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The Salvador/Warts/Hippo pathway controls regenerative tissue growth in *Drosophila melanogaster*

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ABSTRACT

During tissue regeneration, cell proliferation replaces missing structures to restore organ function. Regenerative potential differs greatly between organs and organisms; for example some amphibians can regrow entire limbs whereas mammals cannot. The process of regeneration relies on several signaling pathways that control developmental tissue growth, and implies the existence of organ size-control checkpoints that regulate both developmental, and regenerative, growth. Here we explore the role of one such checkpoint, the Salvador–Warts–Hippo pathway, in tissue regeneration. The Salvador–Warts–Hippo pathway limits tissue growth by repressing the Yorkie transcriptional co-activator. Several proteins serve as upstream modulators of this pathway including the atypical cadherins, Dachsous and Fat, whilst the atypical myosin, Dachs, functions downstream of Fat to activate Yorkie. Using *Drosophila melanogaster* imaginal discs we show that Salvador–Warts–Hippo pathway activity is repressed in regenerating tissue and that Yorkie is rate-limiting for regeneration of the developing wing. We show that regeneration is compromised in *dachs* mutant wing discs, but that proteins in addition to Fat and Dachs are likely to modulate Yorkie activity in regenerating cells. In conclusion our data reveal the importance of Yorkie hyperactivation for tissue regeneration and suggest that multiple upstream inputs, including Fat–Dachsous signaling, sense tissue damage and regulate Yorkie activity during regeneration of epithelial tissues.

Introduction

Regeneration is a process whereby an organism can stimulate tissue growth to replace damaged body parts and re-establish tissue homeostasis. The ability to regenerate varies greatly in the animal kingdom and also between different organs of the same species. For example planarians can regrow completely from a small body remnant, some crustaceans and amphibians can re-grow entire limbs, zebrafish can regenerate hearts and fins, whereas mammals have relatively poor regenerative capacity [reviewed in (Birnbaum and Sanchez Alvarado, 2008)]. Some mammalian organs, like the liver, can regrow whereas most other organs cannot. *Drosophila melanogaster* imaginal discs, the presumptive adult organs, were shown to possess regenerative capacity several decades ago (Hadorn, 1963). They can regenerate during a specific window of larval development in response to either surgically- or genetically-induced wounds (Hadorn, 1963; Smith-Bolton et al., 2009). Several growth-controlling proteins and signaling pathways have been shown to regulate tissue growth not only during development, but also during regeneration; for example fibroblast growth factor in the zebrafish fin (Poss et al., 2000); the Wnt, transforming growth factor β and Hedgehog signaling pathways in planarians (Forsthoefel and Newmark, 2009; Rink et al., 2009; Yazawa et al., 2009); and Wingless, Myc and the Jun N-terminal kinase (JNK) pathway in *D. melanogaster* imaginal discs (Bosch et al., 2005; Mattila et al., 2005; Smith-Bolton et al., 2009).

The Salvador–Warts–Hippo (SWH) signaling pathway has recently emerged as a key modulator of tissue growth during development by regulating key proteins involved in cell proliferation, Cyclin E, and survival, DIAP1 (Tapon et al., 2002). Defective SWH pathway signaling has been associated with a loss of tissue growth control in cancer (Harvey and Tapon, 2007). At the core of the SWH pathway are two serine/threonine kinases, Warts (Wts) and Hippo (Hpo), and two adaptor proteins, Salvador (Sav) and Mob as tumour suppressor (Mats) [reviewed in (Harvey and Tapon, 2007)]. These proteins restrict tissue growth by stimulating Wts to phosphorylate, and thereby repress, the Yorkie (Yki) transcriptional co-activator protein (Huang et al., 2005). The core SWH pathway proteins are regulated by inputs from several upstream regulatory branches, including the Fat–Dachsous (Ft–Ds) branch, the Kibra, Expanded, Merlin (KEM)

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complex, and the apicobasal polarity proteins, Lethal Giant Larvae (Lgl), Crumbs (Crb) and atypical Protein Kinase C (aPKC) [reviewed in (Grusche et al., 2010)]. The Ft–Ds branch of the SWH pathway controls Yki activity by repressing activity of the atypical myosin, Dachs, which in turn is thought to inhibit Warts activity (Cho et al., 2006). Given that the SWH pathway is a key regulator of tissue growth in both insects and mammals during development, and of tissue size homeostasis in adult mice (Camargo et al., 2007; Dong et al., 2007), we postulated that the SWH pathway could also control regenerative tissue growth based on the following logic: SWH pathway activity is repressed in mammalian cultured cells on the edge of physically-induced wounds in vitro (Zhao et al., 2007); SWH pathway activity regulates cell proliferation in a cell-density dependent manner (McPherson et al., 2004); the SWH pathway represses cell proliferation, at least in part, by interactions between the Ft and Ds cadherins on neighbouring cells (Rogulja et al., 2008; Willecke et al., 2008), and therefore we envisaged that these interactions might be abrogated in wounded tissue and this would cause pathway derepression.

To test this hypothesis, we induced tissue ablation in developing *D. melanogaster* imaginal discs and analysed SWH pathway activity. We found that Yki activity was elevated in tissue that was proliferating post wounding due to de-repression by Wts, and that Yki was rate-limiting for wing regeneration. In addition, the Ft–Ds upstream signaling branch of the SWH pathway was partially required for controlling this regenerative response.

Materials and methods

Immunohistochemistry

Imaginal discs were prepared as previously described (Grusche et al., 2009; Harvey et al., 2003). The following antibodies were used: mouse anti-beta Galactosidase (1:200; Sigma #G-4664), rabbit anti-cleaved Caspase 3 (1:400; Cell Signaling Technologies #9661S), mouse anti-Cyclin E (1:50) (Richardson et al., 1995), rabbit anti-Yki (1:400; Oh and Irvine, 2008), rabbit anti-phospho-Histone H3 (1:400; Cell Signaling Technology), goat anti-mouse_555 and goat anti-rabbit_647 (1:600; Molecular Probes, Invitrogen). Images were recorded on an Olympus FV-1000 confocal microscope and processed using Adobe Photoshop CS2.

D. melanogaster strains

w^{1118} flies were used as a wild-type control. Other stocks used were: $hyd^{K3.5}$ (Lee et al., 2002), $vps25^{PB2931}$ (Thompson et al., 2005), $lines^{P14A}$ (K. Harvey, unpublished) ept^{2} (Moberg et al., 2005), $scrib^{1}$ (Bilder and Perrimon, 2000), *diap1-lacZ* (Hay et al., 1995), $dachs^{GC13}$, $dachs^{1}$ (Mao et al., 2006), yki^{B5} (Huang et al., 2005), ex^{MGH1} (Pellock et al., 2007) ex^{e1} and ex^{697} (Boedigheimer and Laughon, 1993). The *UAS–Gal4* system (Brand and Perrimon, 1993) and $Gal80^{ts}$ (McGuire et al., 2003) were used to allow temporal expression of transgenes: *rotund-Gal4, engrailed-Gal4*, (Bloomington), *puckered-Gal4* (Pastor-Pareja et al., 2004), *UAS–GFP* (Bloomington), *UAS-myc–wts* (Feng and Irvine, 2007), *UAS-eiger, UAS-rpr* (Smith-Bolton et al., 2009), *UAS-Debcl* (Colussi et al., 2000).

D. melanogaster genotypes by figure panels:

Figs. 1A–C, 2E, 7H, S1: *y, w, hsFlp/+; ex^{697}/+; FRT 82B, Ubi-GFP/FRT82B, $hyd^{K3.5}$*
Fig. 2A: *y, w, hsFlp/+; ex^{697}/+; Ubi-GFP, FRT 80B/ept^{2}, FRT80B*
Figs. 2B, 7F: *y, w, hsFlp/+; ex^{697}/+; FRT 82B, Ubi-GFP/FRT82B, $scrib^{1}$*
Fig. 2C: *y, w, hsFlp/+; FRT 42D, Ubi-GFP/FRT42D, $lines^{P14A}$; th^{j5c8}/+*
Fig. 2D: *y, w, hsFlp/+; FRT 42D, Ubi-GFP/FRT42D, $vps25^{PB2931}$; th^{j5c8}/+*
Figs. 3A–D, 4A–C, S2: *w; enGal4, UAS–GFP/ex^{697}; tub-$Gal80^{ts}$/UAS-Debcl*
Fig. 3E: *w; UAS–GFP/+; rn-Gal4, tub-$Gal80^{ts}$, UAS-eiger/+*
Figs. 4D, 7J: *w; ex^{697}/+; rn-Gal4, tub-$Gal80^{ts}$, UAS-eiger/+*
Figs. 4E–F, 5A: *w;; rn-Gal4, tub-$Gal80^{ts}$, UAS-eiger/+*
Fig. 5B: *w; UAS-myc–wts/+; rn-Gal4, tub-$Gal80^{ts}$, UAS-eiger/+*
Figs. 6A, C: *w; enGal4, UAS–GFP/FRT42D; tub-$Gal80^{ts}$/UAS-Debcl*
Figs. 6B, D: *w; enGal4, UAS–GFP/FRT42D, yki^{B5}; tub-$G80^{ts}$/UAS-Debcl*
Figs. 7A, B: *w*
Figs. 7C, D: *w; $dachs^{1/GC13}$*
Fig. 7G: *y, w, hsFlp/+; $dachs^{GC13}$, ex^{697}, FRT40A/$dachs^{1}$; FRT 82B, Ubi-GFP/FRT82B, $scrib^{1}$*
Fig. 7I: *y, w, hsFlp/+; $dachs^{GC13}$, ex^{697}, FRT40A/$dachs^{1}$; FRT 82B, Ubi-GFP/FRT82B, $hyd^{K3.5}$*
Fig. 7K: *w; $dachs^{GC13}$, ex^{697}, FRT40A/$dachs^{GC13}$, FRT40A; rn-Gal4, tub-$Gal80^{ts}$, UAS-eiger/+*
Fig. 8A: *w; ex^{e1}, FRT40A/+; rn-Gal4, tub-$Gal80^{ts}$, UAS-eiger/+*
Fig. 8B: *w; ft^{fd}, ex^{e1}, FRT40A/FRT40A, ft^{fd}; rn-Gal4, tub-$Gal80^{ts}$, UAS-eiger/+*
Fig. 8C: *w; ex^{e1}, FRT40A/FRT40A, ex^{MGH1}; rn-Gal4, tub-$Gal80^{ts}$, UAS-eiger/+*
Fig. S3: *w; ex^{697}/UAS–GFP; puc-G4/+.*

Temporal expression of transgenes

To monitor phospho-Histone H3, cleaved Caspase 3, *ex-lacZ* and Yki in *en-Gal4; UAS-Debcl* experiments, larvae were raised at the permissive temperature (either 18 °C or 25 °C), then shifted to the restrictive temperature (29 °C) for 28 h on day 4 AEL (+/−12 h). To monitor *ex-lacZ*, Yki and GFP in *rn-Gal4, UAS-eiger* experiments, larvae were raised at the permissive temperature (18 °C), then shifted to the restrictive temperature (27 °C) for 22 h on day 7 AEL (+/−12 h).

Wing size measurements

For *yki* heterozygosity experiments, eggs were collected on grape plates for 6 h. Equal numbers of larvae (140) were transferred into food vials. Vials were shifted from 25 °C to the restrictive temperature of 29 °C at 96 h (+/−3 h) AEL for 21 h. For irradiation experiments, w^{1118} or $dachs^{GC13/1}$ larvae were raised at RT and irradiated with 40 Gy γ-irradiation at day 7 (+/−12 h) AEL. Wings of 1- to 2-day-old female adults were mounted in Canada Balsam (Sigma, #C1795) and imaged on an Olympus BX-51 microscope. Wing size was quantitated using Adobe Photoshop CS3. The graphs display average wing size, whilst error bars represent standard error of the mean.

Analysis of Yorkie localization

High resolution cross-sections (*x–z*) were taken through *en-Gal4; UAS-Debcl* discs by confocal microscopy. Average staining intensity for Yki in the nucleus of cells in both the anterior and posterior compartments was determined using Metamorph 7.6.3 software. Nuclei were determined by DAPI staining, anterior and posterior compartments were discerned by the presence or absence of GFP, respectively.

Immunoblotting

Between 18 and 22 imaginal discs were dissected from third instar larvae of the appropriate genotypes, lysed in protein sample buffer and subjected to SDS-PAGE. Gels were transferred to PVDF and immunoblotted with either rabbit anti-Yki (KH, unpublished), rabbit anti-Yki-phospho-S168 (Dong et al., 2007). Quantitation of immunoblots was performed using Image J software.