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Article

Regulation of Hippo signalling by p38 signalling

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The Hippo signalling pathway has a crucial role in growth control during development, and its dysregulation contributes to tumorigenesis. Recent studies uncover multiple upstream regulatory inputs into Hippo signalling, which affects phosphorylation of the transcriptional coactivator Yki/YAP/TAZ by Wts/Lats. Here we identify the p38 mitogen-activated protein kinase (MAPK) pathway as a new upstream branch of the Hippo pathway. In Drosophila, overexpression of MAPKK gene *licorne* (*lic*), or MAPKKK gene *Mekk1*, promotes Yki activity and induces Hippo target gene expression. Loss-of-function studies show that *lic* regulates Hippo signalling in ovary follicle cells and in the wing disc. Epistasis analysis indicates that *Mekk1* and *lic* affect Hippo signalling via *p38b* and *wts*. We further demonstrate that the Mekk1-Lic-p38b cascade inhibits Hippo signalling by promoting F-actin accumulation and Jub phosphorylation. In addition, p38 signalling modulates actin filaments and Hippo signalling in parallel to small GTPases Ras, Rac1, and Rho1. Lastly, we show that p38 signalling regulates Hippo signalling in mammalian cell lines. The Lic homologue MKK3 promotes nuclear localization of YAP via the actin cytoskeleton. Upregulation or downregulation of the p38 pathway regulates YAP-mediated transcription. Our work thus reveals a conserved crosstalk between the p38 MAPK pathway and the Hippo pathway in growth regulation.

Keywords: Hippo signalling, p38 signalling, F-actin

Introduction

Over the past decade, the Hippo signalling pathway, which is highly conserved from Drosophila to mammals, has gradually demonstrated its central role in the regulation of growth. Through controlling both cell proliferation and apoptosis, Hippo signalling participates in multiple physiological processes including organ size control, tissue homeostasis, tissue regeneration, and stem cell maintenance. Dysfunction of the Hippo pathway is tightly implicated in tumorigenesis (Harvey and Tapon, 2007; Pan, 2010; Lange et al., 2015; Plouffe et al., 2015). The core kinase cascade of Hippo signalling is composed of four components. The serine/threonine Ste20-like kinase Hippo (Hpo, Mst1/2 in mammals) (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003), in complexed with the scaffold protein Salvador (Sav, Sav1 in mammals) (Kango-Singh et al., 2002; Tapon et al., 2002), phosphorylates and activates the nuclear Dbf-2-related (NDR) family kinase Warts (Wts, Lats1/2 in mammals) (Justice et al., 1995; Xu et al., 1995), which forms a complex with a regulatory protein Mats (Mob1A/B in mammals) (Lai et al., 2005). Very recent studies show that Happyhour/MAP4K kinases act in parallel with Hpo to regulate Wts activation (Meng et al., 2015; Zheng et al., 2015). At the downstream of this kinase cascade, Wts phosphorylates a transcriptional coactivator Yorkie (Yki, YAP/TAZ in mammals) and induces its nuclear export (Huang et al., 2005). In the absence of inhibition by Wts, Yki cooperates with transcription factors to upregulate expression of target genes, such as *expanded* (*ex*), *Diap1*, and *bantam* (*ban*) (Staley and Irvine, 2012; Dong et al., 2015). There are multiple regulatory inputs in the upstream of the core kinase cascade, including Fat-Dachs signalling (Feng and Irvine, 2007; Reddy and Irvine, 2008), Expanded–Merlin–Kibra complex (Hamaratoglu et al., 2006; Baumgartner et al., 2010), Lethal giant larvae (Lgl) complex (Grzeschik et al., 2010; Robinson et al., 2010), and the F-actin cytoskeleton (Fernandez et al.,

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2011; Sansores-Garcia et al., 2011). In addition, studies have disclosed extensive crosstalk between Hippo signalling and other growth control pathways, thereby providing new mechanisms for regulation of growth (Halder and Johnson, 2011; Staley and Irvine, 2012).

The evolutionarily conserved p38 mitogen-activated protein kinase (MAPK) pathway is activated in response to extracellular stimuli, and is involved in a variety of biological processes including cell proliferation, stress tolerance, immune response, and apoptosis (Cuadrado and Nebreda, 2010). Dysregulation of the p38 MAPK pathway has been implicated in a wide range of carcinomas (Koul et al., 2013). Like ERK and JNK signalling, the p38 signalling pathway is composed of three classes of protein kinases: MAPK, MAPK kinase (MAPKK), and MAPK kinase kinase (MAPKKK) (Cuenda and Rousseau, 2007). In Drosophila, p38 MAPK is activated via dual phosphorylation at the Thr-Gly-Tyr motif by a specific MAPKK, Licorne (Lic, MKK3/6 in mammals) (Suzanne et al., 1999). Several MAPKKKs have been proposed to activate the p38 MAPK pathway, including Mekk1 (MEKK4 in mammals), TGF-β activated kinase 1 (TAK1), Apoptotic signal-regulating kinase 1 (ASK1), and Slipper (Slpr) (Inoue et al., 2001; Chen et al., 2010). Existence of multiple MAPKKKs may provide the ability for p38 signalling to respond to a variety of stimuli and to integrate with other pathways. The Drosophila genome encodes three p38 kinases named p38a, p38b, and p38c. However, they may not act redundantly. For example, p38b is proposed to play a central role in Drosophila p38 signalling, while p38c may have specific function in the intestine (Seisenbacher et al., 2011; Vrailas-Mortimer et al., 2011; Belozerov et al., 2012; Chakrabarti et al., 2014; Tian et al., 2014).

In this study, we uncovered a crosstalk between p38 signalling and Hippo signalling. We found that overexpression of *lic*, or its upstream kinase *Mekk1*, enhanced Yki activity and promoted Hippo target gene expression in imaginal discs. *lic* mutant mimicked *yki* mutant to affect Hippo target expression in ovarian follicle cells. Besides, *lic* participated in the regulation of Hippo signalling in the wing disc. Epistasis analysis demonstrated that *wts* and *yki* were epistatic to *lic*, and *p38b* mediated the effects of *lic* and *Mekk1* on Hippo signalling. We further showed that activation of the p38 kinase cascade induced F-actin accumulation, which was essential for the regulation of Hippo signalling by p38 signalling. Interestingly, we found that the Drosophila p38 pathway, unlike their mammalian homologues, regulated F-actin accumulation independent of MK2 and Hsp27. In addition, small GTPases Ras, Rac1, and Rho1 did not act upstream of the p38 pathway to regulate actin filaments and Hippo signalling. Furthermore, we showed that activated p38b phosphorylated the Ajuba LIM protein (Jub), which might link activation of p38 signalling to inhibition of Wts. Lastly, we found that the regulation of the Hippo pathway by the p38 pathway was conserved in mammalian cells. The Lic homologue MKK3 promoted nuclear localization of YAP by inducing F-actin accumulation. Overexpression of *MKK3* enhanced YAP-mediated target gene expression, while knockdown of *p38* downregulated transcription of YAP targets.

Results

Overexpression of lic induces Hippo target gene expression and promotes Yki activity

In a previous study, we showed that overexpressed *lic* could affect Wingless (Wg) target gene expression (Schertel et al., 2013). Consistent with its role downstream of *wg*, overexpression of *lic* in the posterior compartment of the wing disc did not affect the levels of Wg at the dorsal-ventral (D/V) boundary. In contrast, we observed upregulation of Wg levels in the hinge region (Figure 1A and B). Similar result was observed when

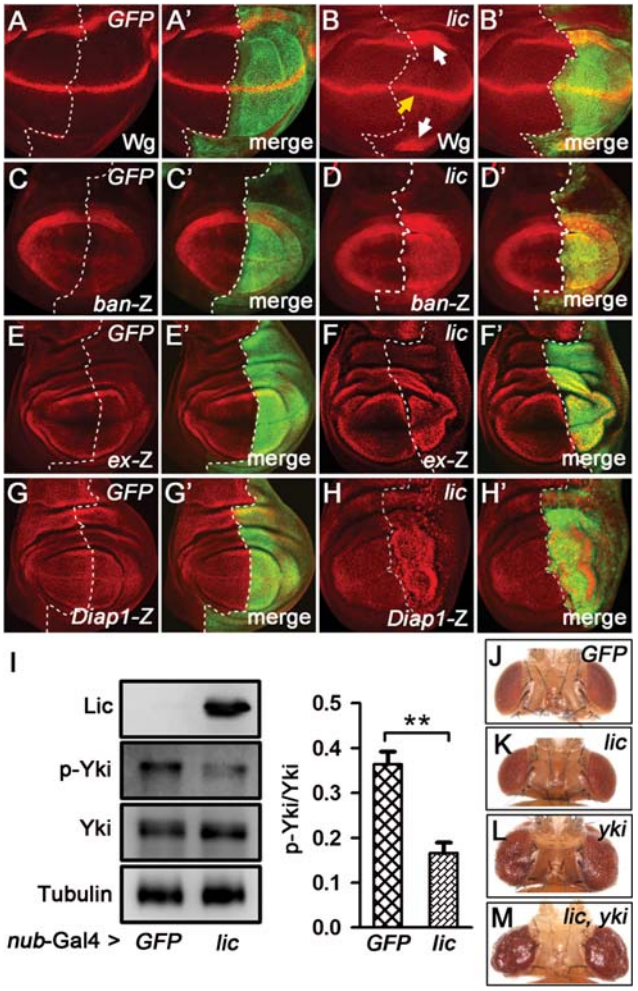

Figure 1 Overexpression of *lic* promotes Yki activities and induces Hippo target gene expression. (**A**–**H**) Immunostaining of Wg (**A** and **B**), *ban*-lacZ (**C** and **D**), *ex*-lacZ (**E** and **F**), and *Diap1*-lacZ (**G** and **H**) in the wing disc expressing *UAS-GFP* control or *UAS-lic* by *en*-Gal4 *tub*-Gal80^{ts}. Wg signal at the hinge region and the D/V boundary is indicated by white and yellow arrows, respectively. (**I**) Total and phosphorylated (S168) levels of Yki in the wing disc expressing *UAS-lic* by *nub*-Gal4. The right panel displays the ratio of p-Yki (S168) levels to total Yki levels. Data represented as mean ± SD (n = 3). Student's t-test, $^{**}P < 0.01$. (**J**–**M**) Drosophila eyes expressing *UAS-GFP* (**J**), *UAS-lic* (**K**), *UAS-yki* (**L**), or *UAS-lic* and *UAS-yki* by *GMR*-Gal4 (**M**).

we expressed *lic* in the dorsal compartment by *ap*-Gal4 (Supplementary Figure S1A and B). As the expression of *wg* in the D/V boundary and the hinge region are regulated by Notch signalling and Hippo signalling, respectively, these results suggest that *lic* may act in the Hippo pathway (Cho et al., 2006). To test this idea, we examined the effects of *lic* overexpression on other Hippo target genes in the wing disc. Expression of *lic* by *en*-Gal4 increased transcription of *ban*, *ex*, and *Diap1*, three well-known target genes of the Hippo pathway (Figure 1C–H). Expression of *lic* by *ptc*-Gal4 or *ci*-Gal4 also showed similar effects on these Hippo target genes (Supplementary Figure S1C–F). A kinase-dead version of *lic* (*lic KD*) failed to affect the levels of *ex* or *Diap1*, indicating that the kinase activity of Lic is required for its functions in Hippo signalling (Supplementary Figure S1G and H). In addition, overexpression of *Mekk1*, which encoded a MAPKKK and activated endogenous Lic, also upregulated *Diap1* expression in the wing disc (Supplementary Figure S1I).

The Hippo signalling pathway represses its target gene expression by mediating phosphorylation and inactivation of the coactivator Yki (Huang et al., 2005). We next examined the effects of *lic* overexpression on Yki. We ubiquitously expressed *lic* in the wing pouch by *nub*-Gal4 and monitored Yki by western blotting. Overexpression of *lic* decreased the phosphorylation levels of Yki without reducing its protein levels (Figure 1I). In addition, although overexpression of *lic* by *GMR*-Gal4 only showed a mild rough eye phenotype, co-expression of *lic* and *yki* caused a much stronger eye overgrowth phenotype than overexpression of *yki* alone (Figure 1J–M). These results indicate that *lic* overexpression increases the activity of Yki.

lic is required for proper expression of Hippo targets in ovarian follicle cells

Next, we investigated whether *lic* was physiologically required to regulate the Hippo pathway targets during Drosophila development. Drosophila modENCODE tissue expression data imply that *lic* is most abundantly expressed in the ovary, where Hippo signalling has been shown to control the maturation of the posterior follicle cells (PFCs) occurring between stages 6 and 7 in egg chambers (Meignin et al., 2007; Polesello and Tapon,

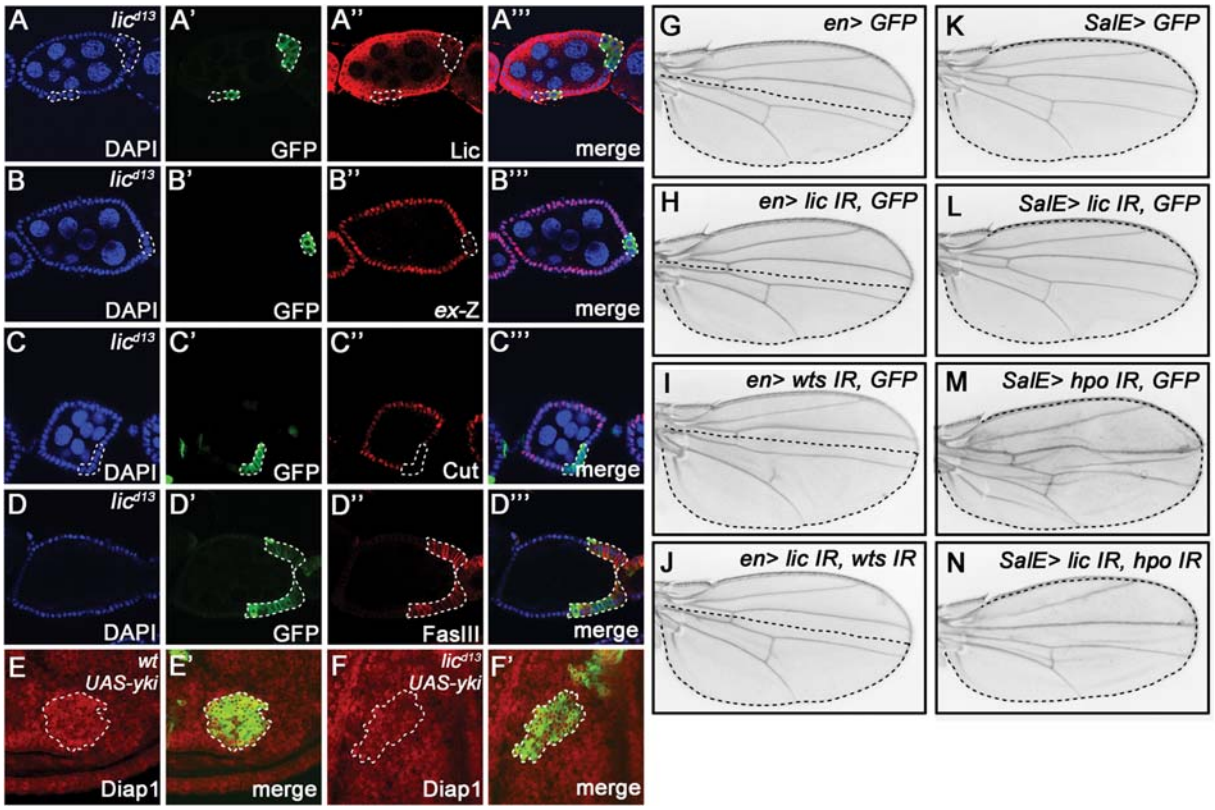

Figure 2 *lic* regulates Hippo signalling in ovary follicle cells and in the wing disc. (**A**–**D**) Immunostaining of Lic (**A**), ex-lacZ (at stages 7–9) (**B**), Cut (at stages 1–6) (**C**), and FasIII (at stages 1–6) (**D**) in the egg chamber containing lic^{d13} MARCM clones. (**E** and **F**) Immunostaining of Diap1 in the wing disc containing wild-type MARCM clones (**E**) or lic^{d13} mutant MARCM clones expressing *UAS-yki* by *Act*-Gal4 (**F**). The MARCM clones are marked by the presence of GFP and outlined by white dashed lines. (**G**–**J**) Adult wings expressing *UAS-GFP* (100% penetrant, $n = 35$) (**G**), *UAS-lic IR* and *UAS-GFP* (100% penetrant, $n = 35$) (**H**), *UAS-wts IR* and *UAS-GFP* (100% penetrant, $n = 33$) (**I**), *UAS-lic IR* and *UAS-wts IR* (91.4% penetrant, $n = 35$) by *en*-Gal4 (**J**). (**K**–**N**) Adult wings expressing *UAS-GFP* (100% penetrant, $n = 35$) (**K**), *UAS-lic IR* and *UAS-GFP* (100% penetrant, $n = 35$) (**L**), *UAS-hpo IR* and *UAS-GFP* (100% penetrant, $n = 30$) (**M**), *UAS-lic IR* and *UAS-hpo IR* (85.7% penetrant, $n = 35$) by *SalE*-Gal4 (**N**). The expression domain of *en*-Gal4 or *SalE*-Gal4 in the adult wing was outlined with black dashed lines.

2007). Using a Lic antibody generated in our lab, we confirmed *lic* expression in the egg chamber and strong reduction of Lic protein levels in lic^{d13} mutant clones (Cully et al., 2010) (Figure 2A). In follicle cells at stages 7–9, we observed downregulation of *ex* expression in *lic* mutant PFC clones (Figure 2B). In follicle cells at stages 1–6, Yki upregulates *cut* expression (Koontz et al., 2013). Also in these cells, active Yki suppresses *FasIII* expression in main-body follicle cell precursors, while reduction of Yki activity allows *FasIII* expression in polar cells (Chen et al., 2011). Similar to *yki* loss-of-function phenotypes, we observed downregulation of *cut* expression and ectopic *FasIII* expression in *lic* mutant clones of follicle cells at stages 1–6 (Figure 2C and D). Therefore, consistent with the results from the gain-of-function analysis, the loss-of-function analysis also suggests that *lic* has a positive effect on Yki-mediated gene expression.

lic participates in the regulation of Hippo signalling in the wing disc

The Hippo pathway also regulates imaginal disc development. We further tested whether *lic* was essential for expression of Hippo targets in this context. However, expression of *ex* or *Diap1* in the wing disc was not affected in *lic* mutant clones (Supplementary Figure S2A and B). Similarly, knockdown of *lic* in the posterior compartment of the wing disc via RNA interference (RNAi) showed no effects on *ex* or *Diap1* expression (Supplementary Figure S2C–H). Thus, the physiological function of *lic* may be more important in controlling egg chamber development by Hippo signalling.

Several upstream branches of the Hippo pathway have shown their regulatory roles in the wing disc. The lack of loss-of-function phenotype of *lic* in this tissue may be due to redundant contributions by other regulatory inputs. If this hypothesis is correct, loss of *lic* may have a stronger influence on Hippo signalling when the upstream control is weakened in the wing disc. Therefore, we performed our analysis in the *yki* gain-of-function background, in which we reduced the dosage of *hpo* or *wts*, or increased the dosage of *yki*. Clonal overexpression of *yki* upregulated expression of *Diap1* in the wing disc. In contrast, expression of *Diap1* was unaltered in lic^{d13} mutant clones that overexpress *yki*, indicating that *lic* was required to regulate increased levels of Yki in the wing disc (Figure 2E and F). We also tested the effects of loss of *lic* on Yki-mediated growth control in the adult wing. As lic^{d13} mutant was lethal, we used the heterozygous *lic* mutant or *lic* RNAi lines for this purpose. Overexpression of *yki* by *ptc*-Gal4 significantly increased the area between the L3 and the L4 veins in the wing. This overgrowth pattern was partially rescued in the heterozygous *lic* mutant background (Supplementary Figure S2I–M). Similarly, knockdown of *wts* or *hpo* in the wing disc, which increased endogenous levels of Yki, resulted in overgrowth of the wing in corresponding domains. Knockdown of *lic* in same domains largely relieved these overgrowth phenotypes caused by knockdown of *wts* or *hpo* (Figure 2G–N, Supplementary Figure S2N and O). These results suggest that *lic* participates in the regulation of Hippo target expression and Hippo signalling-mediated growth control in the wing tissue.

wts and yki are epistatic to lic in the Hippo pathway

We next analysed whether the effects of *lic* on Hippo targets was mediated via the Hippo pathway. We test this notion in both egg chambers and wing discs. In the egg chamber, *wts* RNAi prevented ectopic expression of *FasIII* in *lic* mutant clones (Figure 3A and B). In the wing disc, overexpression of *lic* upregulated *ex* transcription, which was abolished by co-expression of *wts* or by knockdown of *yki* (Figure 3C–H). These results imply that *lic* functions upstream of, or in parallel to, *wts* and *yki* in the Hippo pathway to regulate target gene expression. As *lic* can regulate Hippo signalling in both egg chambers and wind discs, and the wing disc is a better characterized model system than the egg chamber to define genetic relationships, we performed the rest of epistatic analysis in the wing disc.

We noticed that overexpression of *lic* in the wing disc caused apoptosis (Supplementary Figure S3A). Co-expression of *p35*, which eliminated apoptosis, did not affect the upregulation of

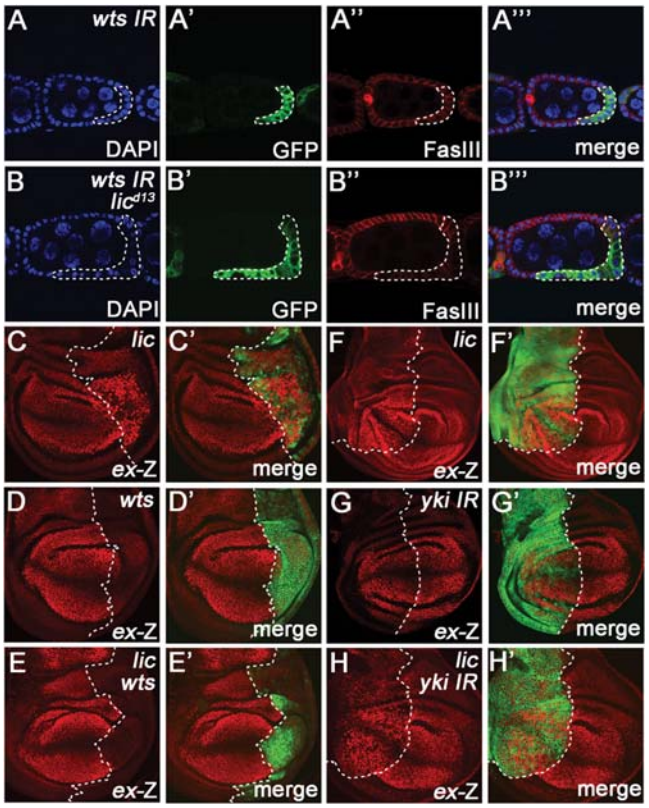

Figure 3 Lic acts upstream of Wts and Yki. (**A** and **B**) Immunostaining of FasIII in follicle cells expressing *UAS-wts IR* in control (**A**) or lic^{d13} MARCM clones (**B**) (marked by the presence of GFP and outlined by white dashed lines). (**C**–**E**) Immunostaining of ex-lacZ in the wing disc expressing *UAS-lic* (**C**), UAS-wts (**D**), or *UAS-lic* and *UAS-wts* by *en*-Gal4 *tub*-Gal80^{ts} (**E**). (**F**–**H**) Immunostaining of ex-lacZ in the wing disc expressing *UAS-lic* (**F**), *UAS-yki IR* (**G**), or *UAS-lic* and *UAS-yki IR* by *ci*-Gal4 (**H**).

Diap1 expression by *lic* overexpression (Supplementary Figure S3B and C). Apoptosis in the wing disc may trigger c-Jun N-terminal kinase (JNK)-mediated activation of Yki (Sun and Irvine, 2011). However, expressing a dominant negative form of *basket* (*bsk*, the Drosophila homologue of *JNK*), or its RNAi construct, did not interfere with *lic*-induced Hippo target gene expression (Supplementary Figure S3D–G). Therefore, our observations of the effects of *lic* on Hippo signalling are not mediated via apoptosis or JNK signalling.

lic affects Hippo signalling via p38b

As *lic* encoded a MAPKK for the p38 kinase, we asked the question whether *lic* affected Hippo signalling through *p38*, or via a mechanism that was independent of *p38*. It was proposed that p38b had a central role in Drosophila p38 signalling (Seisenbacher et al., 2011; Vrailas-Mortimer et al., 2011; Belozerov et al., 2012; Tian et al., 2014). To distinguish these two possibilities, we knocked down *p38b* by RNAi or generated *p38b* mutant clones with a $p38b^{156A}$ allele (Chen et al., 2010). In the wing disc, knockdown of *p38b* had no effects on basal expression of *Diap1* or *ex* (Figure 4A and B, Supplementary Figure S4A). In contrast, *p38b* RNAi blocked the upregulation of *Diap1* and *ex* expression in *lic* overexpression domains (Figure 4C and D, Supplementary Figure S4B). Similarly, knockdown of *p38b* largely eliminated the upregulation of *Diap1* expression by *Mekk1* (Supplementary Figure S4C–F). When we overexpressed *lic* in the posterior compartment of the wing disc by *hh*-Gal4 and generated *p38b* mutant clones, basal expression of *Diap1* in the anterior compartment was not affected, while upregulated *Diap1* expression in the posterior compartment was abolished (Figure 4E). These results suggest that *p38b* mediates the effects of *lic* and *Mekk1* on Hippo target expression.

Is the activation of p38b required by Lic or Mekk1 to affect Hippo signalling? Overexpression of *lic* or *Mekk1*, but not *lic KD*, largely increased the levels of phosphorylated p38 (Figure 4F and G, Supplementary Figure S4G). These observations seem to agree with this notion. To test this hypothesis, we overexpressed p38b in the wing disc. Consistent with a previous study showing that overexpressed p38b was not phosphorylated in S2 cells and Drosophila larvae in the absence of stressful stimuli (Belozerov et al., 2012), overexpression of p38b in the wing disc did not upregulate its phosphorylation level (Supplementary Figure S4H). More importantly, overexpression of this unphosphorylated form of p38b did not affect *Diap1* expression (Supplementary Figure S4I and J). Therefore, Lic and Mekk1 may affect the Hippo pathway via activating p38b.

The p38 pathway regulates F-actin accumulation and Jub phosphorylation

We observed that overexpression of *lic* or *Mekk1* in the wing disc induced F-actin accumulation (Figure 5A and B, Supplementary Figure S5A and B). These effects were also

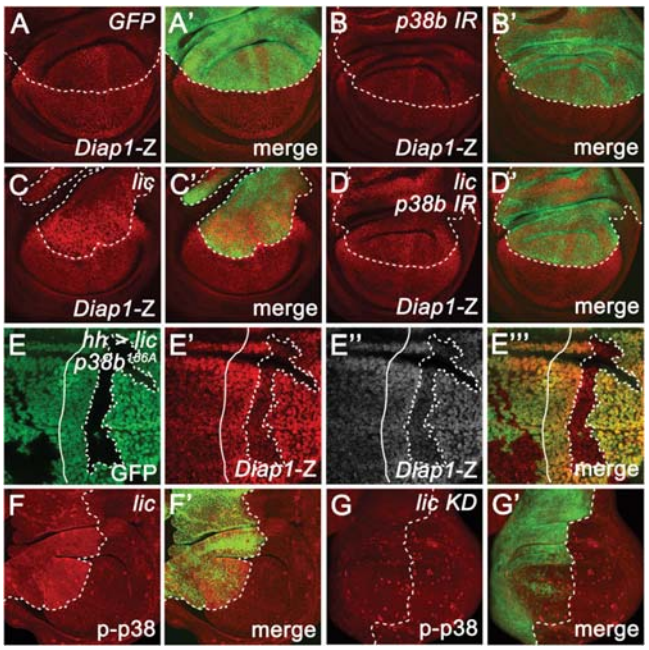

Figure 4 *lic* regulates Hippo signalling via *p38b*. (**A**–**D**) Immunostaining of *Diap1*-lacZ in the wing disc expressing *UAS-GFP* (**A**), *UAS-p38b IR* (**B**), *UAS-lic* (**C**), or *UAS-lic* and *UAS-p38b IR* by *ap*-Gal4 (**D**). (**E**) Immunostaining of *Diap1*-lacZ in the wing disc expressing *UAS-lic* by *hh*-Gal4 and containing $p38b^{156A}$ clones (marked by the absence of GFP and outlined by white dashed lines). (**F** and **G**) Immunostaining of phosphorylated p38 in the wing disc expressing *UAS-lic* (**F**) or *UAS-lic KD* by *ci*-Gal4 (**G**).

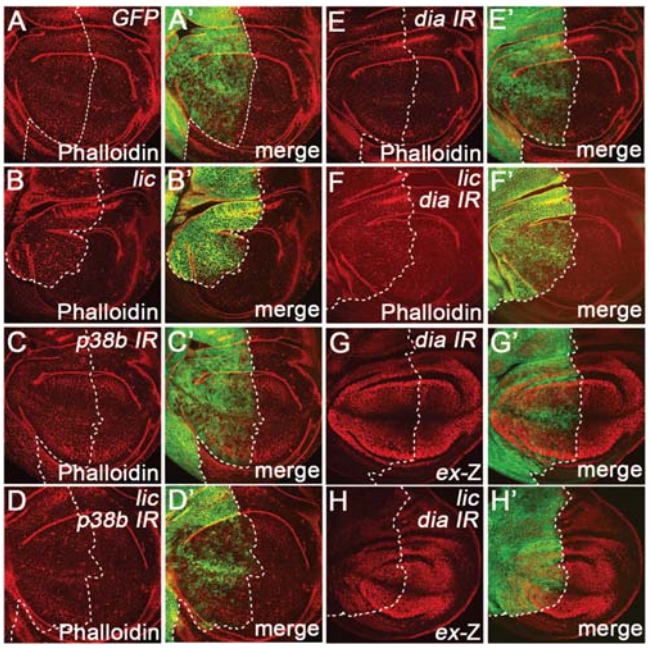

Figure 5 The p38 pathway regulates the Hippo pathway via F-actin accumulation. (**A**–**D**) Staining of F-actin in the wing disc expressing *UAS-GFP* (A), *UAS-lic* (**B**), *UAS-p38b IR* (**C**), or *UAS-lic* and *UAS-p38b IR* by *Ci*-Gal4 (**D**). (**E**–**H**) Staining of F-actin (**E** and **F**) or *ex*-lacZ (**G** and **H**) in the wing disc expressing *UAS-dia IR* (**E** and **G**) or *UAS-lic* and *UAS-dia IR* by *Ci*-Gal4 (**F** and **H**).

dependent on *p38b*, as *p38b* RNAi largely prevented F-actin accumulation in the *lic* or *Mekk1*-overexpressing domain (Figure 5C and D, Supplementary Figure S5C and D). The actin cytoskeleton has been shown to participate in the regulation of the Hippo pathway (Fernandez et al., 2011; Sansores-Garcia, et al., 2011; Gaspar and Tapon, 2014; Rauskolb et al., 2014). Therefore, we investigated whether Lic and Mekk1 regulated Hippo signalling through p38b-dependent F-actin accumulation. The actin nucleation factor Diaphanous (Dia) mediates F-actin polymerization (Sansores-Garcia et al., 2011). As expected, knockdown of *dia* significantly diminished F-actin accumulation induced by *lic* overexpression, indicating that Dia acted downstream of the p38 pathway to modulate actin filaments (Figure 5E and F). More importantly, *dia* RNAi prevented upregulation of *ex* levels by *lic* overexpression, suggesting that *lic* regulated the Hippo pathway by promoting extra F-actin formation (Figure 5G and H).

The Ajuba LIM protein (Jub) is associated with the actin cytoskeleton and closely linked to inhibition of Wts (Das Thakur et al., 2010; Rauskolb et al., 2014). If our hypothesis is correct, reduction of Jub levels should be able to interfere with the crosstalk between p38 signalling and Hippo signalling. Indeed, knockdown of *jub* abolished the upregulation of *Diap1* expression, but not the accumulation of F-actin, by *lic* or *Mekk1* (Figure 6A–F). These results indicate that Jub acts downstream of, or in parallel to, actin filaments to mediate the regulation of the Hippo pathway by the p38 pathway. It has been reported that JNK and ERK can phosphorylate Jub and enhance its ability to block Wts function (Reddy and Irvine, 2013; Sun and Irvine, 2013). Therefore, we tested whether p38 had similar activity as a member of MAPKs. Although overexpressed p38b was not activated in S2 cells, indicated by the lack of phosphorylated p38b in the western blot, co-expression of Lic and p38b ensured p38b activation, and caused apparent mobility shift of Jub (Figure 6G). This effect was eliminated by treating the cell lysate with calf intestinal phosphatase (CIP), indicating that activated p38b was able to phosphorylate Jub (Figure 6H).

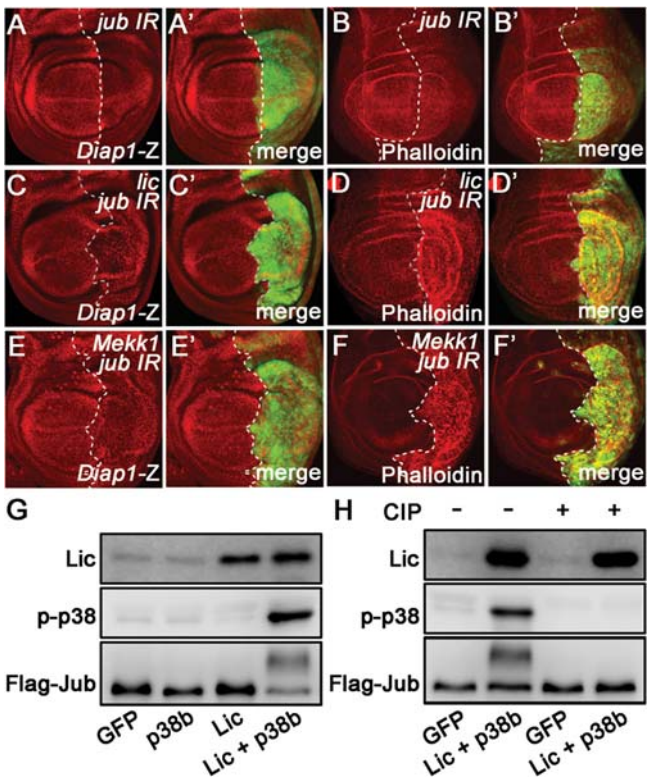

Figure 6 Regulation of Hippo signalling by p38 signalling requires Jub. (**A**–**F**) Staining of *Diap1*-lacZ or F-actin in the wing disc expressing *UAS-jub IR* (**A** and **B**), *UAS-lic* and *UAS-jub IR* (**C** and **D**), or *UAS-Mekk1* and *UAS-jub IR* (**E** and **F**). (**G**) Mobility shift of Jub in the presence of Lic and p38b measured by western blotting. (**H**) Mobility shift of Jub in the absence or presence of CIP measured by western blotting.

We further explored additional factors that transduced the signal from activated p38 to assembly of actin filaments. In mammals, activated p38 can phosphorylate MAP kinase-activated kinase 2 (MK2). Phosphorylation of Heat shock protein 27 (Hsp27) by MK2 is necessary for accumulation of F-actin in mammalian cells (Gerthoffer and Gunst, 2001; Weber et al., 2005). We examined whether similar pathway existed in Drosophila. However, in *lic*-overexpressing domains in the wing disc, the accumulation of F-actin was not affected by *Hsp27* or *MK2* RNAi, or in a $MK2^{5B}$-null mutant background (Chen et al., 2010) (Supplementary Figure S5E–H). Consistently, the effect of *lic* on *ex* expression was not weakened by knockdown of *MK2* (Supplementary Figure S5I and J). Therefore, the Drosophila p38 pathway may adopt an alternative approach, which is independent of MK2 and Hsp27, to modulate F-actin accumulation.

Activation of several small GTPases, including Ras, Rac1, and Rho1, has been shown to promote F-actin accumulation or Hippo target gene expression (Reddy and Irvine, 2013; Regue et al., 2013; Fernandez et al., 2014). We investigated whether any of them acted through the p38 pathway. To test this idea, we overexpressed an activated form of *ras* (ras^{V12}), an activated form of *Rac1* ($Rac1^{V12}$), or *Rho guanine nucleotide exchange factor 2* (*RhoGEF2*) in the wing disc. However, knockdown of *p38b* or *lic* did not prevent F-actin accumulation induced by ras^{V12}, $Rac1^{V12}$, or *RhoGEF2* (Supplementary Figure S6A–C, G–I, M–O). In addition, knockdown of *p38b* or *lic* did not interfere with the upregulation of *ex* or *Diap1* expression by ras^{V12}, $Rac1^{V12}$, or *RhoGEF2* (Supplementary Figure S6D–F, J–L, P–R). These results imply that Ras, Rac1, and Rho1 may regulate actin filaments and Hippo signalling via approaches that bypass the p38 pathway.

Mammalian p38 signalling regulates the Hippo-YAP pathway

Lastly, we investigated whether mammalian p38 signalling played a role in the regulation of the Hippo-YAP pathway through a similar mechanism. In cultured cells, cell densities affect subcellular localization of YAP and Hippo target gene expression through modulating the quantity of F-actin. High cell densities inhibit F-actin polymerization and induce nuclear export of YAP (Wada et al., 2011). We examined the effects of *MKK3* (a mammalian homologue of *lic*) on F-actin assembly and YAP localization. In densely cultured MCF10A cells, transfected

cells overexpressing *MKK3* showed higher levels of F-actin than their neighbouring control cells. Treatment of cells with an anti-actin drug CytoD prevented F-actin accumulation induced by *MKK3* (Figure 7A and B). Also in these cells, *MKK3* expression promoted nuclear localization of YAP1, which could be abolished by CytoD treatment (Figure 7C–E). We also explored the effects of *MKK3* expression on localization of endogenous YAP. At high cell density, endogenous YAP was predominantly located in the cytoplasm. In contrast, endogenous YAP became enriched in the nucleus in cells overexpressing *MKK3* (Supplementary Figure S7). We further analysed the effects of *MKK3* on Hippo target gene expression. In HEK293T cells, transfected *YAP1* upregulated the transcription of a Gal4-TEAD luciferase reporter. Transfected *MKK3* strongly enhanced YAP1-mediated reporter expression. While CytoD treatment did not hamper transcriptional activity of YAP1, it significantly diminished the stimulatory effect of MKK3 on YAP1 activity (Figure 7F). Western blotting analysis confirmed that MKK3 decreased the phosphorylation levels of YAP1, thereby increasing its stability and transcriptional activity (Figure 7G). We also examined endogenous targets of Hippo signalling. MKK3 significantly potentiated YAP1-upregulated transcription of *CTGF* and *Cyr61* (Figure 7H).

Among four mammalian p38 family members, p38α is ubiquitously expressed and highly abundant in most cell types, whereas p38β is expressed at low levels. p38γ and p38δ have restricted expression patterns (Igea and Nebreda, 2015). Elevated p38α activity has been reported in several types of human cancers, including prostate cancer, breast cancer, lung cancer, etc (Koul et al., 2013). To test the idea whether upregulated p38 signalling participates in the regulation of Hippo signalling in cancer cells, we knocked down p38α in A549 lung adenocarcinoma cells with siRNAs. Indeed, transcription of *CTGF* and *Cyr61* was downregulated by siRNAs targeting p38α (Figure 7I). Together with above data, these results suggest that mammalian p38 signalling promotes YAP activity and Hippo target expression via F-actin accumulation.

Discussion

The Hippo tumour suppressor pathway is a major growth control pathway conserved from Drosophila to mammals. The transcriptional coactivator Yki (YAP/TAZ in mammals) integrates upstream inputs to regulate expression of Hippo target genes. Studies in recent years have shown that many pathways can serve as upstream regulatory inputs into Hippo signalling. Among them, two MAPK pathways, ERK and JNK signalling, are closely implicated in the regulation of Hippo signalling (Sun and Irvine, 2011; Reddy and Irvine, 2013). Here we show that the p38 signalling pathway, another major MAPK pathway, also participate in the regulation of Hippo signalling. As all of these MAPK pathways inhibit Hippo signalling at the level of Wts, the molecular mechanisms may share similarities. The activation of ERK or JNK kinase cascade can directly phosphorylate Jub and enhance its inhibitory effect on Wts (Reddy and Irvine, 2013; Sun and Irvine, 2013). In this work, we find that activated p38 also phosphorylates Jub, suggesting that p38 signalling regulates Hippo signalling at least in part by affecting Jub activities

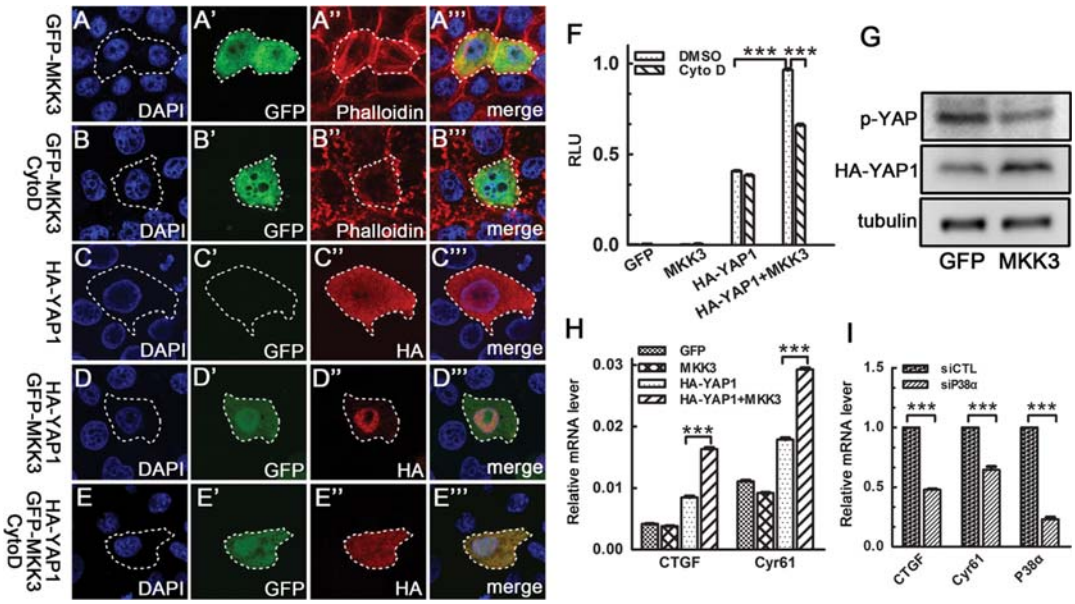

Figure 7 p38 signalling regulates Hippo signalling in mammalian cell lines. (**A**–**E**) MCF10A cells were transfected with indicated plasmids, and cells in B and E were treated with 1 μg/ml CytoD for 1 h. (**A** and **B**) Staining of F-actin. (**C**–**E**) Immunostaining of HA-YAP1. (**F**) Luciferase assays in cells expressing Gal4-TEAD reporter and the indicated plasmids. (**G**) Total and phosphorylated (S127) levels of HA-YAP1 in HEK293 cells expressing *MKK3*. (**H**) Transcription of *CTGF* and *Cyr61* in HEK293T cells transfected with the indicated plasmids. (**I**) Transcription of *CTGF* and *Cyr61* in A549 cells with siRNAs targeting *p38α*. All the experiments were performed with densely cultured cells. Data represented as mean ± SD (n = 3).

(Figure 6). In addition, we show that the Mekk1–Lic–p38b cascade induces F-actin accumulation to regulate Hippo signalling (Figure 5). Although the functional connection between p38 regulation of F-actin and p38 regulation of Jub is not clear, it is conceivable that F-actin accumulation may help to recruit Jub (Rauskolb et al., 2014), and thereby facilitating its phosphorylation by p38b. On the other hand, these MAPK pathways may transduce different upstream signals to regulate Hippo signalling. For example, JNK signalling may inhibit Hippo signalling during regenerative growth (Sun and Irvine, 2011), while p38 signalling may respond to various stress signals to affect the Hippo pathway. Consistent with this speculation, we show that mammalian p38 signalling promotes YAP activity and Hippo target gene expression under the condition of high cell densities (Figure 7), which implies its regulatory role in tumour growth.

Lic function is essentially required for proper expression of Hippo target genes in ovarian follicle cells. In this tissue, *lic* mutant shows phenotypes similar to that of *yki* mutant (Figure 2A–D). In contrast, while ectopic p38 signalling induces Hippo target gene expression in the wing disc, *lic* mutant or *lic* RNAi does not show noticeable effect on Hippo signalling in this tissue in a wild-type background (Supplementary Figure S2A–H). The fact that p38 signalling is essential for the Hippo pathway in the egg chamber, but not in the wing disc, can be attributed to several possibilities. Drosophila modENCODE tissue expression data suggest that *mekk1*, *lic*, and *p38b* are more abundantly expressed in the ovary than in the imaginal disc. Our immunostaining data also reveal high levels of Lic in the egg chamber. Therefore, it is possible that the activity of p38 signalling is higher in the egg chamber and results in stronger influence on the Hippo pathway in this tissue. In addition, there are many upstream regulators of Hippo signalling. They may have different levels of contributions or play redundant roles depending on the tissue type and developmental context. For example, Fat is required to regulate Hippo signalling in the imaginal disc, but not in the follicle cell (Meignin et al., 2007; Polesello and Tapon, 2007; Reddy and Irvine, 2008). Similarly, the EGFR-Ras-ERK pathway is required to regulate Hippo signalling in glial cells, but not in wing discs (Reddy and Irvine, 2013). Therefore, it is conceivable that the p38 signalling pathway may have limited influence on Hippo signalling in the wing disc due to the presence of other regulatory inputs there. Consistent with this hypothesis, we find that *lic* mutant or *lic* RNAi reduces Hippo target expression and Hippo signalling-mediated wing overgrowth in a sensitized background where the dosage of *hpo*, *wts*, or *yki* is altered (Figure 2E–N, Supplementary Figure S2I–O).

In summary, our study suggests the p38 MAPK signalling pathway as a new upstream branch of Hippo signalling in Drosophila and in mammalian cells. We show that a MAPKKK-MAPKK-p38 MAPK kinase cascade regulates Yki/YAP activity and Hippo target expression via modulating the actin cytoskeleton and Jub phosphorylation. Thus our study reveals a conserved crosstalk between two important signalling pathways, the p38 MAPK pathway and the Hippo pathway, and expands our understanding of growth regulation.

Materials and methods

Fly strains

Flies were raised at 25°C under standard conditions unless otherwise indicated. The following stocks were used in this study*: GMR-Gal4*, *en-Gal4*, *hh-Gal4, SalE-Gal4, ptc-Gal4, en-Gal4 tub-Gal80^{ts}, ci-Gal4, ap-Gal4*, *hsflp;sp/cyo;act>CD2>Gal4, hsflp hsGFP FRT19A, hsflp;ubi-GFP FRT40A, hsflp tub-Gal80 FRT19A; Act-Gal4 UAS-GFP*, *UAS-Rac1^{V12}* (Bloomington 6291), *UAS-RhoGEF2* (Bloomington 9386), *UAS-dia IR* (Bloomington 28541), *UAS-jub IR* (Bloomington 32923), *UAS-MK2 IR* (Bloomington 41894), *UAS-lic IR-1* (VDRC 106822), *UAS-lic IR-2* (VDRC 20166), *UAS-lic IR-3* (Bloomington 31643), *UAS-bsk IR* (VDRC 34138), *UAS-yki IR* (VDRC 40497), *UAS-wts IR* (VDRC 106174), *UAS-hpo IR (VDRC 104169), UAS-p38b IR* (NIG 7793R-1), *UAS-Hsp27 IR* (NIG 4466R-2), *UAS-lic* (Fly ORF), *UAS-p38b* (Fly ORF). *UAS-yki*, *ex-lacZ*, *Diap1-lacZ*, and *ban-lacZ* were gifts from Prof. Lei Zhang. *UAS-ras^{V12}* and *UAS-bsk^{DN}* were gifts from Prof. Lei Xue. *UAS-wts* was a gift from Prof. Shian Wu. *p38b^{156A}* and *MK2^{5B}* were gifts from Prof. Jianming Chen. *UAS-Mekk1 and UAS-Lic KD* were generated in our lab. For experiments involving *tub-Gal80^{ts}*, larvae were raised at 18°C to restrict Gal4 activity for 7 days and shifted to 29°C for 2 days.

Immunohistochemistry and western blotting

The following antibodies were used: rabbit anti-HA (sc-805, 1:1000), goat anti-rabbit IgG-HRP (sc-2030, 1:3000), mouse anti-β-galactosidase (sc-65670, 1:800), and rabbit anti-p-p38 (sc-17852-R, 1:400) were from Santa Cruz. Mouse anti-Wg (4D4, 1:500), mouse anti-Cut (2B10, 1:50), and mouse anti-FasIII (7G10, 1:50) were from DSHB. Rabbit anti-Caspase3 (32183, 1:400), rabbit anti-p-YAP (4911, 1:1000), and rabbit anti-YAP (4912, 1:1000) were from Cell Signalling Technology. Phalloidin Alexa Fluor568 (A12380, 1:50), goat anti-mouse Alexa Fluor594 (A11012, 1:500), goat anti-rabbit Alexa Fluor594 (A11005, 1:500), and goat anti-guinea pig Alexa Fluor568 (A11075, 1:200) were from Invitrogen. Mouse anti-Tubulin (T6074, 1:3000) and mouse anti-YAP (WH0010413M, 1:1000) were from Sigma. Rabbit anti-Yki (1:500) was a gift from Prof. Kenneth D. Irvine. Rabbit anti-p-Yki (1:1000) was a gift from Prof. Duojia Pan. Mouse anti-Diap1 (1:50) was a gift from Prof. Lei Zhang. Rabbit anti-Lic (1:40000 for western blot and 1:2000 for immunostaining) was generated with GST-Lic by Abclone technology. To generate follicle cell clones, newly eclosed flies were heat-shocked at 37°C for 1 h in two continuous days, and were dissected 3–5 days later. To generate mutant clones in the wing disc, second-instar larvae were heat-shocked at 37°C for 30 min. Wing discs, ovaries, and MCF10A cells were fixed in phosphate buffered saline (PBS)-T containing 4% formaldehyde for 20 min at room temperature. They were then washed three times in PBS-T and blocked with PBS-T containing 5% bovine serum albumin. Primary antibodies were incubated at 4°C overnight and secondary antibodies were incubated at room temperature for 2 h (Yin et al., 2014).

Cell culture and transfection

S2 cells were cultured in Drosophila Schneider's Medium (Gibco) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. HEK293T cells were cultured in DMEM (Gibco) with 10% FBS. A549 cells were cultured in RMPI1640 (Gibco) with 15% FBS. MCF10A cells (a gift from Prof. Lixing Zhan) were cultured in DMEM/F12 (Invitrogen) supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 mg/ml hydrocortisone, 10 mg/ml insulin, 100 ng/ml cholera toxin, and 50 mg/ml penicillin/streptomycin. Plasmid transfection was performed using Effectene (QIAGEN) or Lipofectamine 3000 (Invitrogen), according to the manufacturer's instructions.

Luciferase reporter assay

GFP or *MKK3* expression construct was co-transfected with the *5XUAS-luc* reporter, *Gal4-TEAD4*, *HA-YAP1*, and *PRL-TK* constructs in HEK293T cells. Two days after transfection, cells were lysed and luciferase activity was measured with the Luciferase Assay System (Promega) following the manufacturer's instructions. All Firefly luciferase activities were normalized to Renilla luciferase activities.

Real-time PCR

Total RNA was extracted with TriZol (ambion). One microgram of total RNA was reverse-transcribed into complementary DNA with the Rever TraAce qPCR RT Kit (TOYOBO), and quantitative PCR was performed with SYBR Green Realtime PCR Master Mix (TOYOBO) and quantified by the StepOne Real-Time PCR System (Applied Biosystems). GAPDH was used as an internal control. The following primer sequences were used for real-time PCR. *GAPDH*: GGCATCCTGGGCTACACTGA and GAGTGGGTGTCGCTGTT GAA; *CTGF*: AAAAGTGCATCCGTACTCCCA and CCGTCGGTACATACT CCACAG; *Cyr61*: GGTCAAAGTTACCGGGCAGT and GGAGGCAT CGAATCCCAGC; *p38α*: TGAAATGACAGGCTACGTGG and CATCTAT AAGGAGGTCCCTGA; siRNA sequences for *p38α*: GGUCUCUGGAGG AAUUCAATT (sense), UUGAAUUCCUCCAGAGACCTT (antisense).

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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