Supplemental Experimental Procedure

***Drosophila* genotypes used for clonal analysis:**

GFP$^-$ *hpo*$^-$ clones:
hs-Flp; FRT42D hpo$^{42\text{-}47}$ / FRT42D Ubi-GFP

GFP$^-$ *wts*$^-$ clones:
hs-Flp; FRT82B wts^{X1} / FRT82B Ubi-GFP
hs-Flp; FRT82B wts^{X1} / FRT82B Arm-lacZ

GFP$^-$ *sav*$^-$ clones:
hs-Flp; FRT82B sav^3 / FRT82B Ubi-GFP

GFP$^-$ *mats*$^-$ clones:
hs-Flp; FRT82B mats^{e235} / FRT82B Ubi-GFP

GFP$^-$ *yki*$^-$ clones:
hs-Flp; FRT42D yki^{B5} / FRT42D Ubi-GFP

GFP$^-$ *Cka*$^-$ clones:
hs-Flp; FRT40A Cka^{s1883}/ FRT40A Ubi-GFP

GFP$^+$ clones overexpressing *yki*:
hs-Flp; actP>CD2>Gal4; UAS-GFP/UAS-yki

GFP$^+$ *sd*$^-$ clones overexpressing *yki*:
tub-Gal80 hs-Flp FRT19A/sd^{47M} FRT19A; UAS-GFP; tub-Gal4/UAS-yki

Molecular biology
Full-length cDNA clones for *cact* (LD10168), *Tl* (RE13664) and *Pll* (LD43152) were obtained from *Drosophila* Genomics Resource Center (DGRC). Tl^{10b} was generated by mutating Cys781 of Tl into Tyr using QuickChange Lighting kit (Agilent). Tl^{10b}-FLAG and FLAG-Cact were constructed by adding a FLAG tag (MDYKDDDDK) to the C-terminus and N-terminus respectively using the pAc5.1/V5-HisB vector (Invitrogen). HA-Pll was generated by adding an N-terminal HA tag (MYPYDVPDYA) using the same vector. FLAG-Cact was cloned into pUASTattB vector and used to generate UAS-Cact *Drosophila* line. V5-Wts, Myc-Hpo and Myc-Hpo^{KD} (kinase dead) have been described previously (Huang et al., 2005; Wu et al., 2003). All plasmids were verified by DNA sequencing (Macrogen).

Cell culture
Mbn2 cells were purchased from DGRC. S2 cells, S2R+ cells and Mbn2 cells were cultured in *Drosophila* Schneider's Medium (Gibco) supplemented with 10% FBS and antibiotics. Gene knockdown in cultured cells was performed following the bathing protocol provided by *Drosophila* RNAi Screening Center (DRSC). To make heat-inactivated bacteria, 4 ml of overnight cultured *S. Aureus* (OD_{600}=2) or *E. Faecalis* (OD_{600}=2) suspension was spun down (5000 g for 5 min). After re-suspending in 1 ml ddH_2O, the bacteria were treated under 65^oC for 1.5 hrs. For western blot in cultured cells, cells were seeded in 12 well culture plate and transfected with Effectene transfection reagent (Promega) after 24 hrs. 48 hrs later, cell lysate was prepared by boiling in standard SDS loading buffer for 10 min and analyzed by SDS-PAGE. Signals were detected using ECL Western Blotting Substrate, SuperSignal Dura Extended Duration Substrate or SuperSignal Femto Maximum Sensitivity Substrate (Pierce).

Antibodies
Anti-Cact antibody 3H12 was described previously (Whalen and Steward, 1993) and was obtained from Developmental Studies Hybridoma Bank (DSHB). Phospho-antibody against the activation loop of MST1/MST2/Hpo (Hpo-Thr^{195}) was obtained from Cell Signaling (#3681). Anti-Hpo antibody has been described (Wu et al., 2003). Phospho-Yki and Yki antibodies were described previously (Dong et al., 2007).

Immunostaining
Third instar larvae were dissected in PBS and fixed with 4% paraformaldehyde for 30 min at 37^{o}C (fat body staining) or 15 min at room temperature (wing disc staining). The tissues were then washed with PBST and blocked in 5% goat serum for 30 min. After incubating with 1:50-1:100 dilution of the primary antibody at room temperature for 2 hrs, the samples were washed for 3 times with PBST and followed by incubating with the corresponding secondary antibody at room temperature for 2 hrs. The samples were washed for 3 times and mount with VECTASHIELD mounting medium containing the nuclear dye DAPI.

RNA preparation and quantitative real-time PCR
Sex-matched adults were infected with *S. Aureus* (OD_{600}=2) or *Ecc15* (OD_{600}=200) and kept at 29^{o}C. After 6 hrs, live flies were collected and frozen in liquid nitrogen until usage. Flies were homogenized in liquid nitrogen to break the tissues. RNA was extracted using Trizol Reagent (Invitrogen) and purified using RNeasy Mini Kit (Qiagen) following the kit manual. For each sample, 1 μg RNA was used for reverse transcription using iScript cDNA synthesis kit (BioRad). 100 ng cDNA was used for real-time PCR with SYBR master mix (BioRad). Real-time PCR was performed using CFX96 real-time system (BioRad).

Luciferase assay
Luciferase assay was performed as previously described (Wu et al., 2008). Briefly, *Drosophila* S2R+ cells were seeded on 48 well plate. After 24 hrs, cells were transfected with the desired luciferase reporter constructs together with Renilla luciferase reporter plasmid (as the internal control) using Effectene transfection reagent (Promega). Luciferase assay was performed at 24 hrs post transfection by using Dual Luciferase Assay system (Promega) following the manufacturer's instructions and a FLUOstar Lumiometer (BMG Lab Technologies).

In Vitro kinase assay
For kinase assay with Hpo as substrate, S2 cells were transfected with FLAG-Pll and FLAG-Tao1 plasmids. 48 hrs post-transfection, cells were lysed with lysis buffer (50 mM Tris at PH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, protease inhibitor cocktail (Roche), 50 mM NaF, 1.5 mM Na_3VO_4) and immunoprecipitated with anti-FLAG agarose beads (Biolegend). The immunoprecipitated Pll and Tao1 were incubated with recombinant GST-Hpo^{KD} in kinase buffer containing 25 mM HEPES PH 7.2, 25 mM $MgCl_2$, 50 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.5 mM sodium vanadate, 10 μM ATP at 37^{o}C for 30 min.

For kinase assay with Cka as substrate, S2R+ cells were transfected with HA-Pll, lysed with lysis buffer (see above) and immunoprecipitated with anti-HA antibody (Sigma). *In vitro* kinase assay was performed as previously described (Allen et al., 2007). Briefly, the immunoprecipitated Pll was subjected to kinase assay in the presence of 1mM ATP-γ S, 10 mM HEPES PH 7.4, 150 mM NaCl, 10 mM $MgCl_2$ and 1 μg GST-Cka. The reaction mixtures were incubated for 30 min at 30^{o}C, which was further supplemented with 2.5 mM PNBM for 2 hrs at room temperature. The mixtures were boiled and analyzed by SDS-PAGE.

Supplemental Table S1. Quantification of clonal phenotypes, related to Figure 2, 3, 4, 5, 6, 7, S2 and S4.

Figure #	Phenotype	Positive/Total
Figure 2A	Reduced Drs-GFP in *wts* mutant cells	13/21
Figure 2B	Normal Dpt-GFP in *wts* mutant cells	18/18
Figure 2C	Increased Cact in *hpo* mutant cells	27/33
Figure 2D	Increased Cact in *sav* mutant cells	23/28
Figure 2E	Increased Cact in *wts* mutant cells	32/34
Figure 2F	Increased Cact in *mats* mutant cells	25/26
Figure 3A	Increased Cact in Yki-overexpression cells	40/41
Figure 3B	Reduced Cact in *yki* mutant cells	7/10
Figure 3C	Normal Cact in *sd* mutant Yki-overexpression cells	23/23
Figure 3D	Increased cact-lacZ in Yki-overexpression cells	39/53
Figure 3E	Increased cact-lacZ in *wts* mutant clones	20/23
Figure 4C	Increased #4-lacZ in Yki-overexpression clones	21/22
Figure 4D	Normal #4M1-lacZ in Yki-overexpression clones	20/20
Figure 4E	Increased #4-lacZ in Yki-overexpression cells	27/35
Figure 4F	Normal #4M1-lacZ in Yki-overexpression cells	45/45
Figure 5C	Reduced cytoplasmic Yki in *hpo* mutant cells	19/24
Figure 6F	Reduced cytoplasmic Yki in *pll* mutant cells	22/28
Figure 6G	Normal Yki localization in Cact-overexpression cells	55/55
Figure 6H	Increased Yki cytoplasmic localization in Tl-overexpression cells	38/51
Figure 6I	Increased Yki cytoplasmic localization in Pll-overexpression cells	43/46
Figure 7B	Increased Yki cytoplasmic localization in *Cka* mutant cells	17/25
Figure 7H	Reduced Cka in Pll-overexpression cells	26/26
Figure 7I	Reduced Cka in Pll-overexpression cells	23/23
Figure 7K	Increased Cka in Pll-knockdown cells	29/35
Figure 7L	Increased Cka in Pll-knockdown cells	32/33
Figure S2A	Increased Cact in *hpo* mutant clones	18/22
Figure S2B	Increased Cact in *sav* mutant clones	21/24
Figure S2C	Increased Cact in *wts* mutant clones	47/48
Figure S2D	Increased Cact in *mats* mutant clones	50/53
Figure S4A	Increased Cact in Yki-overexpression clones	34/34
Figure S4B	Increased Cact in Yki-overexpression cells	29/29
Figure S4C	Increased Cact in Yki-overexpression cells	15/15
Figure S4D	Normal Cact in *sd* mutant Yki-overexpression clones	27/27

Supplemental References

Whalen, A.M., and Steward, R. (1993). Dissociation of the dorsal-cactus complex and phosphorylation of the dorsal protein correlate with the nuclear localization of dorsal. J Cell Biol 123, 523-534.