Supplementary Information

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'-CACCGCCACCACACCAACGGCTG-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 S9: Uncropped western blot scans

Supplementary Table 1: Candidates from the genome-wide screen and enriched hit list

All candidates from the genome-wide RNAi screen that scored at least three standard deviations away from the mean and that were selected for luciferase assay-based re-screening are shown. From this re-screening process, a selection of candidates was further designated as enriched hit list (see Fig. 1e). These candidates were then subjected to biochemical screening using the pS168-Yki antibody. Potential pathway activators displayed a decrease of pS168-Yki levels and, conversely, inhibitors showed increased levels. For these candidates, the gene symbols are annotated. Z-scores are shown for the present screen and a published screen performed with the same dsRNA library using the GAL4-DBD-Yki assay[57]. Note that the readouts of these two screens are opposite. The primary candidate Sik3 is represented as bold. Y = yes, N = no.

Supplementary Table 2: Fly genotypes

All fly genotypes used in this study are listed.