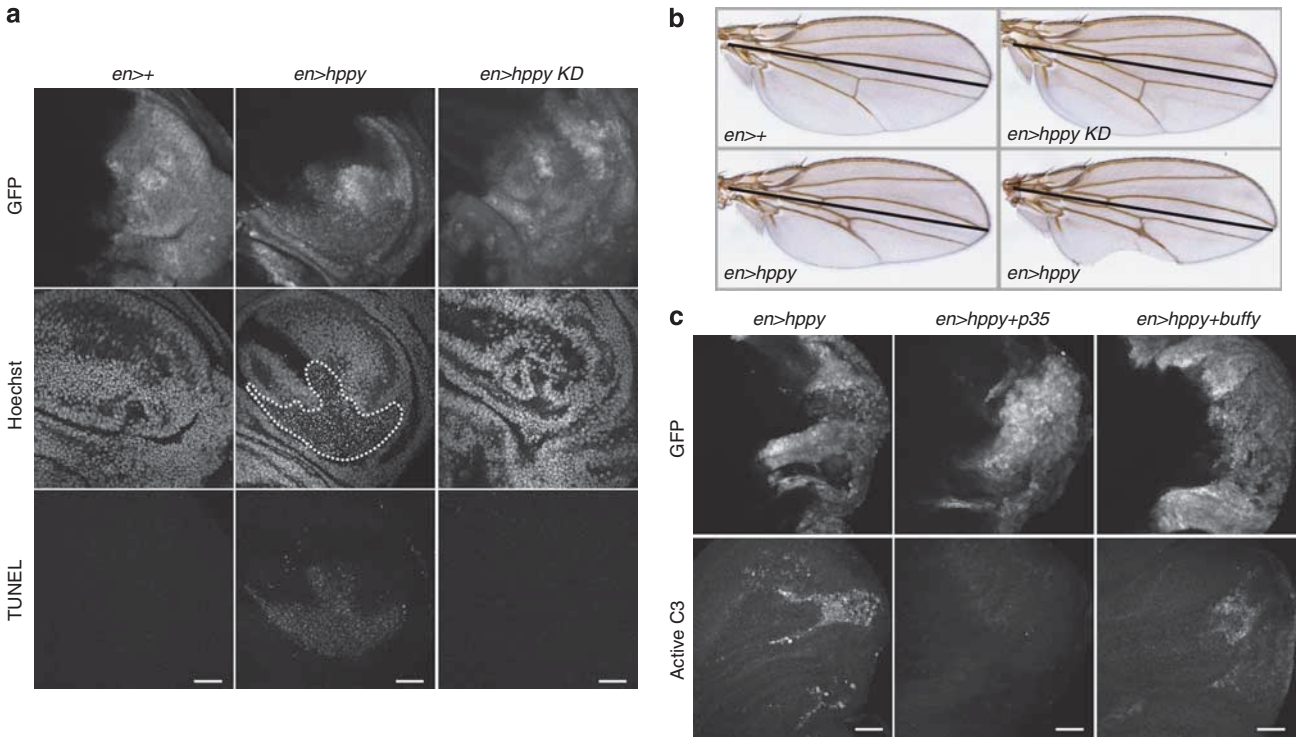


**Figure 2** *hppy* promotes caspase-dependent apoptosis. (**a**) Ectopic expression of *hppy* causes cell death in *Drosophila* wing discs. The area of *en-Gal4*-driven gene expression is marked with GFP fluorescence (top). The region of cells with nuclear chromatin condensation, as revealed by Hoechst staining, is indicated with the dashed line (middle). TUNEL-positive cells colocalise with nuclear DNA condensation (bottom). (**b**) Ectopic expression of *hppy* in the posterior compartment of the wing causes a defect in the wing structure. The black line denotes the boundary between the anterior and posterior compartments of the adult wing. (**c**) *hppy* induces caspase activation. The area of *en-Gal4*-driven gene expression is marked with GFP fluorescence (top). Wing discs from larvae with the indicated genotypes were stained with an anti-active caspase-3 antibody (bottom). The scale bars represent 20 $\mu$m

we analysed the levels of apoptosis in *hppy*-expressing tissues. The overexpression of *hppy* driven by *en-Gal4* in the posterior compartment of the developing wing discs results in the appearance of cells that are terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive and display condensed chromatin (Figure 2a), confirming that this kinase induces apoptotic cell death. Importantly, we failed to detect significant levels of cells displaying nuclear markers of apoptosis in two independent lines expressing the kinase-inactive Hppy (*hppy KD*), indicating that the kinase activity is required for the induction of apoptosis (Figure 2a). Moreover, analysis of the adult wings of flies expressing *hppy* under the control of *en-Gal4* showed a defect in vein formation and a clear reduction of the posterior area of the wing (Figure 2b), further suggesting that the decrease in wing size caused by *hppy* expression is a consequence of cell death in the developing wing.

We previously showed that apoptosis induced by human MAP4K3 can be blocked by expressing inhibitors of the mitochondrial (intrinsic) pathway such as the antiapoptotic B-cell lymphoma protein-2 (Bcl-2) family member B-cell lymphoma-extra large (Bcl-XL).[6] To test the relevance of the intrinsic pathway in *hppy*-dependent apoptosis in flies, we expressed the *Drosophila* antiapoptotic Bcl-2 family protein Buffy [9] and examined the effect on caspase activation. We observed that the ectopic expression of *hppy* driven by *en-Gal4* results in caspase activation in the posterior compartment of the developing wing discs (Figure 2c, left panel). Such caspase activation could be completely suppressed by the expression of the caspase inhibitor p35 [10] (Figure 2c, middle panels), indicating that Hppy-induced cell death is caspase dependent. Buffy expression only modestly suppressed caspase activation (Figure 2c, right panels), however, suggesting that the mechanism of apoptosis induction in flies might be different from that in mammals.

**JNK, but not TOR signalling, is required for Hppy-mediated apoptosis.** Human Map4k3 promotes apoptosis through the post-transcriptional modulation of BH3-only proteins in the JNK and mTORC1 pathways.[6] We therefore sought to determine the effect of *hppy* expression on the activation of the JNK and TOR pathways in *Drosophila*.

Monitoring of JNK activity in the wing disc by *lacZ* expression in a *puckered (puc)-LacZ* enhancer trap line[11] revealed that *hppy* expression results in robust JNK activation and that this activation requires the kinase activity of Hppy (Figure 3a). To verify the involvement of the JNK signalling pathway in the apoptosis induced by *hppy* expression, we coexpressed $bsk^{DN}$, a dominant-negative form of *Drosophila* JNK,[12] or *puckered* (*puc*), a JNK activity inhibitor.[11] Inhibition of JNK signalling by the coexpression of $bsk^{DN}$ or *puc*, marked by the suppression of *puc-lacZ* expression, suppresses the cell death induced by ectopic *hppy* expression (Figures 4a and b), indicating that JNK signalling is required for Hppy-mediated cell death.

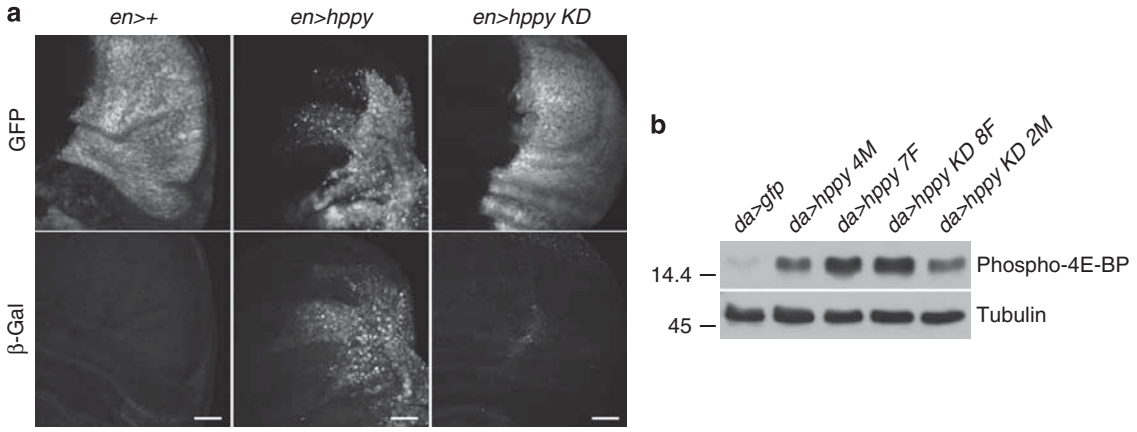


**Figure 3** *hppy* activates JNK and TOR signalling pathways. (**a**) Ectopic expression of *hppy* induces JNK activation in *Drosophila* wing discs. The area of *en-Gal4*-driven gene expression is marked with the GFP fluorescence (top). Wing discs from larvae with the indicated genotypes were stained for JNK activity with an anti-$\beta$-galactosidase antibody (bottom). The scale bars represent 20 $\mu$m. (**b**) Ubiquitous expression of *hppy* enhances phosphorylation of 4E-BP. Phopho-4E-BP levels were analysed with western blots of protein lysates of flies with the indicated genotypes and normalised against levels of tubulin

To assess the ability of Hppy to activate the TOR pathway in flies, we assessed the phosphorylation status of the TOR substrate, 4E-BP, in adult flies expressing *hppy*, as well as in appropriate controls, under the ubiquitous driver *da-Gal4* (Figure 3b). We found that *hppy* expression results in enhanced phosphorylation of 4E-BP. Surprisingly, the expression of *hppy KD* failed to suppress the enhanced phosphorylation of 4E-BP, suggesting that Hppy kinase activity is not critical for TOR activation. To determine the role of the TOR pathway in Hppy-induced apoptosis, we coexpressed *hppy* with either tuberous sclerosis complex (TSC)1/2 (*TSC*), an inhibitor of TOR activity, or the TOR substrate 4E-BP, driven by *en-Gal4*. With both approaches, we failed to observe a significant reduction of cell death in the posterior compartment of the wing discs (Figures 4c and d). Thus, the TOR pathway is unlikely to have a significant role in Hppy-dependent cell death.

Together, these results demonstrate that *hppy* requires the JNK, but not TOR signalling pathway, to induce apoptotic cell death in *Drosophila*.

## Discussion

The work reported here identifies Hppy, the *Drosophila* orthologue of human MAP4K3, as an *in vivo* modulator of apoptosis. In mammalian cells, MAP4K3 promotes apoptosis by inducing the post-transcriptional activation of a subset of BH3-only Bcl-2 family proteins. Expression of MAP4K3 leads to cell death through the mitochondrial (intrinsic) pathway; this action is suppressed by JNK and TOR inhibition. In this study, we provide the first *in vivo* evidence that the fly orthologue of MAP4K3, Hppy, is a death-inducing kinase that promotes caspase-dependent apoptosis. Mitochondria have a crucial role in the intrinsic apoptosis pathway in vertebrates. In *Drosophila*, however, the role of mitochondria in apoptosis is unclear, as mutants of the *Drosophila bcl-2* homologues *debcl* and *buffy* show no obvious defects in developmental apoptosis[12] and this form of cell death is not blocked in cells with depleted Cyt-C.[13] Our data suggest that Hppy-mediated cell death occurs independently of the mitochondrial pathway; *buffy* expression failed to block Hppy-dependent apoptosis, and negative modulation of the TOR pathway in flies failed to suppress Hppy-dependent cell death. Given that the human orthologue of Hppy employs the TOR pathway to stimulate the mitochondrial pathway of apoptosis, we propose that suppression of this pathway in flies fails to affect Hppy-dependent cell death because the mitochondrial apoptosis pathway may be of limited importance in flies. Our genetic analysis suggests that the JNK pathway positively regulates Hppy-dependent cell death. As mentioned above, it is unlikely that the JNK pathway induces cell death by activating the mitochondrial pathway in *Drosophila*. In flies, the genes *reaper*, *grim* and *hid* (RGH) promote caspase activation by antagonising the *Drosophila* inhibitor of apoptosis proteins. Although *reaper* and *grim* are only expressed in cells that are destined for death, *hid* expression is controlled at both the transcriptional and post-transcriptional levels (reviewed in Bilak and Su[14]). Hid has been shown to be transcriptionally activated in a JNK- and Foxo-dependent manner. Additionally, the proapoptotic activity of Foxo is opposed by the action of receptor tyrosine kinases (RTKs) such as EGFR and insulin-like growth factor receptor. Given the recent observation that Hppy is a potent negative modulator of EGFR signalling,[5] it is tempting to propose that this kinase might promote apoptosis by modulating signalling pathways that affect the levels of RGH proteins. This idea is consistent with the observation that another Ste20 family member, Hippo, promotes *hid* expression.[15]

A single amino-acid substitution in MAP4K3 has been associated with pancreatic cancer, suggesting that this kinase might be an important modulator of tumourigenesis.[8] We also previously found that the levels of MAP4K3 are significantly reduced in pancreatic tumour samples.[6] Given that in *Drosophila*, Hppy acts to both stimulate JNK signalling (this study) and dampen the EGFR pathway,[5] it is tempting to propose that mammalian MAP4K3 might act as a tumour suppressor, not only by promoting JNK-dependent apoptosis but also by blocking the prosurvival effects of EGFR signalling mechanisms.

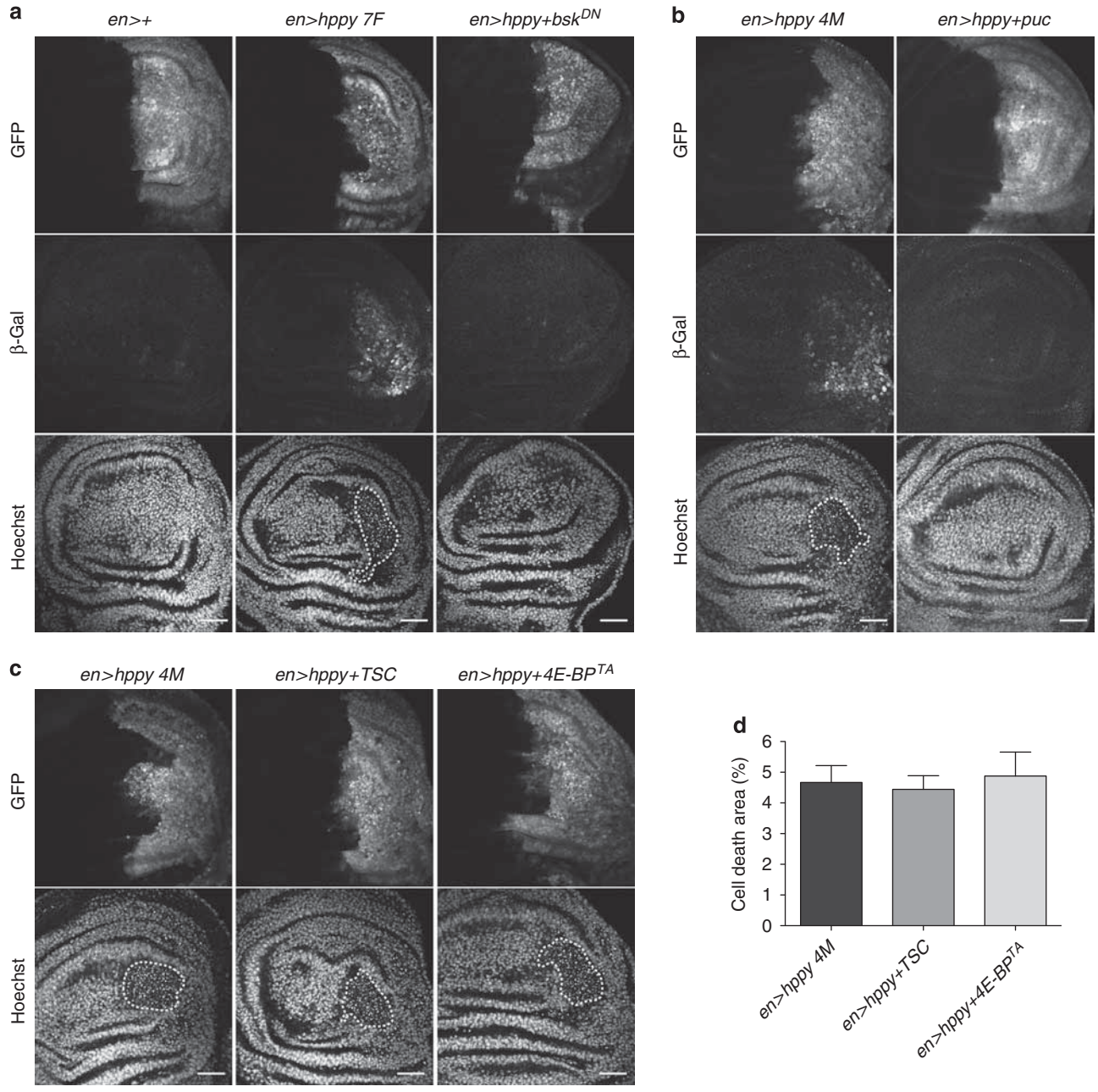


**Figure 4** JNK is required for *hppy*-induced apoptosis. (**a**) Expression of a dominant-negative version of *Drosophila JNK* (*bsk*$^{DN}$) suppresses Hppy-mediated apoptosis. The area of *en-Gal4*-driven gene expression is identified by GFP fluorescence (top). Wing discs from larvae with the indicated genotypes were stained with an anti-$\beta$-galactosidase antibody (middle) and Hoechst (bottom). Condensed chromatin is indicated by the dashed line. (**b**) Expression of the JNK activity inhibitor, puckered (*puc*), suppresses Hppy-mediated apoptosis. The area of *en-Gal4*-driven gene expression is identified by GFP fluorescence (top). Wing discs from larvae with the indicated genotypes were stained with an anti-$\beta$-galactosidase antibody (middle) and Hoechst (bottom). Condensed chromatin is indicated by the dashed line. (**c**, **d**) The expression of either *TSC1/2* (*TSC*) or *4E-BP*$^{TA}$ fails to block Hppy-dependent apoptosis. The area of *en-Gal4*-driven gene expression is identified by GFP fluorescence (top). Wing discs from larvae with the indicated genotypes were stained with Hoechst (bottom). Condensed chromatin is indicated by the dashed line. Quantitative analysis of cell death was performed in wing discs from larvae with the indicated genotypes. The data presented are the mean values $\pm$ S.E.M.; $n \geqslant 14$ in each group. The scale bars represent 20 $\mu$m

## Materials and Methods

**Antibodies.** The primary antibodies employed in this study are listed in Supplementary Table S1.

***Drosophila* strains and phenotypic analysis.** Fly stocks and crosses were maintained on standard cornmeal agar media at 25°C. Genotypes of flies employed in this study are listed in Supplementary Table S2. *Drosophila MAP4K3* (*hppy*) was PCR-amplified from BDGP clone RH10407 and subcloned into the pUAST vector. A kinase-dead *hppy* mutant was made by mutating lysine 55 to glutamic acid in the ATP-binding domain of this kinase using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA, USA). The corresponding transgenic flies were generated by BestGene Inc. (Chino Hills, CA, USA; http://www.thebestgene.com). The *en-Gal4*, *UAS-gfp*/*CyO*, *da-Gal4*, *UAS-puc* and *UAS-TSC1/2* (kind gifts from N Tapon), *UAS-Buffy* (Quinn *et al.*[9]), *UAS-p35* (Hay *et al.*[10]), *UAS-4E-BP*$^{TA}$ (Imai *et al.*[17]) and all others were obtained from the Bloomington Stock Center (Bloomington, IN, USA). Wing phenotypes were

analysed at 25°C. Wings were dissected from 3- to 4-day-old females and mounted for microscopic examination in halocarbon oil. Images were acquired using a Leica (Wetzlar, Germany) stereomicroscope ( $\times$ 40) equipped with a Leica colour digital camera. Images are presented in the orientation of proximal to the left and anterior to the top.

**RNA extraction and real-time PCR.** Isolation of total RNA was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Quantitative real-time RT-PCR was performed on an Mx4000 (Stratagene) real-time cycler using the QuantiTect SYBR Green RT-PCR system (Qiagen). Gene-specic primers for the *actin 79B*, *buffy*, *puc* and *TSC* genes were obtained from Qiagen (QuantiTect Primer Assays). Primers recognising $hppy^{RA}$ and $hppy^{RB}$ transcripts were described in a previous study.[5] The relative transcript levels of the target genes were normalised against *actin* mRNA levels; quantification was performed using the comparative Ct method.[16] The mRNA expression levels of *hppy*, *buffy*, *puc* or *TSC* in transgenic flies used in this study and driven by the ubiquitous *da-Gal4* driver are shown in Supplementary Figure S1.

**Immunohistochemistry and TUNEL.** Inverted third-instar larvae were fixed in 3% paraformaldehyde in PBS for 20 min at room temperature and extensively washed in PBS containing 0.2% Triton X-100 (PBT). Tissues were then blocked for 1 h in PBT containing 1% bovine serum albumin (BSA). Primary antibodies diluted in 1% PBT/BSA were incubated overnight at 4°C. Dissected larvae were washed and then incubated for 4 h at room temperature with secondary antibodies (1/200) and Hoechst 33342 (1/500) (Invitrogen, Carlsbad, CA, USA) diluted in 1% PBT/BSA. After washing, wing discs were dissected and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Fluorescence images were acquired with a Zeiss LSM510 confocal microscope. Images are presented in the orientation of anterior to the left and ventral to the top. TUNEL staining was performed with the ApopTag red *in situ* apoptosis detection kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

**Western blotting.** In all, 100 $\mu$g of protein lysates was resolved by SDS-PAGE and transferred to Immobilon PVDF membranes (Millipore). The membranes were blocked in 5% milk and subsequently incubated with the indicated primary antibody (see Supplementary Table S1) before being incubated with the appropriate HRP-conjugated secondary antibody. Antibody complexes were visualised by enhanced chemiluminescence.

**Statistical analysis.** Mean values are presented with error bars corresponding to $\pm$ S.D. or $\pm$ S.E.M. as indicated. Statistical analysis was performed using Prism statistical analysis software (http://www.graphpad.com). Significance is indicated as $^{***}P<0.001$, $^{**}P<0.01$ and $^{*}P<0.05$.

### Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)