Supplemental Information

SI Materials and Methods

Cell culture and transfection

HEK293T, HeLa, RKO, SW480, and DLD-1 cell lines were purchased from American Type Culture Collection (ATCC). The cell lines were authenticated at ATCC and were used at low (<25) passages. HCT116 cell line was a gift from Dr. Michael Brattain (University of Nebraska Medical Center), who originally established and deposited this cell line to ATCC. RKO cells were cultured in MEM, DLD-1 cells were maintained in RPMI-1640, and HCT116 cells were maintained in McCoy's 5A media. Other cell lines were maintained in DMEM media and all cell lines were supplemented with 10% FBS and 1% penicillin/streptomycin. Attractene and HiPerFect (Qiagen) were used for transient overexpression and siRNA transfections, respectively, following the manufacturer's instructions. Nocodazole (100 ng/ml for 16-20 h) and taxol (0.1 μM for 16 h) were used to arrest cells in G2/M phase unless otherwise indicated. SiRNAs for Zyxin (#1:guguuacaagugugaggac; #2:guccucacacuuguaacac) were purchased from GenePharma. SiRNA for CDK8 (#1:agugcuagcacaguuugug; #2:cacaaacugugcuagcacu) and Cyclin B1 (aagaaauguacccuccagaaa) were obtained from Sigma-Aldrich. VX680 (Aurora-A, -B, -C inhibitor), ZM447439 (Aurora-B, -C inhibitor), and BI2536 (Plk1 inhibitor) were from Selleck Chemicals. U0126 (MEK-ERK inhibitor), SB203580 (p38 inhibitor), LY294002 (PI-3K inhibitor), rapamycin (mTOR inhibitor), and SP600125 (JNK inhibitor) were from LC Laboratories. RO-3306 (CDK1 inhibitor) and roscovitine (CDKs inhibitor) were from ENZO Life Sciences. MK5108 (Aurora-A inhibitor) was from Merck. SB216763 (GSK3β inhibitor) was from Sigma.

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Establishment of cell lines

Ectopic expression of empty vector, YAP-S127A in DLD-1 (CDK8-KO) and SW480 (CDK8-KO) cell lines was achieved by a retrovirus-mediated approach as described previously (1). The transduced cells were then selected with 800 µg/ml of neomycin (at 48 hours post-infection) to establish cell lines stably expressing exogenous proteins. Zyxin downregulation in HCT116, RKO, and SW480 cells was obtained by lentivirus-mediated Zyxin shRNA expression (2). Ectopic expression of β-catenin-S33Y, CDK8, or CDK8-KD was also achieved by a lentivirus-mediated approach (2). The HeLa cell line expressing TetOn-shCDK1 was kindly provided by Dr. Xiaolong Yang (3).

Expression constructs

pcDNA-YAP (no tag) expression constructs have been described (4). Retroviral wild type YAP and YAP mutant constructs have been described (5). The human Zyxin cDNA clone (RFP-Zyxin) was obtained from Dr. Anna Huttenlocher's lab (Addgene, plasmid #26720). To make the shRNA-resistant (sh-Res) Zyxin cDNA, the target sequence (5'-agaaggtgagcagtattgatt-3') was changed into 5'-agaaagtcagtagcattgatt-3' by PCR mutagenesis. The mutated Zyxin cDNA was then cloned into the MaRX^{TM} IV vector (1). The β-catenin-S33Y cDNA was a gift from Shinya Yamanaka (Addgene #13371). The GFP-Cyclin B-R42A and pcDNA3-CDK1-AF (718) plasmids were gifts from Jonathon Pines (Addgene #61849 and #39872). The CDK8 cDNAs were also purchased from Addgene (#19578 and #19579). β-catenin-S33Y, wild type CDK8 and its kinase dead (D173A) mutant (CDK8-KD), and CDK1-AF were then subcloned into lentivirus-based pLVX-EF1α-IRES-mCherry vector (Clontech), which was inserted with an N-terminal

Flag-tag with multiple-cloning-site sequences. All plasmids were confirmed by DNA sequencing. Point mutations were generated by the QuickChange Site-Directed PCR mutagenesis kit (Stratagene) and verified by sequencing.

Recombinant protein purification and *In vitro* kinase assay

The GST-tagged full-length Zyxin proteins were purchased from Abnova. The GST-tagged and His-tagged YAP proteins (pET-21c vector, Novagen/EMD Chemicals) were bacterially expressed and purified on GSTrap FF affinity columns (GE Healthcare) and HisPur™ Cobalt spin columns (Pierce), respectively following the manufacturer's instructions (5). 100 ng of GST-Zyxin was incubated with 10 U recombinant CDK1/cyclin B complex (New England Biolabs) or 100 ng other kinases (SignalChem) in kinase buffer (6) in the presence of 5 μCi γ-^{32}P-ATP (3000 Ci/mmol, PerkinElmer). The reaction was performed at 37°C for 30 min. CDK2, CDK5, CDK8, MEK1, ERK1, p38α, JNK1, and JNK2 active kinases were purchased from SignalChem. The samples were resolved by SDS-PAGE, transferred onto PVDF (Millipore) and visualized by autoradiography followed by Western blotting.

Antibodies

Rabbit polyclonal phospho-specific antibodies against Zyxin S281, S308, and S344 were generated and purified by AbMart, Inc. The peptides used for immunizing rabbits were VASKF-pS-PGAPG (S281), SAGTG-pS-PQPPS (S308), and QNQVR-pS-PGAPG (S344). The corresponding non-phosphorylated peptides were also synthesized and used for antibody purification and blocking assays. Anti-Zyxin, p-YAP S127, YAP, p-β-catenin

S33/S37/T41, p-Cofilin S3, Cofilin, p-H3 S10, Survivin, and p-Aurora-A/B/C were from Cell Signaling Technology. Anti-GST, His, and CDK8 antibodies were from Bethyl Laboratory. Anti-Aurora-A, β-tubulin, and Flag antibodies were from Sigma. Anti-Cyclin B1, β-actin, and β-catenin were purchased from Santa Cruz Biotechnology.

SI References

1. Xiao L, Chen Y, Ji M & Dong J (2011) KIBRA regulates hippo signaling activity via interactions with large tumor suppressor kinases. *J Biol Chem* 286(10): 7788-7796.

2. Zhang L, *et al* (2015) The hippo pathway effector YAP regulates motility, invasion, and castration-resistant growth of prostate cancer cells. *Mol Cell Biol* 35(8): 1350-1362.

3. Zhao Y & Yang X (2015) Regulation of sensitivity of tumor cells to antitubulin drugs by Cdk1-TAZ signaling. *Oncotarget* 6(26): 21906-21917.

4. Dong J, *et al* (2007) Elucidation of a universal size-control mechanism in drosophila and mammals. *Cell* 130(6): 1120-1133.

5. Yang S, *et al* (2013) CDK1 phosphorylation of YAP promotes mitotic defects and cell motility and is essential for neoplastic transformation. *Cancer Res* 73(22): 6722-6733.

6. Xiao L, *et al* (2011) KIBRA protein phosphorylation is regulated by mitotic kinase aurora and protein phosphatase 1. *J Biol Chem* 286(42): 36304-36315.

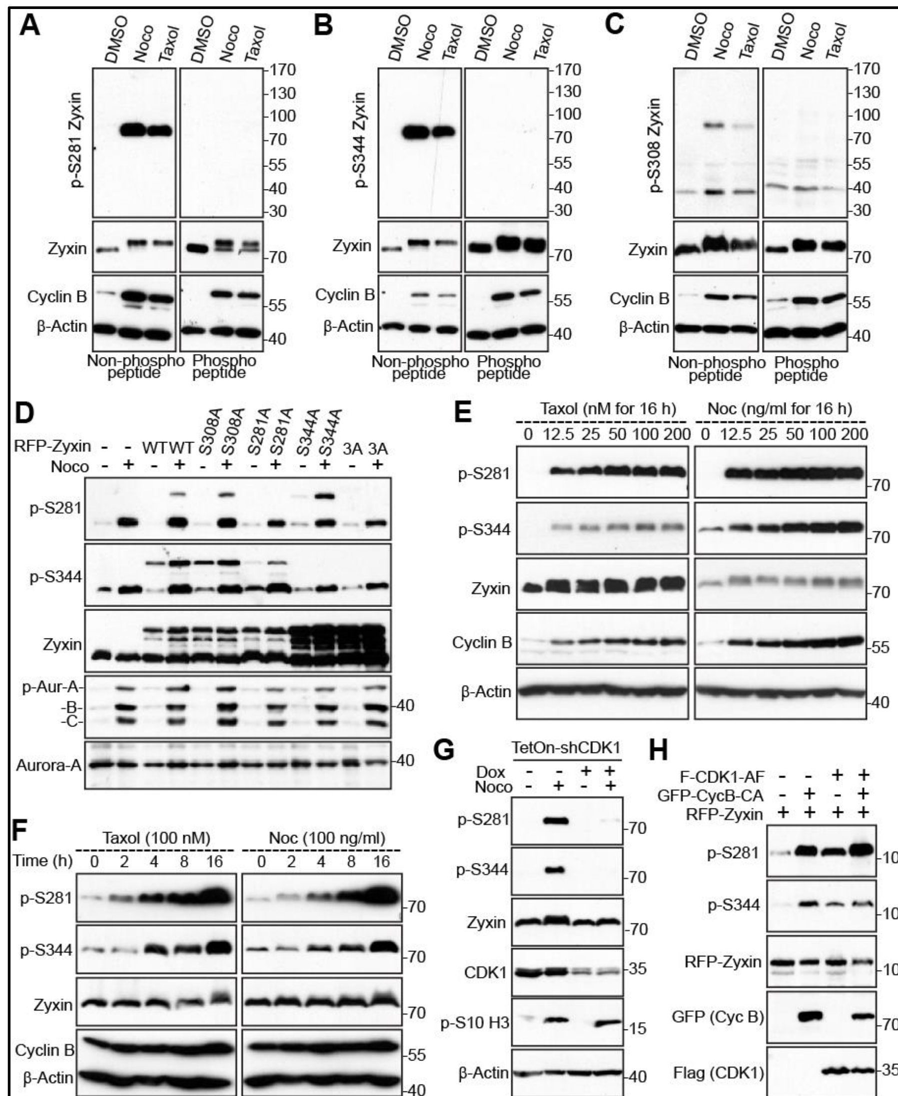

Figure S1. Validation of phospho-specific antibodies of Zyxin

(A-C) HeLa cells were treated with nocodazole (Noco) or taxol for 16 h. Total cell lysates were subjected to Western blotting with the indicated antibodies. Non-phosphopeptide: Western blotting in the presence of control (not phosphorylated) peptide; Phosphopeptide: Western blotting in the presence of corresponding phosphorylated peptide (used for antibody generation). Increased Cyclin B levels mark the cells in mitosis.
(D) HEK293T cells were transfected with RFP-Zyxin mutants as indicated. At 32 h post-transfection, the cells were treated with nocodazole for 16 h. Total cell lysates were subjected to Western blotting with the indicated antibodies. Both endogenous (lower bands) and exogenous (RFP-tagged, upper bands) Zyxin were shown. Increased p-Aurora kinases mark the cells in mitosis.
(E, F) Dose- and time course-dependent phosphorylation of Zyxin. HeLa cells were treated as indicated. Total cell lysates were subjected to Western blotting with the indicated antibodies.
(G) CDK1 knockdown inhibited Zyxin phosphorylation. TetOn-inducible shRNA targeting CDK1 was expressed in HeLa cells. The cells were treated with or without Doxycycline (Dox) for 2 days and were further treated with nocodazole (Noco) for an additional 16 h. Total cell lysates were subjected to Western blotting with the indicated antibodies. Increased p-H3 S10 levels mark the cells in mitosis.
(H) HEK293T cells were transfected as indicated. At 48 h post-transfection, total cell lysates were subjected to Western blotting with the indicated antibodies. F-CDK1-AF indicates the constitutive active form of CDK1 (T14 and Y15 sites were mutated to non-phosphorylatable Alaline and Phenylalanine). GFP-CycB-CA: constitutive active of Cyclin B1 (R42A non-degradable mutant).

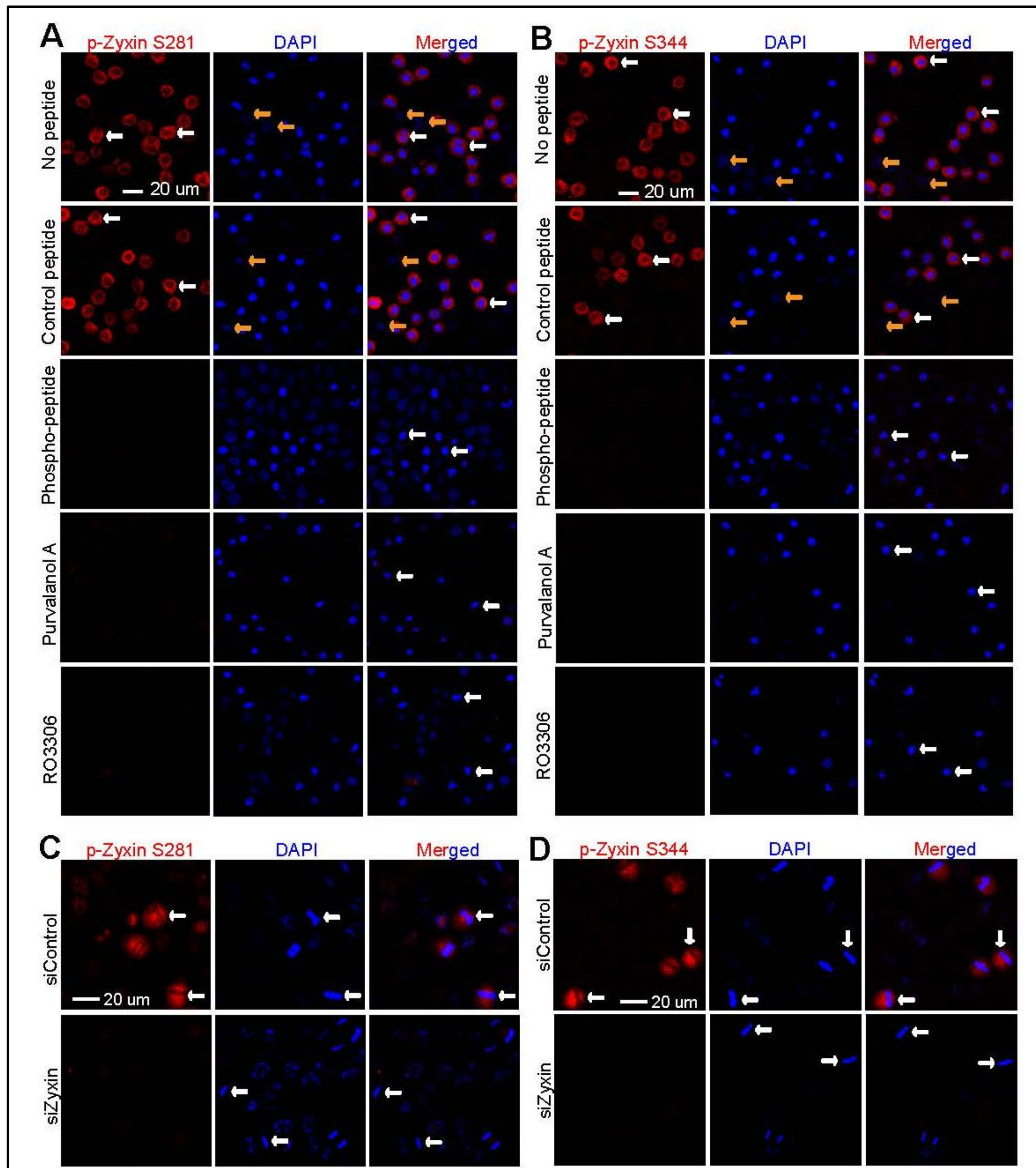

Figure S2. CDK1 phosphorylates Zyxin during mitotic arrest

(A, B) HeLa cells were treated with nocodazole and then fixed. Before the cells were stained with phospho-specific antibody against S281 or S344 of Zyxin, they were pre-incubated with PBS (no peptide control), or non-phosphorylated (control) peptide, or the phosphorylated peptide used for immunizing rabbits. CDK1 inhibitors RO3306 (5 μM) or Purvalanol A (10 μM) together with MG132 (25 μM) were added 2 h before the cells were fixed. White and yellow arrows mark some of the prometaphase cells and the interphase cells, respectively.

(C, D) HeLa cells were transfected with scramble (Control) siRNA or siRNAs targeting Zyxin. At 48 h post-transfection, cells were treated with nocodazole and then fixed for staining with p-S281 Zyxin (C) or p-S344 Zyxin (D) antibodies. White arrows mark some of the metaphase cells.

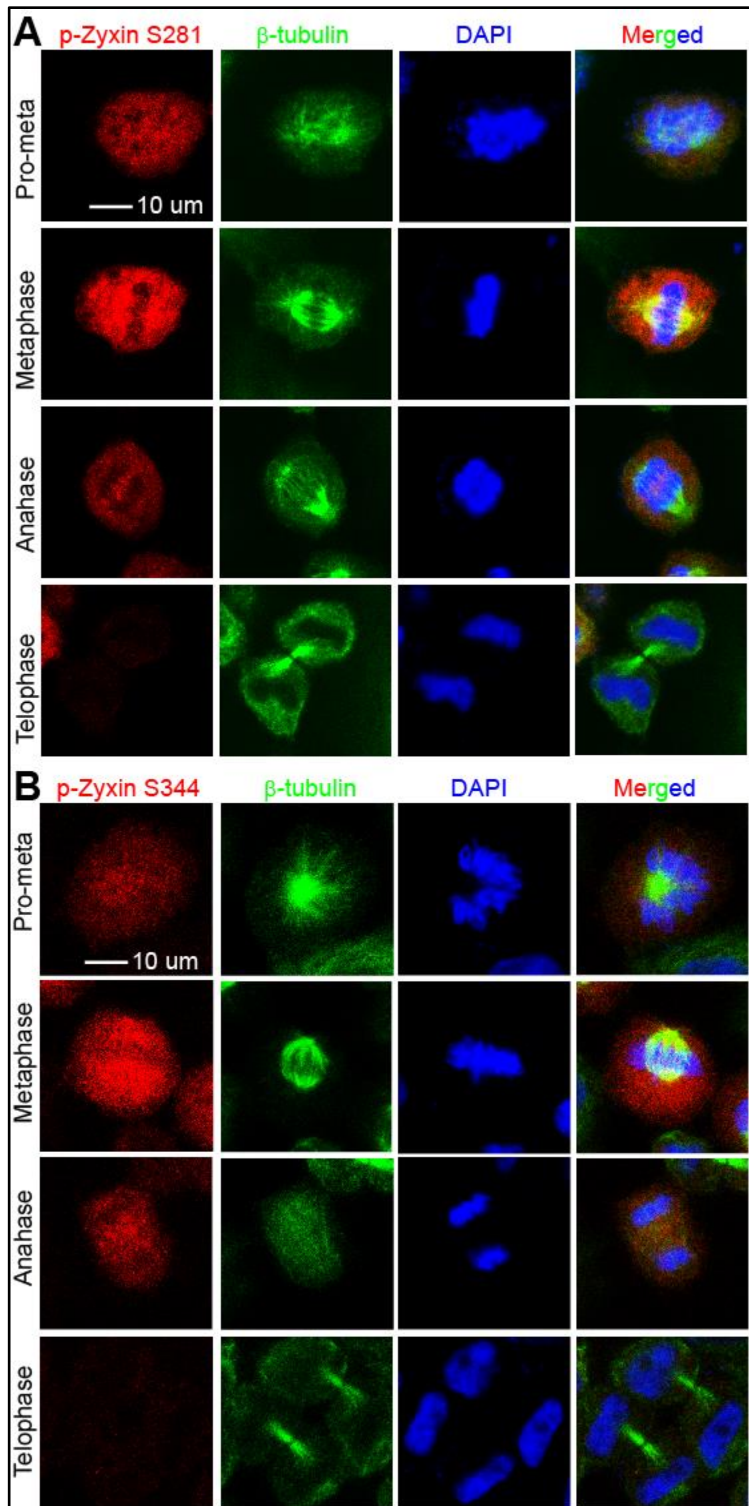

Figure S3. Zyxin is phosphorylated during unperturbed mitosis

(A, B) HeLa cells were synchronized by a double thymidine block and release method. Cells were stained with antibodies against p-Zyxin S281 (A) or p-Zyxin S344 (B) or β-tubulin, or with DAPI. Representative cells in different mitotic phases were shown.

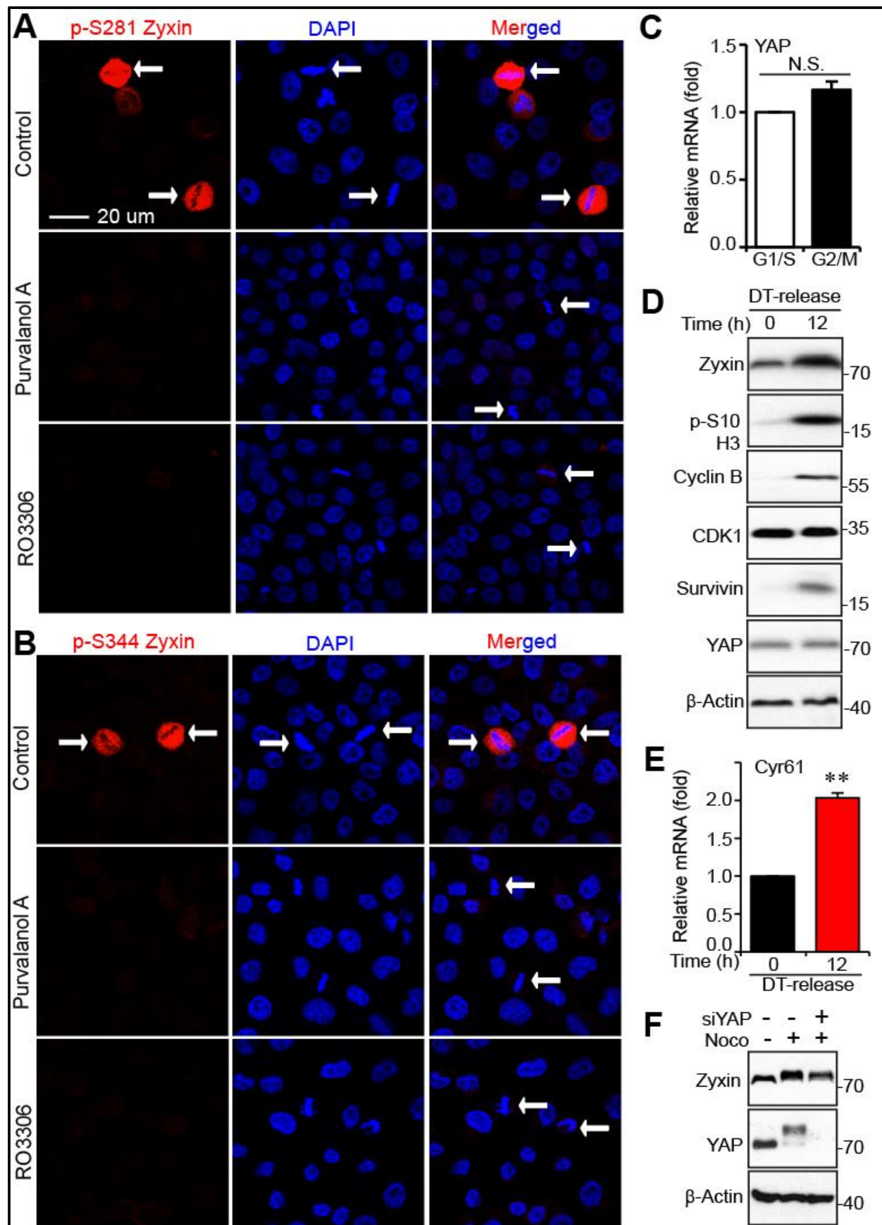

Figure S4. Phosphorylation of Zyxin occurs during normal mitosis

(A, B) Immunofluorescence (IF) staining of p-Zyxin in freely cycling HeLa cells. IF staining procedures were described in the main text. White arrows mark cells in metaphase (condensed and aligned chromosomes). CDK1 inhibitors (Purvalanol A or RO3306) were added 1.5 h before the cells were fixed.

(C) Quantitative RT-PCR revealed that YAP mRNA levels were not induced during mitosis. HeLa cells were synchronized by a double thymidine block and release method and collected at 10 h post release (G2/M). Asynchronized cells (G1/S) were also included. N.S.: not significant.

(D) HeLa cells were synchronized by a double thymidine (DT) block and release method and collected at the indicated time points. Total cell lysates were subjected to Western blotting with the indicated antibodies. Increased p-H3 S10 and Cyclin B levels mark the cells in mitosis. Increased survivin level in mitosis serves as a positive control.

(E) HeLa cells were treated as in D and RNAs were used for quantitative RT-PCR. Data were expressed as mean ± s.e.m from three independent experiments. **: $p<0.01$ (t-test).

(F) HeLa cells were transfected with siRNA targeting YAP (40 nM) for 48 h and were treated nocodazole (Noco) for an additional 18 h. SiRNA for YAP has been validated in our previous study (5).

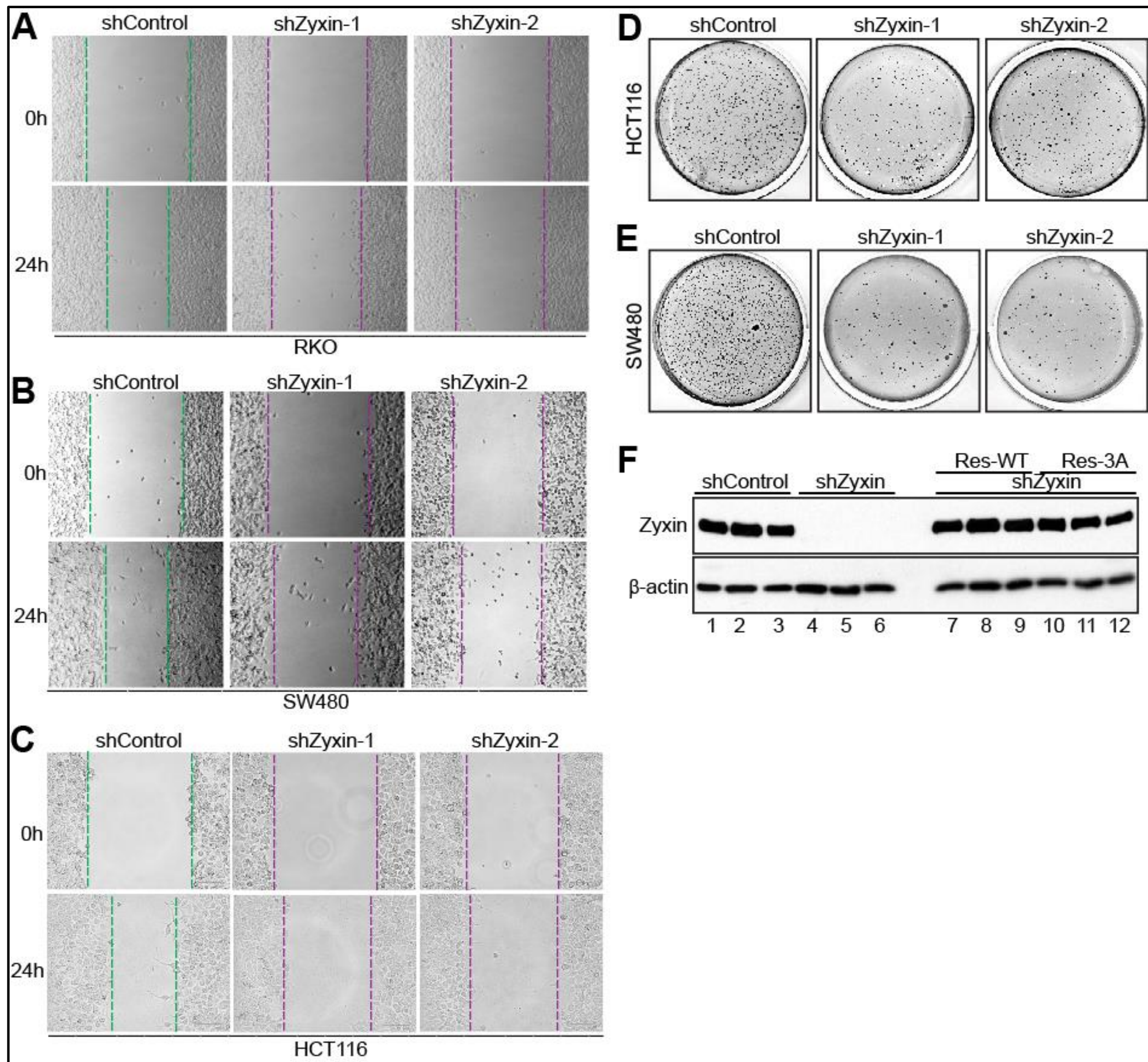

Figure S5. Zyxin knockdown impairs migration and anchorage-independent growth in colon cancer cells

(A-C) Representative images for would healing (migration) assays in RKO, SW480, and HCT116 cells.

(D, E) Representative images for anchorage-independent growth (colony assay in soft agar) SW480 and HCT116 cells.

(F) Zyxin expression in xenograft tumors. Total protein lysates from three tumors of each line were probed with anti-Zyxin and β-actin antibodies.

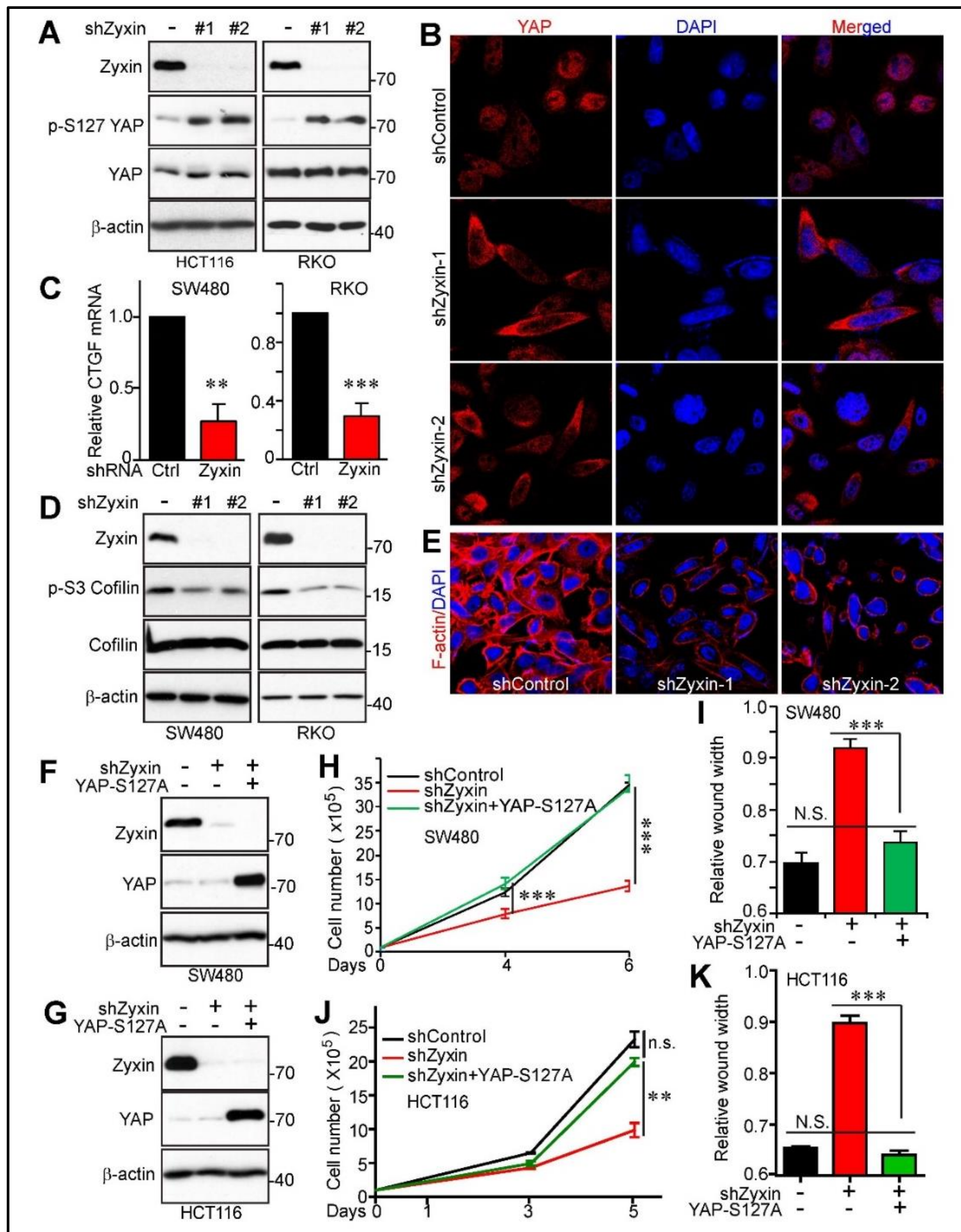

Figure S6. Zyxin regulates F-actin polymerization and YAP activity

(A) Zyxin knockdown increased p-YAP S127 in HCT116 and RKO colon cancer cells.

(B) Zyxin knockdown impaired YAP nuclear localization in SW480 cells. YAP subcellular localization was determined by immunofluorescence confocal microscopy using anti-YAP antibodies.

(C) Zyxin knockdown inhibited CTGF (YAP target gene) expression. CTGF mRNA levels were determined by quantitative RT-PCR.

(D) Zyxin regulates cofilin. Cofilin activity was analyzed by phospho-specific antibodies.

(E) Zyxin knockdown reduced F-actin polymerization (SW480). F-actin activity was determined by immunofluorescence confocal microscopy using anti-F-actin antibodies (Rhodamine Phalloidin (Cytoskeleton).

(F, G) Establishment of cell lines expressing YAP-S127A in Zyxin-knockdown SW480 and HCT116 cells.

(H-K) The effects (impaired migration and proliferation) of Zyxin-knockdown were rescued by re-expression of YAP-S127A in SW480 and HCT116 cells. Cell proliferation (H, J) migration (I, K) and assays using cell lines from (F, G). Data (C, H-K) are from three biological repeats and expressed as the mean ± s.e.m. ***: p<0.001; **: p<0.01 (t-test). N.S.: not significant.

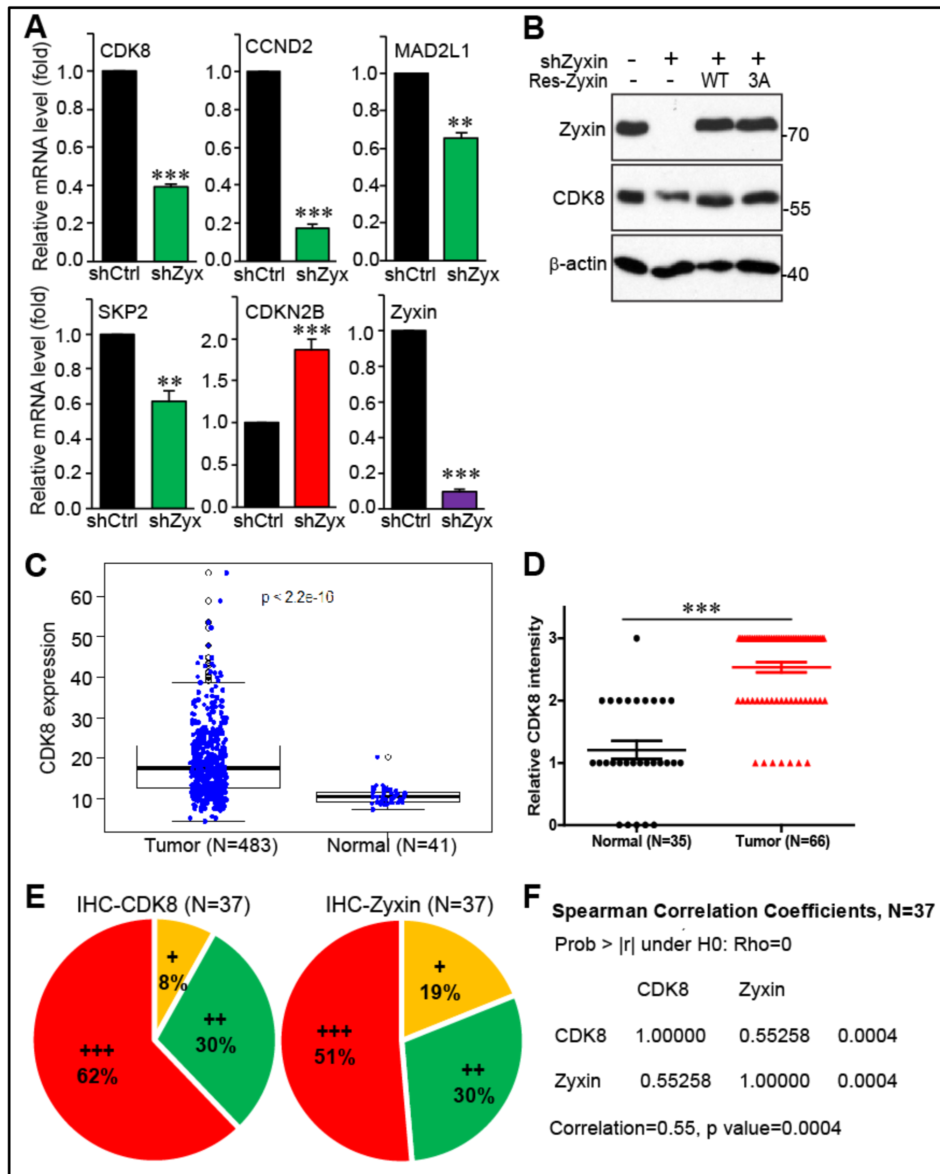

Figure S7. Zyxin regulates CDK8 expression in colon cancer cells

(A) Zyxin knockdown inhibited the expression of multiple cell cycle regulators. Candidates' mRNA levels were determined by quantitative RT-PCR. Zyxin serves as a positive control. Data are from three biological repeats and expressed as the mean ± s.e.m. ***: $p<0.001$; **: $p<0.01$ (t-test).

(B) Re-introduction of wild type (WT) or the non-phosphorylatable (3A) Zyxin rescued CDK8 protein levels in Zyxin-knockdown cells. The CDK8 levels were determined by immunoblotting in various cell lines as indicated.

(C) CDK8 mRNA levels were increased in colon cancer patients (data were extracted from TCGA, the Student t-test was used for statistical evaluation).

(D) CDK8 protein levels were increased in colon cancer patients, as revealed by IHC staining on TMAs (N=31 for normal and N=74 for tumor/cancer). ***: $p<0.001$ (Wilcoxon rank sum test).

(E) Zyxin and CDK8 protein levels are positively correlated in metastatic colon cancer samples. Immunohistochemistry (IHC) staining analysis of Zyxin and CDK8 on colon cancer tissue microarrays. +: low expression; ++: moderate expression; +++: strong expression.

(F) Correlation analysis between CDK8 and Zyxin.

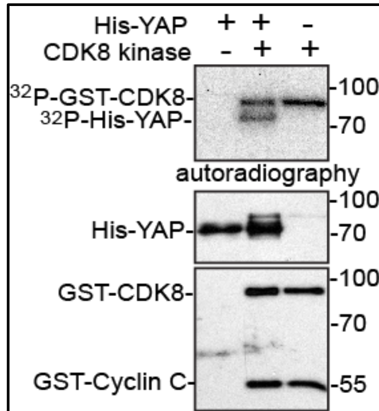

Figure S8. Detection of autophosphorylation of CDK8. *In vitro* kinase assays in the presence (lanes 1 and 2) or absence of His-YAP substrates (the 3rd lane).

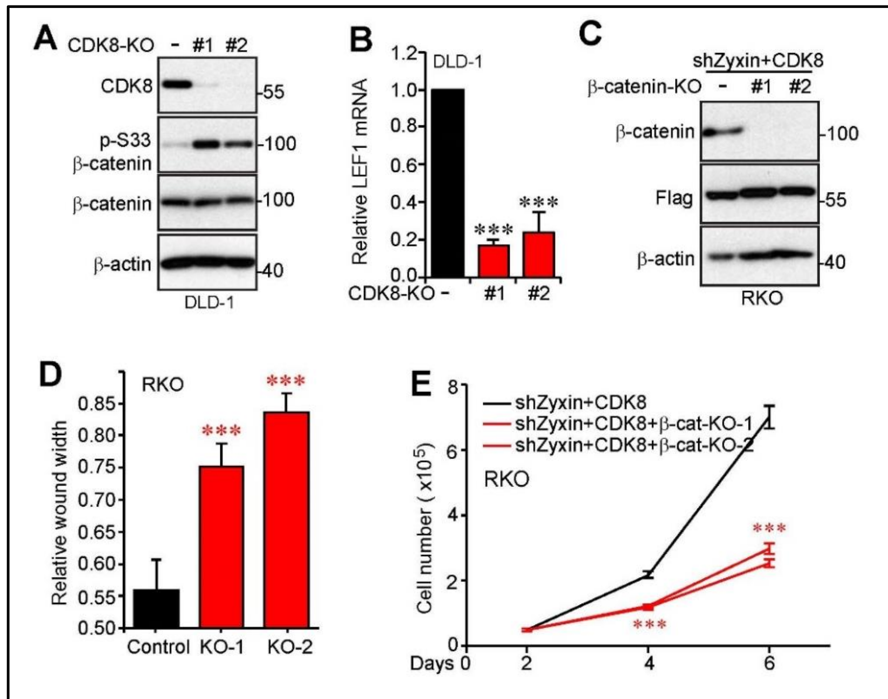

Figure S9. CDK8 regulates β-catenin activity in colon cancer cells

(A, B) CDK8 knockout inhibited β-catenin activity. The β-catenin activity was determined by immunoblotting using the inhibitory phospho-antibody (p-S33 β-catenin) (A). LEF1 mRNA levels (determined by quantitative RT-PCR) were inhibited in CDK8-knockout cells (B). Data were expressed as the mean ± s.e.m. of three independent experiments. ***: p< 0.001(t-test).

(C-E) β-catenin knockout inhibited migration and proliferation in colon cancer cells. (C) Establishment of β-catenin knockout (KO) cell lines. CRISPR-double nickase-mediated (two independent gRNAs were used) knockout of β-catenin in RKO cells where Zyxin was depleted and CDK8 was re-expressed. Cell migration (wound healing) (D) and proliferation (E) assays in cell lines from (C). Data were expressed as the mean ± s.e.m. of three independent experiments. ***: p< 0.001(t-test).

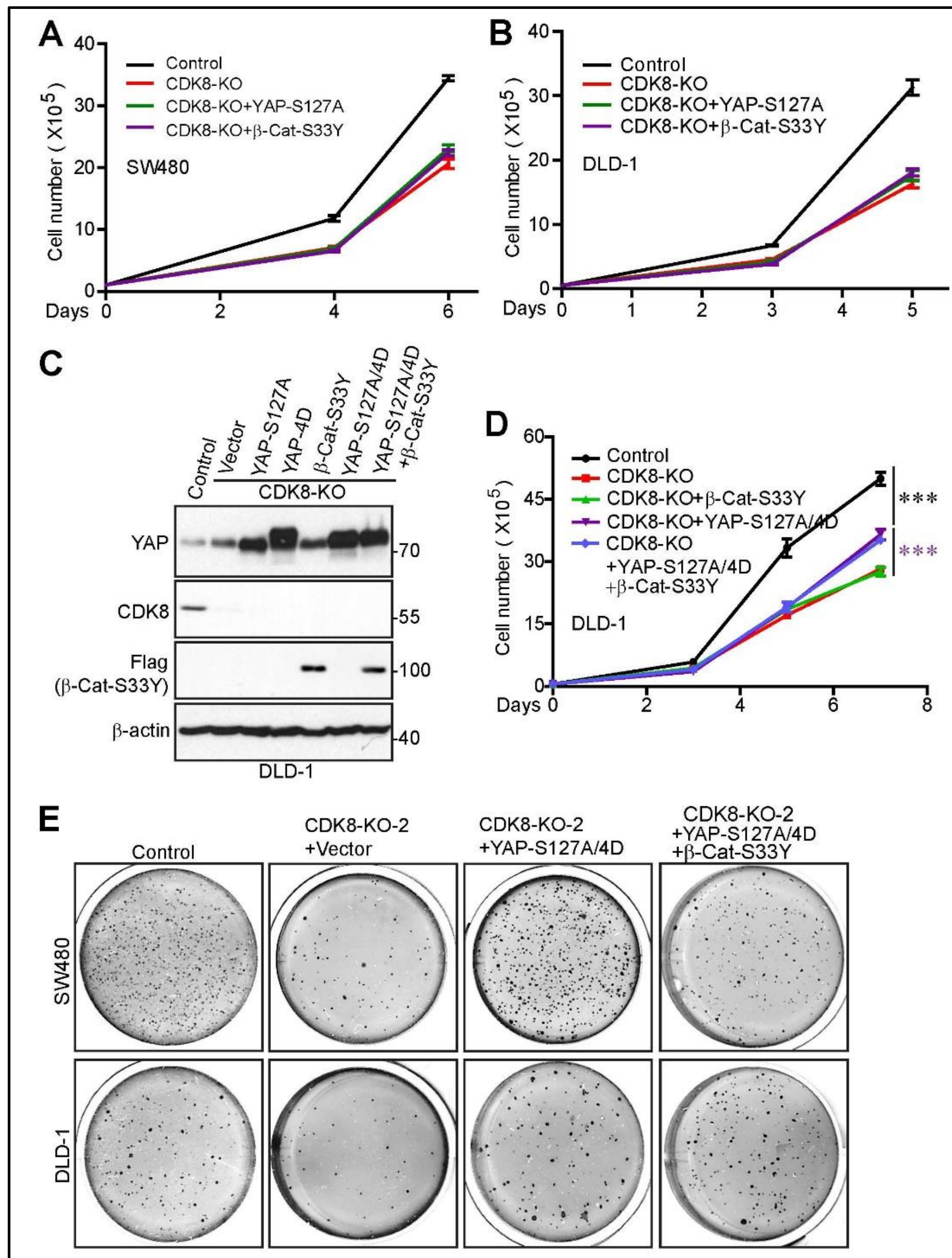

Figure S10. YAP phosphorylation is required for CDK8-driven tumorigenesis in colon cancer cells

(A, B) YAP-S127A or β-catenin-S33Y was not sufficient to rescue the proliferation defects of CDK8-KO in colon cancer cells. YAP-S127A and β-catenin-S33Y are mutants that are the active forms of their corresponding proteins.

(C) Establishment of cell lines expressing various YAP mutants and/or β-catenin in CDK8 knockout DLD-1 cells.

(D) Proliferation assays of cell lines expressing various YAP and/or β-catenin mutants as indicated.

(E) Representative images of anchorage-independent growth (colony assays in soft agar) in cell lines as indicated. Data (A, B, and D) are from three biological repeats and expressed as the mean ± s.e.m. ***: $p<0.001$(t-test).