Puckered, a *Drosophila* MAPK phosphatase, ensures cell viability by antagonizing JNK-induced apoptosis

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**Summary**

MAPK phosphatases (MKPs) are important negative regulators of MAPKs in vivo, but ascertaining the role of specific MKPs is hindered by functional redundancy in vertebrates. Thus, we characterized MKP function by examining the function of Puckered (Puc), the sole *Drosophila* Jun N-terminal kinase (JNK)-specific MKP, during embryonic and imaginal disc development. We demonstrate that Puc is a key anti-apoptotic factor that prevents apoptosis in epithelial cells by restraining basal JNK signaling. Furthermore, we demonstrate that JNK signaling plays an important role in γ-irradiation-induced apoptosis, and examine how JNK signaling fits into the circuitry regulating this process. Radiation upregulates both JNK activity and puc expression in a p53-dependent manner, and apoptosis induced by loss of Puc can be suppressed by p53 inactivation. JNK signaling acts upstream of both Reaper and effector caspases. Finally, we demonstrate that JNK signaling directs normal developmentally regulated apoptotic events. However, if cell death is prevented, JNK activation can trigger tissue overgrowth. Thus, MKPs are key regulators of the delicate balance between proliferation, differentiation and apoptosis during development.

Key words: Apoptosis, JNK, Phosphatase, *Drosophila*

**Introduction**

The balance between cell proliferation, differentiation and death is strictly regulated during development. Genetic or epigenetic disruption of this balance has severe consequences, including both degenerative and hyper-proliferative diseases. To understand the mechanistic basis by which cells balance proliferation, differentiation and apoptosis and correct imbalances triggered by outside stress, we used *Drosophila* as a model system to examine the role of mitogen-activated protein kinase (MAPK) signaling in this process.

Three evolutionarily conserved MAPK pathways have been described. These cascades relay information about the environment to the nucleus via the sequential phosphorylation of a series of kinases, with terminal activation of the MAPKs ERK, Jun N-terminal kinase (JNK) or p38 eliciting changes in gene transcription (reviewed by Schaeffer and Weber, 1999).

MAPKs are key modulators of cell proliferation, differentiation and apoptosis during oncogenesis. The Ras-Raf-ERK pathway has received the most attention for its role in promoting transformation. By contrast, the JNK pathway has both oncogenic and tumor-suppressing roles depending on the cellular context. For example, transformation of leukemia cell lines by Bcr-Abl is dependent on JNK signaling (Dickens et al., 1997; Raitano et al., 1995). By contrast, chemotherapeutic agents promote JNK-dependent apoptosis (Osborn and Chambers, 1996; Seimiya et al., 1997; Stadheim and Kucera, 2002). Thus, JNK signaling can both support and antagonize transformation; however, the molecular mechanisms underlying this choice are not well defined.

JNK signaling is initiated by dual phosphorylation of threonine and tyrosine residues in its activation loop, while removal of either phosphate inactivates MAPKs (reviewed by Davis, 2000). Thus, steady-state levels of MAPK activity depend upon the equilibrium between MEK kinases and protein phosphatases. MAPK phosphatases (MKPs) are one class of proteins tempering MAPK activity (reviewed by Keyse, 1998). At least nine vertebrate MKPs, all very similar in their phosphatase domains, have been identified. One class, typified by MKP-1/CL100, are immediate-early genes: their transcription is activated by JNK signaling and they limit the peak level and duration of MAPK activity by completing a negative-feedback loop. As differences in peak amplitude and duration of MAPK signaling can elicit distinct cellular responses, MKP activity may play an important role in regulating biological responses to stimuli.

Functional redundancy among the three JNK isoforms complicates analysis of JNK signaling in mammals (reviewed by Davis, 2000). By contrast, a single JNK [*basket (bsk)*] exists in *Drosophila* and plays an important role in embryonic development (Riesgo-Escovar et al., 1996; Sluss et al., 1996). Likewise, little is known about the in vivo function of vertebrate MKPs, as functional redundancy among MKPs (Dorfman et al., 1996) and overlapping substrate specificity of distinct MKPs (Franklin and Kraft, 1997; Sun et al., 1993) hinder the assessment of phenotypes in vivo. Again, a single
MKP, Puckered (Puc), antagonizes JNK signaling in Drosophila (Martin-Blanco et al., 1998; Rintalan et al., 2003). Thus, Drosophila has emerged as an excellent model system for characterizing both the function and regulation of JNK signaling in vivo.

Drosophila JNK signaling coordinates dorsal closure, a morphogenetic event that unites the two lateral epidermal sheets. Mutations in any component of the JNK pathway, like hemipterous (the JNK kinase), bsk, kayak (Kay; Drosophila fos) and Dijun (Ira – FlyBase) disrupt dorsal closure (reviewed by Noselli and Agnes, 1999; Stronach and Perrimon, 1999). This may be an evolutionarily conserved mechanism, as JNK signaling regulates vertebrate cell migration and epithelial wound healing (reviewed by Martin and Parkhurst, 2004). puc zygotic mutants also exhibit defects in dorsal closure; increased JNK signaling in puc mutants disrupts alignment of the lateral epidermal sheets (Martin-Blanco et al., 1998). Thus, the tempering of JNK signaling by Puc is crucial during dorsal closure.

In addition to regulating morphogenesis, JNK signaling is also activated by other signaling pathways; in this context, JNK activation triggers apoptosis. In Drosophila, these include TGFβs (Igaki et al., 2002; Moreno et al., 2002b), TGFβ (Adachi-Yamada et al., 1999; Adachi-Yamada and O’Connor, 2002; Adachi-Yamada and O’Connor, 2004) and Myc (de la Cova et al., 2004; Moreno and Basler, 2004). However, the function of Puc during the induction of apoptosis is less clear.

Previous analysis of Puc focused on zygotic mutants retaining maternally contributed Puc. Here, we extend this analysis by completely removing both maternal and zygotic Puc from embryos, and by eliminating Puc function in imaginal disc cells. We find that Puc is required continuously to antagonize JNK-dependent apoptosis in a cell-autonomous manner, suggesting that basal JNK signaling is poised just below a threshold required to eliminate epithelial cells. Second, we find that JNK signaling also plays a key role in promoting apoptosis in response to a diverse array of signals, including both the p53-dependent response to DNA damage and developmentally regulated apoptosis. Finally, we demonstrate that when apoptosis is blocked, Puc prevents tissue overgrowth by preventing unbridled JNK activation. Thus, JNK signaling can promote apoptosis or proliferation in different cellular contexts, and Puc plays an important role in controlling this balance.

Materials and methods

Genetics

Wild-type was CantonS or y w (for immunofluorescence). Sources of stocks: FRT82B pucA251.1 (L. Dobbens); pucR10 (A. Martinez-Arias – it was recombined onto FRT82B); and rpr-11-lacZ (Abrams et al., 1993). UAS-p53Gus 3.1 (Brodsky et al., 2000), p53b1/or (-) (a null allele) and other stocks were from the Bloomington Stock Center (flybase.bio.indiana.edu). Myc-Puc carries six tandem copies of the human MYC epitope in frame with the N terminus of Puc, and is cloned in p[UA3p]. UAS-myc-Puc Dead is identical except for a 185D>A site-directed mutation, targeting the catalytic D conserved in all tyrosine phosphatases. It has previously been shown to inactivate PTP1B (Flint et al., 1997). Overexpression of myc-Puc Dead has no effect on viability. Transgenics were generated by P-element transformation. Maternal-zygotic puc mutants were generated as described by Xu and Rubin (Xu and Rubin, 1993). hs-flp/; FRT82B puc/FRT82B ovoD1-18 larvae were heat-shocked for 3 hours at 37°C, ~72 hours after egg laying, and females mated to wild-type or puc/+ males. MARCM (Lee and Luo, 1999) was used to generate GFP-marked clones. hsflp1; FRT82B pucA251.1/TM6b males were crossed to Act5C-GAL4 UAS-GFP; FRT82B tubP-GAL80.L3 females, larval heat-shocked 1 hour at 37°C, 3-4 days after egg laying, and wing discs dissected from non-Tb wandering 3rd-instar female larvae. Mutant eye discs were derived as described by Stowers and Schwarz (Stowers and Schwarz, 1999). FRT828 w;+o; FRT828 pucA251.1/TM3, FRT828 pucR10/TM3, bsk2/+; FRT828 pucA251.1/TM3, or FRT828 pucR10/TM3 males were mated to y w; eyeless-GAL4 UAS-FLP1(D1); FRT828 GMR-hid554, l(3)/TM2 females.

Irradiation

Wandering 3rd-instar larvae were given 4000 rads of γ-radiation from a 137Cs source, and allowed to recover for 4 hours at 25°C before dissection.

Immunofluorescence

Dechorionated embryos were fixed for 20 minutes in 1:1 3.7% formaldehyde/PBS:heptane and methanol-devitellinized. Discs were fixed with 3.7% formaldehyde/PBS+0.5% Triton-X-100 (PTB5) for 20 minutes on ice. Tissues were blocked for 3 hours at room temperature in PBS+0.1% Triton-X-100 (PTB) (embryos) or PTB5 (discs) + 10% BSA, incubated with primary antibody overnight at 4°C, washed in PTB or PTB5, incubated for 1 hour at room temperature with fluorescent secondary antibodies (1:1000; Molecular Probes) and washed.

Antibodies

Rat-α-DE-cad2 (1:250; DSHB), mouse-α-phosphotyrosine (4G10 1:1000; Upstate), α-Engrailed (4D9 1:50; DSHB), β-α-galactosidase (Promega; 1:1000) and α-Myc (9E10; 1:5) were used. Alexa488- or Alexa568-phalloidin (Molecular Probes) and Hoechst (Sigma) were used at 1:1000.

TUNEL labeling

Samples were post-fixed for 15 minutes in 3.7% formaldehyde in PTB5, permeabilized in 100 mM sodium citrate/0.1% Triton-X-100 at 65°C for 30 minutes, rinsed in PTB5, and free DNA ends fluorescein-labeled using the In Situ Cell Death Detection Kit (Roche). Samples were mounted in Aqua Poly/quick (Polysciences).

Image collection and processing

Images of embryos, wings and legs were captured on a Nikon OPTIPHOT-2 with Kodak Technical Pan film and digitized with a Polaroid SprintScan35. Eyes, thorax and genitalia were photographed with a stereomicroscope-mounted Nikon Coolpix4500 digital camera after immersion in 70% ethanol. z-series were collected with a Zeiss 510 confocal microscope, and brightest-point projections generated with Zeiss LSM software. Brightness, contrast and levels were adjusted in Photoshop 7.0.

Results

Complete loss of Puc activity promotes cell-autonomous apoptosis

puc zygotic mutants die with defects in dorsal closure and inappropriate cell fate choices in the ventral epidermis, which reflect inappropriate activation of JNK signaling (Martin-Blanco et al., 1998) and may also reflect effects on other signaling cascades, such as the Wnt pathway (McEwen et al., 2000).

Maternally contributed mRNA and protein often mask requirements for genes during early Drosophila development; thus, we used the hs-flp/DFS technique (Chou and Perrimon, 1992) to remove both maternal and zygotic Puc function.
Mothers with germlines homozygous for the strongest allele, \( puc^{R10} \), failed to produce eggs, but females with germlines homozygous for \( puc^{A251.1} \), a strong allele, lay reduced numbers of small eggs, allowing us to assess the effect of complete loss of Puc function.

\( puc^{A251.1} \) maternal/zygotic (M/Z) mutant embryos have severe defects in embryonic development, secreting only fragmented scraps of cuticle (compare Fig. 1B with 1A; henceforth, unless noted, puc refers to \( puc^{A251.1} \)). The same phenotype was noted using different paternally contributed puc alleles, suggesting it is due to puc and not another second-site mutation (see Table S1 in the supplementary material). Similar 'scraps'-like phenotypes are observed when epithelial cells initiate apoptosis (e.g. \( crb \) mutants) (Tepass et al., 1990). Thus, we used TUNEL labeling to assess whether the \( puc^{M/Z} \) phenotype resulted from widespread apoptosis. Late-stage \( puc^{M/Z} \) mutant embryos have disorganized epithelia with highly elevated numbers of TUNEL-positive cells (Fig. 1G,H) relative to similarly staged wild-type controls (Fig. 1E,F). A similar increase in apoptosis was observed in living \( puc^{M/Z} \) mutants stained with Acridine Orange (data not shown). Thus, Puc normally prevents the induction of apoptosis throughout the embryonic epidermis.

The cuticle defects and widespread apoptosis of \( puc^{M/Z} \) mutants are suppressed if the embryo receives paternal wild-type Puc (Fig. 1C,I,J), but this fails to rescue embryo viability. \( puc \) maternal-only mutants have patterning defects distinct from those of zygotic mutants, exhibiting segmental deletions and fusions, as evidenced by loss of Engrailed stripes (compare Fig. 1L with 1K) and the corresponding loss of denticle belts (compare Fig. 1C with 1D). Thus, maternal Puc plays a distinct role in establishing segmentation, but we have not pursued this role further.

Given the clear role for Puc in preventing apoptosis in \( puc^{M/Z} \) mutants, we re-examined the ventral segment-polarity phenotype of \( puc \) zygotic mutants (McEwen et al., 2000) to see if this might also be, in part, the result of apoptosis. Increased numbers of apoptotic cells were observed in a segmentally repeated pattern in both the ventral and lateral epidermis (compare Fig. 1M with 1F); JNK signaling is upregulated in a similar segmental pattern (Fig. 1M). Thus, the \( puc \) zygotic mutant phenotype also reflects an increase in JNK-dependent apoptosis.

To further characterize the role of Puc during epithelial development, we examined its function in the imaginal discs, precursors of the adult epidermis. Clones of \( puc \) mutant tissue were generated in the wing disc, with mutant cells positively marked by GFP (Fig. 2A-D). Although clones were readily derived from wild-type controls (Fig. 2A), neither \( puc^{A251.1} \) (Fig. 2B) nor \( puc^{R10} \) (Fig. 2C) mutant clones were recovered...
anywhere throughout the wing disc. We did not observe puc mutant clones even when we looked as soon as 8 hours after clone induction, when wild-type clones were only a few cells in size. We cannot rule the possibility that loss of Puc may reduce proliferation, but given the clear induction of apoptosis in the embryonic epidermis following Puc loss, we think apoptosis is the most likely explanation for clone loss in the wing.

To confirm that loss of epithelial cells resulted from loss of Puc, a Myc-epitope tagged puc transgene (myc-puc) was used to rescue puc clones. Addition of Puc+ activity back to puc clones at the time of clone induction, using this transgene, rescued cell viability (e.g. Fig. 2D). Rescued puc mutant clones were recovered throughout the wing disc, though at lower frequencies than wild-type controls; we suspect that the delay in myc-puc transgene expression after clone induction (Gal4-mediated Myc-Puc accumulation requires degradation of Gal80) means that many puc cells are committed to die before rescue can occur.

Next, we assessed whether Puc plays a similar role in eye discs. Removal of Puc function from the entire eye imaginal disc results in nearly complete loss of eye tissue (puc+/H9253, Fig. 2F; pucR10, Fig. 2G). To assess whether loss of puc mutant eye tissue resulted from inappropriate JNK activation, puc mutant eyes were generated in flies with reduced levels of JNK. Heterozygosity for bsk2 partially rescued puc mutant eye tissue (Fig. 2H).

Together, these data demonstrate a crucial role for Puc in preventing apoptosis in embryonic and larval epithelial cells. Apoptosis occurred in the absence of any outside stress (other than the loss of Puc) that might stimulate JNK activity, suggesting that Puc prevents apoptosis by buffering basal levels of JNK signaling.

**JNK and MKP activities regulate radiation-induced apoptosis in a p53-dependent manner**

Next, we explored whether Puc plays a broader role in regulating JNK-induced apoptosis by examining γ-radiation-induced cell death. To assess whether JNK activity is upregulated in response to γ-irradiation, β-galactosidase (β-gal) expression from an enhancer trap in puc (Martin-Blanco et al., 1998) was monitored. puc enhancer traps have been widely used to monitor JNK activity in vivo (e.g. Adachi-Yamada et al., 1999; Igaki et al., 2002; Tateno et al., 2000). For simplicity we subsequently refer to this readout as JNKREP activity. In the wing disc, JNKREP activity is normally limited to the peripodial membrane overlaying the notal region of the wing disc (Fig. 3B). However, γ-irradiation strongly induced both JNKREP activity (Fig. 3C) and apoptosis (Ollmann et al., 2000) throughout the wing disc within 4 hours.

To confirm that JNKREP activation reflects JNK activation in situ, we used Myc-tagged Puc (Myc-Puc) to antagonize JNK activity. Expression of Myc-Puc in the posterior compartment of the wing disc (Fig. 3A) prevented radiation-induced JNKREP activation (Fig. 3D). Interestingly, it also substantially reduced radiation-induced apoptosis (Fig. 3G,G'). By contrast, expression of a catalytically-inactive form of Puc (Myc-PucDEAD) failed to block either JNKREP activity (Fig. 3E) or apoptosis (Fig. 3H,H'). Thus, in *Drosophila*, radiation-induced apoptosis depends, at least in part, on JNK signaling.

Discontinuities in morphogen gradients induce JNK-dependent apoptosis, perhaps by promoting discontinuities in a JNK activation gradient (Adachi-Yamada et al., 1999; Adachi-Yamada and O’Connor, 2002). However, lowering JNK activity by expressing Myc-Puc in the posterior compartment did not trigger apoptosis along the anteroposterior (AP) compartment border (Fig. 3F). Furthermore, expression of Myc-Puc in a stripe anterior to the A-P compartment boundary using patched-Gal4 or in clonal patches throughout the wing disc by flip-out mediated gene induction did not induce autonomous or non-autonomous apoptosis, but it blocked radiation-induced apoptosis (data not shown).

p53 is a key regulator of cell cycle progression and apoptosis in mammals, and is mutated in more than half of all tumors. Like its ortholog, *Drosophila* p53 is required for radiation-induced apoptosis and can induce apoptosis when overexpressed (Brodsky et al., 2000; Brodsky et al., 2004; Ollmann et al., 2000; Jassim et al., 2003; Lee et al., 2003;
Sogame et al., 2003). However, unlike mammalian p53, overexpression of fly p53 does not induce G1 arrest (Ollmann et al., 2000) and it is not required for G2/M arrest in response to radiation (Brodsky et al., 2000).

To assess how JNK signaling and p53 function are integrated in the hierarchy of signaling pathways regulating radiation-induced apoptosis, we asked if p53 is required for JNKREP induction. Radiation-induced JNKREP expression in wing discs requires p53 (compare Fig. 3I with 3C), whereas developmentally regulated JNKREP expression in the peripodial membrane does not. Furthermore, p53 mis-expression in the posterior compartment of the wing disc is sufficient to induce both JNK activation, as evidenced by induction of the JNKREP (Fig. 3J′), and apoptosis (Fig. 3J′′). Thus, p53 is both necessary for radiation-induced JNKREP activity and sufficient to promote JNK activation and apoptosis. However, co-expression of Myc-Puc failed to block apoptosis induced by sustained expression of p53 (Fig. 3K,K′,K″).

Although p53 is not required for cell viability, it is thought to monitor genomic integrity during normal development. We thus asked whether the p53 and JNK pathways interact in the developing eye disc in the absence of stress. Although inactivation of puc in the eye disc results in ablation of the adult eye (Fig. 2F,G), concomitant loss of p53 suppressed cell death to some extent (Fig. 2I). As loss of p53 alone has no effect on normal eye development, this may suggest that p53 plays a more general role in cell viability by regulating basal JNK activation.

Reaper functions downstream of JNK signaling
Reaper (Rpr), Head Involution Defective (Hid) and Grim (collectively referred to as RHG proteins) are key regulators of apoptosis that promote caspase activation via IAP degradation. Transcriptional upregulation of RHG genes precedes developmental and stress-induced apoptosis, whereas mis-expression of any these genes induces apoptosis. In developing
embryos, rpr-11-lacZ has been used to assess rpr induction in response to both developmental defects as well as radiation (Nordstrom et al., 1996).

To test whether JNK signaling can regulate rpr reporter expression in wing discs, we examined the effects of JNK activation on rpr-11-lacZ expression. In un-irradiated rpr-11-lacZ/+ wing discs, basal levels of reporter are seen, with β-gal expression highest at the wing margin and along the anterior side of the AP compartment boundary (Fig. 4A). γ-Radiation triggers a substantial increase in β-gal expression throughout the wing pouch (Fig. 4B), consistent with previous work. To determine whether this induction by radiation is JNK dependent, we overexpressed Myc-Puc throughout the AP compartment blocked induction of the reporter by irradiation (Fig. 4D); it also blocked β-gal expression along the wing margin in un-irradiated discs; Fig. 4C). Together, these results suggest that JNK signaling may promote apoptosis by transcriptionally upregulating rpr expression; this is subject to the caveat that the rpr-11-lacZ reporter does not perfectly reflect endogenous rpr expression (e.g. Nordstrom et al., 1996). However, JNK-induced upregulation of hid was observed in eye imaginal discs (Moreno et al., 2002b).

Previous work suggested that caspase activation might regulate JNK signaling in Drosophila (Kuranaga et al., 2002; Ryoo et al., 2004). To test directly whether RHG protein-mediated induction of apoptosis triggers JNK activation, we examined JNKREP activity in response to rpr and hid misexpression. When we used en-Gal4 to direct rpr expression in embryos, cell death was observed in en stripes, without concomitant JNK activation (Fig. 1O,P). As en-GAL4-driven rpr is embryonic lethal, we could not use this to examine wing discs. We thus used heat-shock to express hid in wing discs. A one-hour heat-shock of puc4251.1/hs-hid larvae induced apoptosis throughout the wing disc (data not shown), but did not elevate JNKREP activity (Fig. 4G,H; a slight increase in JNKREP was sometimes seen in the overlying peripodial membrane, as determined by the superficial position and size of the nuclei in H). This contrasts with the effect of γ-irradiation (Fig. 3C). Heat-shock of control puc4251.1/+ wing discs did not induce JNKREP (compare Fig. 3B to Fig. 4E,F) or apoptosis (data not shown). Thus, RHG-induced apoptosis can occur without JNK activation.

Caspase-mediated cleavage of cellular substrates can promote activation of mammalian JNK and p38 (Graves et al., 1998). To examine whether caspase activity is required for JNK activation, we blocked caspase activation in the posterior compartment. en-Gal4/UAS-p35; puc/+ larvae were irradiated and JNKREP activity assessed 4 hours later when apoptosis is readily detectable in the anterior but not the posterior compartment (Fig. 6N). JNKREP activity was observed throughout the developing disc (Fig. 6M), suggesting that radiation-induced JNK activation is independent of effector caspase activity.

**JNK signaling regulates programmed cell death during normal development**

In Drosophila, apoptosis plays crucial roles in establishing tissue architecture and removing excess cells during normal development. For example, inactivation of hid leads to mis-orientation of the external male genitalia (Abbott and Lengyel, 1991), and inactivation of either dark (Drosophila Apaf) or Traf1 (Drosophila tumor-necrosis factor receptor-associated factor1) leads to extra scutellar bristles (Kanuka et al., 1999; Kuranaga et al., 2002; Rodriguez et al., 1999). To test whether JNK signaling is required for developmentally regulated apoptosis, we blocked JNK signaling in the genital and wing discs.

Inhibition of apoptosis by expressing the baculovirus caspase inhibitor p35 resulted in genital mis-orientation (Fig. 5B; quantified in 5D), phenocopying reduction in Hid function.
Mis-expression of Myc-Puc had a similar effect (Fig. 5C, quantified in 5D), while control constructs (GFP or Myc-PucDEAD) failed to affect genital orientation (Fig. 5D). Extra scutellar bristles were produced when we prevented apoptosis in the scutellar region of the notum by expressing p35 with ptc-Gal4 (Fig. 5F; mean=5.4 bristles/fly, range=4-10, compared with four bristles in wild-type). ptc-Gal4-mediated expression of Myc-Puc also increased bristle number (Fig. 5G; mean=6.71 bristles/fly; range=4-10), whereas neither GFP nor Myc-PucDEAD altered the number of scutellar bristles (Fig. 5H; means=4.07 and 4.15 bristles/fly, respectively). While our manuscript was in preparation, Macias et al. (Macias et al., 2004) also reported that Puc or p35 mis-expression both block genital rotation. Together, these results suggest that JNK signaling is required for developmentally regulated, caspase-dependent apoptosis.

**When cell death is blocked, inappropriate activation of JNK signaling can promote overgrowth**

Growth of different compartments of the wing disc is strictly coordinated so that the adult wing has a defined shape and precisely matched surfaces. In normal wings, proliferation and apoptosis are coordinated to correct experimental perturbations, and presumably to correct defects arising during normal development (de la Cova et al., 2004; Neufeld et al., 1998). However, apoptosis does not appear to play an essential role in patterning of the wild-type wing, as over-expression of the caspase inhibitor p35 in the posterior compartment has little or no effect on wing pattern (over 96% of the wings of en-Gal4/UAS-p35 flies appear normal; Fig. 6B) (see also Pérez-Garijo et al., 2004), although altering apoptosis may affect wing size (de la Cova et al., 2004).

By contrast, when apoptosis is blocked in the posterior compartment of puc heterozygotes (en-Gal4/UAS-p35; puc/+), we observed tissue overgrowths at random positions throughout the posterior compartment in >90% of the flies (Fig. 6C-G). Overgrowths arising in the interior of the wing blade resemble wing blisters; however, there appear to be excess cells in only one compartment, as outgrowths project from the dorsal or ventral surfaces, but not both (Fig. 6C,E). When overgrowths arise along the wing margin, excess tissue is readily apparent as an extension of the wing (Fig. 6D,F). Cells within overgrowths respond to developmental cues, adopting appropriate cell fates (Fig. 6E-G). Thus, when cell death is blocked and restraints on JNK activation are relaxed by puc heterozygosity, small groups of cells escape regulation by the machinery that normally synchronizes growth of different cell populations in wing discs.

Overgrowths could already be observed in wing discs from en-Gal4/UAS-p35;puc/+ larvae (Fig. 6I-L). Multiple, randomly-positioned focal disruptions of disc architecture were observed in the posterior compartment where apoptosis was selectively blocked (Fig. 6I,L). These foci contain excess cells, resulting in abnormal folding of the disc epithelium. Furthermore, cells associated with the foci invariably exhibited elevated JNKREP activity (Fig. 6J-L). Most cells within the foci were not apoptotic (Fig. 6J,K); however, occasional TUNEL-positive cells were observed. While JNKREP activity and disc overgrowth were sometimes confined to small foci (Fig. 6I-K), at other times activation of the JNKREP and areas of overgrowth were more extensive (Fig. 6L).

While our manuscript was in preparation, three groups reported that when they induced apoptosis by various stimuli and blocked caspase activity, it also triggered overgrowth (Ryoo et al., 2004; Pérez-Garijo et al., 2004; Huh et al., 2004). Each group found that some of the ‘undead’ cells expressed the morphogen and disc growth factor Wg. We examined whether this was also the case in our situation. In wing discs...
from en-Gal4/UAS-p35;puc/+ larvae, a subset of cells in JNK\textsuperscript{REP}-positive foci in the wing pouch ectopically expressed Wg (Fig. 6P); however, in control discs Wg is only expressed in a stripe at the dorsal/ventral compartment boundary (Fig. 6O). Ectopic Wg expression, like JNK\textsuperscript{REP} activation, was confined to the posterior compartment where p35 was expressed.

The correlation between foci with activated JNK signaling in discs and outgrowths in adults suggested that JNK activation promotes overgrowth when apoptosis is blocked. To test this, we examined whether reducing the dose of kayak (previously D-fos) affected outgrowth frequency. Consistent with a role for JNK signaling in promoting overgrowth, reducing kayak by 50% resulted in a 25% decrease in outgrowth frequency (P<0.001, Student’s t-test). Thus, when apoptosis is blocked, JNK signaling promotes tissue overgrowth.

**Discussion**

We characterized the role of MAPK/MKP interactions in Drosophila epithelial tissues by examining the function of Puc, a MKP that selectively antagonizes JNK signaling. Puc plays a key role in regulating cell viability in response to a diverse array of normal and abnormal developmental signals.

**Puc restraints apoptosis in the absence of stress**

Mammalian JNK signaling promotes apoptosis in response to both extrinsic (e.g. TNF\textalpha) and intrinsic (e.g. DNA damage) cues (Davis, 2000). Drosophila JNK signaling plays a similar role in apoptosis triggered by extrinsic cues (Adachi-Yamada et al., 1999; Adachi-Yamada and O’Connor, 2002; Burke and Basler, 1996; Iguiki et al., 2002; Kuranaga et al., 2002; Martin et al., 2004; Moreno et al., 2002a; Moreno et al., 2002b), but its role in response to intrinsic cues remained to be established. The role of Puc in regulating responses to either sort of signal was also unclear.

We found that removal of Puc triggers apoptosis in epithelia, even in the absence of stress. Thus, basal JNK signaling exists in the absence of stress in embryonic and larval epithelia, and if Puc is not present to restrain this intrinsic JNK activity, it exceeds a threshold and triggers apoptosis. This suggests cells may regulate apoptosis without exogenous JNK stimulation, by regulating MKP levels. Biochemical studies suggest a possible mechanism. Several MKPs are labile proteins, whose half-lives can be shortened or lengthened by post-translational modification (Brondello et al., 1999; Lin et al., 2003). Thus, cell signals may influence apoptosis by modulating MKP accumulation.

Although Puc is crucial to restrain JNK activity and prevent apoptosis in most epithelia, high-level JNK signaling does not always induce apoptosis. JNK signaling is required for embryonic dorsal closure. High JNK activity is normally restricted to the dorsal-most epithelial cells, though in puc mutants it extends into adjacent cells (Martin-Blanco et al., 1998). However, this JNK signaling does not trigger apoptosis (Fig. 1N). By contrast, ectopic JNK signaling in ventral and lateral epithelial cells in puc mutants does trigger apoptosis. Thus, cells where JNK activity is normally high are somehow refractory to JNK-induced apoptosis. JNK signaling normally activates Dpp in dorsal epithelial cells, with ectopic Dpp activation in more lateral cells in puc mutants – perhaps this is anti-apoptotic, as Dpp is a survival factor in wing discs (e.g. Moreno et al., 2002a). Interplay between death and survival
Development

JNK, as revealed by its effect on the JNKREP, while loss of p53 response to DNA damage. Thus, we investigated how JNK overwhelming the ability of Puc to restrain JNK.

apoptotic effects of Puc overexpression, perhaps artificially prolonged p53 overexpression overcomes the anti-

the Puc-defined threshold may be exceeded. Indeed, however, if damage persists and JNK stimulation continues,
time, cells could attempt to repair radiation-induced damage.

damage-triggered activation of p53 would induce JNK

signaling induces expression of MKPs such as Puc. Our data provide an instance of how this may regulate the DNA damage response. puc is upregulated by γ-irradiation in a JNK-dependent manner, and artificially prolonged Myc-Puc expression prevents radiation-induced apoptosis. Thus, the initial increase in Puc expression following irradiation may create a `grace period' during which Puc elevates the threshold of JNK activation required to induce apoptosis. During this time, cells could attempt to repair radiation-induced damage. However, if damage persists and JNK stimulation continues, the Puc-defined threshold may be exceeded. Indeed, artificially prolonged p53 overexpression overcomes the anti-apoptotic effects of Puc overexpression, perhaps overwhelming the ability of Puc to restrain JNK.

p53 is a key regulator of decisions between life and death in response to DNA damage. Thus, we investigated how JNK signaling and p53 are integrated. Expression of p53 activated JNK, as revealed by its effect on the JNKREP, while loss of p53 prevented radiation-induced JNK activation. These results suggest that p53 acts upstream of JNK signaling in response to cellular stress. Several mechanisms are possible; e.g. p53 might upregulate transcription of JNK pathway components, whose overexpression can induce apoptosis (Adachi-Yamada et al., 1999; McEwen et al., 2000).

p53 normally monitors genome integrity. Loss of p53 significantly, although not completely, suppresses apoptosis induced by Puc inactivation. One model to explain this suggests that basal levels of DNA damage or replication errors act through p53 to regulate basal JNK activity during normal development. This basal activity is kept below the apoptotic threshold by Puc. In p53 mutants, basal JNK activity would be lowered enough that it could not exceed the apoptotic threshold, even in the absence of Puc. Alternatively, p53 may also function downstream of JNK. Consistent with this, p38 can activate p53 in response to UV (Bulavin et al., 1999; Huang et al., 1999), and JNK can regulate p53 stability/activity via direct phosphorylation (Buschmann et al., 2001). This would set up a positive-feedback loop: DNA damage-triggered activation of p53 would induce JNK activation, which would further elevate p53 activity. Additional experiments are required to test these alternate hypotheses.

Signal transduction pathways regulate apoptosis, at least in part, by regulating transcription of *rpr*, *hid* and *grim* (the RHG proteins), the key developmental effectors of apoptosis in *Drosophila* (reviewed by Martin, 2002). The relationship between JNK signaling, RHG proteins and caspase activation are not well understood. Caspase 3 cleavage of Mst1, an upstream regulator of JNK and p38, has been suggested to amplify apoptotic responses (Graves et al., 1998), while other data suggest that Mst1 activates caspases via a JNK-dependent pathway (Ura et al., 2001). Likewise, in *Drosophila*, initiation of an apoptotic response by inactivation of DIAP1 may lead to caspase-independent induction of a JNKREP (Kuranaga et al., 2002; Ryoo et al., 2004).

We directly assessed regulatory relationships between JNK activation, RHG proteins and caspase activation. Our data suggest that JNK signaling acts through RHG proteins and caspases to induce apoptosis. JNK signaling is required for *rpr*-reporter induction in response to radiation. Thus, RHG proteins may be JNK-responsive target genes upregulated to elicit apoptosis. Consistent with this hypothesis, mis-expression of *eiger*, a known JNK activator, promotes *hid* expression and apoptosis in eye discs (Moreno et al., 2002).

To assess whether JNK signaling can be triggered by caspase activation, we examined JNKREP activity in response to Rpr or Hid expression. Both induced apoptosis without concomitant JNK activation. Furthermore, our data suggest that, in at least some contexts, caspases act downstream of JNK: p35-mediated caspase blockade allows cells with elevated JNK signaling to survive in imaginal discs, but does not prevent JNK activation in response to irradiation. Thus, RHG proteins elicit caspase-mediated apoptosis downstream of JNK activation.

### JNK-induced apoptosis during normal development

Work on JNK-induced apoptosis in *Drosophila* has focused on its role in the complex processes shaping organ size. Wing discs cells make decisions about whether to die or proliferate by integrating levels of different developmental signals they receive, and comparing their status with that of their neighbors. This occurs in part by competition for survival signals like the TGFB family member Dpp (Burke and Basler, 1996; Martin et al., 2004; Moreno et al., 2002a). Complex crosstalk among this and other signaling pathways precisely regulates the size and pattern of the wing, in a process that is very resistant to tissue damage or developmental errors. Discontinuities in smooth morphogen gradients, which may arise from errors in patterning or tissue injury, are corrected in part by JNK-dependent apoptosis (e.g. Adachi-Yamada and O’Connor, 2002).

Our data clarifies roles for JNK signaling in adult development (Agnes et al., 1999). Our failure to recover *puc* clones anywhere in the wing disc suggests that basal JNK activity is sufficient to promote cell-autonomous apoptosis independent of other signals activating JNK. However, JNK signaling does not play a crucial role in wing patterning, as JNK inactivation in the posterior compartment (by Myc-Puc mis-expression) did not have drastic consequences. We did identify roles for JNK signaling in genitalia and thoracic bristles, where it regulates developmentally programmed apoptosis; this was also reported by Macias et al. (Macias et al., 2004).
Can MKPs act as tumor suppressors?
JNK signaling plays complex roles during oncogenesis. It can prevent tumorigenesis by promoting apoptosis, and it can promote tumorigenesis by supporting Ras- (Behrens et al., 2000; Smeal et al., 1991) or BCR-Abl-mediated (Dickens et al., 1997; Raitano et al., 1995) transformation. As each tumor type has a unique set of mutations in oncogenes and/or tumor suppressors, the phenotypic effects of JNK activation probably differ depending on the activity of other pathways.

Inhibition of apoptosis is one prerequisite for tumorigenesis. We thus examined the consequences of JNK activation when apoptosis was blocked. When cell death was blocked in the posterior compartment of the wing and the restraints on JNK activation were relaxed by puc heterozygosity, tissue overgrowth occurred. Groups of posterior cells, presumably of clonal origin, exhibited elevated levels of JNK activation, and formed small overgrowth both in developing imaginal discs and in the resulting adult wings and legs. Thus, when apoptosis is suppressed, JNK activation can lead to tissue over-growth.

Ryoo et al. (Ryoo et al., 2004), Pérez-Garjio et al. (Pérez-Garjio et al., 2004), and Huh et al. (Huh et al., 2004) recently reported related results, inducing apoptosis by diap inactivation, irradiation or Hid expression, while simultaneously blocking caspase activation. This triggered non-autonomous proliferation of neighboring cells, presumably to compensate for cell loss by apoptosis. Furthermore, some of the ‘undead’ cells produced by these treatments show JNK-dependent upregulation of Wg or Dpp. Ryoo et al. show that Wg signaling is required for compensatory proliferation. Our results extend theirs, as our experiment differed in one significant way: we did not actively induce apoptosis, but simply blocked caspase activation in puc heterozygotes. Thus, when apoptosis is inhibited and Puc repression is reduced, cells become susceptible to runaway JNK activation. It is likely that analogous focal JNK activation occurs in cells in which apoptosis is not blocked (e.g. anterior compartment cells in our experiment), but JNK-induced apoptosis rapidly eliminates them. We also found that at least a subset of the ‘undead’ cells activated expression of Wg, which may promote excess growth. Interestingly, however, this ectopic Wg expression does not alter cell fates, at least in those situations where overgrowths survive to be observed in the adult wing.

Together, these data have interesting implications. In tumor cells in which apoptosis is prevented, JNK signaling might switch from promoting apoptosis to promoting proliferation by inducing Wnt or TGFβ, suggesting how JNK signaling can be both pro- and anti-tumorigenic. Future experiments should address mechanisms by which JNK activation triggers Wnt and TGFβ signaling. Our data also suggest that runaway JNK activation can promote cell-autonomous proliferation, as groups of cells with elevated JNK activity are associated with overgrowths. Thus, JNK activation or loss of MKP-mediated JNK repression may play multiple roles in tumors whose cells have lost the ability to die.

Ectopic JNK activation occurred in small groups of cells in random positions throughout the posterior compartment. The event(s) that initiate unrestrained JNK activation in these cells remain to be defined. Perhaps there are stochastic variations in JNK signaling that are normally below the threshold triggering apoptosis. However, when Puc activity is reduced, some cells may exceed this threshold, triggering runaway JNK activation in the initial cell and its descendents. Variations in JNK activity may also be induced by spontaneous DNA damage. Normally, such damage would trigger JNK-dependent apoptosis; however, if apoptosis is blocked, the cells proliferate. Finally, loss-of-heterozygosity at the puc locus could create cells lacking restraints on JNK signaling. As mitotic recombination in somatic tissue can occur (Baker et al., 1978), Puc might act in a manner analogous to classic tumor suppressor genes. Additional studies will be required to distinguish between these possibilities.

In summary, our work establishes that Puc is a key negative regulator of apoptosis throughout Drosophila development. In its absence, basal JNK activity is poised to eliminate cells from the developing epithelium. Our results position JNK signaling in the hierarchy of events regulating radiation-induced apoptosis. Finally, our data support the possibility previously suggested by cytogenetics (Armes et al., 2004; Furukawa et al., 2003) that MKPs may act as tumor suppressor genes. These data prompt many new mechanistic questions regarding the role of JNK signaling in apoptosis and oncogenesis.

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