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Regulation of Hippo signaling by Jun kinase signaling during compensatory cell proliferation and regeneration, and in neoplastic tumors

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ABSTRACT

When cells undergo apoptosis, they can stimulate the proliferation of nearby cells, a process referred to as compensatory cell proliferation. The stimulation of proliferation in response to tissue damage or removal is also central to epimorphic regeneration. The Hippo signaling pathway has emerged as an important regulator of growth during normal development and oncogenesis from *Drosophila* to humans. Here we show that induction of apoptosis in the *Drosophila* wing imaginal disc stimulates activation of the Hippo pathway transcription factor Yorkie in surviving and nearby cells, and that Yorkie is required for the ability of the wing to regenerate after genetic ablation of the wing primordia. Induction of apoptosis activates Yorkie through the Jun kinase pathway, and direct activation of Jun kinase signaling also promotes Yorkie activation in the wing disc. We also show that depletion of neoplastic tumor suppressor genes, including *lethal giant larvae* and *discs large*, or activation of aPKC, activates Yorkie through Jun kinase signaling, and that Jun kinase activation is necessary, but not sufficient, for the disruption of apical-basal polarity associated with loss of *lethal giant larvae*. Our observations identify Jnk signaling as a modulator of Hippo pathway activity in wing imaginal discs, and implicate Yorkie activation in compensatory cell proliferation and disc regeneration.

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Introduction

When cells in a tissue are damaged, proliferation of neighboring cells can be induced, enabling tissue repair. This phenomenon is central to epimorphic regeneration, which enables the regrowth and replacement of body parts after injury or amputation. The capacity of tissues to undergo epimorphic regeneration has been known for centuries and exists throughout the metazoa, although it varies between organisms, organs, and developmental stages. An important insight into epimorphic regeneration was provided by the observation that when cells initiate apoptosis, they produce mitogenic signals, thereby stimulating the proliferation of neighboring cells. This process, termed compensatory cell proliferation, was first characterized in the developing imaginal discs of *Drosophila*, but similar phenomena occur in other systems (reviewed in Bergantinos et al., 2010b; Fan and Bergmann, 2008).

Compensatory cell proliferation has been observed in *Drosophila* imaginal discs upon induction of cell death by X-irradiation, by expression of pro-apoptotic genes, or by mutation of the anti-apoptotic gene *thread* (*Diap1*) (Haynie and Bryant, 1977; Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004). Compensatory cell proliferation is associated with the induction of signaling molecules that have been linked to the promotion of cell proliferation, including Wingless (Wg) and Decapentaplegic (Dpp) (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004). Another common and essential feature of compensatory cell proliferation is the activation of Jun-kinase (Jnk) signaling (Fan and Bergmann, 2008; Perez-Garijo et al., 2009; Ryoo et al., 2004). Jnk signaling is a MAPK signaling pathway regulated by diverse cellular stresses, including irradiation, reactive oxygen species, infection, aging, disruption of cell polarity, cytoskeletal changes, and induction of apoptosis (reviewed in Bogoyevitch et al., 2010; Igaki, 2009; Karin and Gallagher, 2005). Jnk signaling has distinct outcomes in different contexts. It is crucial for morphogenesis during embryogenesis and wound healing (Martin and Parkhurst, 2004), and has an important pro-apoptotic function (Igaki, 2009; Kanda and Miura, 2004). However, when apoptosis is blocked, Jnk signaling can promote cell proliferation (Hariharan and Bilder, 2006; Igaki et al., 2006; McEwen and Peifer, 2005; Ryoo et al., 2004).

In addition to its role in compensatory cell proliferation, Jnk signaling has been linked to proliferative and metastatic features of tumors associated with disruptions of apical-basal polarity in epithelial cells (Igaki et al., 2006; Uhlirova and Bohmann, 2006). Genes that, when mutated, result in over-proliferation coupled to loss of normal tissue architecture are classified in *Drosophila* as neoplastic tumor suppressors (reviewed in Hariharan and Bilder, 2006). Three of the best studied neoplastic tumor suppressors, *lethal giant larvae* (*lgl*), *discs large* (*dlg*) and *scribbled* (*scrib*), form a junctional complex that contributes to apical-basal polarity in epithelial cells (Bilder et al., 2000). Their effects on growth are complex. When an entire disc is mutant for one of these genes, it can overgrow and form a tumorous mass of unpolarized cells (Agrawal et al., 1995; Bilder et al., 2000). However, when clones of cells

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mutant for these genes are induced by mitotic recombination in wing discs, they generally fail to survive, and are eliminated by Jnk-dependent apoptosis. But if combined with other oncogenic mutations, such as expression of Myc or activated-Ras or Notch, clones of cells mutant for *lgl*, *dlg* or *scrib* can survive and form large tumors that are prone to metastasis; the growth and metastasis of these tumors also depends on Jnk signaling (Brumby and Richardson, 2003; Froldi et al., 2010; Igaki et al., 2006, 2009; Pagliarini and Xu, 2003).

The Hippo pathway controls growth during normal development, and its dysregulation is associated with oncogenesis (reviewed in Reddy and Irvine, 2008; Zhao et al., 2010). Hippo signaling is mediated by a transcriptional co-activator protein, Yorkie (Yki) (reviewed in Oh and Irvine, 2010). When Hippo signaling is active, Yki is kept inactive, retained in the cytoplasm through the action of upstream tumor suppressor genes in the Hippo pathway. The key, direct repressor of Yki activity is the kinase Warts (Wts), which phosphorylates Yki (Huang et al., 2005). In the absence of Wts, unphosphorylated Yki accumulates in the nucleus (Dong et al., 2007; Oh and Irvine, 2008), and in conjunction with DNA-binding proteins, regulates the transcription of downstream genes. Recently, mutation of *lgl*, or activation of aPKC, was reported to result in activation of Yki, and Yki was functionally linked to over-proliferation phenotypes in these genotypes (Grzeschik et al., 2010; Menéndez et al., 2010). The mechanism by which Yki becomes activated by these manipulations is not known, although it was suggested that it might involve mis-localization of Hippo and a Hippo-interacting protein, dRassf.

Here, we characterize the regulation and role of the Hippo pathway in compensatory cell proliferation and regeneration. Damage to the epithelial cells of the *Drosophila* wing imaginal disc by expression of pro-apoptotic genes results in activation of Yki. This Yki activation is mediated by the Jnk signaling pathway. We further determined that disruption of apical-basal polarity by depletion of neoplastic tumor suppressor genes, or activation of aPKC, activates Yki through Jnk signaling, and that Yki is required for wing disc regeneration after genetic ablation of the wing primordia. Our results identify Jnk signaling as a mechanism for regulating Hippo pathway activity in wing imaginal discs, and establish a fundamental role for Hippo signaling in regenerative responses to tissue damage.

Materials and methods

Drosophila genetics

Stocks used included *ex-lacZ en-Gal4 UAS-GFP/CyO; UAS-dcr2/TM6b*, *UAS-lgl-RNAi (VDRC 51249)*, *UAS-dlg-RNAi (VDRC 41136)*, *UAS-bsk-RNAi (VDRC 104569)*, *UAS-yki-RNAi (VDRC 104532)*, *UAS-wg-RNAi (VDRC 104579)*, *rn-Gal4 UAS-egr tub-Gal80^{ts}/TM6b,Gal80* (Smith-Bolton et al., 2009), *rn-Gal4 UAS-rpr tub-Gal80^{ts}/TM6b,Gal80* (Smith-Bolton et al., 2009), *puc-lacZ[A251.1F3]ry/TM3 (Bloomington 11173)*, *UAS-myc:wts.2*, *FRT42D yki^{B5}/CyO,Act-GFP*, *UAS-puc, salPE-Gal4 UAS-GFP/CyO; Dronc^{129} FRT2A/TM6b* (Perez-Garijo et al., 2009), *UAS-hep.CA/CyO; Dronc^{129} FRT2A/TM6b* (Perez-Garijo et al., 2009), *UAS-wts:myc[2-2]* (gift of Tian Xu), *UAS-GFP[T-2] (Bloomington 1521)*, *UAS-aPKC:CAAX* (Lee et al., 2006), *UAS-yki:V5* (Oh and Irvine, 2009), *tub-Gal80^{ts}/TM6B, tub>CD2>Gal4 UAS-CD8:GFP/CyO; tub-Gal80^{ts}/TM6B* (Buttitta et al., 2007), *UAS-hep.CA (Bloomington 6406)*, *UAS-rpr[14] (Bloomington 5824)*, *rn-lacZ^{89}*, *ry^{+}*, *ry^{506}/TM3, Sb* (St Pierre et al., 2002), *UAS-egr* (Moreno et al., 2002), *UAS-lacZ* (Brand and Perrimon, 1993). The specificity of *lgl* RNAi has been described previously (Grzeschik et al., 2010). The specificity *yki* RNAi was confirmed by rescue using a *UAS-yki* line to over-express Yki. Because we lacked a direct test for *bsk* RNAi specificity, all experiments with *bsk* RNAi were repeated using *UAS-puc*, which gave similar results.

Larvae from crosses of *rn-Gal4 UAS-egr tub-Gal80^{ts}/TM6b,Gal80* or *rn-Gal4 UAS-rpr tub-Gal80^{ts}/TM6b,Gal80* were kept at 18 °C for 8 days and shifted to 30 °C for 30 h to induce cell death. After cell death induction, larvae were either dissected or put back to 18 °C for 24, 48, and 72 h recovery. To make *rpr* or *hep.CA* clones, *yw hs-flp; UAS-rpr/CyO,GFP* or *yw hs-flp; UAS-hep.CA/CyO,GFP* flies were crossed to *tub>CD2>Gal4 UAS-CD8:GFP/CyO; tub-Gal80^{ts}/TM6B*. Larvae were maintained at 25 °C for 3 days and clones were induced by heat shock at 38 °C for 10 min. Larvae were then kept at 18 °C for 3 days to let clones grow larger, and then cell death was induced by temperature shift to 30 °C for 12–14 h.

To evaluate the effect of *yki^{B5}/+* on development timing, *rn-Gal4 UAS-egr tub-Gal80^{ts}/TM6b,Gal80* females were crossed to Oregon-R or *FRT42D yki^{B5}/CyO,Act-GFP* males. Eggs were collected at 25 °C in 8 h intervals on grape juice plates covered with yeast, and subsequently kept at 18 °C. The dates of larvae hatching and pupa formation were recorded.

For adult wings, larvae were maintained at 18 °C after tissue damage until eclosion. Wings were mounted in Gary's Magic Mountant, and measured using NIH ImageJ software.

Immunofluorescent staining

Wing discs of third instar larvae were fixed in 4% paraformaldehyde and stained as described previously. Primary antibodies used include rabbit anti-Yki (1:400), mouse anti-Wg (1:800, 4D4, Developmental Studies Hybridoma Bank (DSHB), mouse anti-DLG(1:400, 4F3, DSHB), rat anti-Fat (1:800) (Feng and Irvine, 2009), goat anti-β-gal (1:1000, Biogenesis), rabbit anti-GFP (1:400, Molecular Probes), rat anti-DE-cadherin (1:450, DCAD2, DSHB), rabbit anti-active Caspase 3 (1:200, Asp175, Cell Signaling Technology), rabbit anti-phospho-JNK (1:100, 81E11, Cell Signaling Technology), mouse anti-Myc (1:400, 9E10, Babco), mouse anti-Nubbin (1:100, gift from Steve Cohen). EdU labeling was performed using Click-it™ EdU Alexa Fluor Imaging Kit (Molecular Probes). Images were captured on a Leica TCS SP5 confocal microscope.

Results

Activation of Yki adjacent to apoptotic cells

To investigate the potential involvement of Hippo signaling in compensatory cell proliferation and regeneration, we examined the sub-cellular localization of Yki in wing imaginal discs after localized induction of cell death. In one approach, we adopted a system developed by Smith-Bolton et al. (2009) for analysis of regenerative growth in imaginal discs, which involves expressing pro-apoptotic genes throughout the wing primordia of the developing disc under the control of *rotund* (*rn-Gal*), and then controlling the timing of expression using a temperature-sensitive repressor of Gal4 (*Gal80^{ts}*). The pro-apoptotic genes expressed were *reaper* (*rpr*), an inhibitor of the *Drosophila* apoptosis inhibitor Diap1 (Goyal et al., 2000; Wang et al., 1999; White et al., 1994), or *eiger* (*egr*), a *Drosophila* TNFα that is a ligand for the Jnk pathway (Igaki et al., 2002; Kanda and Miura, 2004; Moreno et al., 2002).

Normally, Yki is predominantly cytoplasmic within imaginal disc cells (Fig. 1A). However, if Hippo signaling is impaired, then Yki can be detected in the nucleus (Dong et al., 2007; Oh and Irvine, 2008). When *rpr* or *egr* was expressed for 30 h under *rn-Gal4* control, most of the wing primordia was ablated, although a small, irregular region of *rn-Gal4* expression persists, which includes both dying cells in which relatively stable marker proteins (β-galactosidase or GFP) are still detectable, and some cells that appear viable (Fig. 1B,C and data not shown) (Smith-Bolton et al., 2009). Wing discs in which wing pouch cells have been ablated by expression of *rpr* or *egr* exhibit a striking re-localization of Yki to the nucleus, both in cells adjacent to the *rn-Gal4* domain, as well as among surviving cells within the *rn-Gal4* domain (Fig. 1B,C). This re-localization implies that ablation of cells and/or induction of apoptosis results in a strong, local activation of Yki. Consistent with this inference, a downstream target of Yki, *expanded*

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