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Supplemental Data

Elucidation of a Universal Size-Control Mechanism in *Drosophila* and Mammals

Jixin Dong, Georg Feldmann, Jianbin Huang, Shian Wu, Nailing Zhang, Sarah A. Comerford, Mariana F. Gayyed, Robert A. Anders, Anirban Maitra, and Duojia Pan

Supplemental Experimental Procedures

Cell Transfection and Western Blotting

S2 cells were propagated in *Drosophila* Serum Free Medium (SFM, Invitrogen) supplemented with L-Glutamine and antibiotics. Myc-Hpo, Flag-Sav, V5-Wts and HA-Yki constructs were described previously (Huang et al., 2005). HA-myrAkt was gift from Morris Birnbaum (Verdu et al., 1999). For insulin treatment, S2 cells were incubated with 10μg/ml bovine insulin (Sigma) for 20 min. Cell fractionation was carried out according to a previous report (Kim and Chen, 2000). Antibodies against 14-3-3 zeta/leonardo and dSREBP were kindly provided by Efthimios Skoulakis and Robert Rawson, respectively. Other antibodies were: P-Akt(S473) (Cell Signaling), P-S6K(T389) (Cell Signaling), HA (Sigma) and V5 (Invitrogen). In vitro kinase assay was conducted as described previously (Huang et al., 2005), except that the P-Yki(S168) antibody was used to visualize reaction products in place of ^{32}P.

HPNE cells were maintained in 75% DMEM and 25% M3 base medium (InCell Corporation) supplemented with 5% FBS and 10ng/ml of EGF (Invitrogen). All other cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum and antibiotics (Invitrogen). The Lats1/2, Mst1/2 and hWW45 expression plasmids were gift of Herman Sillje (Chan et al., 2005), and the GFP-YAP plasmid was gift of Marius Sudol. Cells were lysed in RIPA buffer (150mM sodium chloride, 50mM Tris-HCl at pH7.4, 1% NP-40, 0.5% sodium-deoxycholate, 0.1% SDS, 1 mM PMSF) with protease

inhibitors (Roche). The proteins were separated on SDS-polyacrylamide gels and transferred onto PVDF membranes (Millipore). The blots were probed with antibodies against Myc (Santa Cruz Biotechnology), Flag (Sigma), YAP (Cell Signaling), cleaved caspapse-3 (Asp175) (Cell Signaling), PARP (Clone C2-10, BD PharMingen), β-actin (Chemicon International).

To establish the YAP-overexpressing cell line, 0.5μg of plasmid (pcDNA3.1-hYAP or –hYAP^{S127A}) was transfected with SuperFect (QIAGEN) in HPNE cells in 6-well plates and the cells were selected with G418-selective medium (400μg/ml) 48 hours after transfection for 3-4 weeks. For RNAi knockdown of BIRC5/survivin, shRNAs targeting control and *BIRC5*/*survivin* (Open Biosystems) were transfected into YAP-HPNE cells to establish stable cell lines. For YAP-knockdown stable cell lines, shRNAs specifically targeting human YAP and control shRNA (Open Biosystems) were transfected with Effectene in H2227 and PK-9 human cancer cell lines and the cells were selected with the antibiotic medium (puromycin, 0.5-2μg /ml) 48 hours after transfection for 3 weeks.

Soft Agar Assay and Tumorigenic Growth in Mice

Two ml of medium containing 1% agarose (Invitrogen) were poured into each well of 6-well plates. After the agarose had solidified, another 2 ml of medium containing 0.6% agarose with 10,000 (for Figure 4E) or 20,000 (for Figure 6I) tester cells was applied on top of the first layer. Finally, 2 ml of full medium was pipetted on top and the plates incubated for 3 (for Figure 4E) or 4 (for Figure 6I) weeks. The medium was changed weekly. To assess colony formation, the cells were stained with 1.5 ml of 0.5% Wright's staining solution. Colonies were visualized by trans-UV illumination and counted using the analysis software Quantity One (BioRad).

For assessment of *in vivo* growth of human cancer cells with YAP knockdown, 2.5x10^6 cells in 100 μl PBS and 50 μl matrigel (BD Biosciences) were injected subcutaneously into male athymic CD1 *nu/nu* mice. Tumor volumes V were determined after measuring the larger (a) and smaller (b) diameters as $V = \frac{a \cdot b^2}{2}$.

Mouse Microarray Analysis

Male mice (3 transgenic and 3 non-transgenic from the same litter) were induced by Dox for 2 weeks. Liver total RNA was isolated with TRIzol reagent (Invitrogen) and further cleaned up by RNeasy kit (QIAGEN). RNA from control and experimental tissues were processed using the RNA amplification protocol described by Affymetrix (Affymetrix Expression Manual) and 10μg of total fragmented cRNA were hybridized to the Affymetrix GeneChip arrays (Mouse genome 430 2.0). Affymetrix Fluidics Station 450 was then used to wash and stain the Chips. Fluorescence was detected using the Affymetrix GS3000 GeneArray Scanner and image analysis of each GeneChip was done through the GeneChip Operating System software from Affymetrix (GCOS1.3) using the standard default settings. To estimate the gene expression signals, data analysis was conducted on the chips' CEL file probe signal values at the Affymetrix probe pair (perfect match (PM) probe and mismatch (MM) probe) level, using the statistical technique RMA (Robust Multi-array expression measure) with Affy. Principle component analysis was performed to assess sample variability. An empirical Bayes method with the Gamma-Gamma modeling was used to estimate the posterior probabilities of the differential expression of genes between the control and YAP samples. The criterion of the posterior probability > 0.5 was used to produce the differentially expressed gene lists. Hybridization and raw data analysis were done by the Microarray Core Facility at the Johns Hopkins University.

Quantitative Real-Time PCR

Total liver cellular RNA was extracted using TRIzol reagent (Invitrogen). RNA was reverse transcribed with oligo-dT primers at 42°C for 50 min using the Superscript 1st Strand System for RT-PCR (Invitrogen). For real-time quantitative PCR (Q-PCR), 1 μl of the RT reaction was amplified using the Quantitect SYBR Green master mix (QIAGEN) in a total volume of 25 μl on a 7300 Real-Time PCR System (Applied Biosystems) with 35 cycles of denaturation at 95 ˚C for 15 sec, annealing at 61 ˚C for 30 sec and extension at 72 ˚C for 30 sec. Q-PCR was done in triplicate, using PGK1 as a housekeeping control. Relative fold differences in expression of candidate genes expressed in control and YAP

upregulated livers were determined using 2 -ΔΔCt method. Murine primer sequences are available on request.

TUNEL Staining and BrdU Labeling

To induce hepatocytes apoptosis, 4-week old mice (induced by Dox for 1 week) were injected intraperitoneally with 10 μg of monoclonal agonistic anti-Fas antibody (Clone Jo-2, BD PharMingen) essentially as described (Comerford et al., 2003). Liver samples were harvested 3 hours after injection. Apoptosis was detected by In Situ Cell Death Detection Kit, TMR Red (Roche) following the manufacturer's protocol with some modifications. Briefly, the slides were heated at 55°C for 30 min followed by washing in xylene and rehydration through a graded series of ethanol and distilled water. Tissue sections were then treated with 15μg/ml of proteinase K (Roche) in 10 mM Tris-HCl (pH7.4) for 30 min at room temperature. After washing with PBS, TUNEL reaction mixture was added to the section and incubated for 1 h at 37°C in humidified atmosphere in the dark.

For BrdU labeling, mice were injected intraperitoneally with 50mg BrdU/kg body weight. Livers were harvested, fixed and sectioned as described above 1 hour postinjection and incorporated BrdU was detected by immunostaining with anti-BrdU antibody and Cy3-labeled secondary antibody.

Immunohistochemical Analysis of YAP Protein Expression on Human Cancer Tissue Microarrays

Tissue microarrays (TMAs) were obtained from the Johns Hopkins TMA core facility. One hundred and fifty tumors corresponding to 25 cancers each from the lung, pancreas, prostate, ovary, colorectum and liver were stained for YAP expression. Unmatched non-cancerous specimens from all six anatomic sites were available on these TMAs as "control", and were used for assessment of YAP expression in normal tissues. Immunohistochemistry was performed using an anti-YAP antibody (Cell Signaling, 1:100 dilution), following citrate buffer antigen retrieval. YAP labeling was assessed as

with or without prominent nuclear accumulation, and tabulated independently for each cancer type.

Supplemental References

Chan, E.H., Nousiainen, M., Chalamalasetty, R.B., Schafer, A., Nigg, E.A., and Sillje, H.H. (2005). The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. Oncogene *24*, 2076-2086.

Comerford, S.A., Clouthier, D.E., Hinnant, E.A., and Hammer, R.E. (2003). Induction of hepatocyte proliferation and death by modulation of T-Antigen expression. Oncogene *22*, 2515-2530.

Huang, J., Wu, S., Barrera, J., Matthews, K., and Pan, D. (2005). The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. Cell *122*, 421-434.

Kim, J.E. and Chen, J. (2000). Cytoplasmic-nuclear shuttling of FKBP12-rapamycin-associated protein is involved in rapamycin-sensitive signaling and translation initiation. Proc. Natl. Acad. Sci. U. S. A *97*, 14340-14345.

Verdu, J., Buratovich, M.A., Wilder, E.L., and Birnbaum, M.J. (1999). Cell-autonomous regulation of cell and organ growth in Drosophila by Akt/PKB. Nature Cell Biol. *1*, 500-506.

Figure S1

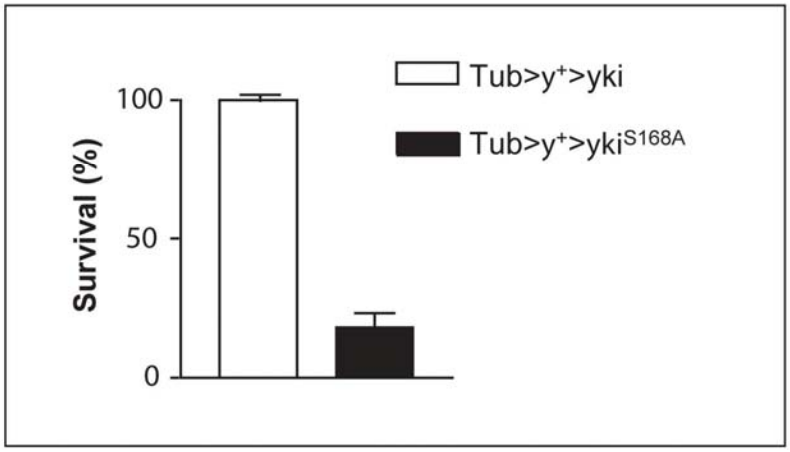

Figure S1. Flp-out of Tub>yki^{S168A} in the eye resulted in substantial animal lethality.

ey-FLP flies were crossed to Tub>y^{+}>yki/TM3 or Tub>y^{+}>yki^{S168A}/TM3 flies at room temperature. The number of F1 progeny of the genotype ey-FLP; Tub>y^{+}>yki or ey-FLP; Tub>y^{+}>yki^{S168A} were calculated as a percentage of ey-FLP; TM3 siblings from the same cross. Only 18% of ey-FLP; Tub>y^{+}>yki^{S168A} flies survived to adults. Even fewer ey-FLP; Tub>y^{+}>yki^{S168A} flies (<2%) survived to adulthood if the crosses were done at 25^{O}C.

Figure S2

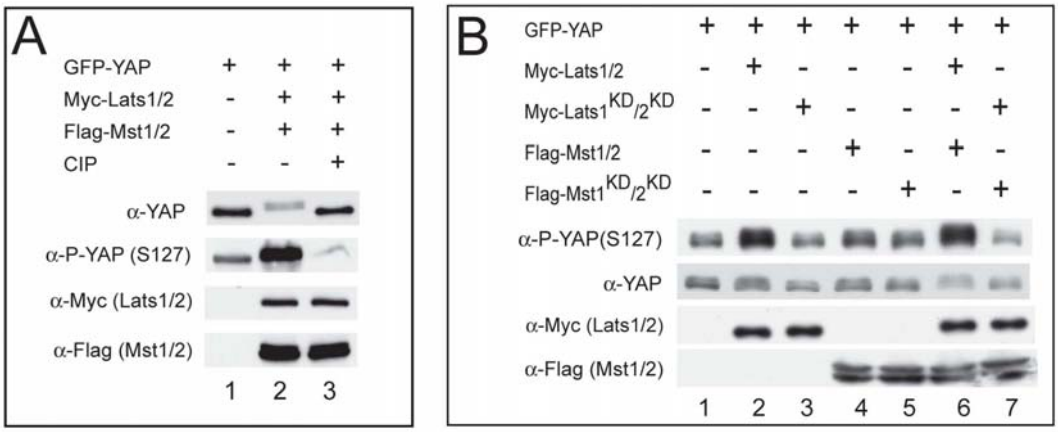

Figure S2. YAP phosphorylation by Mst1/2 and Lats1/2.

(A) Phosphatase (CIP) treatment reversed the mobility shift of YAP induced by Mst1/2 and Lats1/2 in HEK293 cells. Compare lanes 2 and 3.

(B) Linking Mst1/2-Lats1/2 to YAP phosphorylation in HPNE cells. Lysates from HPNE cells expressing various epitope-tagged proteins were probed with the indicated antibodies. Note the increased YAP(S127) phosphorylation resulting from Lats1/2 (lane 2), Mst1/2 (lane 4) or both (lane 6), but not the respective kinase-dead forms or their combinations (lanes 3, 5 and 7).

Figure S3

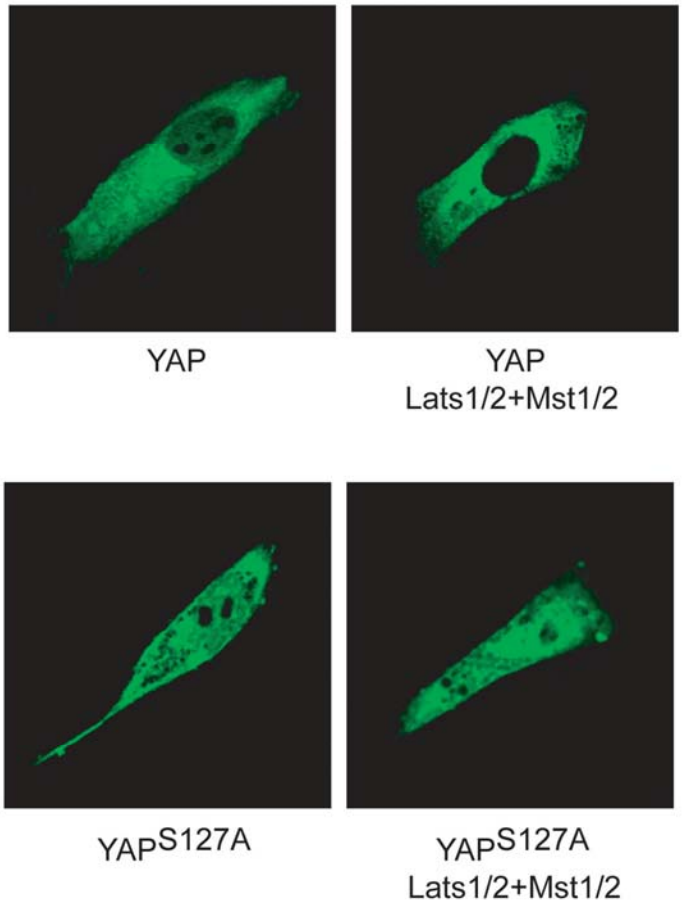

Figure S3. The mammalian Hippo pathway promotes the cytoplasmic localization of YAP via S127 phosphorylation.

GFP-YAP or GFP-YAP^{S127A} was expressed in HPNE cells with or without Lats1/2 and Mst1/2, and visualized for GFP localization. While both YAP (upper left) and YAP^{S127A} (lower left) can be detected in the nucleus as well as the cytoplasm, YAP^{S127A} showed a more intense nuclear signal. Upon co-expression of Lats1/2 and Mst1/2, YAP showed exclusive localization to the cytoplasm (upper right), whereas the localization of YAP^{S127A} was unchanged (lower right).

Figure S4

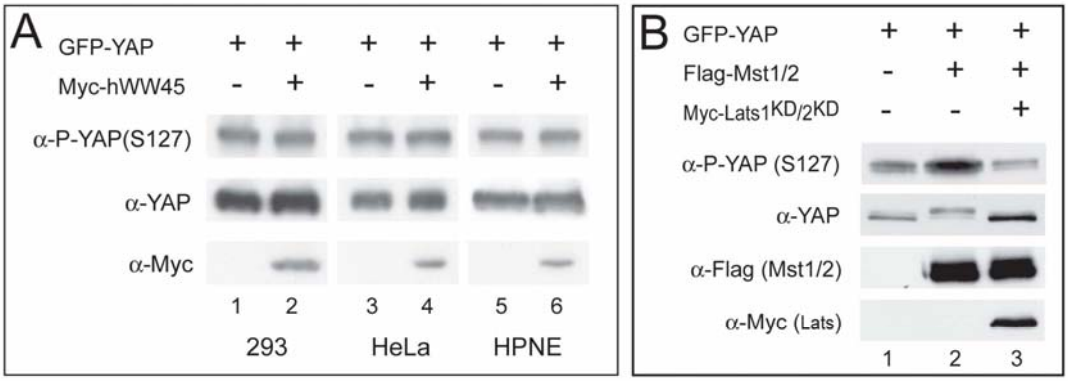

Figure S4.

(A) Overexpression of hWW45 does not stimulate YAP(S127) phosphorylation in hWW45-positive cell lines. HPNE, Hela or HEK293 cells were transfected with YAP with or without Myc-tagged hWW45 and probed with α-P-YAP(S127), α-YAP and α-Myc antibodies. Note the similar YAP(S127) phosphorylation levels with (lanes 2, 4, 6) or without (lanes 1, 3, 5) hWW45 overexpression.

(B) Expression of dominant negative Lats1/2 reversed Mst1/2-induced YAP phosphorylation. Lysates from HEK293 cells expressing the indicated constructs were analyzed. The suppression of Mst1/2-induced phosphorylation is evident from the P-YAP (S127) signal and the mobility shift (compare lanes 2 and 3).

Figure S5

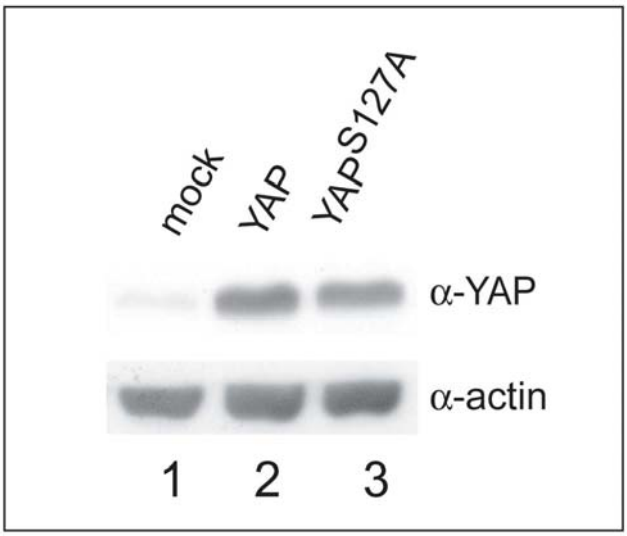

Figure S5. Establishment of stable HPNE cell lines expressing similar levels of YAP and YAP^{S127A}.

HPNE cells stably transfected with an empty vecotr (lane 1), YAP (lane 2) or YAP^{S127A} (lane 3) were probed with antibodies against YAP and actin. Multiple independent lines were established, and only representative lines were analyzed here.

Figure S6

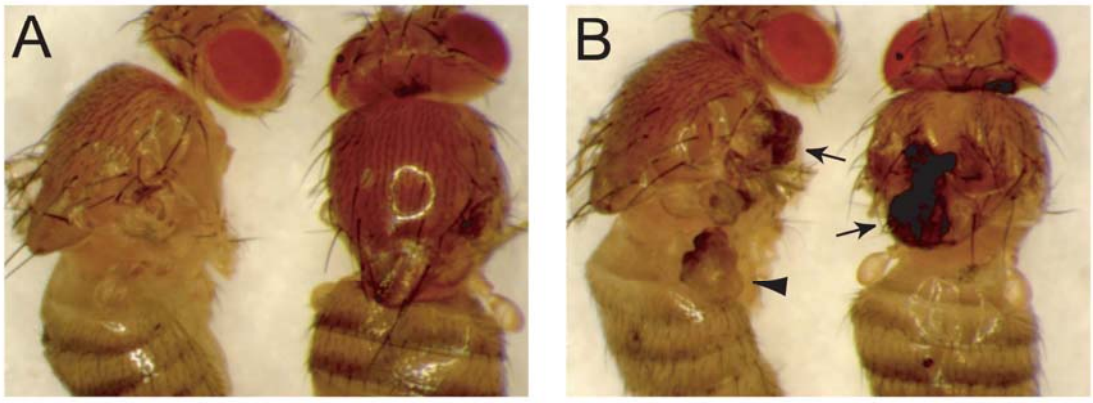

Figure S6. Gain-of-function activity of YAP^{S127A} in *Drosophila*.

Flies of the genotype hsflp; Act>CD2>Gal4; UAS-YAP (A) or hsflp; Act>CD2>Gal4; UAS-YAP^{S127A} (B) that had been heatshocked at first instar larval stage. Wings had been removed for photographic purpose. Note the enlarged haltere (arrowhead) and thoracic overgrowth (arrows) in (B) only.

Figure S7

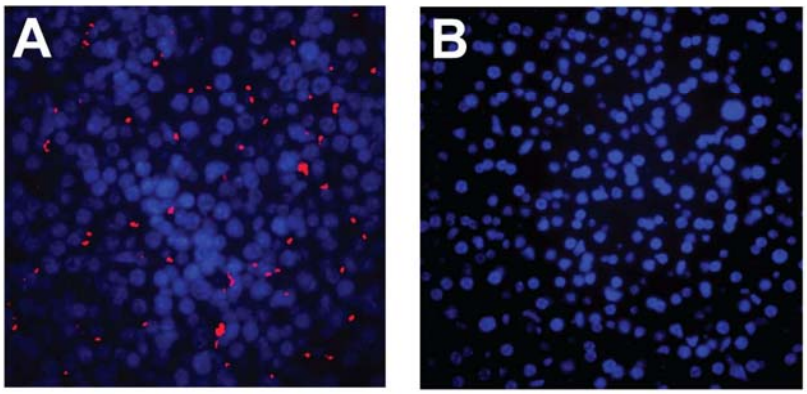

Figure S7. The regression of liver size in the YAP transgenic mice is accompanied by apoptosis. The ApoE/rtTA-YAP transgenic mice were fed Dox-containing water for 2 weeks starting at 3 weeks of age. Dox was either withdrawn (A), or continued (B), for three days, at which point the livers were analyzed by TUNEL (red). Note the occurance of apoptosis in A, but not in B.

Figure S8

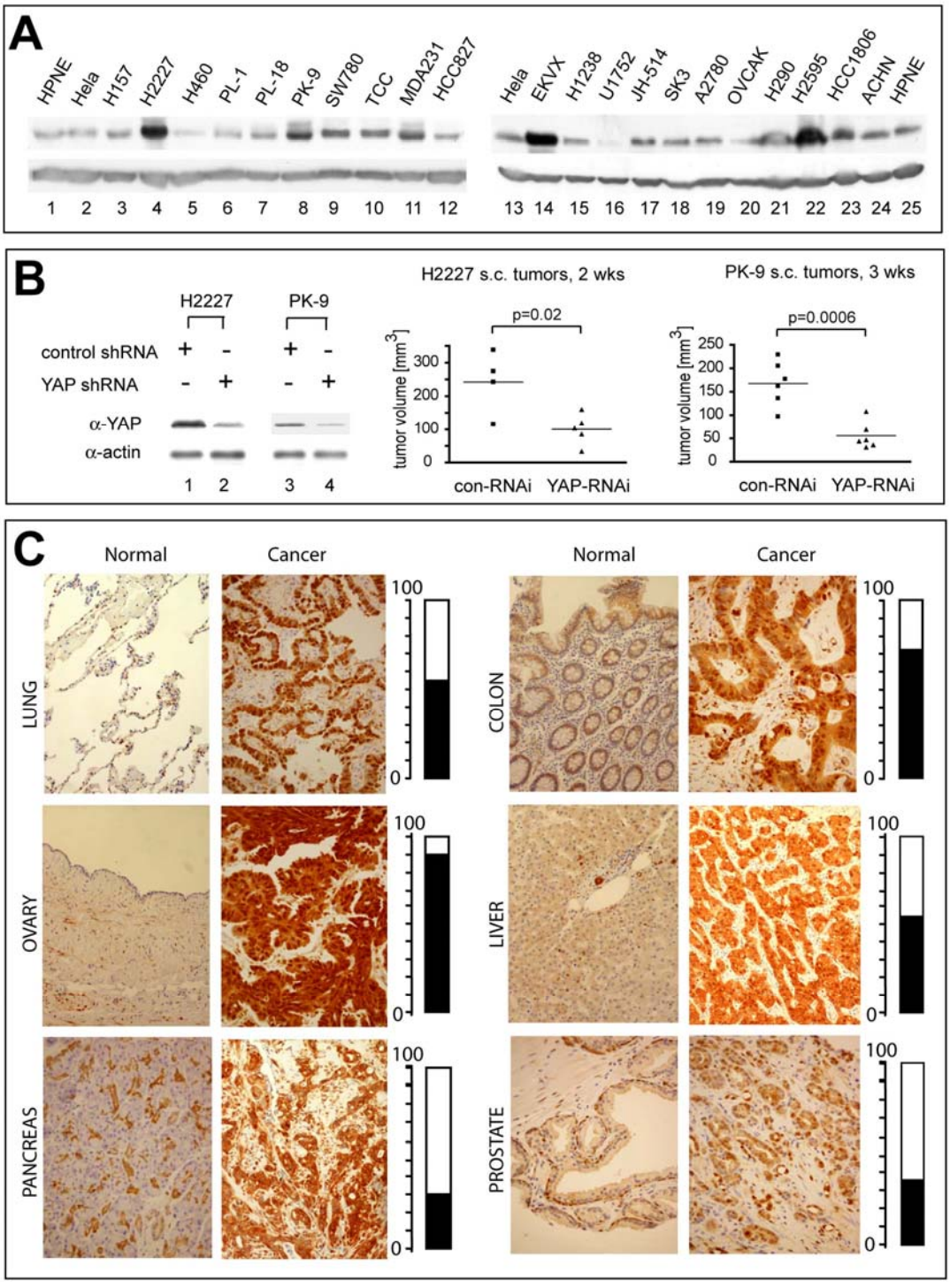
A
HPNE
Hela
H157
H2227
H460
PL-1
PL-18
PK-9
SW780
TCC
MDA231
HCC827
Hela
EKVX
H1238
U1752
JH-514
SK3
A2780
OVCAK
H290
H2595
HCC1806
ACHN
HPNE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
B
H2227
PK-9
control shRNA
YAP shRNA
α-YAP
α-actin
1 2 3 4
H2227 s.c. tumors, 2 wks
p=0.02
tumor volume [mm3]
con-RNAi
YAP-RNAi
PK-9 s.c. tumors, 3 wks
p=0.0006
C
Normal
Cancer
LUNG
COLON
OVARY
LIVER
PANCREAS
PROSTATE
100
0

Figure S8. The prevalence of YAP overexpression in human cancer.

(A) A series of 22 human cancer cell lines from a variety of anatomic sites were probed for YAP protein expression by western blot (upper gel: YAP; lower gel: actin). The cancer cell lines are: Hela (cervical); H157, H2227, H460, EKVX, H1238, U1752 (lung); PL-1, PL-18, PK-9 (pancreatic); SW780, TCC (bladder); MDA231, HCC827, HCC1806 (breast); JH-514, SK3, A2780, OVCAK (ovarian); H290, H2595 (mesothelioma); ACHN (renal). HPNE cells were used as a "control" line to normalize for YAP expression. YAP is overexpressed in approximately ~25% of cancer cell lines, including H2227, PK-9, MDA231, EKVX, and H2595 cells.

(B) RNAi knockdown of YAP decreased the tumorigenicity of YAP high-expressing cancer cells. Stable cell lines of PK-9 or H2227 expressing YAP shRNA constructs were established. Greater than 80% knockdown is observed in the shRNA transfected cells compared to control shRNA transfected controls (left). Subcutaneous xenografts were generated from each paired control and YAP shRNA expressing cell line, and tumor volumes measured at 2 or 3weeks post-injection. In both PK-9 and H227 xenografts, there is significant inhibition of in vivo growth upon downregulation of YAP expression (right).

(C) Tissue microarrays containing human cancer specimens from six anatomic sites (lung, ovary, pancreas, colorectum, liver, and prostate) and non-neoplastic tissues were stained for immunohistochemical assessment of YAP protein expression. YAP overexpression was frequently detected in lung (18/18 examined), ovarian (20/20 examined), pancreatic (19/20 examined), colorectal (16/19 examined), hepatocellular (13/20 examined), and prostate (19/19 examined) carcinomas. For each anatomical site, the left image shows YAP expression in the normal tissue and the right image shows YAP expression in the cancer tissue, while the corresponding histogram shows the proportion of cancer cells with ("+N") or without ("-N") prominent nuclear accumulation. In the histogram, black = "+N" and white = "-N". Note that the proportion of "+N" and "-N" YAP patterns varies by cancer types. Nearly all ovarian cancers display the "+N" pattern, while 2/3 of pancreatic cancers have a "-N" pattern of expression.