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Akt is negatively regulated by Hippo signaling for growth inhibition in *Drosophila*

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ABSTRACT

Tissue growth is achieved through coordinated cellular growth, cell division and apoptosis. Hippo signaling is critical for monitoring tissue growth during animal development. Loss of Hippo signaling leads to tissue overgrowth due to continuous cell proliferation and block of apoptosis. As cells lacking Hippo signaling are similar in size compared to normal cells, cellular growth must be properly maintained in Hippo signaling-deficient cells. However, it is not clear how Hippo signaling might regulate cellular growth. Here we show that loss of Hippo signaling increased Akt (also called Protein Kinase B, PKB) expression and activity, whereas activation of Hippo signaling reduced Akt expression in developing tissues in *Drosophila*. While *yorkie* (*yki*) is sufficient to increase Akt expression, Akt up-regulation caused by the loss of Hippo signaling is strongly dependent on *yki*, indicating that Hippo signaling negatively regulates Akt expression through Yki inhibition. Consistently, genetic analysis revealed that Akt plays a critical role in facilitating growth of Hippo signaling-defective tissues. Thus, Hippo signaling not only blocks cell division and promotes apoptosis, but also regulates cellular growth by inhibiting the Akt pathway activity.

Introduction

During development, cell growth, cell division and apoptosis determine cell size, cell number, and ultimately the size of organ and organism. Over the past three decades, much has been learned about the molecular basis and cell signaling mechanisms involved in regulating these fundamental biological processes. However, how cell growth, cell division and apoptosis can be properly coordinated has not been completely understood.

Under certain mutant background, cell growth has been shown to be separable or uncoupled from cell division. When cell proliferation rate is accelerated by over expressing specific cell-cycle regulators, such as dE2F, in cell clones or wing compartments in the developing *Drosophila* wing disc, more cells are generated but they are smaller in size because cell growth is unaffected (Neufeld et al., 1998). Therefore, although the cell numbers were increased 4- to 5- folds, clone or compartment sizes were not changed much (Neufeld et al., 1998). In other words, cell division acceleration is insufficient to drive cell growth (Nurse, 1975; Johnston et al., 1977). Loss of Hippo signaling, however, has been shown to cause dramatic increase of cell number and tissue overgrowth due to an increased cell proliferation and block of apoptosis (reviewed by Chan et al., 2011; Halder and Johnson, 2011; Oh and Irvine, 2010; Pan, 2010; Zhao et al., 2010). Because cells lacking Hippo signaling are similar in size compared to normal cells (e.g. Harvey et al., 2003; Lai et al., 2005), cell growth must be properly maintained in Hippo signaling-deficient cells. This observation also suggests that Hippo signaling negatively regulates cell growth in addition to its role in cell division and apoptotic control. However, it is not clear how the increase in cell growth is coupled to cell cycle acceleration to achieve tissue overgrowth in Hippo pathway mutants.

Protein synthesis comprises the main part of animal growth during development. Among the signaling pathways that regulate the protein translation apparatus, insulin receptor (Inr)/Akt signaling pathway (we use the term of Akt signaling for simplicity in the writing below) is the most prominent one. Akt signaling is known to play a critical role in growth control by regulating cell proliferation, cell growth and apoptosis and also in other biological processes, such as metabolism, reproduction and longevity (Oldham and Hafen, 2003; Hietakangas and Cohen, 2009; Tumaneng et al., 2012). In response to ligand binding, insulin receptor (Inr) is activated to recruit Chico, *Drosophila* homolog of Insulin Receptor Substrate (IRS), and the lipid kinase PI3K (phosphoinositide 3-kinase) to the plasma membrane (Bohni et al.,

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1999; Leevers et al., 1996; Weinkove et al., 1999). PI3K functions by converting PIP2 (phosphatidylinositol (4,5)-bisphosphate) into PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) via phosphorylation. This effect is reversed by lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10), which dephosphorylates PIP3 back to PIP2 (Maehama and Dixon, 1999). PIP3 at the membrane recruits pleckstrin homology (PH) domain-containing protein serine/threonine kinases, Akt (also called protein kinase B, PKB) and PDK1 (phosphoinositide-dependent kinase), to colocalize (Coffer et al., 1998; Brazil and Hemmings, 2001). Upon Ser/Thr phosphorylation by PDK1 and TOR (target of rapamycin), Akt kinase is activated (Alessi et al., 1997; Sarbassov et al., 2005). Like in vertebrate animals, *Drosophila* Tsc1 and Tsc2 (tuberous sclerosis complexes 1 and 2) proteins were identified as negative growth regulators (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001), playing a role in inhibiting TOR complex 1 (TORC1) activity. Although Akt kinase directly phosphorylates both Tsc1 and Tsc2, it turned out that such phosphorylation is not essential for normal animal growth (Dong and Pan, 2004; Schleich and Teleman, 2009). Therefore, PI3K/Akt appears to be able to regulate normal cell and tissue growth independent of the Tsc1/2 pathway. TOR also exists in another rapamycin-insensitive complex, TOR complex 2 (TORC2), which acts upstream of Akt as a positive regulator (Loewith et al., 2002). Activated TORC1 subsequently phosphorylates the eukaryotic initiation factor 4E binding protein (4E-BP), which blocks its function as inhibitor of cap-dependent translation (Lawrence and Abraham, 1997). TOR and PDK1 also activate ribosomal S6 kinase (S6K), thereby controlling the activity of protein translation machinery (Dufner and Thomas, 1999). Mutations of the positive components or overexpression of the inhibitory components of this pathway can result in reduced cellular growth rate (Hietakangas and Cohen, 2009).

In this work, we aimed at testing a hypothesis that Hippo signaling negatively regulates cell growth by reducing Akt pathway activity. We found that loss of Hippo signaling increased Akt expression as well as Akt activity, whereas activation of Hippo signaling reduced Akt expression in *Drosophila* developing tissues. The activity of a negative downstream component of the Akt pathway, 4E-BP, can be downregulated by loss of Hippo signaling. Genetic analysis indicated that reduction of Hippo signaling activity enhanced the ability of Akt kinase to promote cell growth and Akt plays a critical role in promoting tissue overgrowth induced by the loss of Hippo signaling activity. Acting downstream of the Hippo pathway, *yorkie* (*yki*) is sufficient to increase Akt expression. Moreover, Akt up-regulation caused by the loss of Hippo signaling is dependent on Yki, which supports a critical role of Akt in promoting cell and tissue growth and Yki is critical for Akt activation. Therefore, Hippo signaling negatively regulates cell and tissue growth by reducing Akt expression and activity through Yki inhibition.

Results

Genetic interaction between Akt and mats in regulating cell and organ size

Akt signaling plays a critical role in tissue development by promoting cell growth in *Drosophila* (Verdu et al., 1999;

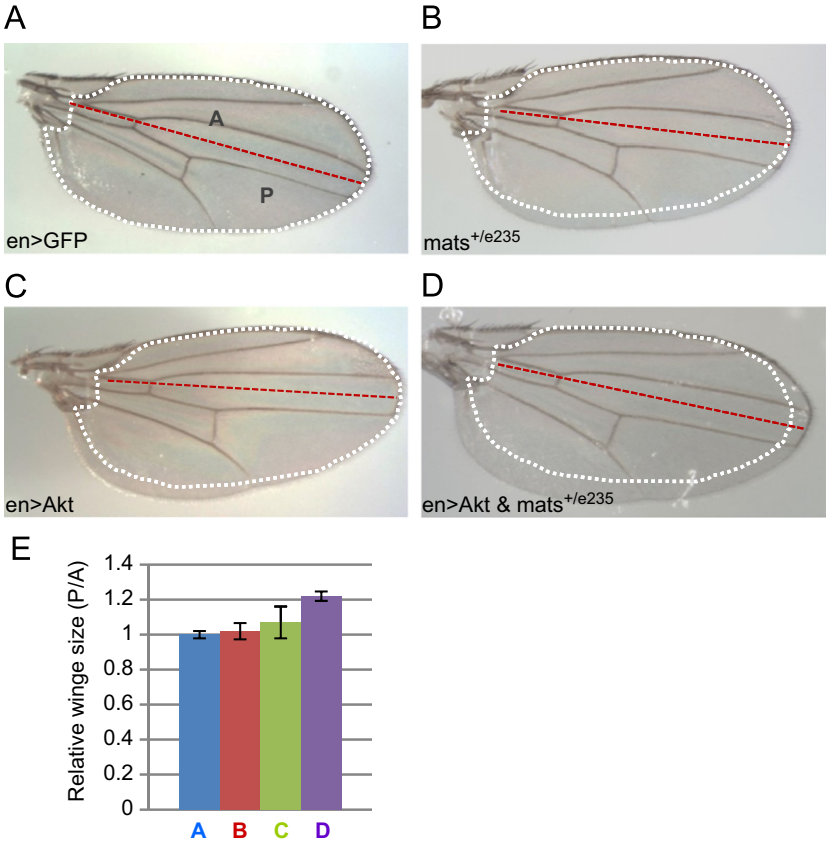

Fig. 1. The enlarged wing phenotype induced by Akt was dominantly enhanced by *mats* mutation. (A) The wing of *en-Gal4/UAS-GFP* adult fly, serving as a wild-type control ($n=21$). (B) $mats^{+}/mats^{e235}$ flies, the posterior/anterior (P/A) size of adult wing does not have significant change ($n=17$). (C) In *en-Gal4/UAS-Akt* flies, the posterior/anterior size of adult wing was increased by 5% ($n=35$). (D) In *en-Gal4/UAS-Akt*; $mats^{+}/mats^{e235}$ flies, the posterior/anterior size of adult wing was further increased to 22% ($n=13$) of that of wild-type flies. (E) Data from the statistic analysis. The white dots outline the size of a wild-type wing displayed in (A). The red-dot lines indicate the boundary between posterior (P) and anterior (A) compartments. The area of P is normalized to that of A to calculate the relative size of the P compartment (P/A). *en-Galt4* drives expression of *UAS* transgenes in the P compartment.