

Foxo and Fos regulate the decision between cell death and survival in response to UV irradiation

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Cells damaged by environmental insults have to be repaired or eliminated to ensure tissue homeostasis in metazoans. Recent studies suggest that the balance between cell survival signals and pro-apoptotic stimuli controls the decision between cell repair and death. How these competing signals are integrated and interpreted to achieve accurate control over cell fate *in vivo* is incompletely understood. Here, we show that the Forkhead Box O transcription factor Foxo and the AP-1 transcription factor DFos are required downstream of Jun-N-terminal kinase signaling for the apoptotic response to UV-induced DNA damage in the developing *Drosophila* retina. Both transcription factors regulate the pro-apoptotic gene *hid*. Our results indicate that UV-induced apoptosis is repressed by receptor tyrosine kinase-mediated inactivation of Foxo. These data suggest that integrating stress and survival signals through Foxo drives the decision between cell death and repair of damaged cells *in vivo*.

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Introduction

Metazoans are able to maintain tissue homeostasis over a long period of time while under constant exposure to environmental challenges that damage macromolecules like DNA and proteins. Tight control of the decision between repair and salvage of damaged cells or elimination of such cells by programmed cell death (apoptosis) is crucial for this ability. It is becoming increasingly recognized that the integration and interpretation of extracellular death and survival cues by complex signal transduction networks ultimately controls such life or death decisions (Janes *et al*, 2005). However, it remains a challenge to identify and characterize the molecu-

lar mechanisms employed by cells to decode and respond to such competing signals *in vivo*.

The developing *Drosophila* retina is an ideal system to study mechanisms that control cellular life/death decisions genetically. In the developing eye, tyrosine kinase signaling (mainly via the EGF receptor (EGFR)) is required to provide survival cues during photoreceptor differentiation (Bergmann *et al*, 1998b, 2002; Baker, 2001; Baker and Yu, 2001; Freeman and Bienz, 2001; Freeman, 2002; Brown and Freeman, 2003). EGFR signaling leads to activation of the *Drosophila* Ras homologue and protects cells against apoptosis. This effect is in part mediated by the MAPK ERK, which phosphorylates the pro-apoptotic protein Head Involution Defective (Hid) and thus induces its degradation (Bergmann *et al*, 1998a). A second mechanism to repress apoptosis downstream of EGFR signaling involves transcriptional repression of *hid* (Kurada and White, 1998).

Apoptosis in the developing retina occurs naturally to shape the adult morphology of this highly ordered tissue (Baker, 2001), but can also be induced by genotoxic stress, for example by UV-induced DNA damage (Jassim *et al*, 2003). When repair of UV-induced DNA damage by photoreactivation is impaired, excessive apoptosis ensues and results in extensive damage to the fly's compound eye. Overexpression of caspase inhibitors reduces the morphological disruptions observed after UV irradiation, establishing the role of apoptosis in this effect. Furthermore, transcriptional upregulation of *hid* in response to UV-irradiation in the pupal retina has been reported, suggesting transcriptional induction of pro-apoptotic molecules as an important part of the DNA damage response in the eye (Jassim *et al*, 2003).

An evolutionarily conserved regulatory system that influences cell survival and death in response to extracellular as well as intracellular cues is the stress-responsive Jun-N-terminal kinase (JNK) signaling pathway. A multitude of studies using cell culture systems and genetic model organisms have demonstrated that JNK plays an important role in apoptosis. However, JNK does not act universally as a pro-apoptotic signaling pathway, as JNK activation can also exert antiapoptotic activity in certain situations (Weston and Davis, 2002; Lamb *et al*, 2003; Lin, 2003; Liu and Lin, 2005; Ventura *et al*, 2006). The mechanism(s) by which the cellular response to JNK activation is determined is a subject of intense investigation. In a recent study, signaling interactions in cancer cells have been modeled using a systems biology approach, resulting in the identification and prediction of parameter sets that influence cellular life/death decisions as a function of JNK, EGF and insulin signaling activity (Janes *et al*, 2005). The models developed in these studies suggest that the strength of survival signals (EGF, insulin) determines the pro or antiapoptotic effect of JNK activation.

Work in *Drosophila melanogaster* indicates that the pro-apoptotic as well as the prosurvival functions of JNK are evolutionarily conserved (Adachi-Yamada *et al*, 1999; Igaki *et al*, 2002; Moreno *et al*, 2002; Adachi-Yamada and

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O'Connor, 2004; de la Cova *et al*, 2004; Ryoo *et al*, 2004; McEwen and Peifer, 2005; Perez-Garijo *et al*, 2005; Uhlirva *et al*, 2005). As compared with mammals, the *Drosophila* system exhibits reduced genetic redundancy, which makes it easier to derive definitive answers to questions regarding signaling specificity as well as context-dependent responses to JNK activation. The JNK signaling pathway in flies consists, at its core, of the JNK kinase hemipterous (Hep), which is phosphorylated and activated by a variety of upstream JNKK kinases, and in turn activates the JNK Basket (Bsk) by phosphorylation. Bsk is known to phosphorylate transcription factors of the AP-1 family (mainly Djun and Dfos), thus regulating transcription. *Drosophila* AP-1 induces the transcription of the JNK-specific phosphatase Puckered (Puc), limiting the activity of JNK in a negative feedback loop.

As in mammals, activation of the JNK pathway in flies is not always sufficient to induce apoptosis. JNK activity is, for example, required for morphogenetic processes during development, in which it regulates cellular shape changes (Harden, 2002). Moreover, JNK activation increases stress tolerance and longevity of flies (Wang *et al*, 2003, 2005). The molecular mechanisms that determine context- or cell type-specific cellular responses to JNK activation remain largely unknown.

Transcription factors of the Forkhead Box O (Foxo) family play an important role in mediating apoptosis in a variety of cellular contexts (e.g. in B lymphocytes and neurons; Dijkers *et al*, 2000, 2002; Linseman *et al*, 2002; Accili and Arden, 2004; Greer and Brunet, 2005). Foxo factors are phosphorylated in response to cell survival signals by active Akt or IKK, and are consequently retained in the cytoplasm. When cells are deprived of survival signals, the Akt signal is reduced and Foxo translocates to the nucleus, where it can induce the expression of pro-apoptotic molecules such as the BH3-only Bcl2 family member Bim (Dijkers *et al*, 2000; Greer and Brunet, 2005). Foxo can, however, also induce gene expression programs with protective functions for the cell (Kops *et al*, 2002; Murphy *et al*, 2003). The mechanism that switches the cellular response to Foxo activation from apoptosis to survival remains unclear.

Recently, a function for JNK in the regulation of Foxo activity has been identified in mammalian cells, *Caenorhabditis elegans* and *Drosophila* (Essers *et al*, 2004; Oh *et al*, 2005; Wang *et al*, 2005). This activation was shown to enhance oxidative stress resistance and longevity of the animal, but its role in the control of apoptosis has not been addressed. Interestingly, JNK signaling is known to induce Bim expression in neurons (Harris and Johnson, 2001; Whitfield *et al*, 2001), suggesting that Foxo might act downstream of JNK to promote apoptosis. Foxo is thus a prime candidate for an integrator of survival and stress signals in the control of cell homeostasis.

In this study, we use UVC-mediated disruption of retinal morphogenesis of the fly eye as an *in vivo* model system for DNA damage-induced apoptosis (Jassim *et al*, 2003). We present genetic evidence that this apoptotic response is mediated by JNK/Foxo signaling. Furthermore, we show that Foxo and the AP-1 transcription factor Dfos are required for UV-induced apoptosis, and that both factors control the transcription of *hid*. Consistent with the notion of Foxo acting as an integration point for competing survival/death signals in *Drosophila* cells, we find that UV-induced apoptosis is

suppressed by cellular survival signaling through the EGFR/Ras and the InR/PI3K/Akt pathways.

Results

Induction of DNA damage by UVC irradiation of the pupal retina results in loss of eye tissue in the adult fly (Jassim *et al*, 2003). This phenotype is observed only when DNA repair by photoreactivation is impaired, and when the retina is exposed to UV between 23 and 24 h of pupal development. At this time point, the retina consists mainly of post-mitotic, differentiating cells. Studying UV-induced cell death in this system thus allows one to genetically dissect signaling pathways involved in DNA damage-induced cell death of post-mitotic tissues.

Apoptotic response to UV-induced DNA damage in the *Drosophila* retina is mediated by JNK/Foxo signaling

JNK is activated by UV irradiation, and studies in cell culture suggest that JNK signaling might play an important role in the control of apoptosis after DNA damage (van Dam *et al*, 1995; Tournier *et al*, 2000; Jassim *et al*, 2003; Hamdi *et al*, 2005). To test this hypothesis genetically, we examined whether JNK signaling is required for apoptosis in the retinal DNA damage response. We exposed the pupal retina of flies carrying the *hep* loss-of-function alleles *hep*¹ or *hep*^{r75} to mild UVC irradiation (5 mJ/cm² at 254 nm) and prevented photorepair by allowing subsequent pupal development to proceed in the dark.

Comparing the phenotypic effects of this treatment on *hep* mutants and the corresponding wild-type controls (OreR) showed that reduction of *hep* function prevents excessive tissue loss (Figure 1A and B; the phenotypic effects of UV were quantified by calculating the ratio between the sizes of irradiated and non-irradiated eyes of the same head (Figure 1E). The resulting values are highly reproducible, and were identical in males and females of the same genotype. See also Materials and methods). This suggests that the JNK signaling pathway is required for apoptosis in the pupal retina in response to UV-induced DNA damage. This interpretation is supported by the observation that mutant animals heterozygous for the JNK phosphatase *puc*^{E69} (in which JNK signaling activity is increased) show increased tissue loss upon mild irradiation (Figure 1C and E). The involvement of JNK signaling in the retinal UV response was further confirmed by the induction of *puc* transcription (detected using RT-PCR, as well as by lacZ staining of *puc*^{E69} reporter flies) in response to UV irradiation (Figure 1D). Induction of the JNK target gene *puc* is a well-described indicator of JNK activation.

UV irradiation induces *hid* expression in a JNK-dependent manner

To specify the outcome of JNK signaling activity *in vivo*, additional signaling inputs have to exist that control pro-apoptotic signal transduction downstream of JNK. The mechanism(s) by which JNK induces apoptosis in flies is only beginning to be understood. Although direct activation of the mitochondrial pathway by JNK cannot be ruled out, recent studies in *Drosophila* support the notion that JNK signaling induces the transcription of pro-apoptotic molecules of the RGH (reaper, grim, *hid*) family, which inactivate the

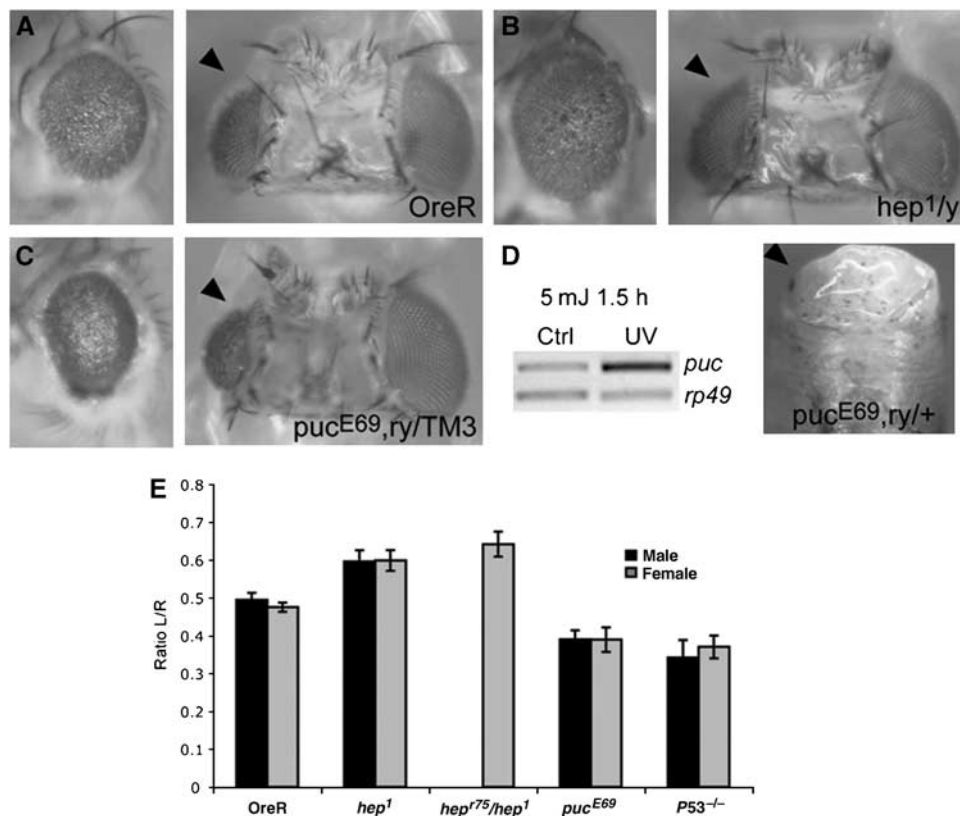


Figure 1 JNK signaling regulates UV-induced apoptosis in the retina. (A–C) JNK signaling regulates the apoptotic response to UV irradiation in the developing retina. Pupal cases were removed from 24-h-old pupae to expose the developing eye. One of the eyes was subjected to UVC irradiation (5 mJ/cm²), whereas the other eye was shielded. After subsequent incubation at 25°C in the dark, morphological defects are observed in the irradiated eye (A, arrowhead). This phenotype is caused by DNA-damage-induced apoptosis (Jassim *et al*, 2003). (B, C) DNA damage-induced apoptosis requires JNK signaling. Loss of JNKK (Hep) function (in hemizygotes for *hep*¹) protects eyes from UV-induced apoptosis (B), whereas increased JNK activity owing to loss of the JNK-phosphatase *puc* results in strongly increased defects (C). *puc* transcription (an indicator of JNK activation in flies) is induced in response to UV irradiation (D). Left panel: RT-PCR demonstrating rapid induction of *puc* transcripts in the retina within 1.5 h after UV irradiation; right panel: whole-mount X-gal staining showing activation of the *puc* gene in the irradiated part of the pupal head (arrowhead). The *puc*^{E69} allele contains a JNK-responsive lacZ P-element that serves as JNK reporter *in vivo*. (E) Quantification of tissue loss in irradiated eyes can be used to quantify the extent of apoptosis in a given genotype. The ratio between the area of left (L, irradiated) and right (R, control) eyes for each head was determined for *n* = 10 heads of each genotype (see Materials and methods for details). Means and standard deviations are shown here. Differences between each group are statistically significant (*P* < 0.001, Student's *t*-test). Quantification is shown for wild-type flies (OreR), *hep*¹ hemizygous mutant males or homozygous mutant females, *hep*⁷⁵/*hep*¹ transheterozygous females, *puc*^{E69} heterozygotes and *p53*^{5A-1-4} (Rong *et al*, 2002) homozygotes. A requirement for p53 in protection against UV-induced apoptosis was demonstrated by Jassim *et al* (2003). P53 mutants are included here as control.

Drosophila inhibitor of apoptosis (DIAP) and thus induce caspase-mediated cell death (Moreno *et al*, 2002; McEwen and Peifer, 2005).

We tested whether the requirement for JNK in UV-mediated apoptosis would correlate with transcriptional upregulation of pro-apoptotic molecules. Using a lacZ reporter line (Russell *et al*, 1998; Cox *et al*, 2000; Cullen and McCall, 2004; Sen *et al*, 2004), we found rapid induction of *hid* transcription in response to genotoxic UV irradiation (Figure 2A). This induction, which we validated using RT-PCR on RNA extracted from irradiated retinæ (Figure 2B), was not observed in *hep*¹ mutant animals (Figure 2B). Thus, JNK signaling is required for pro-apoptotic gene expression in response to DNA damage.

We tested genetically whether *hid* might act downstream of JNK to induce apoptosis. Excessive JNK signaling during development of the retina, experimentally induced by the expression of constitutively active Hep (Hep^{act}) in differentiating photoreceptors and cone cells (under the control of sepGal4), induces morphological defects that are caused by

apoptosis (determined by TUNEL staining; Figure 2C and D). The distribution of apoptotic figures in these eye imaginal discs correlates with the strong upregulation of *hid* transcription, indicating that JNK activation is sufficient to induce *hid* (Figure 2E and F). Reducing the gene dose of *hid* (*W*⁰⁵⁰¹⁴ and *W*¹), as well as of all three RGH genes (*Df*(H99)), was sufficient to significantly reduce the extent of damage observed in adult eyes expressing constitutively active Hep (Figure 2G and J). These results confirm a requirement for *hid* downstream of JNK in the induction of apoptosis.

Transcriptional control of apoptosis by Foxo and Fos downstream of JNK signaling

Direct transcriptional control of *hid* downstream of JNK is likely to be achieved by the activation of JNK-responsive transcription factors. The AP-1 family members Jun and Fos (in *Drosophila* encoded by the *jra* and *kayak* genes, respectively) are canonical downstream transcription factors that respond to JNK signaling in a variety of physiological situations (Kockel *et al*, 2001). In addition, recent studies from our

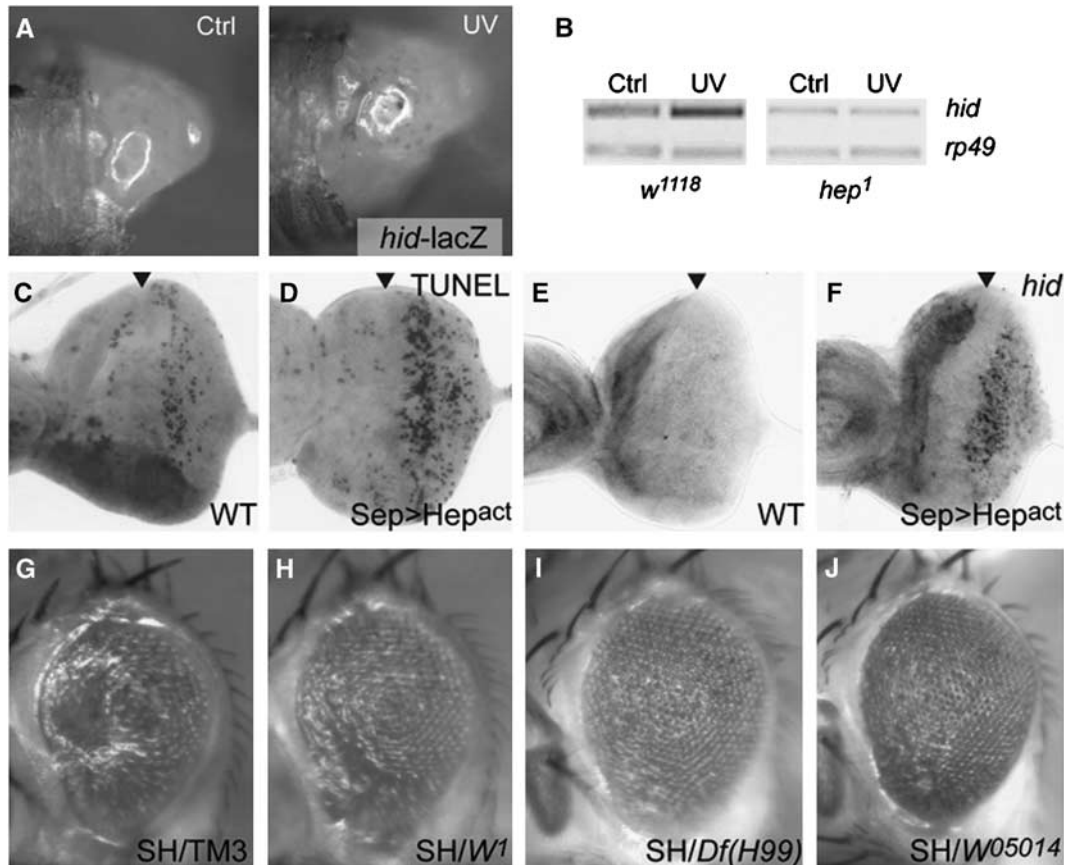


Figure 2 JNK-mediated *hid* induction induces apoptosis in the retina in response to UV irradiation. (A, B) *hid* is induced by UV irradiation in the *Drosophila* retina in a JNK-dependent manner. (A) *hid* expression 2 h after UV irradiation was detected by monitoring lacZ expression from a *hid* reporter line (Russell *et al*, 1998; Cox *et al*, 2000; Cullen and McCall, 2004; Sen *et al*, 2004). The irradiated eye is shown on the right (UV) and the shielded eye on the left (Ctrl). (B) Similarly, *hid* induction in wild-type animals (*w¹¹¹⁸*, left panel) could be observed using RT-PCR on retina dissected from pupae 2.5 h after irradiation (*rp49* transcript levels serve as internal controls). Induction of *hid* was not observed in *hep¹* hemizygous males (right panel), indicating a requirement for JNK signaling in the transcriptional response to UV. (C, D) Overexpression of constitutively active Hep (Hep^{act}) in developing photoreceptors and cone cells causes apoptosis. Increased TUNEL-positive cells were observed in larval eye imaginal discs expressing Hep^{act} under the control of Sep-Gal4 (D), compared to wild-type discs (C). Sep-Gal4 is active in the developing photoreceptors and cone cells posterior to the morphogenetic furrow (arrowhead; Jasper *et al*, 2002). (E, F) JNK activation is sufficient to induce *hid* expression in the developing retina. *In situ* hybridization demonstrating *hid* induction in response to overexpression of Hep^{act} under the control of Sep-Gal4 (F). (G–J) The resulting adult eye phenotype (G; sep-Gal4, UAS-Hep^{act} is abbreviated as SH) is reduced in *hid* mutant backgrounds (in heterozygous conditions for *hid* (alleles *W⁰⁵⁰¹⁴* and *W¹*) as well as all three RGH genes (*Df(H99)*).

and other laboratories have established the forkhead transcription factor Foxo as a downstream effector of JNK signaling in flies, mice and worms (Essers *et al*, 2004; Oh *et al*, 2005; Wang *et al*, 2005). A role for Foxo in inducing apoptosis has been described in mammalian cells, in which Foxo-mediated transcriptional induction of pro-apoptotic molecules, such as Bim, has been found (Accili and Arden, 2004; Greer and Brunet, 2005). *In vivo* confirmation of such a pro-apoptotic role of Foxo has been elusive.

To test whether AP-1 or Foxo acts downstream of JNK signaling in the control of apoptosis, we assessed whether JNK gain-of-function phenotypes in the eye are modulated when the gene dose of either *dfoxo* or the Dfos-encoding gene *kayak* (*kay*) is reduced. We found that introducing the *dfoxo* loss-of-function allele *dfoxo²¹* (Junger *et al*, 2003; Puig *et al*, 2003) reduced the JNK gain-of-function phenotype (Figure 3A and B). Similar effects were observed when introducing mutations in *kay* (*kay²*; Figure 3E) or in both *dfoxo* and *kay* (Figure 3G), as well as when expressing a dsRNA molecule directed against the *fos* transcript (Fos^{RNAi}; Hyun *et al*, 2006; Figure 3F). In contrast, overexpression of Foxo dramatically

enhanced the JNK gain-of-function background (Figure 3C; overexpression of Foxo alone under the control of the weak SepGal4 driver had no apparent effect, Figure 3D). These results indicate that Foxo and Fos are both required for JNK-induced apoptosis in the eye. We tested whether this requirement would also be observed in the apoptotic response to UV irradiation, and found that both heterozygotes (not shown) and homozygotes for the *dfoxo* loss-of-function alleles *dfoxo²¹* (not shown) and *dfoxo²⁵*, as well as transheterozygotes of *dfoxo²¹* and *dfoxo²⁵* have significantly reduced morphological defects after UV irradiation (Figure 3H, I and N). Similarly, decreasing Fos activity resulted in reduced apoptosis (Figure 3J, K, L and N). Increasing Fos expression mildly (using a heat-shock-inducible Fos transgene from which Fos is expressed at low levels at normal temperatures; Zeitlinger and Bohmann, 1999), on the other hand, led to hypersensitivity of the developing retina to UV (Figure 3M and N).

Transcriptional regulation of *hid* by Foxo and Fos

The requirement for Fos and Foxo in the UV-induced apoptotic response suggested that these transcription factors are in-

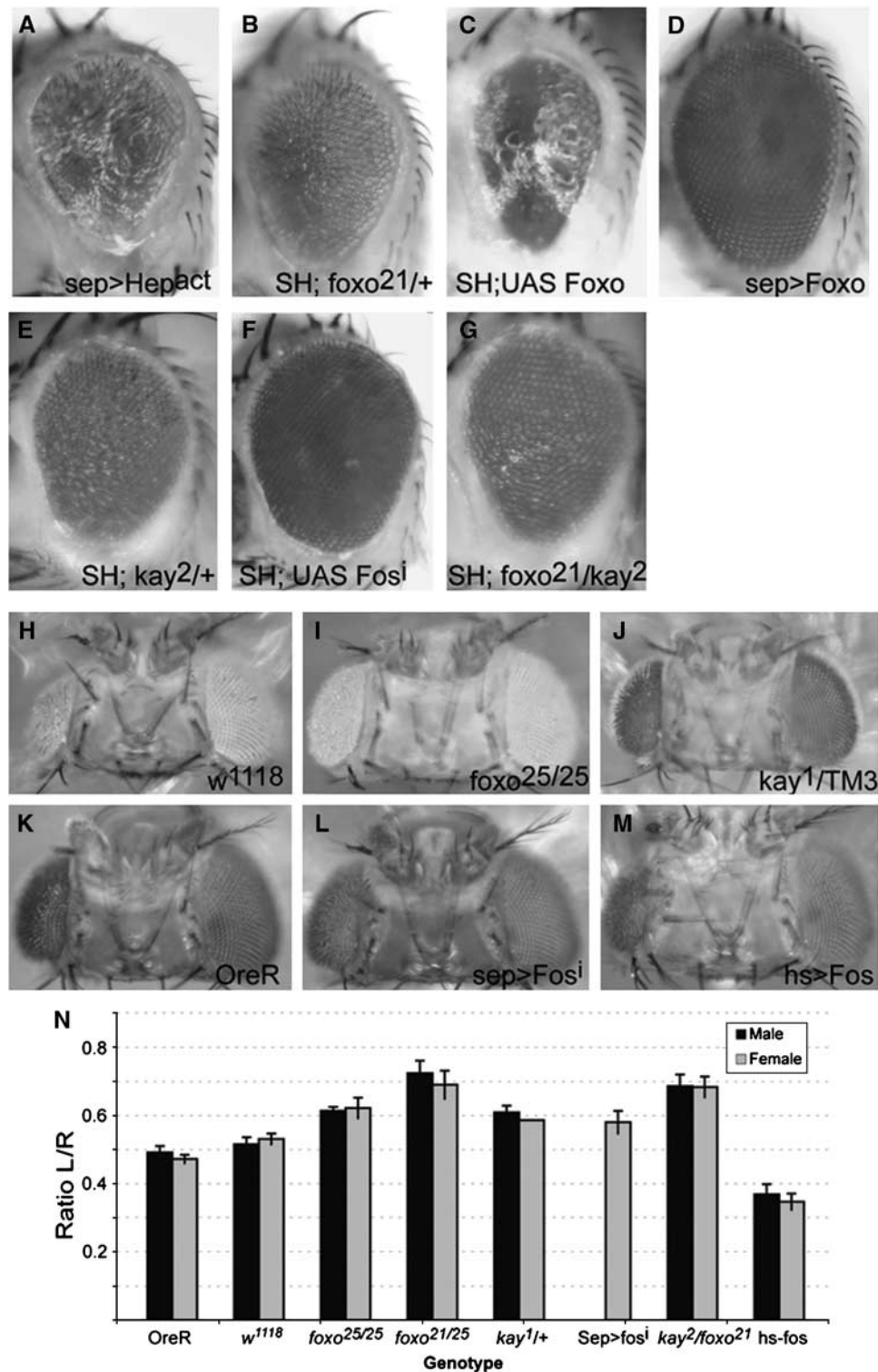


Figure 3 The transcription factors Fos and Foxo are required for UV-induced apoptosis in *Drosophila*. (A) Constitutive activation of JNKK (Hep) in the fly retina results in a ‘rough’ eye phenotype (expressed in photoreceptors and cone cells under the control of sep-Gal4). This eye phenotype (referred to as SH throughout) requires the downstream kinase, JNK (not shown). (B–D) The forkhead transcription factor FOXO genetically interacts with the JNK pathway. Heterozygosity for the loss-of-function allele *dfoxo*²¹ suppresses the JNK gain-of-function phenotype (B), whereas co-overexpression of Foxo greatly enhances the eye phenotype (C). Overexpression of Foxo alone with sep-Gal4 does not impact eye morphology (D). (E–G) Similarly, decreasing Fos function by introducing the loss-of-function mutation *kay*² (E), or by co-overexpressing dsRNA against Fos (F), rescues the sep>Hep^{act} phenotype. Reducing both *dfoxo* and *kay* gene dose (G) results in an almost full recovery of normal eye morphology. (H, I, N) Homozygosity for the *dfoxo* loss-of-function allele *dfoxo*²⁵ reduces UV-induced apoptosis. Treatment and quantification were performed as described in Figure 1. (J–N) Increased Fos expression (M, N) enhances, and loss of *fos* function (J, L, N) reduces UV-induced apoptosis in the eye, as compared to wild-type controls (K, N).

involved in the UV-induced transcriptional induction of *hid*. Supporting such a role of Foxo, we observed activation of the *hid* gene when wild-type Foxo or FoxoTM (a constitutively nuclear form of Foxo which can not be phosphorylated by Akt; Junger *et al*, 2003; Puig *et al*, 2003; Hwangbo *et al*, 2004) was overexpressed in the larval retina (*hid* induction was observed using either *hid-lacZ* (Figure 4A) or by RNA *in situ* hybridization (Figure 4B). We further found that *hid* is rapidly induced in response to Foxo activation in imaginal disc cells, mimicking its response to Hep^{act} overexpression (Figure 4C).

These experiments were performed using the TARGET system (McGuire *et al*, 2003). The TARGET system uses a temperature-sensitive Gal80 to inhibit Gal4 at the permissive temperature. Combining this with the Gal4-UAS system in flies allows for temporal control of transgene expression. Temporal control is important for these experiments, as constitutive expression of Hep^{act} or FoxoTM results in cell death, preventing transcriptional analysis of JNK/Foxo target genes. Importantly, our results indicate that the induction of *hid* by JNK signaling requires Foxo activity, as *hid* induction in

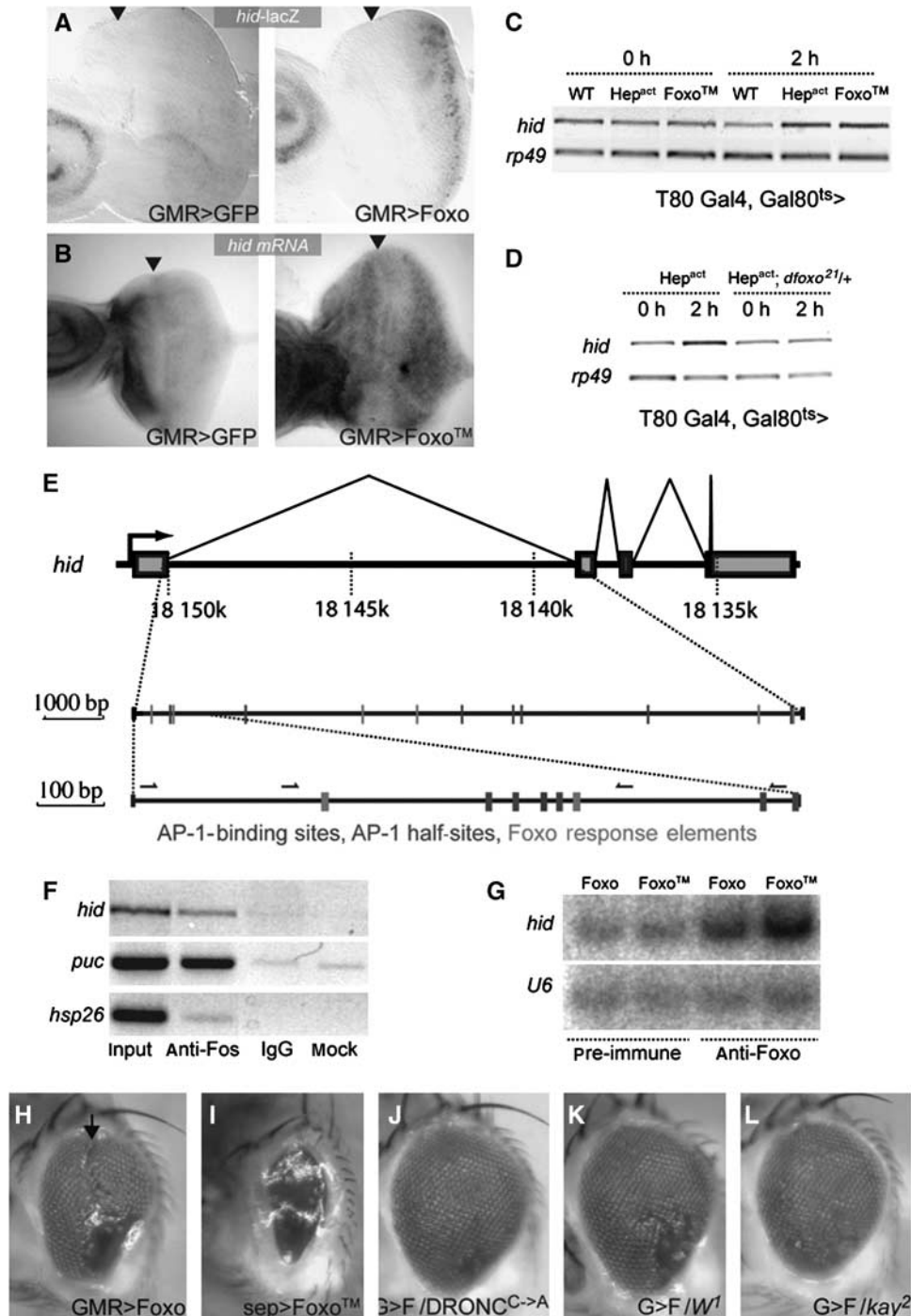


Figure 4 For caption see page 386

response to Hep^{act} expression in imaginal discs was not observed in a *dfoxo*²¹ heterozygous background (Figure 4D).

As clusters of Foxo and AP-1 response elements are present in the first intron of the *hid* gene (Figure 4E), we tested whether Fos and Foxo would bind to the *hid* locus. Using chromatin-immunoprecipitation (ChIP) against endogenous Fos in S2 cells, we found that Fos specifically and selectively binds to sites in the first *hid* intron (Figure 4F). Similarly, immunoprecipitation of transfected wild-type or constitutively active Foxo demonstrated binding of Foxo to the *hid* intron. Binding was more efficient for constitutively nuclear FoxoTM, supporting a role for Akt-mediated regulation of Foxo in the control of *hid* expression (Figure 4G, and see below). Control ChIPs targeting the second intron of *hid* yielded negative results (not shown), supporting the notion that Foxo and Fos selectively bind to the first intron of the *hid* locus.

Increasing Foxo expression in the fly retina (using the retinal driver GMR-Gal4) is sufficient to induce a weak phenotype that includes loss of ommatidia in the ventral areas as well as in the central midline of the eye (Figure 4H, arrow; see also Junger *et al*, 2003; Puig *et al*, 2003). When constitutively active Foxo (FoxoTM) is overexpressed in the retina, complete ablation of the ommatidia is observed (Figure 4I; sep-Gal4 is used here, as overexpression of FoxoTM with GMRGal4 leads to ablation of most head structures and pupal lethality), indicating that the pro-apoptotic function of wild-type Foxo is repressed under normal conditions, most likely by survival signals (see below). The loss of ommatidia observed in GMR>Foxo eyes was reduced when a dominant-negative form of the *Drosophila* caspase 9 homologue DRONC (Meier *et al*, 2000) was co-overexpressed, further supporting a pro-apoptotic role for Foxo in the fly (Figure 4J). Similarly, heterozygosity for *W¹* reduces this phenotype, indicating that *hid* is required downstream of Foxo to induce apoptosis (Figure 4K).

The genetic and biochemical results presented here suggest that binding by both Foxo and Fos to the first *hid* intron is required for JNK-induced apoptosis. Supporting this notion, the Foxo gain-of-function phenotype in the eye is reduced in a *kay*² mutant background (Figure 4L).

Modulation of the apoptotic response by survival signaling

Our data suggest that JNK-induced activation of Fos and Foxo and subsequent transcriptional induction of *hid* are required for DNA damage-induced apoptosis. At the same time, JNK-mediated activation of Foxo has beneficial effects for the organism, promoting stress tolerance and extending lifespan (Oh *et al*, 2005; Wang *et al*, 2005). These protective and pro-apoptotic roles of JNK and Foxo seem contradictory. Two possible explanations could reconcile these observations: (i) stress-induced apoptosis by JNK/Foxo signaling might be beneficial owing to the elimination of damaged cells that would otherwise contribute to deregulated overgrowth and consequent senescence of the organism. (ii) The effects of JNK/Foxo signaling might be context-dependent. Thus, activation of JNK and Foxo might elicit apoptotic responses only in conditions of severe cellular damage, whereas when cells are stressed, but able to adapt to the environment, the JNK/Foxo signaling axis might promote protective gene expression. This latter model has gained credence in recent years with the observation that JNK- as well as Foxo-induced apoptosis can be modulated by survival signals (Brunet *et al*, 2004; Janes *et al*, 2005).

Foxo is negatively regulated by a number of survival signaling pathways, most notably signaling through Akt. Cells that receive abundant survival signals are thus expected to be resistant to JNK-mediated apoptosis. We tested this by analyzing whether the activity of EGFR or insulin receptor (InR) signaling would modulate JNK/Foxo-mediated apoptosis in the retina. EGFR signaling plays a crucial role in cell division and photoreceptor determination in the developing fly retina, but is also an important source for survival stimuli in differentiating neurons (Freeman and Bienz, 2001; Brown and Freeman, 2003). Similarly, InR signaling has been proposed to provide cell survival signals in the fly (Scanga *et al*, 2000). EGFR signals through Ras, which in turn activates a number of downstream signaling pathways, including phosphatidylinositol 3 kinase (PI3K)—Akt signaling. Supporting a role for EGFR in preventing excessive apoptosis after JNK and Foxo activation, we found that a reduction in the EGFR or Ras gene dose dominantly enhanced JNK and Foxo

Figure 4 Transcriptional regulation of *hid* by Fos and Foxo. (A, B) Foxo overexpression is sufficient to induce *hid* expression in the developing retina. (A) LacZ staining of flies overexpressing GFP (as control, left panel) or Foxo (right panel) posterior to the morphogenetic furrow in a *W⁰⁵⁰¹⁴* (*hid-lacZ*) background. (B) RNA *in situ* hybridization detecting *hid* transcript in eye imaginal discs of flies overexpressing GFP (left panel) or constitutively nuclear Foxo (FoxoTM; right panel) posterior to the morphogenetic furrow (arrowheads). (C) Foxo and JNK signaling induces *hid* expression in wing imaginal discs. *hid* induction was detected using RT-PCR in wing imaginal discs in which Hep^{act} or Foxo expression was induced using the TARGET system (McGuire *et al*, 2003). In this system, a temperature-sensitive allele of Gal80 inhibits Gal4-mediated transcription until flies are heat-shocked. Heat shock was performed for 30 min at 37°C. At 2 h after induction of Hep^{act} (HA) or of a constitutively nuclear form of Foxo (FoxoTM), increased *hid* expression can be detected. Lanes are as follows: WT, wild-type wing discs (T80Gal4/CyO; Gal80^{ts}/TM3); HA, Hep^{act}-expressing discs (T80Gal4/UASHeP^{act}; Gal80^{ts}/TM3); 0 h, dissected immediately after heat shock; 2 h, dissected 2 h after heat shock. (D) *hid* induction in wing imaginal discs in response to JNK activation is lost in a *dfoxo* heterozygous mutant background. Experiment was performed as in (B), with flies of the following genotypes: *w*; T80-Gal4/UASHeP^{act}; Gal80^{ts}/+ (left lanes) or *w*; T80-Gal4/UASHeP^{act}; Gal80^{ts}/*dfoxo*²¹. (E) Structure of the *hid* locus. Coordinates for chromosome 3L are listed. The first intron of *hid* contains multiple AP-1-binding sites (TGANTCA, blue), as well as AP-1 half-sites (TGNNTCA, brown) and Foxo response elements (TTGTTTAC, FRES, red). Arrows indicate binding sites for primers used for ChIPs in panels F and G. (F) Chromatin IP demonstrating binding of Fos to the *hid* locus in S2 cells. PCR on the same ChIP material against the *puc* locus is included as positive control and against the *hsp26* locus as negative control (Lee *et al*, 2005). Primers used for PCR flank the 5' cluster of AP1 and Foxo-binding sites in the first *hid* intron and are indicated as arrows in panel E. (G) Chromatin IP demonstrating binding of Foxo to the *hid* locus in S2 cells. PCR on the same ChIP material against the *arrows* snRNA promoter is included as negative control. Wild-type Foxo (WT) or constitutively nuclear Foxo (TM) was transfected into S2 cells and immunoprecipitated using anti-Foxo antibody or pre-immune serum as control (see also Puig *et al*, 2003). Note that constitutively nuclear Foxo (TM) binds more efficiently to the *hid* locus than wild-type Foxo. Primers used for PCR flank the 5' cluster of AP1 and Foxo-binding sites in the first *hid* intron and are indicated as arrows in (E). (H, I) Overexpression of wild-type (H) or constitutively nuclear (I) Foxo in the developing retina leads to ommatidia loss in the adult eye. (J) The phenotype caused by Foxo overexpression in the eye can be reduced by co-overexpression of a dominant-negative form of the *Drosophila* Caspase DRONC (Meier *et al*, 2000). GMR>Foxo is abbreviated here as G>F for clarity. (K) Similarly, reduction in the gene dose of *hid* (*W1*) leads to a partial rescue of the Foxo gain-of-function phenotype. (L) Foxo-induced apoptosis requires Fos function. Reduced apoptotic defects in eyes expressing wild-type Foxo in a *kay*² heterozygous background.

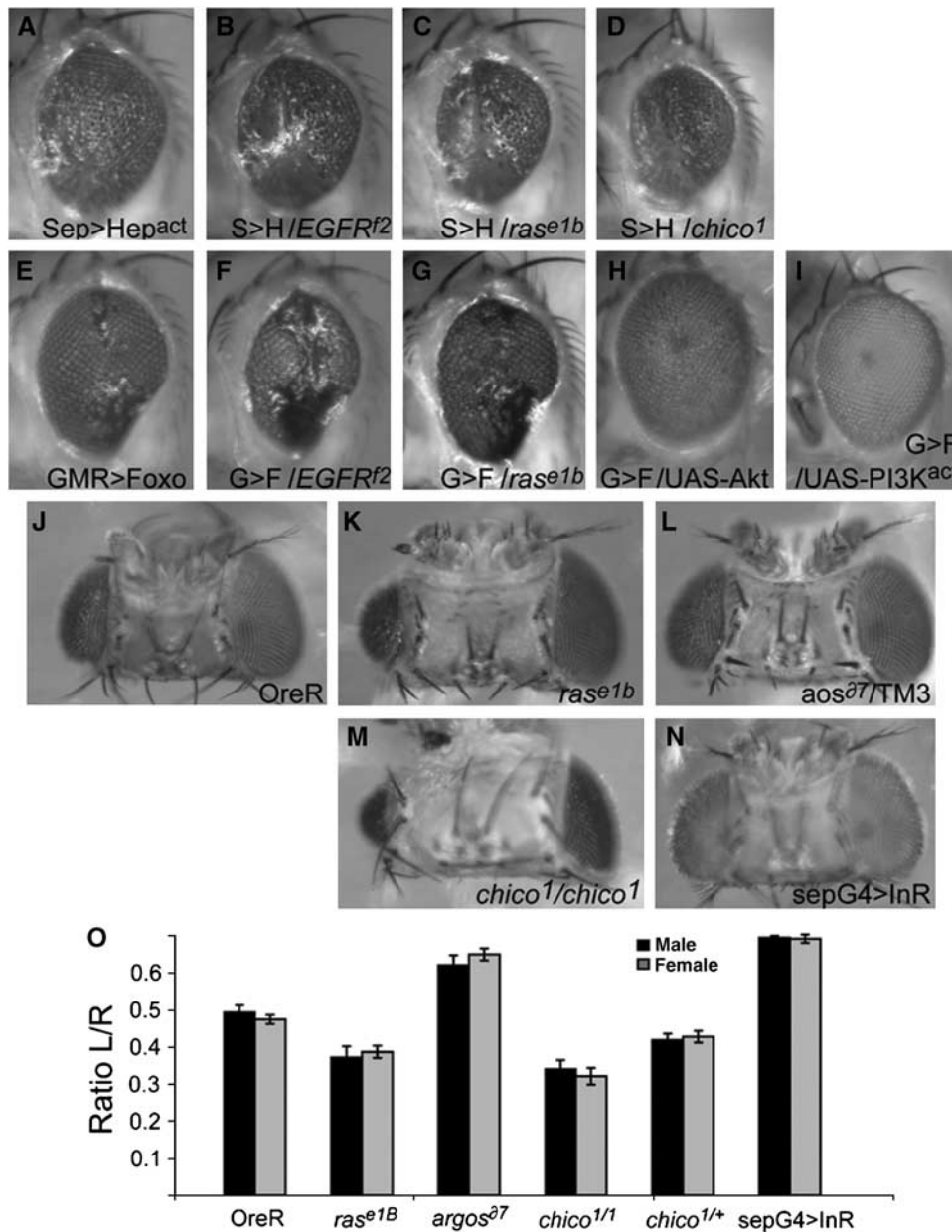


Figure 5 EGFR and Insulin signaling suppress JNK-mediated apoptosis. Decreased level of EGFR activity by mutations in either *EGFR* (B) or *ras* (C) and reduced insulin signaling owing to loss of *chico* (D) substantially enhances the apoptotic phenotype induced by overexpression of *Hep^{act}* (A). Similarly, the eye phenotype induced by Foxo overexpression (E) is enhanced by reduced *EGFR* (F) or *ras* gene dose (G). Conversely, blocking Foxo activity by co-overexpressing Akt or active PI3K blocks the Foxo gain-of-function phenotype (H, I). (J–O) UV-induced apoptosis is reduced by EGF/insulin-mediated survival signaling. Reduced activity of *ras* (K, O) or *chico* (M, O) results in strongly increased UV-induced defects in the eye. Conversely, loss of *aos*, a negative regulator of EGFR, or increased expression of InR, decreases UV-induced apoptotic defects (L, N, O). Genotypes are as follows: A, *w*; *sep-Gal4*, UAS-*Hep^{act}*/+; B, *w*; *sep-Gal4*, UAS-*Hep^{act}/egfr^{Δ2}*; C, *w*; *sep-Gal4*, UAS-*Hep^{act}*/+; *ras^{e1B}*/+; D, *w*; *sep-Gal4*, UAS-*Hep^{act}/chico¹*; E, *w*; *GMR-Gal4*, UAS-*Foxo*/+; F, *w*; *GMR-Gal4*, UAS-*Foxo/egfr^{Δ2}*; G, *w*; *GMR-Gal4*, UAS-*Foxo*/+; *ras^{e1B}*/+; H, *w*; *GMR-Gal4*, UAS-*Foxo/UAS-Akt*; I, *w*; *GMR-Gal4*, UAS-*Foxo/UAS-PI3K^{act}*; J, *OreR*. K: *ras^{e1B}/TM3*; L, *aos^{Δ7}/TM3*; M, *chico¹/chico¹*; N, *w¹¹¹⁸*; *sep-Gal4/UAS-InR*.

gain-of-function phenotypes in the eye (Figure 5A–C and E–G). A similar enhancement was observed when the gene dose of *chico*, which encodes the *Drosophila* IRS-1 homologue, was reduced (Figure 5D). *Chico* relays signals from the *Drosophila* insulin receptor to PI3K, ultimately activating Akt. Both EGFR and InR signaling might therefore have survival functions in the eye that are mediated by Akt-induced Foxo inactivation. Accordingly, Foxo gain-of-function phenotypes were rescued when Akt or activated PI3K were overexpressed (Figure 5H and I). The role of Akt

phosphorylation in inhibiting Foxo-induced apoptosis is supported by the complete ablation of normal ommatidial structures when constitutively nuclear Foxo (FoxoTM, in which the Akt phosphorylation sites are mutated) was overexpressed in the retina (Figure 4I).

To test whether survival signals initiated by receptor tyrosine kinases would influence the apoptotic response to DNA damage, we assessed the extent of UV-induced morphological defects in the retina of EGFR and InR pathway mutants. We observed a marked increase in UV-induced

apoptosis in flies heterozygous for *ras* or homozygous mutant for *chico* (Figure 5J, K and M–O). Furthermore, reduction in the gene dose of the EGF antagonist *argos* (thus increasing EGFR activity in the eye), or increased expression of *InR*, was sufficient to reduce the extent of UV-induced apoptosis in the retina (Figure 5L, N and O).

Discussion

Our results indicate that transcriptional regulation of *hid* is a crucial component to the DNA damage-induced apoptotic response in flies. The JNK-responsive transcription factors Foxo and Fos are both required for this response, suggesting that binding of both factors to their binding sites in the *hid* locus is required for *hid* induction. Thus, negative regulation of either of these factors would lead to repression of JNK-induced apoptosis (Figure 6). Accordingly, we show that RTK-induced Akt signaling, which inhibits Foxo, desensitizes cells towards DNA damage-induced apoptosis.

The requirement for Foxo in JNK-mediated apoptosis allows fine tuning of the decision between life and death of a cell, as the balance between Akt-mediated survival stimuli that inhibit Foxo activity and JNK-induced pro-apoptotic activation of Foxo would ultimately decide the cellular response to DNA damage. This interpretation is supported by our observation that UV-induced apoptosis in the pupal retina is influenced by the activities of the EGFR and insulin-signaling pathways and by the fact that overexpression of a mutant of Foxo that cannot be phosphorylated by Akt (and is therefore constitutively active) results in complete ablation of ommatidial structures. Mathematical models of signaling networks in cancer cells have predicted the importance of balancing survival signals and JNK activity for accurate control of the cellular decision between death and survival

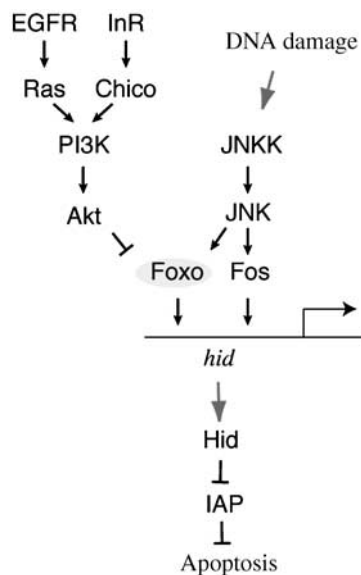


Figure 6 Model for the regulation of UV-induced apoptosis in the *Drosophila* retina. JNK activation results in Foxo- and Fos-dependent transcriptional upregulation of *hid*. *Hid* inhibits IAP and induces caspase-dependent apoptosis. Induction of *hid* can be blocked by *InR* or EGFR-initiated survival signals that inhibit Foxo activity. The relative balance between stress (JNK) and survival (RTK) signaling determines the cellular response to UV-induced DNA damage.

(Janes *et al*, 2005). Our work presented here validates these predictions *in vivo* and identifies the action of Foxo and Fos on the *hid* promoter as a crucial sensor and readout of this balance.

How is the cellular response to JNK/Foxo signaling regulated?

Our model proposes that JNK-mediated activation of Foxo leads to its nuclear translocation, where it activates pro-apoptotic gene expression. Interestingly, JNK signaling and Foxo activity have also been found to induce protective stress response molecules and damage repair proteins in flies as well as in *C. elegans* and mammalian cells, suggesting a role for Foxo in promoting cell repair and survival in response to stress (Kops *et al*, 2002; Murphy *et al*, 2003; Brunet *et al*, 2004; Wang *et al*, 2005). Evidently, the cellular context determines how a JNK/Foxo signal is interpreted. A variety of mechanisms governing the decision between Foxo-induced protective gene expression and Foxo-induced apoptosis can be envisioned. One candidate determinant of Foxo function is its acetylation status, which is influenced by the protein deacetylase Sir2 and which is believed to affect its promoter specificity (Brunet *et al*, 2004; Motta *et al*, 2004).

Specific responses to JNK/Foxo activity could equally be achieved by the availability of transcriptional cofactors of Foxo that are required to selectively activate the expression of certain genes. Our results suggest that Fos is a pro-apoptotic transcriptional cofactor of Foxo in the fly. Among other recently identified cofactors of Foxo in mammalian cells and *C. elegans* are β catenin and SMK-1, both of which appear to be required for selected functions of Foxo and are thus prime candidates for additional specificity-providing inputs (Essers *et al*, 2005; Wolff *et al*, 2006).

An alternative mechanism by which the decision between death and survival downstream of JNK could be controlled *in vivo* has been proposed (McEwen and Peifer, 2005). JNK-induced cell death might be governed by a timing mechanism, in which short-term activation of JNK (which is normally inhibited by a negative feedback loop involving Puc) would allow cell repair, whereas long-term activation would lead to cell death. Such a time-dependent cellular response to JNK activation has been observed in mammalian cells (Karin and Gallagher, 2005; Ventura *et al*, 2006). It could explain why apoptosis in the eye is observed only when photorepair is deficient (and thus JNK is active for a long period of time), but not when JNK is activated transiently by UV, but DNA is repaired. In this model, the activity of EGFR/Akt signaling might change the threshold that distinguishes between pro-apoptotic and prorepair functions of JNK/Foxo signaling.

Accurate understanding of the above-mentioned mechanisms and their importance for the regulation of cell fate *in vivo* is required to gain insight into how organisms fend off environmental insults and is imperative for understanding disease processes like cellular senescence during aging or cancer.

Materials and methods

Fly strains and handling

The following fly stocks were obtained from the Bloomington Stock Center: *OreR*, *w¹¹¹⁸*, *W⁵⁰¹⁴*, *Df(H99)*, *ras^{EB}*, *chico¹* and *argos^{delta-7}*. *hep¹/FM6* was a gift from S Noselli; *puc^{E69}* was a gift from E Martín-

Blanco; UAS^{Hep}^{act}, *GMRGal4* and *sepGal4* were gifts from M Mlodzik; *Dfoxo*²¹/*TM3* and *Dfoxo*²⁵/*TM3* were gifts from E Hafen; UAS^{pro}.Dronc^{CtoA} was a gift from G Evan; UAS^{Dfoxo} is described in Puig *et al* (2003); UAS^{Dfoxo}TM was a gift from Marc Tatar (Hwangbo *et al*, 2004). Other fly strains are as described: UAS^{fos}^{RNAi} (Hyun *et al*, in preparation); *hs-fos* and *kay*²/*TM3* (Zeitlinger and Bohmann, 1999). In the transient overexpression experiments, flies were raised at room temperature (21°C). Wandering third instar larva were collected and heat-shocked at 37°C for 1 h and subsequently kept at room temperature for another 2 h.

Pupal UV irradiation and phenotype quantification

Mid-aged pupae (24 h after puparium formation) were collected and subjected to surgical removal of the pupal shell surrounding the head area. UV irradiation was carried out on larvae that were immobilized on the side, so that only one retina was exposed to UV. A UV crosslinker (Stratalinker, 1800) was used with energy set at 5 mJ/cm². After irradiation, pupae were kept in the dark until being processed.

The images of UV-damaged adult eyes were taken from the top so that both eyes were visible. The boundary of each eye was outlined using Photoshop. The eye size was determined by measuring the number of pixels contained within this area. Ratios between the area of irradiated and non-irradiated eyes were then determined.

TUNEL staining

TUNEL stainings were performed using the ApopTag kit (Chemicon International) following the instructions.

RT-PCR

Twenty wing or eye imaginal discs were collected for each RNA extraction. Total RNA was isolated using TRIzol (Invitrogen), and RT-PCR was performed as previously described (Jasper *et al*, 2001; Wang *et al*, 2003). Primers include *hid* (sense, 5'-TGCGAAATA CACGGTTCA-3' and antisense 5'-CCAATATCACCCAGTCCCG-3') *puc* (sense, 5'-CGAGGATGGTTGATTACGA-3', and antisense,

5'-TCAGTCCCTCGTCAAATTGCT-3') and *rp49* (sense, 5'-TCCTAC CAGCTTCAAGATGAC-3' and antisense, 5'-CACGTTGTGCACCAG GAAC-3')

Beta-gal staining

Samples were fixed in 1% glutaraldehyde at room temperature and washed with PBS + 2 mM MgCl₂. The staining solution contained 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆ and 0.1% X-gal. Stainings were performed at 30 or 37°C for 48 or 24 h, respectively.

Chromatin immunoprecipitation

Chromatin from continuously dividing S2 cells was collected according to the protocol of a commercial ChIP assay kit (Upstate Biotechnologies). Rabbit anti-D-Fos antibody (Ciapponi *et al*, 2001) was used for immunoprecipitation. Promoter regions with or without AP-1 sites of *puc*, and *hsp26* were detected in the precipitated material by PCR using the following primer sets: *hid* (5'-ATTGTGTGGGTTAATCAGGA-3' and 5'-TTGTAAGATTCCAC TTGG-3'); *hsp26* (5'-TTAATAAAGAGGAAAACCAG-3' and 5'-AA AAATAAACTAACTAACCTT-3'); and *puc* (5'-GGTTTGAGCCCGA GATAA-3' and 5'-ACTGAAGACTTTGCGGTTGAA-3'); see also Lee *et al* (2005).

ChIP assays for Foxo were performed as described (Puig *et al*, 2003), using an anti-DFoxo antibody and the following primers: *hid* (5'-TTGTGATAAGCTGACAAAGTGCTG-3' and 5'-CAAGAAGGA TTTTCATAGGATCTCCTG-3') and *U6 snRNA* (5'-GCAGAGGGTTCT TAAGACCATTTGCC-3' and 5'-GCTTCACGATTTGCGTGCATCC-3').

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