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Modulating F-actin organization induces organ growth by affecting the Hippo pathway

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The Hippo tumour suppressor pathway is a conserved signalling pathway that controls organ size. The core of the Hpo pathway is a kinase cascade, which in *Drosophila* involves the Hpo and Warts kinases that negatively regulate the activity of the transcriptional coactivator Yorkie. Although several additional components of the Hippo pathway have been discovered, the inputs that regulate Hippo signalling are not fully understood. Here, we report that induction of extra F-actin formation, by loss of Capping proteins A or B, or caused by overexpression of an activated version of the formin Diaphanous, induced strong overgrowth in *Drosophila* imaginal discs through modulating the activity of the Hippo pathway. Importantly, loss of Capping proteins and Diaphanous overexpression did not significantly affect cell polarity and other signalling pathways, including Hedgehog and Decapentaplegic signalling. The interaction between F-actin and Hpo signalling is evolutionarily conserved, as the activity of the mammalian Yorkie-orthologue Yap is modulated by changes in F-actin. Thus, regulators of F-actin, and in particular Capping proteins, are essential for proper growth control by affecting Hippo signalling.

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Introduction

The Hippo (Hpo) tumour suppressor pathway has emerged as a key signalling pathway that controls tissue size in *Drosophila* and vertebrates (Pan, 2010; Zhao *et al*, 2010; Halder and Johnson, 2011). Hpo signalling inhibits growth by suppressing cell proliferation and by promoting apoptosis. Thus, fruit flies that lack Hpo pathway activity in imaginal discs, the precursors of adult structures, have severely overgrown discs and corresponding adult structures. The Hpo pathway therefore regulates tissue size during development. However, signals that control the activity of the Hpo pathway are poorly understood (Pan, 2010; Zhao *et al*, 2010; Halder and Johnson, 2011).

Several components of the Hpo pathway have been discovered and a signal transduction pathway from the plasma membrane into the nucleus has emerged (Pan, 2010; Zhao *et al*, 2010; Halder and Johnson, 2011). Central to the Hpo pathway is a kinase cascade involving the Hpo (Harvey *et al*, 2003; Jia *et al*, 2003; Pantalacci *et al*, 2003; Udan *et al*, 2003; Wu *et al*, 2003) and Warts (Wts) kinases (Justice *et al*, 1995; Xu *et al*, 1995) and their adaptor proteins Salvador (Sav) (Kango-Singh *et al*, 2002; Tapon *et al*, 2002) and Mob as tumour suppressor (Mats) (Lai *et al*, 2005). Active Hpo phosphorylates and activates Wts (Wu *et al*, 2003), which inhibits the activity of the transcriptional coactivator Yorkie (Yki) by phosphorylation, leading to 14-3-3 binding and cytoplasmic retention (Huang *et al*, 2005; Dong *et al*, 2007; Zhao *et al*, 2007; Oh and Irvine, 2008, 2009). When unphosphorylated, Yki translocates to the nucleus where it binds to transcription factors, such as Scalloped (Sd) or Homothorax, and induces the expression of target genes that drive cell proliferation and cell survival (Goulev *et al*, 2008; Wu *et al*, 2008; Zhang *et al*, 2008; Zhao *et al*, 2008; Peng *et al*, 2009). Thus when active, Hpo and Wts suppress cell proliferation by suppressing the activity of Yki.

Several components are known that act upstream of Hpo and Wts (Pan, 2010; Zhao *et al*, 2010; Halder and Johnson, 2011) such as the atypical Cadherin Fat (Bennett and Harvey, 2006; Cho *et al*, 2006; Silva *et al*, 2006; Willecke *et al*, 2006; Tyler and Baker, 2007), which transduces signals from Dachsous (Ds), an atypical cadherin related to Fat, and Four-jointed (Fj), a Golgi-resident kinase that phosphorylates Fat and Ds (Cho and Irvine, 2004; Ishikawa *et al*, 2008; Rogulja *et al*, 2008; Willecke *et al*, 2008), and Crumbs (Crb), a transmembrane protein regulating apical–basal cell polarity in epithelial cells (Bazellieres *et al*, 2009; Chen *et al*, 2010; Grzeschik *et al*, 2010; Ling *et al*, 2010; Robinson *et al*, 2010). Fat signal transduction involves the FERM-domain adaptor protein Expanded (Ex), the atypical myosin Dachs (D), and the kinase Discs overgrown (Dco), although through poorly understood processes (Cho and Irvine, 2004; Bennett and Harvey, 2006; Cho *et al*, 2006; Hamaratoglu *et al*, 2006; Mao *et al*, 2006; Silva *et al*, 2006; Willecke *et al*, 2006; Feng and Irvine, 2007, 2009; Tyler and Baker, 2007; Sopko *et al*, 2009), while Crb regulates the activity of the Hpo pathway by directly recruiting Ex to the plasma membrane (Chen *et al*, 2010; Ling *et al*, 2010; Robinson *et al*, 2010). Although Fat, Crb, and other proteins have been identified as critical upstream regulators of the Hpo pathway, the regulation of the pathway is not fully understood (Pan, 2010; Zhao *et al*, 2010;

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Halder and Johnson, 2011). Here, we show that loss of function of Capping proteins A and B (Cpa and Cpb), which leads to inappropriate F-actin polymerization, induces phenotypes resembling those caused by inactivation of the Hpo pathway, namely overgrowth, excess cell proliferation, and the induction of Hpo pathway target genes. These effects depend on normal Yki levels, indicating that actin dynamics regulate the activity of the Hpo pathway.

Results

Actin modulators affect Yorkie activity in S2 cells

In order to identify novel regulators of the Hpo pathway, we performed a genome-wide RNAi screen in S2 cells using a Yki-dependent luciferase assay (Huang *et al*, 2005). In this assay, Yki is fused to the DNA-binding domain of Gal4 (GDBD), which recruits Yki to the promoter of a *UAS-luciferase* reporter construct inducing luciferase expression. To activate the Hpo pathway, we coexpressed Ex, an upstream activator of the pathway that attenuates Yki activity in this assay (Hamaratoglu *et al*, 2006) (Figure 1A). As a secondary assay, we rescreened positive hits using a similar reporter assay that used a Scute–GDBD fusion protein instead of Yki-GDBD, which is not regulated by Hpo signalling but induces luciferase expression levels comparable to the Yki-GDBD construct (Supplementary Figure S1). A gene was considered a positive hit when the dsRNA increased the activity of the Yki- but not the Scute-dependent readout. In this screen, we identified the known Hpo pathway components Hpo, Ex, Wts, Kibra, Sav, and Mats as regulators of Yki activity (Supplementary Table S1). As potential novel regulators of Hpo signalling, we identified the actin regulators Cpa and Cpb, Capulet (Capt), Twinstar (Tsr), Actin5C (Act5C), and Actin87E (Act87E) (Figure 1A). To exclude possible effects of actin on the expression of the Yki-GBDB or the Renilla luciferase transfection control plasmids, which use an actin responsive actin promoter, we validated these hits using another assay that expressed Yki and Renilla luciferase under the control of a metallothionein gene promoter and measured the activity of Yki by measuring the amount of luciferase produced by a construct that drives firefly luciferase by multimerized Sd-binding sites (3xSd2-Luc) (Zhang *et al*, 2008). In this assay, dsRNAs targeting *cpa*, *cpb*, *capt*, and *tsr* tested positive as modifiers of Yki activity (Figure 1B). Notably, these four genes have in common that mutations in them lead to extra F-actin accumulation (Gunsalus *et al*, 1995; Benlali *et al*, 2000; Delalle *et al*, 2005; Janody and Treisman, 2006). In addition to these four genes, we identified Wasp and Arc-p20, which stimulate F-actin formation and for which the dsRNA inhibited Yki activity (Supplementary Table S1). To confirm that lowering F-actin levels reduce Yki activity, we treated S2 cells with the F-actin destabilizing drug, cytochalasin D. Using a construct expressing Yki and Renilla luciferase under control of the methallothionein promoter and using the 3xSd2-Luc reporter as a readout for Yki activity, we found that treatment of cells with cytochalasin D lead to the inhibition of Yki activity, but did not affect Renilla luciferase expression (Figure 1C and D). Together, these data show that changing F-actin levels modulates the activity of Yki in cultured *Drosophila* cells. We next wanted to test whether F-actin organization also affects the Hpo pathway *in vivo*.

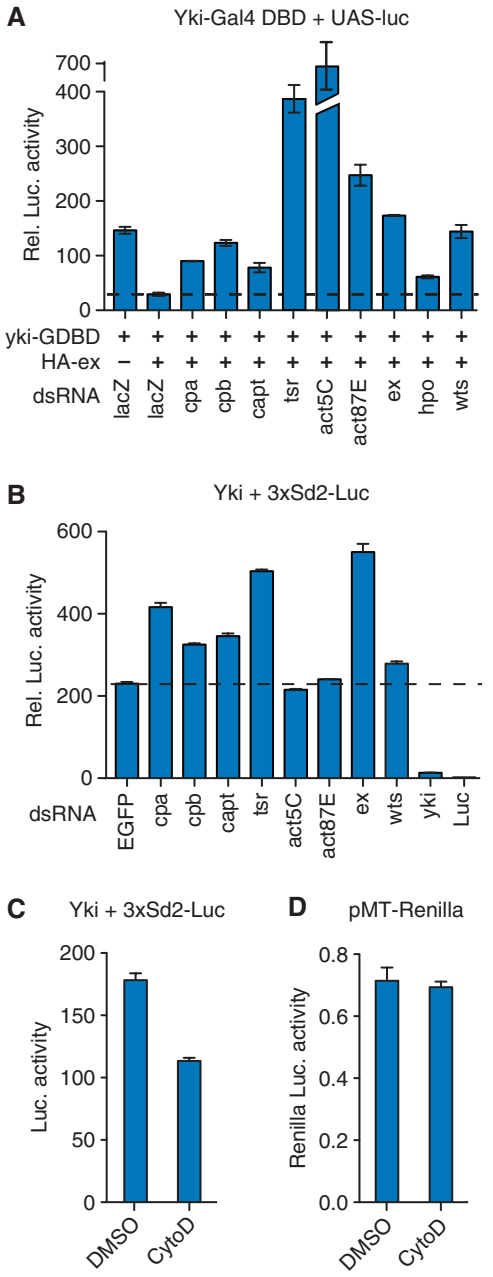

Figure 1 Actin modulators regulate Yorkie activity *in vitro*. Luciferase activity assays normalized with a Renilla luciferase control for transfection efficiency. (**A**) *Drosophila* S2 cells were transiently transfected with plasmids expressing *UAS-Luciferase* and *Yki-GDBD* alone or cotransfected with *HA-Ex* constructs in the presence of the dsRNAs as indicated. (**B**) *Drosophila* S2 cells were transiently transfected with the Yki-reporter construct *3xSd2-Luciferase* and *pMT-Yki* in the presence of the dsRNAs as indicated. (**C**) Luciferase activity produced by *pMT-Yki* driving the *3xSd2-Luciferase* reporter construct in vehicle (DMSO) and cytochalasin D (CytoD) treated S2 cells. (**D**) Renilla Luciferase activity produced by the transfection control plasmid is the same in vehicle (DMSO) and cytochalasin D (CytoD) treated S2 cells. The error bars represent 'standard error of the mean' (s.e.m.).

Extra F-actin polymerization causes tissue overgrowth in vivo

To analyse potential effects of F-actin on Hpo signalling *in vivo*, we expressed transgenic double-stranded RNAs to knockdown actin modulators during wing disc development. In addition,

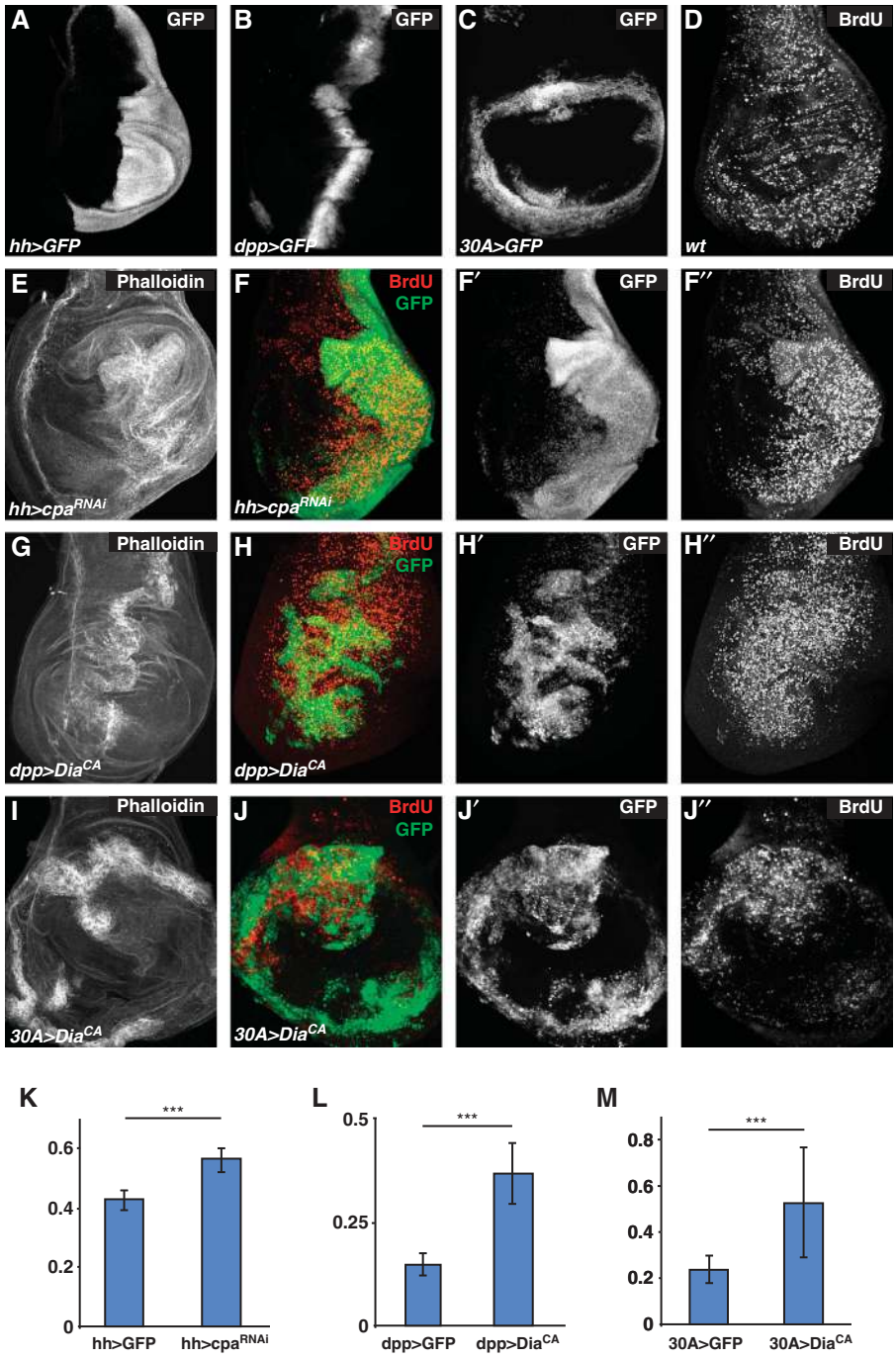

Figure 2 Induction of F-actin polymerization induces overgrowth of imaginal discs. (**A**–**J**) Confocal images of third instar wing imaginal discs showing (**A**) *UAS-GFP* expression driven by *hh-Gal4*, (**B**) *UAS-GFP* expression driven by *dpp-Gal4*, and (**C**) *UAS-GFP* expression driven by *30A-Gal4*. (**D**) BrdU incorporation in a wild-type disc. (**E**) Phalloidin staining of *hh-Gal4*, *UAS-GFP*, *UAS-cpa*^{RNAi}. (**F**) BrdU (red, grey in **F**″) and GFP (green, grey in **F**′) staining of *hh-Gal4*, *UAS-GFP*, *UAS-cpa*^{RNAi}. (**G**) Phalloidin staining of *dpp-Gal4*, *UAS-GFP*, *UAS-dia*^{CA}. (**H**) BrdU (red, grey in **H**″) and GFP (green, grey in **H**′) staining of *dpp-Gal4*, *UAS-GFP*, *UAS-dia*^{CA}. (**I**) Phalloidin staining of *30A-Gal4*, *UAS-GFP*, *UAS-dia*^{CA}. (**J**) BrdU (red, grey in **J**″) and GFP (green, grey in **J**′) staining of *30A-Gal4*, *UAS-GFP*, *UAS-dia*^{CA}. (**K**–**M**) Quantification of the size of the GFP expression domains normalized to the size of the entire imaginal disc of the indicated genotypes. *** indicates that the two populations are different with $P<0.001$. Note that Gal4 in the *dpp-* and *30A-Gal4* driver lines is not stably expressed in cell lineages but is lost when progenitor cells move away from the domain where Gal4 expression was induced. Therefore, non-GFP expressing cells may show upregulated BrdU due to earlier Gal4 expression driving Dia^{CA}. Thus, seemingly non-autonomous effects may still be due to cell autonomous action of Dia^{CA}. Similar effects may apply to analysis of the expression of *ex-lacZ* in Figure 3.

we artificially induced F-actin formation by overexpressing an activated version of the actin nucleation factor Diaphanous (Dia^{CA}), which triggers extra F-actin polymerization (Somogyi and Rorth, 2004; Mulinari *et al*, 2008). We used three different Gal4 drivers: *hh-Gal4*, which expresses in the posterior compartment of wing discs, *dpp-Gal4*, which expresses along the anterior–posterior compartment boundary, and *30A-Gal4*, which drives expression in the presumptive hinge region (Figure 2A–C).

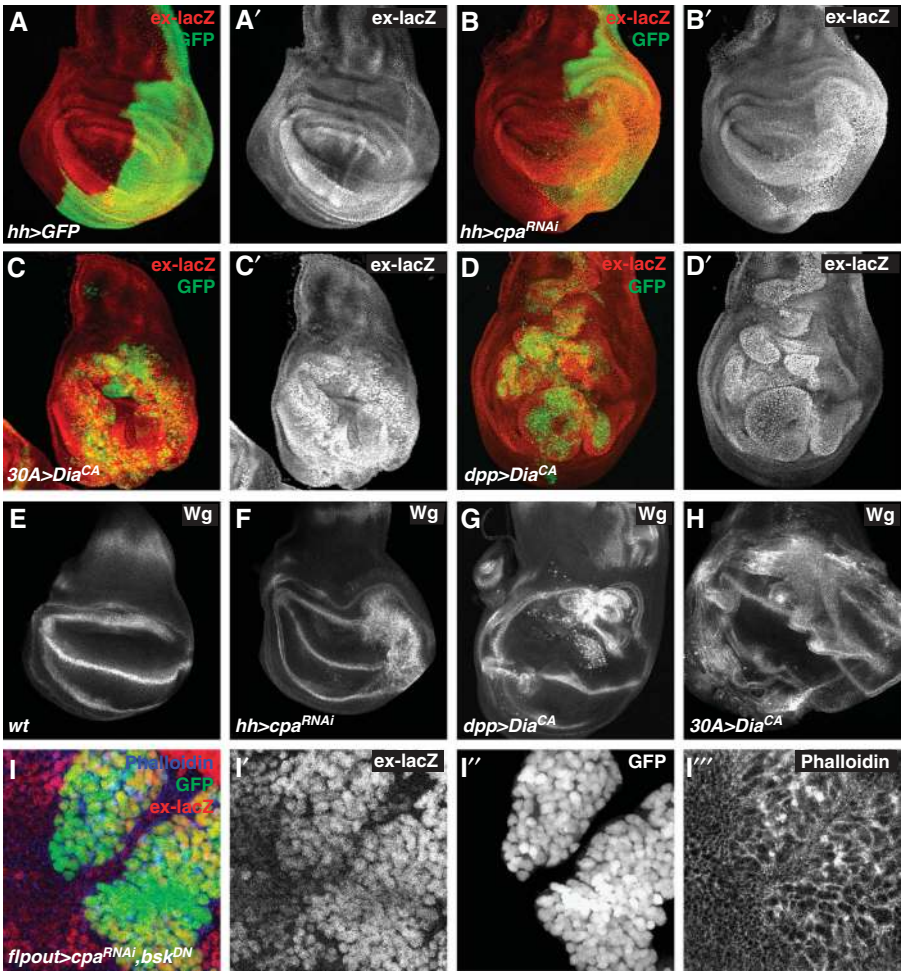

Figure 3 Actin modulators regulate Hippo pathway target genes. Confocal images of third instar wing imaginal discs. (**A**–**D**) GFP (green) and *ex-lacZ* (red, grey in **A**′–**D**′) staining of (**A**) *hh-Gal4*, *UAS-GFP*, (**B**) *hh-Gal4*, *UAS-GFP*, *UAS-cpa*^{RNAi}, (**C**) *30A-Gal4*, *UAS-GFP*, *UAS-dia*^{CA}, and (**D**) *dpp-Gal4*, *UAS-GFP*, *UAS-dia*^{CA}. (**E**–**H**) Wingless (Wg) antibody staining of (**E**) wild type, (**F**) *hh-Gal4*, *UAS-GFP*, *UAS-cpa*^{RNAi}, (**G**) *dpp-Gal4*, *UAS-GFP*, *UAS-dia*^{CA}, and (**H**) *30A-Gal4*, *UAS-GFP*, *UAS-dia*^{CA}. (**I**) Close-up image of a clone of cells in the wing pouch region of a wing disc expressing *UAS-cpa*^{RNAi}, *UAS-bsk*^{DN}, and *UAS-GFP* driven by *Flip-out-Gal4* stained for *ex-lacZ* (red, grey in **I**′) and Phalloidin (blue, grey in **I**‴) and showing GFP expression (green, grey in **I**″).

Knockdown of *cpa* and *cpb in vivo* resulted in overgrowth phenotypes in wing discs (Figure 2E and F; Supplementary Figure S2), which was especially evident in the presumptive hinge region (Supplementary Figure S3). Similarly, overexpression of Dia^{CA} lead to overgrowth (Figure 2G–J), which was even stronger than that caused by Cpa or Cpb knockdown. These overgrowths were characterized by increased F-actin accumulation as expected and extra folding of the imaginal discs (Figure 2E, G and I). We quantified the amount of overgrowth by measuring the relative size of the Gal4 expression domains and found that the knockdown of *cpa* or overexpression of Dia^{CA} induced overgrowth ranging from 31% (*hh-Gal4*, *UAS-cpa*^{RNAi}) to over 140% (*dpp-Gal4*, *UAS-Dia*^{CA}) (Figure 2K–M). This overgrowth is characterized by an increase in proliferation as knockdown of *cpa* or overexpression of Dia^{CA} elevated the levels of BrdU incorporation in the domain in which these transgenes were overexpressed (Figure 2D, F″, H″ and J″). We conclude that the induction of ectopic F-actin formation by loss of Capping proteins or by activated Dia causes extra cell proliferation and overgrowth in imaginal discs. Thus, Cpa and Cpb act as tumour suppressors during wing disc development.

Changes in actin organization affect Hippo pathway target gene expression

The observed overgrowth and proliferation phenotypes prompted the question of whether these phenotypes were caused by the deregulation of the Hpo pathway, as the overgrowth phenotypes resembled those caused by loss of Hpo signalling. We thus analysed the expression of two Yki reporters, namely *ex-lacZ*, a lacZ enhancer trap insertion in the *ex* locus (Boedigheimer and Laughon, 1993) and *diap1-GFP*, a *diap1* reporter transgene (Zhang *et al*, 2008). We found that both Yki reporters were upregulated upon the expression of *cpa*^{RNAi}, *cpb*^{RNAi}, or Dia^{CA} (Figure 3A–D; Supplementary Figures S2 and S4). Importantly, we found that *ex-lacZ*, which is regulated by the Hpo pathway in various imaginal discs (Hamaratoglu *et al*, 2006), was upregulated in wing and leg discs in response to Dia^{CA} overexpression (Figure 3C and D; Supplementary Figure S4). In addition, we analysed Wingless (Wg) expression, which is regulated by Hpo signalling in the presumptive wing hinge region (Cho and Irvine, 2004). Expression of both *cpa*^{RNAi} and Dia^{CA} caused ectopic Wg expression in the presumptive hinge (Figure 3E–H). These results show that loss of Capping proteins, or induction

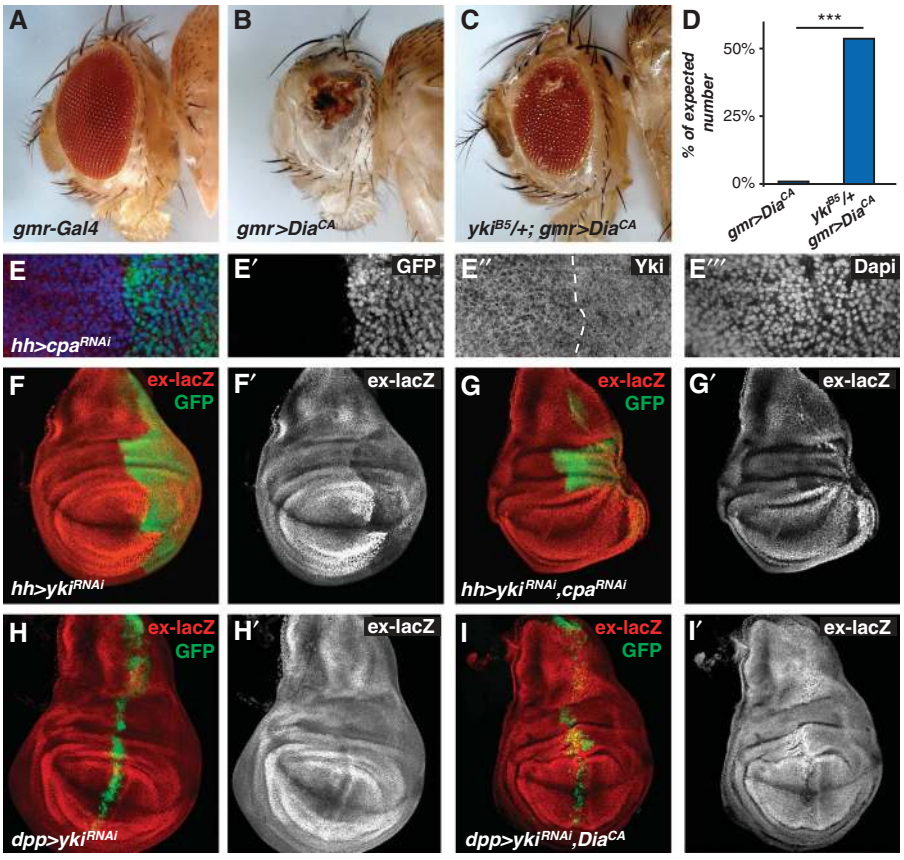

Figure 4 Yorkie is required for actin dynamics induced overgrowth and upregulation of Hippo target genes. (**A**–**C**) Adult flies of the indicated genotypes. (**D**) Quantification of the number of enclosed adult flies of the genotypes in (**B**, **C**) relative to (**A**). (**E**) Confocal image of a third instar wing imaginal disc stained for GFP (green, grey in **E**′), Yki (red, grey in **E**″), and Dapi (blue, grey in **E**‴) of *hh-Gal4*, *UAS-GFP*, *UAS-cpa*^{RNAi}. (**F**, **I**) Third instar wing imaginal discs stained for GFP (green) and *ex-lacZ* (red, grey in **F**′–**I**′) of (**F**) *hh-Gal4*, *UAS-GFP*, *UAS-yki*^{RNAi}, (**G**) *hh-Gal4*, *UAS-GFP*, *UAS-yki*^{RNAi}, *UAS-cpa*^{RNAi}, (**H**) *dpp-Gal4*, *UAS-GFP*, *UAS-yki*^{RNAi}, and (**I**) *dpp-Gal4*, *UAS-GFP*, *UAS-yki*^{RNAi}, *UAS-dia*^{CA}.

of F-actin polymerization by Dia^{CA}, leads to strong upregulation of different Yki downstream genes, thus mimicking Wts loss of function (Supplementary Figure S4). This suggests that Capping proteins restrict growth by affecting the Hpo pathway.

To test whether the upregulation of Yki target genes was a cell autonomous response to elevated F-actin levels, we analysed *ex-lacZ* expression in clones of cells that had a knockdown of Cpa and coexpressed a dominant negative version of the Basket JNK *(bsk*^{DN}*)* to prevent JNK-mediated apoptosis, known to be induced in *cpa* mutant cells (Janody and Treisman, 2006). We found that *ex-lacZ* was upregulated cell autonomously in such mutant clones, including cells abutting the clone border, but not in cells outside the clones (Figure 3I). Control clones expressing *bsk*^{DN} alone did not effect *ex-lacZ* expression (Supplementary Figure S5). These results indicate that extra F-actin activates Yki cell autonomously. Notably, coexpression of *bsk*^{DN} with *cpa*^{RNAi} either in Gal4 expressing clones (Supplementary Figure S5), or in the entire posterior compartment driven by hh-Gal4, caused upregulation of *ex-lacZ* expression (Supplementary Figure S5), indicating that the upregulation of Yki activity does not depend on JNK activity, although the overgrowth caused by *cpa*^{RNAi} was partially suppressed by *bsk*^{DN}. Therefore, extra F-actin may regulate Yki activity in JNK-dependent, in addition to JNK-independent mechanisms.

F-actin-induced overgrowth and upregulation of Hippo target genes requires Yorkie

To investigate the interaction between actin organization and Hpo signalling, we first tested for dominant genetic interaction between overexpression of Dia^{CA} and reduction of *yki*. In the developing eye, overexpression of Dia^{CA} with *gmr-Gal4* caused high mortality at the pupal stage (1% survival of expected frequency; Figure 4D), and the few survivors had severely compromised, amorphous eye structures (Figure 4A and B). Removal of one copy of *yki* in this background significantly rescued the eye phenotype and mortality (54% of expected ratio; Figure 4C and D). These data indicate that Dia^{CA}-induced phenotypes are sensitive to Yki levels.

Next, we tested whether F-actin affects Yki localization. We found that knockdown of *cpa* caused redistribution of Yki from a largely cytoplasmic localization to a more uniform distribution that included the nucleus (Figure 4E), similar to the phenotypes caused by *wts* and *hpo* mutations (Dong *et al*, 2007; Oh and Irvine, 2008). We then tested whether the upregulation of Hpo pathway reporters in response to increased F-actin accumulation requires Yki. As reported previously, expression of *yki*^{RNAi} leads to a decrease in *ex-lacZ* expression and reduced growth (Figure 4F and H) (Zhang *et al*, 2008). In contrast, expression of *cpa*^{RNAi} or Dia^{CA} leads to a strong increase in *ex-lacZ* and increased growth (Figure 3B–D). However, coexpression of *yki*^{RNAi} with *cpa*^{RNAi} or

Dia^{CA} suppressed the upregulation of *ex-lacZ* and overgrowth caused by *cpa*^{RNAi} or Dia^{CA} expression (Figure 4G and I), while coexpression of an irrelevant *w*^{RNAi} or adding a second UAS construct (*UAS-GFP*) had no effect (Supplementary Figure S6). In fact, the observed phenotypes were similar to those caused by expression of *yki*^{RNAi} alone, indicating that the effects of *cpa*^{RNAi} or Dia^{CA} expression depend on normal Yki levels. Similarly, knockdown of Yki reduced the upregulation of Wg in the hinge region caused by expression of *cpa*^{RNAi} or Dia^{CA} (Supplementary Figure S6). Altogether, these experiments show that Yki is required for the overgrowth and the induction of Hpo pathway target genes in response to conditions that stimulate F-actin formation.

Modulation of Hippo signalling is a specific downstream effect of changes in actin organization

The observation that increased F-actin polymerization caused overgrowth and deregulation of Hpo signalling is striking but raised the question of the specificity of this effect. Actin is required for many processes, for example for the localization of adherence junction components, an important step in the maintenance of epithelial cell polarity (Li and Gundersen, 2008). Loss of epithelial cell polarity can deregulate the Hpo pathway (Grzeschik *et al*, 2010; Menendez *et al*, 2010; Sun and Irvine, 2011) and cause excess proliferation (Dow and Humbert, 2007). We therefore analysed cell polarity upon the induction of ectopic F-actin formation. We investigated the localization of different markers for the apical (Crb and Patj) and baso-lateral (Dlg) membranes and adherens junction (Armadillo and E-cadherin) (Dow and Humbert, 2007). We did not find significant changes in their subcellular localization in the domain that had knockdown of Cpa (Supplementary Figure S7). Overexpression of Dia^{CA} caused more excessive overgrowth and severe folding of the affected region, such as the presumptive hinge region in the 30A-Gal4 crosses. While some cells overexpressing Dia^{CA} were extruded from the epithelium and showed piknotic nuclei, many remained in the epithelium and showed relatively normal localization of the polarity markers E-cadherin, Patj, Crb, and Dlg (Supplementary Figure S8). Therefore, the effects of actin dynamics on growth are unlikely due solely to defects in cell polarity. We next investigated whether increased F-actin deregulates other pathways involved in growth of imaginal discs. We assayed readouts for Hedgehog (Hh) and Decapentaplegic (Dpp) signalling, two major signalling pathways that operate during wing development (Neto-Silva *et al*, 2009). We assayed Cubitus interuptus whose levels indicate the activity of Hh signalling (Chen *et al*, 1999), and the phosphorylation status of Mad, which correlates with the activity of Dpp signalling (Teleman and Cohen, 2000). We found that patterns and levels of these two readouts were not significantly affected by *cpa*^{RNAi} or Dia^{CA} expression (Supplementary Figures S7 and S8). Altogether, we conclude that extra F-actin formation does not cause general defects in cell signalling and thus that the effects on Hpo signalling are a specific downstream effect of the loss of Capping proteins and Dia^{CA} expression.

Overexpression of Warts suppresses diaphanous-induced phenotypes

To gain insight into how F-actin affects Hpo signalling, we first tested whether changes in actin organization affect the localization of Hpo pathway components. We tested the localization of Ex, Mer, and Hpo, none of which was significantly affected upon downregulation of Cpa, although Ex protein levels were upregulated (Supplementary Figure S9), mirroring the increase in *ex-lacZ* expression. We then set out to test at what level in the pathway Dia^{CA} affects Hpo signalling. To do this, we used constructs overexpressing Ex, Hpo, and Wts which are able to activate Hpo signalling (Udan *et al*, 2003; Hamaratoglu *et al*, 2006), resulting in the downregulation of *ex-lacZ* (Figure 5A, C and E). Wts overexpression significantly suppressed the Dia^{CA}-induced overgrowth (Figure 5E–G) and the induction of Hpo pathway target genes *ex-lacZ* (Figure 5E and F) and Wg (Supplementary Figure S10). Notably, Ex and Hpo overexpression had only limited effects on the Dia^{CA}-induced phenotypes (Figure 5A–D and G; Supplementary Figure S10), although in wild type, overexpression of Ex and Hpo have stronger effects on growth than Wts overexpression (Supplementary Figure S10). Neither Wts, Ex, nor Hpo overexpression significantly suppressed the accumulation of F-actin caused by Dia^{CA} overexpression (Supplementary Figure S11). These data suggest that F-actin affects the Hpo pathway upstream of Wts but in parallel to Ex and Hpo, although other possibilities cannot be excluded.

Changes in actin organization affect Yap activity in mammalian cells

The factors that regulate actin dynamics and the components that make up the Hpo pathway are highly conserved in vertebrates (Pan, 2010; Zhao *et al*, 2010; Halder and Johnson, 2011). We therefore tested whether the interaction between actin dynamics and Hpo signalling is also present in mammalian cells. First, we plated HeLa cells at high density and transfected them with a construct that expressed an activated mDia (mDia^{CA}) protein to induce extra F-actin polymerization (Watanabe *et al*, 1999; Copeland and Treisman, 2002) and quantified nuclear localization of Yap, a mammalian orthologue of Yki. The expression of mDia^{CA} lead to a significant increase in the number of cells exhibiting nuclear Yap (46% increase; Figure 6A, B and E), indicating that Hpo signalling was compromised in these cells. Next, we tested whether the disruption of F-actin in HeLa cells by cytochalasin D (Prentki *et al*, 1979) causes the inverse effect, namely a reduction of nuclear Yap localization. Indeed, 1 h after cytochalasin D treatment, the number of cells with nuclear Yap localization was decreased 2.1-fold (Figure 6C, D and F). Since the localization of Yap affects its activity, we next assayed the transcriptional activity of Yap. Yap induces expression of target genes by binding to TEAD transcription factors (Vassilev *et al*, 2001; Ota and Sasaki, 2008; Zhao *et al*, 2008). Yap activity can thus be measured by comparing the activity of a TEAD-responsive reporter that contains TEAD-binding sites (8xGTIIc-d51) with a non-responsive reporter (d51) (Ota and Sasaki, 2008). Under control conditions, the TEAD-responsive reporter had a two-fold higher expression than the non-responsive reporter (Figure 6G). When F-actin polymerization was increased by overexpression of mDia^{CA}, the activity of the TEAD-responsive promoter was nine-fold higher compared with the non-responsive promoter. Thus, increase of F-actin formation increases Yap activity. Next, we wanted to test whether reduction of F-actin reduces Yap activity. Indeed, the higher expression level of the TEAD-responsive promoter under control conditions was abolished

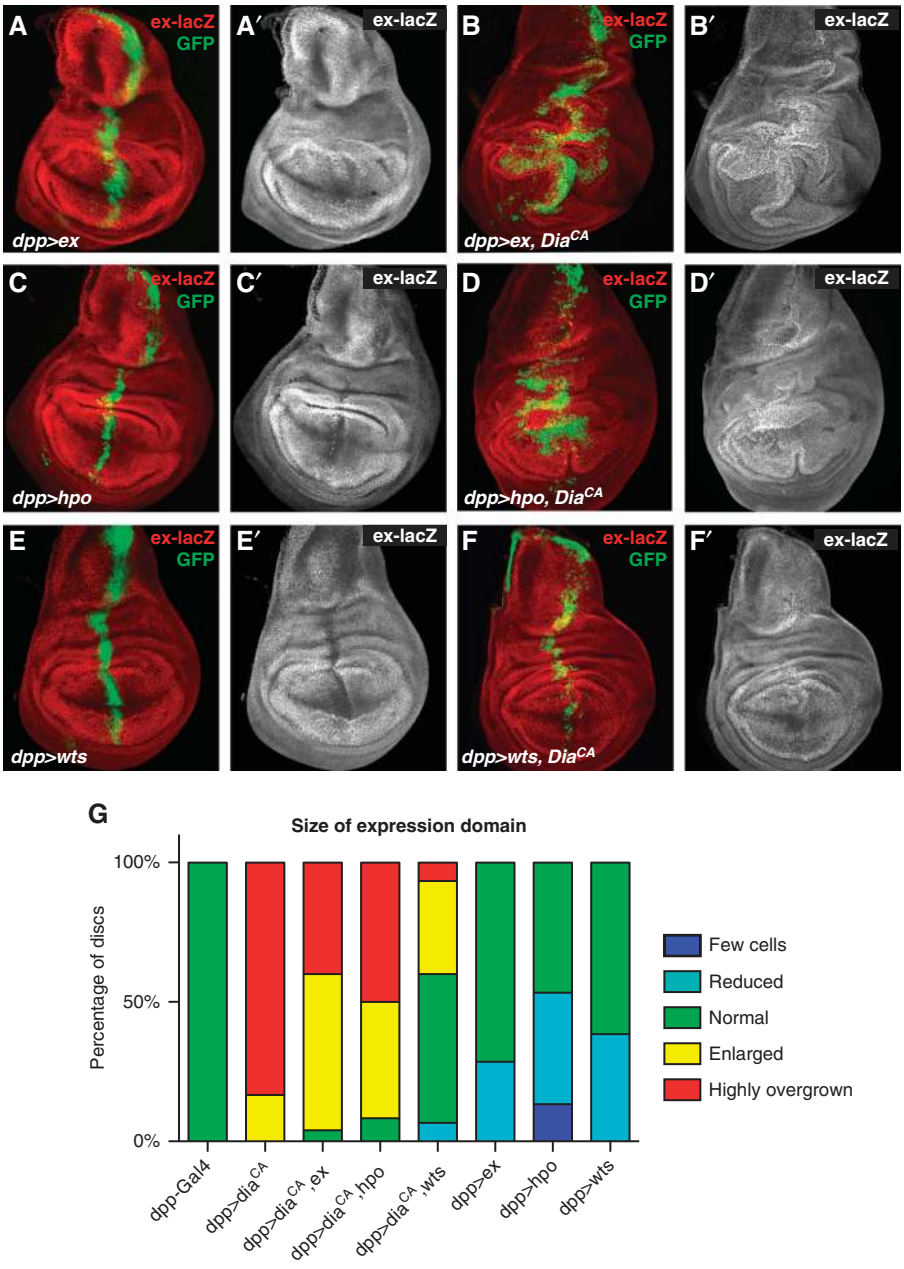

Figure 5 Overexpression of Warts suppresses Diaphanous-induced phenotypes. Confocal images of *ex-lacZ* (red and grey **A′**–**F′**) and GFP (green) stainings of third instar wing imaginal discs of (**A**) *dpp-Gal4*, *UAS-GFP*, *UAS-ex*, (**B**) *dpp-Gal4*, *UAS-GFP*, *UAS-ex*, *UAS-dia*^{CA}, (**C**) *dpp-Gal4*, *UAS-GFP*, *UAS-hpo*, (**D**) *dpp-Gal4*, *UAS-GFP*, *UAS-hpo*, *UAS-dia*^{CA}, (**E**) *dpp-Gal4*, *UAS-GFP*, *UAS-wts*, and (**F**) *dpp-Gal4*, *UAS-GFP*, *UAS-wts*, *UAS-dia*^{CA}. (**G**) Semi-quantification of the sizes of the expression domains of discs with the genotypes shown in this figure.

upon the disruption of F-actin by cytochalasin D (Figure 6H). These results show that disruption of F-actin decreases Yap activity. We conclude that also in mammalian cells, changes in actin organization affect Hpo signalling activity.

Discussion

In this study, we investigated a role of actin Capping proteins and changes in actin organization on tissue growth. We found that changing the organization of the actin cytoskeleton affects growth by modulating the activity of the Hpo pathway. Several observations support this conclusion. First, loss of Capping proteins, or induction of extra F-actin by overexpression of Dia^{CA}, induced strong overgrowth of *Drosophila* imaginal discs. Second, changes in actin organization lead to the upregulation of Hpo pathway target genes, which depended on normal Yki activity. Third, the effects of Dia^{CA} or loss of Capping proteins on Hpo signalling are specific downstream effects and not the cause of general defects in cellular organization and signalling. Fourth, actin dynamics and the Hpo pathway interact with each other in evolutionary distant species. Therefore, F-actin regulates growth in different species through effects on the Hpo pathway.

Several observations were striking. First, our data suggest that the effects on Hpo signalling are specific effects of F-actin accumulation. Given the crucial role for F-actin in numerous cellular processes (Jacinto and Baum, 2003), it might have been expected that imbalances in F-actin organization lead to

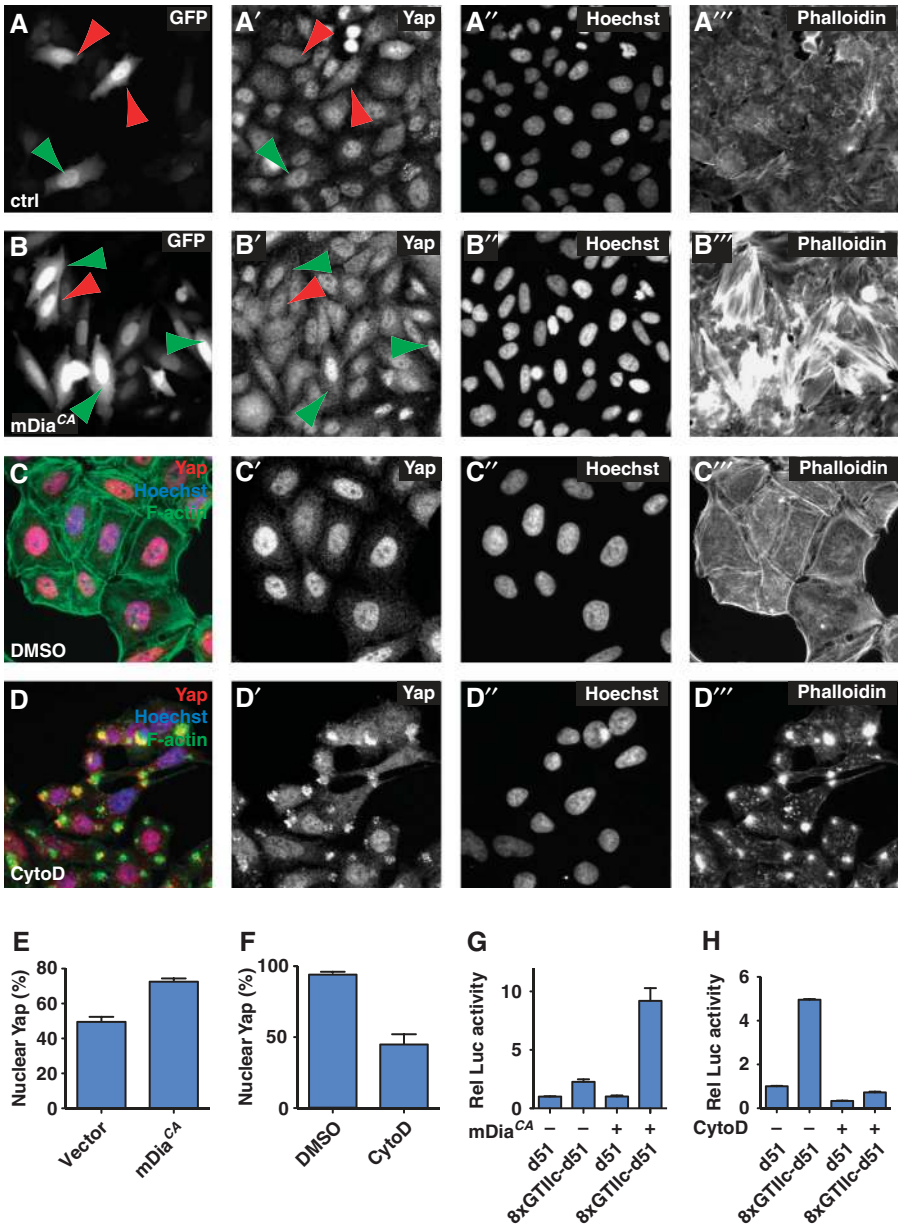

Figure 6 Actin dynamics regulate Hippo signalling in mammalian HeLa cells. (**A**–**D**) *In vitro* culture of HeLa cells. (**A**) GFP construct transfection as a control and staining of (**A**) GFP, (**A′**) Yap, (**A″**) Hoechst, and (**A‴**) Phalloidin. Red arrowheads indicate dispersed Yap staining, green arrowheads indicate nuclear Yap staining. (**B**) Cotransfection of GFP and *mDia*^{CA} and staining for (**B**) GFP, (**B′**) Yap, (**B″**) Hoechst, and (**B‴**) Phalloidin. (**C**) Cells treated with vehicle (DMSO) and stained for (**C′**) Yap, (**C″**) Hoechst, and (**C‴**) Phalloidin. (**D**) Cells treated with cytochalasin D (CytoD) and stained for (**D′**) Yap, (**D″**) Hoechst, and (**D‴**) Phalloidin. (**E**) Quantification of nuclear localization of Yap upon transfection with a plasmid expressing *mDia*^{CA}. (**F**) Quantification of nuclear localization of Yap upon CytoD treatment. (**G**) Quantification of Luciferase expression from a *TEAD-response luciferase* construct (8xGTIIc) in the presence and absence of *mDia*^{CA} expression. (**H**) Quantification of Luciferase expression from a *TEAD-response luciferase* construct (8xGTIIc) with vehicle or CytoD treatment. The error bars in all graphs represent 'standard error of the mean' (s.e.m.).

defects in many different signalling pathways. Surprisingly, however, while changing F-actin organization had strong effects on Hpo signalling, it did not significantly affect epithelial cell polarity, or Hh and Dpp signalling, indicating a specific molecular effect. Second, given the pleiotropic functions of F-actin, it might have been expected that knockdown of Capping proteins would lead to reduced growth. On the contrary, loss of Capping proteins or higher levels of F-actin induced by Dia^{CA} lead to increased proliferation and overgrowth, although mutant regions showed some dying cells (data not shown). We therefore conclude that Capping proteins act as tumour suppressors that affect growth through the Hpo pathway.

Our observations that both loss of Cpa and Cpb, as well as overexpression of activated Dia-induced overgrowth indicate that their effects on growth are due to F-actin accumulation. We currently do not know whether the observed effects involve a specific pool of F-actin or whether any increase in F-actin induces growth. Our screen in S2 cells identified several other genes involved in F-actin formation that modulated Yki activity. It remains to be seen whether these also modulate Hpo signalling *in vivo*.

The effect of modulating F-actin organization on the Hpo pathway may be evolutionary conserved as we see strong effects on Yap localization and activity in mammalian cells. Therefore, proteins that restrict F-actin formation may be tumour suppressors in humans and associated with cancer. Indeed, one example of an inhibitor of F-actin polymerization that is downregulated in several cancers is Gelsolin. Gelsolin is known to sever F-actin filaments and to cap them, which inhibits F-actin polymerization (Kwiatkowski, 1999). Thus, modulators of the F-actin cytoskeleton affect cell proliferation in mammals and may be involved in the development of cancer.

To gain insight into the mechanism by which F-actin affects the Hpo pathway, we analysed the localization of different Hpo pathway components. Mer and Ex, which contain FERM (4.1 protein–ezrin–radixin–moesin) domains and are known to bind F-actin (Bretscher *et al*, 2002), localized normally in cells that lost Cpa function, and similarly Hpo localization was unaffected. However, Yki localization was affected such that more Yki protein localized to the nuclei in cells that lost Capping protein function. Therefore, the F-actin status affects growth upstream of Yki, but might not affect growth by regulating the localization of upstream components in the Hpo pathway. Our *in vivo* data show that overexpression of Ex or Hpo did not significantly rescue Dia^{CA}-induced phenotypes in contrast to their ability to rescue *fat* and *ex;mer* mutant phenotypes (Hamaratoglu *et al*, 2006; Willecke *et al*, 2006). Overexpression of Wts, however, significantly suppressed Dia^{CA}-induced overgrowth and Hpo pathway target gene expression. Interestingly, *ex* mutant cells have increased levels of F-actin, although not as much as cells depleted for Capping proteins (Supplementary Figure S12). Thus, Ex could regulate Hpo signalling indirectly through its effect on F-actin. However, two observations argue against this possibility. First, overexpression of Hpo can rescue *ex* mutant phenotypes (Hamaratoglu *et al*, 2006), but not those caused by Dia^{CA}. Second, Ex and Mer directly interact with the Hpo cofactor Sav (Yu *et al*, 2010). Altogether, these data suggest that F-actin affects growth in parallel to Ex and Hpo but upstream of Yki.

Recent work showed that a small fraction of the mammalian homologues of Hpo, MST1, and MST2, localize to apical actin filaments (Densham *et al*, 2009). Upon disruption of the actin filaments, MST1/2 were activated, although it is not known whether this involves a relocalization of MST1/2. Consistent with MST activation, we found that under similar conditions in which we sever F-actin bundles, Yap is exported from the nucleus and its activity is downregulated. It is not known whether the same or different mechanisms are engaged to regulate Hpo signalling in response to severing or inducing actin filaments, but elucidation of the molecular mechanisms involved will answer this question.

Our data reveal an interaction between F-actin organization and the Hpo pathway in the regulation of growth. A possible connection between F-actin and growth may involve the sensing of mechanical forces. *In vitro*, cells change their rate of proliferation in response to external mechanical forces, which requires an intact actin cytoskeleton (Klein *et al*, 2007; Assoian and Klein, 2008). *In vivo*, the actin cytoskeleton might act as a sensor to couple mechanical forces to growth control (Wang and Riechmann, 2007). While it is not clear whether these effects depend on the Hpo pathway, it is an exciting possibility to be tested in the future.

Materials and methods

Fly stocks

A detailed description of the genotypes used is presented in Supplementary data. The UAS-Gal4 system (Brand and Perrimon, 1993) was used for overexpression using the following stocks: *hh-Gal4*, *dpp-Gal4*, *30A-Gal4*, *en-Gal4*, *ap-Gal4*, *gmr-Gal4*, *UAS-yki* (Huang *et al*, 2005), *UAS-*Dia^{CA} (Somogyi and Rorth, 2004), *UAS-*cpa^{RNAi} (VDRC: TID 100773), *UAS-*cpb^{RNAi} (VDRC: TID 45668), *UAS-GFP*, *UAS-hpo* (Udan *et al*, 2003), *UAS-*ex^{e1} (Boedigheimer *et al*, 1997), *UAS-wts* (Udan *et al*, 2003). Other stocks used were yki^{B5} (Huang *et al*, 2005), ex^{697} (Boedigheimer and Laughon, 1993), and *diap1-GFP* (Zhang *et al*, 2008).

Quantification of mutant areas

The quantification of the overgrowth phenotypes were performed using ImageJ, marking the GFP-positive region or the entire disc with the 'threshold' function and measuring the surface with the 'analyse particle' function. The ratio of GFP-positive surface over total disc surface was calculated.

S2 cell experiments

Drosophila S2 cells, cultured in Schneider's medium containing 10% fetal bovine serum and antibiotics, were transiently transfected using Cellfectin (Invitrogen) according to the manufacturer's protocol. For the genome-wide RNAi screens, we followed the protocols of the DRCS with minor modifications (http://www.flyrnai.org/DRSC-PRR.html). Briefly, cells were seeded into 384-well plates containing the dsRNA library (Ambion) and then transfected with the *HA-Ex*, *Yki-GDBD*, *Renilla luciferase* (all in the pAc5.1 vector), and *UAS-luciferase* (from K Basler) constructs. Plates were assayed for luciferase activity 4 days after transfection using the Promega Dual-Glo kit and were shaken not stirred. For the experiments with Yki in an inducible expression vector, Yki was inserted into the pMT vector (Invitrogen) between the *Eco*RI and *Xba*I sites. For the Renilla luciferase transfection control plasmid, we PCR amplified the Renilla open reading frame from a *tub-Renilla* construct (from K Basler) and inserted it as an *Eco*RI–*Xba*I fragment into the *Eco*RI and *Xba*I sites in the pMT vector. For this experiment, we used a multimerized Sd DNA-binding sites luciferase reporter (Zhang *et al*, 2008). To test individual RNAis, *Drosophila* S2 cells were seeded in 48-well plates in the presence of dsRNA and then transiently transfected using Cellfectin (Invitrogen) according to the manufacturer's protocol. At 24 h after transfection, cells were induced with $CuSO_4$ to a final concentration of 500 μM and cells were assayed for luciferase activity 3 days after induction using the Promega Dual-Glo kit. For cytochalasin D treatment, S2 cells were seeded in 48-well plates at a concentration of 125 000 cells/ml the day before transfection. Transfection was then performed with the *Yki-pMT* and *Renilla-pMT* plasmids and the *3xSd2-Luc* reporter plasmid. At 24 h after transfection, cells were induced with $CuSO_4$ to a final concentration of 500 μM. At 20 h after induction, cells were treated with 3 μg/ml of cytochalasin D or DMSO for the control cells. Luciferase activity assay was performed the following day. Firefly luciferase activity was normalized with Renilla luciferase activity given in relative light units.

Antibody stainings

Antibody stainings of imaginal discs were done as described previously (Kango-Singh *et al*, 2002). The following antibodies were used (source and dilutions in parentheses): mouse anti-Wg (DSHB, 1/50), mouse anti-Dlg (DSHB, 1/300), mouse anti-Arm (DSHB, 1/200), mouse anti-Dlg (DSHB, 1/300), rat anti-DE-Cad (DSHB, 1/50), mouse anti-Crb (K Choi, 1/200), mouse anti-Patj (H Bellen, 1/500), mouse anti-BrdU (Becton-Dickinson, 1/50), rat anti-Ci (R Holmgren, 1/150), mouse anti-β-Gal (Promega, 1;2000), rabbit anti-Vg (S Carroll, 1/20), rabbit anti-phospho-Mad (E Laufer, 1/2000), guinea pig anti-Mer (R Fehon, 1/4000), rabbit anti-Ex (A Laughon, 1/2000), and guinea pig anti-Hpo (1/2000). To mark the F-actin, Phalloidin conjugated to alexa555 or alexa647 was used (Invitrogen; 1/50). BrdU incorporation was carried out as described (Kango-Singh *et al*, 2002) by incorporating BrdU for 1 h.

HeLa cell culture

HeLa cells were cultured in a DMEM containing 10% FCS (D10). HeLa cells (0.5×10^6/35 mm dish) were seeded 1 day before

transfection. Transfection of DNA was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. The transfection mixture was prepared as follows: total 2 μg DNA/35 mm dish pFL-C1 or pFL-mDia/dN3:1 μg, pCAG-EGFP:1 μg, opti-MEM:125 μl Lipofectamine 2000:5 μl, opti-MEM:125 μl. Three hours after transfection, cells were washed five times with PBS, and refed with D10. One day after transfection, cells were fixed and processed for immunofluorescent staining. Rabbit anti-Yap antibody (1:300) was described in Ota and Sasaki (2008). Following secondary antibody and reagents were used: anti-rabbit-alexa647 (Invitrogen) 1:2000, Hoechst 0.5 μg/ml, Phalloidin-alexa568 (Invitrogen) 1:40. Transfected cells were identified by fluorescence of cotransfected GFP. From five random fields, the top 10 strongly GFP-positive cells were selected to be analysed for Yap distribution. A total of 50 cells were counted for each sample. Transfections were performed independently twice ($n=2$). Statistical analysis was performed with Prism5 statistical software (GraphPad), using a one-way ANOVA followed by Tukey's multiple comparison test. For cytochalasin D and cell density experiments, HeLa cells were seeded in following densities 0.1×10^5/35 mm (low density and cytochalasin D treatment) 1×10^6/35 mm (high density). One day after plating, cells were treated with 0.05% DMSO with or without 1 μM cytochalasin D for 3 h, followed by immunofluorescent staining as described above.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: LSG, WB, and CT performed the experiments except Figure 6. KIW performed all the experiments in Figure 6. LSG, WB, and GH designed and analysed the experiments except Figure 6. KIW and HS designed and analysed the experiment in Figure 6. SY provided materials and discussion for experiments in Figure 6. LSG, WB, and GH wrote the article.

Conflict of interest

The authors declare that they have no conflict of interest.

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