Salt-inducible kinases regulate growth through the Hippo signalling pathway in *Drosophila*

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The specification of tissue size during development involves the coordinated action of many signalling pathways responding to organ-intrinsic signals, such as morphogen gradients, and systemic cues, such as nutrient status. The conserved Hippo (Hpo) pathway, which promotes both cell-cycle exit and apoptosis, is a major determinant of size control. The pathway core is a kinase cassette, comprising the kinases Hpo and Warts (Wts) and the scaffold proteins Salvador (Sav) and Mats, which inactivates the pro-growth transcriptional co-activator Yorkie (Yki). We performed a split-TEV-based genome-wide RNAi screen for modulators of Hpo signalling. We characterize the *Drosophila* salt-inducible kinases (Sik2 and Sik3) as negative regulators of Hpo signalling. Activated Sik kinases increase Yki target expression and promote tissue overgrowth through phosphorylation of Sav at Ser 413. As Sik kinases have been implicated in nutrient sensing, this suggests a link between the Hpo pathway and systemic growth control.
Figure 1  A split TEV screen for modulators of the Yki/14.3.3 interaction. (a) Schematic representation of the Yki–NTEV/14.3.3–CTEV split TEV interaction readout to monitor Hpo pathway activity. (b,c) Overexpression (b) or RNAi-mediated depletion (c) of known Hpo pathway components results in the modulation of the split-TEV-based Yki/14.3.3 readout. S2R+ cells were transfected with Yki–NTEV, 14.3.3–CTEV, GV–2ER, UAS–Fluc and a Renilla luciferase under the control of the piZ promoter (pIZRL). Cells were lysed 72 h later and assayed. RLU denotes average of relative luciferase units; all error bars represent s.d., n = 6. (d) Schematic overview of the genome-wide RNAi screen in Drosophila S2R+ cells using the Yki–NTEV/14.3.3–CTEV split TEV assay. Yki–NTEV and 14.3.3–CTEV plasmids along with the reporter plasmids GV–2ER (cytosolic TEV reporter), UAS–Fluc and pIZ–RL were transfected into S2R+ cells, and seeded onto a genome-wide dsRNA library in 384-well plates. After three days of incubation, cells were lysed and subjected to a Dual Luciferase Reporter Assay (Promega). (e) Schematic overview of the secondary screening involving the Yki–NTEV/14.3.3–CTEV split TEV assay, the complementary GAL4–DBD–Yki/Ex assay and the cross-correlation of potential candidates to generate an enriched hit list. See the main text for details. To further eliminate cell-intrinsic variations from the hit list, the pIZ–Rluc used in the genome-wide screen was replaced with an Actin5C-promoter-driven Renilla luciferase (pAc–Rluc) in the re-screening process. Furthermore, candidates exhibiting Rluc values less than 25% of the mean reading per plate were removed as these tended to have distorted Fluc/Rluc ratios.

RESULTS
The split TEV technique as a tool to monitor protein–protein interactions in Drosophila
Cell-based RNAi screens based on transcriptional readouts of Yki activity have already proved a powerful means of identifying Hpo pathway members. To increase the sensitivity and specificity of Hpo pathway readouts, we sought to adapt the split TEV protein–protein interaction technique to Drosophila cell culture.

We used Hpo dimerization through the carboxy-terminal SARAH (Salvador RASSF Hippo) domain to test the split TEV technique in Drosophila cells. We fused the amino-terminal part (amino acids 1–118) and the C-terminal part (amino acids 119–221) of an improved TEV protease mutant (TEV-S219P) to Hpo. In S2R+ cells, dimerization of Hpo led to the functional reconstitution of TEV protease activity, which in turn strongly activated a specific cytosolic TEV reporter.

overgrowth and upregulation of the Yki transcriptional target ex. We show that the Hpo partner Sav is the target of Sik2 and Sik3 in the Hpo pathway. Sik2 and Sik3 associate with and phosphorylate Sav, reducing its activity.
To design a split TEV assay for Hpo signalling activity, we chose the interaction between Yki and 14.3.3, which is dependent on phosphorylation of Yki at Ser 168 by Wts (refs 13,14). Using an interaction module consisting of Yki–NTEV and 14.3.3–CTEV (Fig. 1a), we could detect the kinase-cassette-induced Yki/14.3.3 association. Hpo and Wts expression, either alone or in combination, increased the readout (Fig. 1b). Likewise, using RNAi to deplete known pathway inhibitors, such as Rassf and the PP2A catalytic subunit mts (ref. 57), increased the signal (Fig. 1c). Conversely, pathway inactivation by RNAi-mediated depletion of Wts and Mats decreased the luciferase signal (Fig. 1c). These data suggest that the Yki/14.3.3 split TEV pair is a faithful reporter of Hpo pathway activity in cell culture.

Next, we performed a genome-wide RNAi screen in S2R+ cells using the Yki/14.3.3 split TEV readout and the Ambion Silencer Drosophila RNAi Library, which comprises 13,059 unique double-stranded RNAs (dsRNAs) targeting 92% of the Drosophila genome (Fig. 1d). From this screen, we selected a primary hit list of candidates that were more than 3 s.d. away from the mean (Fig. 1e and Supplementary Fig. S2a). These candidates were then subjected to re-screening using a set of non-overlapping dsRNA amplicons to eliminate off-target effects.

For secondary screening, we used a Yki transcriptional reporter based on a GAL4 DNA-binding domain–Yki fusion protein (GAL4–DBD–Yki; ref. 11). To improve the sensitivity and reproducibility of the assay, we co-transfected a plasmid encoding the upstream Hpo pathway member Ex (ref. 53; Supplementary Fig. S2b). This GAL4–DBD–Yki/Ex assay, known Hpo pathway members behaved as expected when depleted using RNAi (Supplementary Fig. S2c).

Using the complementary GAL4–DBD–Yki/Ex assay for re-screening we assembled a refined hit list by efficiently removing false positives (Fig. 1e). In this cross-correlation analysis, potential pathway activators exhibited a high score in the GAL4–DBD–Yki/Ex assay and a low score in the Yki–NTEV/14.3.3–CTEV assay, and vice versa for inhibitors. The candidate list was then refined using an anti-phospho-Ser168-Yki (pS168-Yki) antibody, which detects Wts-dependent Yki phosphorylation14,28. S2R+ cells were individually depleted for candidate genes and western blots of the lysates were probed for pS168-Yki levels (summarized in Supplementary Table S1).

### Identification of salt-inducible kinase as a Hpo pathway regulator

From this screening approach we recovered the gene CG42856, which encodes a serine/threonine kinase that is the Drosophila orthologue of human salt-inducible kinase 3 (Sik3; Supplementary Fig. S2a). Sik3 and the closely related Sik2 (CG4290) have been shown to regulate gluconeogenesis both in flies and humans39–41. Humans have three salt-inducible kinases, SIK1–3. In flies, Sik2 and Sik3 represent the only two members of the salt-inducible kinase family, Sik2 being the orthologue of human Sik1 and Sik2 (refs 62,63).

### A split TEV assay for Yki/14.3.3 binding and its use in an RNAi screen

To examine the effect of Sik activity on the Hpo pathway in vivo, we generated transgenic fly lines expressing Sik2 and Sik3-PA, both wild-type and activated forms mutant for a regulatory PKA site (Sik2-S1032A and Sik3-PA-S563A; ref. 62), under the control of the

As Siks were identified as potential negative regulators of the Hpo pathway in our screen, we wanted to confirm the effect of Sik2 and Sik3 on Hpo signalling using the pS168-Yki antibody. S2R+ cells depleted for Sik3 show a moderate increase in Yki phosphorylation levels (Fig. 2a). Moreover, on depletion of Ex, where baseline pS168-Yki levels are reduced, we observed a marked increase in Yki phosphorylation levels for Sik3-depleted cells and a modest increase for Sik2-depleted cells, supporting the notion that Siks negatively regulate Yki activity (Fig. 2b,c). On re-testing using the Yki/14.3.3 split TEV and GAL4–DBD–Yki/Ex assays, we confirmed the inhibitory effect for both Sik2 and Sik3 (Fig. 2d,e).

### Sik2 regulates wing tissue size by restricting Hpo signalling

To examine the effect of Sik activity on the Hpo pathway in vivo, we generated transgenic fly lines expressing Sik2 and Sik3-PA, both wild-type and activated forms mutant for a regulatory PKA site (Sik2-S1032A and Sik3-PA-S563A; ref. 62), under the control of the
GAL4/UAS system. Ex is both an upstream Hpo pathway regulator and a transcriptional target of Yki, acting in a negative feedback loop. Thus, we can use an enhancer trap fly line bearing a lacZ reporter inserted in the ex locus to readout of Yki transcriptional activity.

Hpo depletion using RNAi under the hedgehog-GAL4 driver line (hh-GAL4) leads to ex-lacZ upregulation in the wing disc posterior half (Fig. 3a,e,i,b,f,j)). Interestingly, overexpression of Sik2-S1032A, but not wild-type Sik2, induced a robust increase in the level of ex-lacZ expression (Fig. 3c,g,k,d,h,l). Apically localized Ex protein levels were also increased in both Hpo-depleted (Fig. 4a,d,g,j) and Sik2-S1032A-overexpressing clones (Fig. 4c,f,i,l) but not in Sik2 wild-type clones (Fig. 4b,e,h,k). In contrast, staining for the septate junction component Discs-large was unaffected, indicating that cellular architecture was not altered (Fig. 4m–r). Expression of Sik3-PA led to severe defects and cell delamination both under the hh-GAL4 driver and in clones. However, when cells were rescued by expression of the caspase inhibitor p35, we obtained small flip-out clones of Sik3-PA-expressing cells, which showed an increased level of Ex protein staining (Supplementary Fig. S3k,l). Thus, the Sik kinases can promote Yki activity in vivo, in accordance with our cell-based data.

To examine the effects of Sik2 and Sik3 on growth, we measured the wings of adult flies expressing our transgenes. Interestingly, overexpression of activated Sik2-S1032A, but not wild-type Sik2, leads to an increase in posterior compartment size (Fig. 4s–v), consistent with the Ex stainings. Overexpression of Sik3-PA and activated Sik3-PA-S563A caused collapse of the posterior part of the wing (Supplementary Fig. S4i,l). Similarly to other AMPK family members, Hpo RNAi (b,f,j)), Sik2 wild type (c,g,k) and Sik2-S1032A (d,h,l) were expressed under the control of hh-GAL4. Planar sections depict anti-βGal nuclear staining in grey; e-h are merge images of βGal staining (magenta) and GFP (green); i-l are magnifications from a to d. Scale bars, 10 μm.

Depletion of Sik2 and Sik3 reduces wing size
To overcome the potential redundancy between Sik2 and Sik3 in loss-of-function studies, we took advantage of the VDRC sik2 RNAi line KK103739, which is predicted to target both Sik2 and Sik3. We verified this by co-expressing this RNAi line, or a sik3 RNAi line, with either activated Sik2 (Supplementary Fig. S4a–c) or wild-type Sik3 (Supplementary Fig. S4f–k). Whereas the sik3 RNAi line could revert the Sik3 but not the Sik2 overexpression phenotype, the sik2 RNAi line could suppress both the Sik2 and Sik3 phenotypes, confirming that it can be used to silence both kinases. Depleting Sik2 and Sik3 with this RNAi line in the posterior part of the wing significantly reduced posterior compartment size, whereas Sik3 depletion alone had no effect on size (Fig. 5f–i). The Sik kinases therefore promote growth in the wing, consistent with their antagonistic effect on Hpo pathway activity.

Sik2 and Sik3 bind to Sav
To clarify the molecular mechanism of the Siks’ input into Hpo signalling, we investigated whether Siks associate with Hpo pathway components, using co-immunoprecipitation experiments in S2 cells between Sik2 and core Hpo pathway members. We examined a number of pathway components (such as Hpo, Sav, Wts and Mer, Fig. 6a,b). Sik2 associates robustly with Sav, but not with the other tested proteins (Fig. 6b). Interestingly, we also observed a modest
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Figure 4 Activated Sik2 induces wing growth through the Hpo pathway. (a–l) Expression of Hpo RNAi (a, d, g, j) and Sik2-S1032A (c, f, i, l), but not Sik2 (b, e, h, k), in flip-out clones leads to the upregulation of Ex protein levels at the apical membrane of imaginal wing discs. (m–r) Dlg protein levels at the apical membrane are not affected by Hpo RNAi (m, p). Sik2 (n, q) or Sik2-S1032A (o, r) expression. Planar (a–f) and transverse (g–r) sections showing Ex (a–f) or Dlg (g–r) protein levels (grey/magenta) in hsFlp, Act>CD2>GAL4>>Sik2-S1032AAct>CD2>GAL4>>Sik2Act>CD2>GAL4>>Hpo RNAi

co-immunoprecipitation between Sik2 and Mer, which was largely abolished on Sav depletion (Fig. 6b, lanes 6 and 7). Sav contains an N-terminal FERM-binding motif (FBM, Fig. 7a), which binds to the FERM-domain of Mer (ref. 29), supporting the notion that the Sik2/Mer interaction is bridged by Sav.

For Sik3, there are multiple splice isoforms to consider. Sik3-PA, a short isoform, was initially described55, but two longer isoforms (PB and PC) with extended C termini were recently annotated in Flybase. By performing PCR with reverse transcription on RNA extracted from larval preparations we isolated complementary DNAs coding for a total of six isoforms (PA to PF, see Supplementary Fig. S5). Interestingly, we compared the short PA isoform with one of the longer isoforms (PB), and found that only the latter could bind Sav, suggesting that the extended C terminus is required for Sav binding (Fig. 6a).

Next, we mapped the domains in Sik2 and Sav that mediate their interaction. The Sik2-binding domain in Sav is located within the C-terminal part, covering the two WW domains and the region located in between the WW domains and the SARAH domain (Supplementary Fig. S6a,b). The Sav-binding region in Sik2 is located near the C terminus, covering a region from amino acids 1,054 to 1,250 (Supplementary Fig. S6c,d).

Sik2 and Sik3 phosphorylate Sav
Sav contains two potential Sik phosphorylation sites (Fig. 7a), prompting us to investigate whether Sik can phosphorylate Sav. Overexpression of either Sik2 or Sik2-S1032A leads to a Sav mobility shift, whereas a kinase-dead form had no effect, and depletion of Sik2-S1032A by RNAi reverted this band shift (Fig. 7b). Both Ser 162 and Ser 413 of Sav fit the Sik phosphorylation consensus site61. A Sav-S413A mutant showed a decreased mobility shift on Sik2 expression, whereas Sav-S162A was unaffected (Fig. 7c). The Sav-S162/413A double mutant did not further decrease the shift (Supplementary Fig. S7a). These results indicate that Ser 413 is phosphorylated by Sik2.

To validate this finding, we raised a phospho-specific antibody against Sav-S413. This antibody detected a robust signal when wild-type Sav but not Sav-S413A was co-expressed with Sik2-S1032A (Fig. 7d). In addition, lambda phosphatase treatment abolished the phospho-signal and upshift, confirming the antibody’s specificity (Supplementary Fig. S7b). To characterize the Ser 413 site in more detail, we used the truncations we generated to map the Sav/Sik2 interaction (Supplementary Fig. S6a,b). Sav fragment 294–492, which associates with Sik2, was shifted and phosphorylated at Ser 413 on the phospho-signal and upshift, confirming the antibody’s specificity.
Sik2 kinase expression, whereas the S413A mutation reverted the shift (Fig. 7e). However, the band shift reversal in the full-length Sav-S413A mutant was incomplete, so there are probably other Sik sites in Sav. The Sav N terminus (amino acids 1–300) did not shift on Sik2 co-expression, presumably because it does not bind to the kinase (Supplementary Fig. S6b, second lane), but may still harbour phosphorylation sites. We examined eight other potential Sik sites in Sav, but no mutant other than S413A showed a decreased mobility shift (Supplementary Fig. S7c,d), supporting the notion that Ser 413 is the critical Sik site in Sav. Similarly to Sik2, Sik3-PB strongly phosphorylated Sav, whereas Sik3-PA had little effect (Fig. 7d). Together, our data suggest that Sik2 and Sik3 associate with and phosphorylate Sav at Ser 413.

Sik2 antagonizes Hpo pathway activity by phosphorylating Sav

We investigated whether Sav phosphorylation by Sik accounts for its ability to promote Yki activity. Co-expression of Sav with Yki in S2 cells induced a modest increase in Yki phosphorylation on Ser 168 (Fig. 8a). Co-expression of activated Sik2 abolished baseline, Sav-induced and Mer-induced Yki phosphorylation, whereas kinase-dead Sik2 had no effect (Fig. 8a,b). In contrast, activated Sik2 had no effect on Yki phosphorylation induced by Hpo or Wts (Fig. 8b), suggesting that Sik2 acts at the level of Sav, downstream of Mer, and upstream of the kinases Hpo and Wts.

Sav is a scaffold protein known to associate with Hpo through its SARAH domain\(^6,8\), Mer through an FBM (ref. 29), and Wts through its WW domains\(^10\). To gain further insight into how Sik2 regulates the Hpo pathway core kinase cassette, we characterized the effect of Sik2 on binding of Sav to its partners. First, Hpo association with Sav was not perturbed by Sik2 expression (Supplementary Fig. S7e), nor did Hpo or kinase-dead Hpo expression prevent the Sav/Sik2 interaction (Supplementary Fig. S7f). The Sav-S413A mutation did not affect Hpo/Sav or Sik2/Sav binding (Supplementary Fig. S8a,b). Second, the Sav/Mer association was unchanged on Sik2 co-expression (Supplementary Fig. S8c). Finally, we investigated the ability of Sav to scaffold the Hpo/Wts kinase cassette. In the absence of Sav, the interaction between co-expressed Hpo and Wts was barely detectable (Fig. 8c, top panel, lane 2). On Sav overexpression, this interaction was markedly increased (Fig. 8c, lanes 2 and 5). Co-expression of activated Sik2 significantly reduced the Sav-mediated Hpo/Wts association (Fig. 8c and quantified in Fig. 8d). Furthermore, depletion of Sik2 and Sik3 resulted in a modest increase in baseline Hpo/Wts binding.

Figure 5 Depletion of the Sik kinases reduces the level of wing growth. (a–e) Expression of lkb1 RNAi rescues Sik2-S1032A-induced overgrowth (e) in comparison with expression of Sik2-S1032A alone (d). Expression of lkb1 RNAi alone does not decrease the level of wing growth (c). (a) Quantification of the wing phenotypes shown in b–e. (f–i) Expression of sik2 RNAi (h) reduces the level of wing growth, but sik3 RNAi does not affect wing size (i). (f) Quantification of the wing phenotypes shown from g to i. Transgenes were driven using hh-GAL4. The values in a and f represent the ratio of the posterior compartment divided by the total wing area. *** P < 0.001. Error bars represent s.d. In a, n = 25 (GFP and lkb1 RNAi), n = 29 (Sik2-S1032A), n = 35 (Sik2-S1032A, lkb1 RNAi). In f, n = 8 for each genotype.
Figure 6 Sik kinases bind to and phosphorylate the Hpo pathway core member Sav. (a) Sik2 and Sik3-PB, but not Sik3-PA, interact with Sav. (b) Sik2 interacts with Mer in a Sav-dependent manner. Co-immunoprecipitation experiments were performed in S2 cells transfected with the indicated plasmids, and cells were lysed 48 h later. Lysates and FLAG-purified immunoprecipitates (IP) were subjected to western blot analysis using the indicated antibodies. For samples treated with RNAi, cells were pre-incubated with indicated dsRNA before transfection. The asterisk denotes a nonspecific band, and the arrowheads indicate the IgG heavy chain. Uncropped images of blots are shown in Supplementary Fig. S9.

Ser 413 in Sav is critical for Hpo signalling

If Sav phosphorylation by Sik2 affects the assembly of the core complex, this would be expected to affect Yki phosphorylation, which is dependent on Hpo's ability to activate Wts. Indeed, the reduction in pS168-Yki levels observed on activated Sik2 expression was prevented by co-expression of the non-phosphorylatable Sav-S413A mutant (Fig. 8f). Finally, we investigated the biological activity of the Sav-S413A mutant in vivo by making transgenic lines expressing wild-type Sav or Sav-S413A under the control of the GAL4/UAS system. hh-driven expression of wild-type Sav modestly reduced the size of the posterior compartment of the wing, whereas the Sav-S413A mutant had a significantly stronger effect (Fig. 8g–j). In fact, whereas expression of wild-type Sav had no effect on viability, the Sav-S413A mutant strongly reduced the number of progeny. Thus, phosphorylation of Sav on Ser 413 reduces its biological activity in Hpo signalling and growth control.

To investigate whether the Sik-mediated phosphorylation is conserved in humans, we co-transfected Drosophila Sik2 with human SAV1. We detected a moderate upshift of SAV1, although the phosphorylation site is not conserved, as the pS413-Sav antibody failed to detect SAV1 phosphorylation (Supplementary Fig. S8d). However, human SIK2 can increase YAP-mediated transcription in a GAL4-DBD–TEAD4 luciferase readout, which has been widely used to assess Hpo pathway activity in mammalian cells20 (Supplementary Fig. S8e). These data suggest that human SIK kinases functionally inhibit Hpo signalling, but it is unclear whether the exact mechanism is conserved.

DISCUSSION

In this study we combine the protein–protein interaction detection method split TEV and RNAi screening in Drosophila cell culture to identify Hpo pathway modulators. Split TEV was first developed in mammalian cells55, and subsequently shown to be a valuable tool to monitor phosphorylation-dependent interactions of proteins56. Here, we applied split TEV in Drosophila cell culture, both for constitutive (Hpo dimerization) and regulated (Yki/14.3.3) interactions. The success of our screening approach suggests that split TEV will prove invaluable in mapping signalling pathways by providing functional readouts that can be combined with RNAi or pharmacological approaches.

Our results identify Sik kinases as Hpo upstream regulators. In particular, activated Sik2 can induce overgrowth and activation of...
Figure 7 Sik kinases phosphorylate the Hpo pathway core member Sav.
(a) Structure of Sav indicating potential Sik2 phosphorylation sites. Below, the Sik consensus phosphorylation sequence19 and the corresponding sites in Sav are shown. FBM, FERM-binding motif; W, WW domain; SARAH, SARAH domain; Φ, hydrophobic residue. (b) Sav phosphorylation is dependent on Sik2 and its kinase activity. (c) Sik2 phosphorylates Sav on Ser 413 but not Ser 162. S2 cells were transfected with the indicated plasmids, and cells were lysed 48 h later and subjected to western blot analysis using a 7.5% SDS–PAGE gel and the indicated antibodies. (d) Sav is phosphorylated at Ser 413 by Sik2 and Sik3. Indicated plasmids were transfected into S2R+ cells, and lysates (upper panel) and FLAG-purified immunoprecipitates (IP; lower panel) were subjected to western blot analysis using the indicated antibodies. The asterisk indicates a nonspecific band. (e) The Sav fragment 294–492 does not shift when harbouring the S413A mutation. S2R+ cells were transfected with the indicated plasmids, and cells were lysed 48 h later and subjected to western blot analysis using a 7.5% SDS–PAGE gel and the indicated antibodies. Sik2-S1032A, active Sik2; Sik2-D263A, kinase-dead form. When samples were treated with RNAi, cells were pre-incubated with the indicated dsRNA. Uncropped images of blots are shown in Supplementary Fig. S9.

Yki transcriptional activity (Figs 3 and 4), whereas depletion of Sik2/3 in the wing leads to undergrowth (Fig. 5). Interestingly, Sik3 can also antagonize Hpo signalling, but in an isoform-specific manner. The relative contribution of Sik2 and Sik3 to growth in various tissues needs to be addressed to appreciate the possible redundancy between these two kinases, and potentially other AMPK family kinases. A recent report shows that LKB1, which regulates all AMPK family members66, may inhibit YAP activity independently of the core Hpo cassette, suggesting a complex interplay between LKB1/AMPKs and the Hpo pathway67.

The effect of Sik2/3 on Hpo signalling is mediated, at least in part, by Sav phosphorylation on Ser 413 (Figs 7 and 8). Our data suggest that Sav phosphorylation by Sik reduces its ability to efficiently scaffold the Hpo/Wts core kinase complex, thereby reducing Yki inhibitory phosphorylation (Fig. 8c–e). In agreement with this notion, Sav-S413A exhibited an enhanced ability to reduce the level of growth in vivo (Fig. 8g–j). Siks play a major role in inhibiting gluconeogenesis in the liver in response to high glucose levels through inhibitory phosphorylation of the transcriptional co-activator CRTC2 (CREB-regulated transcription co-activator 2)/TORC2, and activatory phosphorylation of the histone deacetylase HDAC4, a function that seems to be conserved in Drosophila59,63,65,68. The Drosophila Siks have mostly been studied in the context of energy balance in the brain69 and fat body (the Drosophila equivalent of the liver and adipose tissue), where they prevent the mobilization of fat and glycogen stores by antagonizing CRTC and Foxo-activated transcription51,65,68. The Siks are under hormonal control by insulin receptor (InR) signalling through the downstream kinase Akt, which phosphorylates and activates Sik2/3, and the glucagon homologue adipokinetic hormone (AKH), which signals through
a G-protein-coupled receptor and PKA, which phosphorylate and inhibits Siks (refs 65,69). Under fasting conditions, low insulin and high AKH activity combine to shut down Sik3, thereby promoting gluconeogenesis and inducing mobilization of fat-body lipid stores to restore circulating glucose levels.65 Our work suggests a role for Sik2/3 in growth control during development. Analogously to InR signalling in Drosophila, which promotes both nutrient storage in the fat body and developmental growth of peripheral tissues, the Sik kinases might couple Hpo pathway activity to nutrient or energy availability, ensuring that Yki is able to drive tissue growth only under favourable conditions. Recent work has shown that Drosophila InR signalling can promote cell proliferation through Yki (ref. 70). Furthermore, mammalian liver cells stimulated with glucagon or the PKA activator forskolin exhibit reduced levels of phosphorylated YAP and increased YAP activity, an effect that may conceivably be mediated by the SIKs (ref. 71).

Recent work has established the Sik kinases as candidate oncogenes in ovarian and lung cancer.72-74 We showed that Sik2 promotes YAP-dependent transcription in human cells (Supplementary Fig. S8e). However, because Sav-S413 is not conserved, the molecular mechanism by which Sik2 regulates the mammalian Hpo pathway may differ and should be investigated further. Interestingly, YAP, the Yki orthologue, has also been reported to function as an ovarian cancer oncogene.75,76 Sik2 inhibitors may therefore prove attractive candidates to boost Hpo pathway activity in ovarian tumour cells, although this strategy may be less effective in tumours harbouring YAP amplifications.77
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**AUTHOR CONTRIBUTIONS**

M.C.W. designed the study and experiments and performed most of the experiments. M.V.H. cloned and analysed the full-length Sks3 ORFs and performed the experiments in Figs S1–f and S8–j and Supplementary Figs S3,k and S4–l. I.G. performed the experiments in Fig. S5–e and Supplementary Fig. S4a–e and contributed western blot data. T.M.M. contributed phosphatase-treated western blot data, and E.C. helped with cloning. R.E.S., M.J., R.I. and M.H. helped perform and analyse the screen. M.J.R. provided the split TEV technology and supported the study. N.T. conceived and supervised the study. M.C.W. and N.T. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Cloning of expression constructs. For split TEV fusions, the pAWF and pAHW vectors from the Drosophila Gateway Vector Collection were modified by introducing an NTEV or a CTEV fragment to yield pAWF–NTEV or pAHW–CTEV vectors. The NTEV fragment (amino acids 1–118 of the TEV protease), followed by a flexible linker (5′-GGGGSGGGG-3′), was amplified by PCR and introduced into the pAWF vector by using the SacI restriction enzyme. The CTEV fragment (amino acids 119–221, with a S219P point mutation for a more stable TEV protease variant77), also followed by the flexible linker, was PCR-amplified and ligated into the pAHW vector using the enzymes SacI and SalI. The open reading frame (ORF) for 14.3.3 epsilon (14.3.3eps; CG31196-PA) was amplified from Drosophila embryonic cDNA (the ORFs for Sik2 (CG1920, clone RH42017) and Sik3-Pa (CG14856-PA, clone LDO7105) were obtained from the DGRG collection. Sik3-P was cloned from cDNA generated from larval fly preparations. For a detailed procedure, see Supplementary Fig. S5. All ORFs were amplified by PCR using primers bearing flanking attB1 and attB2 sequences and subjected to BP recombination reactions using the pDONR/Zevo vector (Invitrogen) to generate Entry vectors. Expression vectors were obtained through LR recombination reactions using the pAWF, pAHW, and pAHW vectors from the DGRG Gateway collection. Vectors for Hpo, Wts, Mats, Sav, Rassf, Kibra, Ex and Mer were previously described82,83. Point mutations and, if required, ORF corrections (that is, for RH42017, Sik2) were generated by using site-directed mutagenesis (Agilent Technologies). The following primers were used for site-directed mutagenesis: Sik2-S1032A (constitutively active), 5′-CGGGAGGAGCAGGGGCGTGGTGGCCTGC-3′; Sik2-D263A (kinase-dead), 5′-GGCGATACTGCGGCGCTCACCAGGCGAAACC-3′; Sik3-S563A (constitutively active), 5′-CATTGTTGGGTCGTTGGCCAGACCGGCGGCGACATC-3′; Sik3-D163A (kinase-dead), 5′-GGTGGTGAGATCGCGCTCACAAGGCGGAGAACCC-3′; Sik3-A263A (constitutively active), 5′-GGATGCGGCGTCGCGGCTG-3′; Sav-S162A, 5′-GGATGCGGCGTCGCGGCTG-3′; Sav-S413A, 5′-GGATGCGGCGTCGCGGCTG-3′; Sav-S162A, 5′-GGATGCGGCGTCGCGGCTG-3′; Sav-S413A, 5′-GGATGCGGCGTCGCGGCTG-3′.

For transfection, the pFW vector from the DRGC Gateway collection and a Gateway-compatible pUAST–attB vector75 allowing site-specific integration into the fly genome were used. Human MST1 was PCR-amplified from image clone 7939613 (Imagenes) and cloned into pENTR2 (Invitrogen). For split TEV fusions in mammalian cells, the previously described N/CTEV vector was used75,82. All PCRs were run using proofreading polymerases (Pwo Master, Roche; Pfu Turbo, Stratagene), and all PCR-amplified ORFs were sequence-verified.

Split TEV and GAL4–DBD–Yki luciferase assays. For individual split TEV assays, plasmids encoding N- and CTEV protein fusions along with the cytosolic transcriptional split TEV reporter GV–2ER and pUAS–Fluc were used. For GAL4–DBD–Yki assays, vectors encoding GAL4–DBD–Yki, Ex and the reporter UAS–Fluc were used. For Drosophila cell culture, luciferase assays were run in 96-well formats for 72 h using S2R+ cells transfected using FugeneHD with a Renilla luciferase as an internal control (pActin5C–hRL). Equal amounts of DNA were transfected into each well (20 ng for each overexpressing plasmid, 30 ng for pUAS–Fluc and 3 ng for pActin5C–hRL). pAWF was used to adjust DNA amounts.

For mammalian cell culture, luciferase assays were performed in 96-well formats using HEK293T cells transfected with Lipofectamine LTX and a mix of Renilla luciferase plasmids (CMV–RL, TK–RL, SV40–RL at 1:2:10 molar proportions) as an internal control. Equal amounts of DNA were transfected into each well (20 ng for all plasmids, including pUAS–Fluc, and 1 ng for Renilla mix). pCDNA3 was used to adjust DNA amounts. After 24 h of incubation, cells were lysed using 1×PassiveLysis buffer (Promega), followed by the Dual Luciferase Reporter Assay (Promega). The readouts for both channels (firefly, Renilla) were recorded using an EnVision Multilabel Reader (PerkinElmer) and a PHERAStar Plus Reader (BMG Labtech).

Cell culture. S2 cells were maintained in Schneider’s medium (Sigma) supplemented with 10% FCS. S2R+ cells were maintained in Sheds and Sang M3 Insect Cell culture. S2 cells were maintained in Schneider’s medium supplemented with 10% FCS, and GlutaMAX (Invitrogen) at 25°C. Drosophila S2R+ cells were maintained in Shields and Sang M3 Insect Cell culture.

METHODS

Controls were manually added into empty wells of this 384-well format. DsRed was used as a baseline control, yki as a functional control, wts and mats as negative controls, and mts and rassf as positive controls. The genome-wide RNAi screen was run in triplicate, using plasmids encoding Yki–NTEV, 14.3.3eps–CTEV, GV–2ER (15 ng per well), UAS–Fluc (20 ng per well) and a Renilla luciferase under the baculoviral OpI2 promoter (pIz–Rluc, 3 ng per well) to normalize for transfection efficiency and cell number.

For the screen, S2R+ cells were batch-transfected with all plasmids (Yki–NTEV, 14.3.3eps–CTEV, GV–2ER, UAS–Fluc, pIz–RL) using FugeneHD (Roche), and 6,000 cells were plated per well of a 384-well plate using a Matrix WellMate (Thermo Scientific). After 72 h of incubation, cells were lysed and processed for the Dual Luciferase Reporter Assay (Promega).

Analysis of the screening data. Readings for firefly and Renilla luciferases were processed and analysed using R and the CellHITS2 bioconductor package (http://www.bioconductor.org/packages/devel/bioc/html/cellHITS2.html). Both firefly and Renilla data were first normalized by dividing each value by the plate median of sample wells to account for plate-to-plate variation. Interpretation of firefly/Renilla ratios suggested a bias towards wells with extreme effects on cell number (either increased or decreased Renilla values). To correct for this, less regression was used to define an expected relationship between the values for Yki alone and the firefly/Renilla ratio, and Renilla values were then corrected by subtracting this expected value. The corrected firefly/Renilla ratios were then divided by the experimental median absolute deviation of ratios to calculate a robust Z-score, indicating the number of standard deviations away from the screen mean. Triplicate plates were summarized by taking the median of the replicate Z-scores, and hits were defined as those genes with median Z-scores greater than +3 or less than −3.

For the secondary screening process, all hits were subjected to a second round of Yki–NTEV/14.3.3eps–CTEV split TEV and GAL4–DBD–Yki/Ex assays, using new dsRNA amplics for each candidate. To normalize for promoter-dependent off-targets, we used an actin-promoter-driven Renilla luciferase (pA WF–hRL) in the secondary assays. In addition, we removed all hits that exhibited a Renilla value that was below 25% of the mean Renilla value across a given plate. In the following cross-correlation process (that is, for pathway inhibitors: high values in the split TEV Yki/14.3.3 assay and low values in the GAL4–DBD–Yki/Ex assay), further potential off-target hits were removed, yielding 46 final candidates (see Supplementary Fig. S3 and Table S1 for details).

These hits were then subjected to a pS168-Yki western blot analysis using individual dsRNAs targeting each candidate. Inhibitors exhibited enhanced levels of pS168-Yki staining, and conversely, activators showed decreased levels. Controls for each set-up were mer (for activators) and aka (for inhibitors).

Co-immunoprecipitations and western blots. S2 and S2R+ cells were transfected with indicated plasmids using the Effectene transfection kit (Promega). In the case of RNAi treatments, cells were incubated with 15 μg dsRNA per well 24 h before transcription. After 48 h of expression, cells were lysed in 1% Triton X-100 lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100 and 1 mM EDTA) supplemented with 0.1 M NaF, phosphatase inhibitor cocktail 2 and 3 (Sigma) and protease inhibitor cocktail (Roche). Cell extracts were spun for 10 min at 4°C at 17,000g. FLAG-tagged proteins were purified using anti-FLAG M2 affinity gel (Sigma). After 2 h of incubation, the FLAG immunoprecipitates were washed four times in lysis buffer. Lambda (λ) phosphatase (NEB) treatment was performed in lysates for 30 min using 2,000 units. Protein gels were run and blotted using the Mini-PROTEAN Tetra Electrophoresis System (BioRad). Detection of purified and associated proteins was performed by western blot analysis using Chemiluminescence (Western Lightning Plus- ECL, PerkinElmer). Densitometric analysis of western blots was done using ImageJ, using a protocol from lukemiller.org (http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/). Significance was calculated using one-way analysis of variance in GraphPad Prism, followed by Bonferroni tests. Western blots were probed with antibodies against pS168-Yki (ref. 28), 14.3.3eps (ref. 34), pYki (Yki-Tyr118) (Sigma, T9026), using dilutions of 1:1,000 for all antibodies.

Immunostainings. Wing imaginal discs from third-instar larval larvae were dissected, fixed for 25 min in 4% PFA, washed three times in PBS supplemented with 0.1% Triton X-100 (PBS-T), permeabilized for 15 min in PBS supplemented with 0.3% Triton X-100, washed once with PBS-T, and pre-blocked in PBS-T containing

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10% normal goat serum (NGS) for 30 min. Discs were incubated in the primary antibody overnight diluted in PBS-T containing 10% NGS, followed by three washes with PBS-T, and an incubation with a secondary antibody in PBS-T/10% NGS for 3 h at room temperature. After four further washes, discs were mounted in Vectashield mounting medium with DAPI (Vectorlabs). Dilutions for the antibodies against β-galactosidase (Promega, Z2788), Ex (a gift from A. Laughon, University of Wisconsin, Madison, USA), Dlg (DSHB collection, monoclonal 4F3) and rhodamine red-X (Jackson ImmunoResearch, 111-295-003) were 1:500.

**Analysis of the it Drosophila wing.** Adult wings were mounted using Euparal mounting medium (Agar Scientific) and imaged using a Zeiss Axio-plan 2 imaging system (using Optovar optics, ×1.6) and a ×2.5 objective (Plan-NEOFLUAR, Zeiss) connected to a Leica DFC420C camera. Wing sizes were quantified by measuring the posterior area divided by total wing area using ImageJ. Significance was determined using the one-way analysis of variance statistic tool in GraphPad Prism, followed by Bonferroni post hoc tests.

**Accession number.** The RNAi screening data were deposited into GenomeRNAi under the accession number: GR00218-S.

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Figure S1 Using the split TEV technique to monitor Hpo dimerisation in Drosophila cell culture. (a) Schematic representation of the Hpo dimerisation split TEV assay. Hpo is fused to the N-terminal fragment of the TEV protease (NTEV) and C-terminal fragment (CTEV). Hpo dimerisation induces reconstituted TEV protease activity, leading to the activation of the cytosolic transcriptional reporter GV-2ER. This reporter is composed of an artificial transcriptional co-activator unit (GAL4-VP16-GV) flanked on either side by two TEV protease cleavage sites and two mutant ligand-binding domains of the estrogen receptor (ERT2), which restrict the non-cleaved reporter to the cytosol. Upon activation, the transcriptional co-activator GV translocates to the nucleus to induce a reporter gene of choice, i.e. a firefly luciferase reporter gene through binding to upstream activating sequences (UAS-Fluc). tevS, TEV protease cleavage site. (b) Domain organisation of Hpo and MST1 variants used in the split TEV assays. NTEV and CTEV fragments are C-terminally fused to Hpo/MST1. Hpo-ΔC, SARAH domain deletion, aa 1-601. MST1-ΔC, aa 1-432. Kinase domain (red), SARAH domain (blue). (c, d) Hpo (c) and MST1 (d) dimers strongly activate split TEV reporters, but no activation was seen with SARAH-domain deletions (ΔC) or mutations (Hpo-L619P, MST1-L444P). S2R+ cells (c) or HEK293 cells (d) were transfected with plasmids as indicated, with the cytosolic TEV reporter GV-2ER, UAS-Fluc and a Renilla luciferase expressed under the OpIE2 promoter (pIZ-Rluc) as control. Cells were lysed 48h later and assayed. GCN4cc, coiled-coiled dimerisation domain of yeast GCN4 (used as baseline control). RLU denote average of relative luciferase units, all error bars represent s.d., n=6. (e) Cleavage of the cytosolic TEV reporter GV-2ER induced by Hpo-N/CTEV dimerisation analysed by western blotting using an ERα antibody. The first cleavage of GV-2ER results in either “GV-ER” or “ER-GV” fragments, while a doubly cleaved reporter yields two ER fragments and one GV moiety. S2R+ cells were transfected as indicated and lysed 48h later. Expression of indicated NTEV (FLAG-tagged) and CTEV (HA-tagged) fusion constructs were confirmed using anti-FLAG and anti-HA antibodies. Tubulin levels served as a loading control. Asterisks denote unspecific bands recognised by the ERα antibody.
Figure S2 Primary and secondary screening for Hpo pathway regulators. (a) Graphic visualisation of the primary screen data. All counts (13,059 single dsRNAs from the Ambion library and 612 controls) from the Yki/14.3.3 split TEV genome-wide RNAi screen were plotted against the Z-score using Mondrian, with pathway inhibitors displaying high values and activators low values. For the secondary analysis, we selected all candidates that were at least three standard deviations away from the mean (depicted in blue). The recovered hit, sik3 (CG45856), wts positive and mts negative controls are shown in red. (b) Schematic representation of the complementary GAL4-DBD-Yki/Ex readout to monitor Hpo pathway activity. When the pathway is activated (ON), Hpo and Wts-mediated phosphorylation of Yki leads to the association of GAL4-DBD-Yki and 14.3.3 in the cytosol resulting in basal luciferase signals only (left). When the pathway is OFF, GAL4-DBD-Yki and 14.3.3 do not interact, allowing GAL4-DBD-Yki to translocate into the nucleus to activate a UAS-driven firefly luciferase reporter gene (Fluc) (right). (c) RNAi-mediated depletion of known Hpo pathway components results in the modulation of the GAL4-DBD-Yki/Ex readout. S2R+ cells were transfected with GAL4-DBD-Yki, Ex-HA, UAS-Fluc and a Renilla luciferase under the control of the Actin promoter (pAFW-hRL). Cells were lysed 72h later and assayed. RLU denote average of relative luciferase units, all error bars represent s.d., n=6.
**Figure S3** Active Sik2 upregulates the Hpo Pathway target Expanded. (a-j) Clonal expression of Sik2-S1032A, but not Sik2, leads to an increase in *ex-lacZ* transcript levels in imaginal wing and eye discs. Planar sections depicting anti-βGal staining (grey) in *hsFlp, act>>hpo RNAi* (a, f), *hsFlp, act>>sik2* (b, g), and *hsFlp, act>>sik2-S1032A* (c-e, h-j) in 3rd instar wing (a-d, f-i) and eye (e, j) discs. f-j are merge images of βGal staining (magenta), GFP (green) and DAPI (blue). (k, l) Active Sik3 upregulates the Hpo pathway target Expanded. Expression of Sik3-PA-S563A in flip-out clones leads to an increase in apical Ex protein levels in wing discs. Transverse section showing Ex protein levels (grey/magenta) in *hsFlp, Act>>Sik3-PA-S563A, p35* 3rd instar wing discs. The merged image includes βGal staining (magenta), GFP (green) and DAPI (blue). Scale bars, 10µm.
**Figure S4** Verification of sik RNAi specificity. (a-e) Expression of sik2 RNAi (d) but not sik3 RNAi (e) rescues the overgrowth caused by Sik2-S1032A. Transgenes were driven by hh-GAL4. (a) Quantification of wing phenotypes shown from (b) to (e). Values represent the ratio of the posterior compartment divided by the total wing area. 3 asterisks indicate p<0.001; n.s., not significant. Error bars represent s.d., n=25 (GFP), n=16 (Sik2-S1032A), n=12 (Sik2-S1032A, sik2 RNAi), n=14 (Sik2-S1032A, sik3 RNAi). (f-k) Expression of either sik2 RNAi (j) or sik3 RNAi (k) can rescue the crumpled wing phenotype observed upon Sik3-PA (i) overexpression using the hh-GAL4 driver.
Figure S5 The CG42856 gene encodes multiple isoforms of Sik3. (a) The Sik3 gene (CG42856, blue arrow) encodes multiple transcripts, shown below the gene (orange and grey boxes, representing coding and non-coding exons respectively). Transcripts CG42856-RA, -RB and -RC have been previously described; transcripts CG42856-RD, -RE and -RF are uncharacterised. The long isoform used in our co-IP experiments corresponds to transcript -RB or -RC (the coding sequences of these isoforms are identical). The position of other nearby genes is also shown (smaller blue arrows). The single-exon gene CG15071 is located within the longest intron of Sik3; we have been unable to detect Sik3 transcripts incorporating this exon. The Sik3-PB ORF was cloned by extracting RNA from wandering stage larvae using the Qiagen RNeasy Mini kit. cDNA was prepared using the Roche AMV First Strand cDNA Synthesis kit. The long isoforms of Sik3 were amplified using Phusion High Fidelity DNA Polymerase (Finnzymes) and primers 5'-CACCATCCGCAAGAATCCTCATCCTC-3' and 5'-TTAGCCTGCCACCTGCTGC-3' and separated on an agarose gel, then TOPO-cloned and sequenced. A clone was selected whose coding sequence matched that of transcript CG42856-RB. This Sik3 coding sequence was then re-amplified without its 5’ UTR or start codon using forward primer 5’-CACCGCCACCAAGCCACCAGGCTG-3’ and the same reverse primer as above. Sik3 (without ATG start codon) was cloned into the pENTR/D-TOPO Gateway vector (Life Technologies), and Gateway recombination cloning was used to shuttle the coding sequence into the tag-bearing expression vectors pAFW, pAHW and pAMW. (b) An alignment of transcripts CG42856-RC, -RD, -RE and -RF in the variable region to better show the splice sites used by each isoform. Exon 9 is missing from transcripts -RE and -RF; transcripts -RD and -RF use an alternative splice acceptor site 9 bp downstream of the start of exon 10. (c) RT-PCR of the variable region of Sik3. The isoforms that incorporate exon 9 are more abundant than those that lack it. Approximate primer positions are indicated by small red arrows beneath transcript -RC.
Figure S6 Mapping the interaction domains for Sav and Sik2. (a, b) Sav binds to Sik2 with its inter-WW/SARAH domain region. Structure of Sav and fragments thereof used in the Sav/Sik2 binding assay (a). Binding strength to Sik2 is classified as weak (+), intermediate (++), and strong (+++). Mobility shifts of Sav and its fragments are also indicated (s). FBM, FERM-binding motif; W, WW domain; SARAH, SARAH domain. Full-length Sav and fragments containing the inter-WW/SARAH domain region bind to Sik2 (b). (c, d) Sik2 binds to Sav with a region close to the C-terminus. Structure of Sik2 and fragments thereof used in the Sik2/Sav binding assay (c). Binding to Sav is indicated by (+). Sik2-ΔKIN, Sik2 without kinase domain. Full-length Sik2 and fragments containing the C-terminal region encompassing amino acids 1054 to 1250 bind to Sav (d). Co-IP experiments (b, d) were performed in S2 cells transfected with indicated plasmids, and cells were lysed 48h later. Lysates and FLAG-purified immunoprecipitates were subjected to western blot analysis using the indicated antibodies. The arrowheads indicate the IgG heavy chain (at 50kD) and the IgG light chain (at 25kD), the asterisk denotes an unspecific band.
Figure S7 Sik2 phosphorylates Sav on S413. (a) Sik2 causes the Sav double mutant S162/413A to shift comparably to the single S413A mutant. (b) Sik2-induced phosphorylation at Sav-S413A can be reverted by λ phosphatase treatment. (c, d) Testing putative Sik2 phosphorylation sites in Sav. The Sav mutants S382A, S380-T381-S382A (c) and T39A (d) do not decrease the shift observed in wt Sav. S2 cells (a–d) were transfected with the indicated plasmids, and cells were lysed 48h later and subjected to western blot analysis using a 7.5 % (a), 8% (b) or 4-15% gradient (c, d) SDS-PAGE gel and the indicated antibodies. (e, f) Sik2 forms a complex with Hpo and Sav. The association between Hpo and Sav is not altered by Sik2 co-expression (e), and Hpo expression does not affect the Sav/Sik2 interaction (f). Co-IP experiments in (e) and (f) were performed in S2 cells transfected with indicated plasmids, and cells were lysed 48h later. Lysates and FLAG-purified immunoprecipitates were subjected to western blot analysis using the indicated antibodies. The arrowheads indicate the IgG heavy chain (at 50kD). Asterisks denote unspecific bands.
Figure S8 Sav’s Hpo/Wts bridging function is modulated by Sik kinases.
(a) The association between Sav and Hpo is neither altered by Hpo kinase activity, nor by the Sav-S413A mutation. (b) The association between Sav and Sik2 is neither altered by Sik2 kinase activity, nor by the Sav-S413A mutation. (c) The Sav/Mer interaction is not modulated by Sik2. Co-IP experiments in (a) to (c) were performed in S2 cells transfected with indicated plasmids, and cells were lysed 48h later. Lysates and FLAG-purified immunoprecipitates were subjected to western blot analysis using the indicated antibodies. The arrowheads indicate the IgG heavy chain (at 50kD) and the IgG light chain (at 25kD). Asterisks denote unspecific bands, and open arrowheads indicate bands (a, Hpo) from a previous exposure. (d) The phosphorylation site S413 in Sav is not conserved in human SAV1. S2 cells were transfected with indicated plasmids, lysed 48h later, and subjected to western blot analysis using the indicated antibodies. The arrowhead indicates the IgG heavy chain (at 50kD), the asterisk denotes an unspecific band. (e) Human SIK2 induces YAP-dependent transcription in a GAL4-DBD-TEAD4 luciferase assay. HEK293 cells were transfected with indicated plasmids, lysed 24h later and analysed using a Dual Luciferase Reporter Assay. RLU denote average of relative luciferase units, and all error bars represent s.d., n=6.
Figure 2a

Figure 2b

Figure 6a

Figure 6b

Figure 7b

Figure S9 Uncropped western blot scans
Figure 7c

FLAG | HA | Tub
---|---|---

Figure 7d

Lysates, pS413-Sav | Lysates, FLAG | Lysates, HA | Lysates, Tub
---|---|---|---

IP:FLAG, IB: pS413-Sav | IP:FLAG, IB: pFLAG | IP:FLAG, IB: pHA

Figure 7e

pS413-Sav | FLAG | HA | Tub
---|---|---|---

Figure 8a

pS168-Yki | Yki | FLAG | HA | Tub
---|---|---|---|---

Figure 8b

pS168-Yki | FLAG | HA | Tub
---|---|---|---

Figure S9 continued
**Figure 8c**

IP: FLAG, V5  
Lysates, V5  

IP: FLAG, Myc  
Lysates, Myc  

IP: FLAG, HA  
Lysates, HA  

IP: FLAG, FLAG  
Lysates, FLAG  

**Figure 8e**

IP: FLAG, IB: HA  
Lysates, IB: HA  

IP: FLAG, IB: Myc  
Lysates, IB: Myc  

IP: FLAG, IB: FLAG  
Lysates, IB: FLAG  

**Figure 8f**

pS168-Yki  
HA  

FLAG  

Tub  

Lane 4 3 2 1  
Lane 4 3 2 1  
Lane 4 3 2 1  
Lane 4 3 2 1  

Figure S9 continued
Figure S7c

Figure S7d

Figure S7e

Figure S7f

Figure S9 continued
Figure S8a
IP: FLAG, IB: FLAG
IP: FLAG, IB: HA
Lysates, IB: HA
Lysates, IB: FLAG

Figure S8b
IP: FLAG, IB: HA
IP: FLAG, IB: FLAG
Lysates, IB: HA
Lysates, IB: FLAG

Figure S8c
IP: FLAG, IB: HA
IP: FLAG, Myc
IP: FLAG, FLAG
Lysates, IB: HA
Lysates, IB: Myc
Lysates, IB: FLAG

Figure S8d
pS413-Sav
FLAG
HA
Tub

Figure S9 continued