Supplementary Materials & Methods

Drugs and antibodies

Latrunculin B, forskolin, 3-isobutyl-1-methylxanthine (IBMX), 2′,5′-dideoxyadenosine (DDA), dopamine, and epinephrine were obtained from Sigma. 8-CPT-2'-O-Me-cAMP (007) was obtained from Santa Cruz. PKI (5-24) was obtained from Calbiochem.

Antibodies used in immunoblot analyses included those against LATS1 (Bethyl); LATS2, LATS2 phospho-Ser909 (LATS pAL), LATS2 phospho-Thr1079 (LATS pHM), phospho-PKA substrate, Flag (rabbit anti-Flag), YAP, YAP phospho-Ser127, CREB, CREB phospho-Ser133 and cleaved caspase-3 (Cell Signaling); β-actin (Sigma); lamin B, NF2, and PKACα (Santa Cruz); HA (Covance); and Flag (WAKO). For phospho-PKA substrate antibody, we purchased two antibodies, Cell Signaling #9621 (Designated as Ab #1) and Cell Signaling #9624 (Designated as Ab#2). Unless specified, all western blot against phosphorylated PKA substrate refers to Ab #2. The WW45 antibody was generated using the C-terminal portion of WW45 as an antigen (Lee et al., 2008).

Antibodies used in immunofluorescence studies included those against YAP (Novus), Sox2 (Santa Cruz), and phospho-H3 (Cell Signaling).

Peptide synthesis

Peptide sequences for scrambled 11R-PKI were described previously (Matsushita et al, 2001). Peptide sequences for scRIAD-11R, RIAD-11R, 11R-scSuperAKAP-IS and 11R-SuperAKAP-IS were described previously (Carlson et al, 2006; Gold et al, 2006). All peptides were synthesized in 80% purity (Peptron). As scrambled control for 11R-PKI, we used 1:1 mixture of scRIAD-11R and 11R-scSuperAKAP-IS.

Antibody generation

Phospho-specific antibodies against YAP Ser381 were generated by immunizing rabbits with

CYHSRDEpSTDSGL peptides. Antibodies reactive to unphosphorylated peptides were removed by first passing the serum through a column linked with non-phosphorylated peptides. Antibodies specific for YAP phospho-Ser381 were purified from the flow through of the first column using a phospho-peptide linked affinity column.

Protein purification

Full-length YAP was cloned into pGEX-4T-1 plasmid and used to transform the ROSETTA strain. Recombinant protein expression was induced by incubating with 1 mM IPTG (isopropylthio-β-galactoside) at 25°C for 6 hours. Harvested cells were lysed by incubation with lysozyme, 1% Triton X-100, 0.1% sarcosyl, and sonication. Lysates were cleared by centrifugation and filtration. Cleared lysates were incubated with GST-agarose beads (Sigma), and GST-YAP protein was eluted with reduced gluthione. Eluted samples were dialyzed against PBS and stored at -70°C.

RNAi transfection

siRNA transfection was performed using RNAi MAX from Invitrogen, as described by the manufacturer. Experiments were performed 48 hours after transfection.

siRNA sequences for LATS1 and LATS2 were previously described (Dupont et al., 2011). The sense strand targeting sequences for human NF2 are as follows: NF2 siRNA-1, 5'-GAGGAAGCAACCCAAGACGUU-3'; NF2 siRNA-2, 5'-UGGCCAACGAAGCACUGAU-3'. RNA oligonucleotides were synthesized by Samchully Pharmaceutical Co.

Subcellular fractionation

Harvested cells were first re-suspended in hypotonic buffer (10 mM HEPES pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid [EGTA], 1 mM DTT). After incubating for 20 minutes on ice, cells were lysed by incubating with 0.3% NP-40 for 10 seconds. Samples were microcentrifuged for 1 minute at 13,000 rpm. Supernatants (cytosolic

fraction) were transferred to a fresh tube. The remaining pellet was washed once with hypotonic buffer and extracted using hypertonic buffer (20 mM HEPES pH 7.8, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM NaCl, 420 mM NaCl) to yield the nuclear fraction.

Immunofluorescence

Cells were fixed by incubating with 4% paraformaldehyde in PBS for 20 minutes at room temperature. For YAP immunofluorescence, fixed cells were permeabilized with 0.3% Triton X-100 and 0.1% saponin. Cells were blocked with 3% BSA and 5% goat serum. Primary antibody was prepared by diluting 1:100 in blocking buffer. Mouse Alexa 488-conjugated secondary antibody was diluted 1:500 in PBS. Coverslips were mounted with mounting solution containing 4',6-diamidino-2-phenylindole (DAPI; Vectorfield). For phalloidin staining, fixed cells were incubated in FITC (fluorescein isothiocyanate)-phalloidin (Sigma; diluted 1:1000 in PBS) and mounted. Samples were examined under a fluorescence microscope (Zeiss).

Western blot quantification

Western blot results were quantified using Image J software.

Supplementary Figure Legends

Supplementary Figure S1. Specificity of YAP phospho-Ser381 antibody. (A) NIH3T3 cells were treated with dimethylsulfoxide or 5 μM latrunculin B for 1 hour, or detached and maintained in suspension for 1 hour. Lysates were fractionated by SDS-PAGE, and proteins were transferred to nitrocellulose membranes. Membranes were treated with lambda-PPase or mock-treated for 1 hour and blotted. (B) Cells were treated as in (A). Lysates were treated with lambda-PPase or mock-treated for 30 minutes. Reaction products were analyzed by Western blotting.

Supplementary Figure S2. Sav1 is dispensable for YAP phosphorylation induced by actin cytoskeletal damage. Wild-type or Sav1-deficient MEFs were treated as indicated.

Supplementary Figure S3. Effect of 11R-PKI on YAP phosphorylation induced by latrunculin B. NIH3T3 cells were pre-treated with scrambled peptide (1:1 mixture of scRIAD-11R and 11R-scSuperAKAP-IS, 25 μM each) or 50 μM 11R-PKI for 30 minutes followed by 5 μM latrunculin B treatment for indicated times.

Supplementary Figure S4. Inhibition of adenylyl cyclase does not affect cytoskeletal damage-induced YAP Ser381 phosphorylation. (A) Cells were pre-treated with 50 μM DDA for 1 hour followed by addition of latrunculin B. Unlike H-89, DDA did not attenuate YAP Ser381 phosphorylation. (B) NIH3T3 cells were treated with forskolin/IBMX or 007 (10, 20, 40 μM) for 1 hour. (C) NIH3T3 cells were treated as indicated for 1 hour. Paraformaldehyde-fixed cells were stained with FITC-phalloidin to reveal the actin cytoskeletal structure. BF, bright field. Scale bar, 10μm.

Supplementary Figure S5. Partial loss of LATS1/2 reduces Ser381 phosphorylation. RPE cells were transfected with control siRNA, 3 μM LATS1 siRNA plus 1.5 μM LATS2 siRNAs (lane 2), or 10 μM LATS1 plus 5 μM LATS2 siRNAs (lane 3). Forty eight hours after transfection, cells were treated with 5 μM latrunculin B for 1 hour. Knockdown efficiencies of LATS1/2 and YAP phosphorylation levels were determined.

Supplementary Figure S6. Effects of H-89 on LATS activation-loop and hydrophobic-motif phosphorylation. Samples from Figure 3C were immunoblotted with phospho-specific antibodies for the activation loop (AL) and hydrophobic motif (HM).

Supplementary Figure S7. Sequential kinase assay with buffer including PKI (5-24). HA-LATS2 WT or KD was co-transfected with Flag-Mob1A to 293T cell. 48 hours after

transfection, cells were harvested and proceeded as in figure 3D except that 1 μM PKI (5-24) was included in the second kinase reaction buffer to inhibit residual PKA activity (Lane 1,2,3,4 and 6). Comparison of lane 5 and 6 indicates that PKA activity is efficiently blocked by PKI (5-24). SE, short exposure; LE, long exposure.

Supplementary Figure S8. Interaction between LATS2 4SA and Mob1A. 293T cells were transfected as indicated. Fourty-eight hours after transfection, equal amounts of cell lysates were immunoprecipitated with Flag antibody. Co-immunoprecipiated Mob1A was examined by anti-HA western blot. WCL, whole cell lysate. *, non-specific signal.

Supplementary Figure S9. dnPKA does not cooperate with YAP WT in cells. (A) NIH3T3 cells were infected with either empty (left) or YAP WT (right) retroviruses with or without dnPKA co-infection. Selected cells were seeded onto poly-HEMA-coated dishes for the indicated times. Cell death was examined by western blotting for cleaved caspase-3. (B) Cells from (A) were starved with 0.1% FBS for the indicated days and cell death was examined.

Supplementary Figure S10. dnPKA and YAP cooperate to induce ectopic proliferation in the developing chick neural tube. *Top*: The left side of the developing chick (+) was transfected with the indicated plasmids and the right side was left untransfected (-); proliferating cells were examined by immunostaining for phospho-H3. Arrows indicate ectopic proliferating cells outside the neural tube. Scale bar, 75μm. *Bottom:* Quantification of the numbers of ectopic phospho-H3-positive cells. ***$p < 0.001$. Error bars indicate SEMs (two-tailed Student's t-test).

Supplementary Figure S11. Induction of ectopic neural progenitors by transfection of YAP 2SA is not inhibited by PKA-CA. The indicated plasmids were transfected along with LacZ to mark the transfected side (left). Neural progenitors were detected by immunostaining for the marker, Sox2. Scale bar, 75μm.

Supplementary Figure S12. PKA-LATS-YAP signaling following Gs-coupled GPCR activation. (A) MDA-MB231 cells were transfected with empty vector or HA-LATS2 plasmids. Forty-eight hours after transfection, cells were stimulated with 10 μM epinephrine for 1 hour. Harvested cells were lysed in RIPA buffer followed by immunoprecipitation with anti-HA antibody. Phosphorylation by PKA was examined by Western blotting using a phospho-PKA substrate antibody. (B) MDA-MB231 cells were infected with empty or Flag-dnPKA retrovirus. Selected cells were stimulated with 10 μM epinephrine for 1 hour. (C) U2OS cells were infected with empty or Flag-dnPKA retrovirus. Selected cells were stimulated with 10 μM dopamine for 1 hour. Because epinephrine and dopamine signal through PKA, PKA inhibition abolished both Ser127 and Ser381 phosphorylation induced by these agonists.

Supplementary Figure S13. AKAP is involved in PKA-LATS-YAP signaling. (A) LATS2 and regulatory subunits associate in intact cells. 293T cells were transfected with HA-LATS2 and Flag-tagged murine regulatory subunits. Forty-eight hours after transfection, LATS2 was immunoprecipitated with an anti-HA antibody and regulatory subunit interaction was tested by Western blotting using a rabbit anti-Flag antibody. WCL, whole cell lysate. (B) NIH3T3 cells were pre-treated with 50 μM of indicated peptides for 30 minutes followed by addition of 5 μM latrunculin B for indicate time. For combinatorial treatment, 50 μM of RAID-11R and 11R-SuperAKAP-IS each (Or their respective scrambled peptides) were used. (C) RPE cells expressing SBP-LATS2 were pre-incubated with scrambled or combined AKAP inhibitor peptides for 30 minutes. After additional 1-hour with 5 μM latrunculin B, cells were harvested and SBP-LATS2 was pulled-down using streptavidin-agarose bead. Samples were fractionated by SDS-PAGE followed by western blotting for indicated antibodies. Band intensities were quantified using Image J software and amount of PKA phosphorylated LATS2 was normalized by total LATS2. Relative levels of PKA phosphorylated LATS2 in each sample are shown below.

Supplementary Figure S14. NF2 interacts with both Type Ⅰ and Type Ⅱ Regulatory

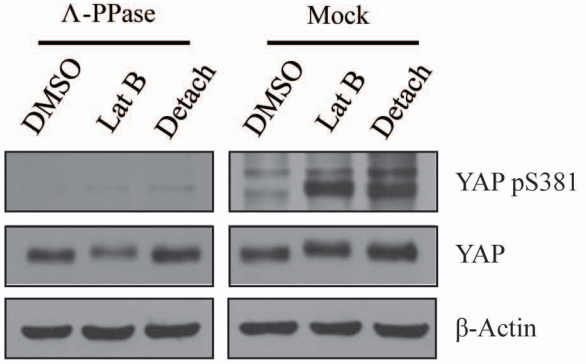
Subunits. 293T cells were transfected with Myc-NF2 and indicated HA tagged Regulatory Subunits and subjected to co-immunoprecipitation procedures as indicated. WCL, whole cell lysate; SE, short exposure; LE, long exposure.

Supplementary Figure S15. NF2 is dispensable for YAP phosphorylation induced by latrunculin B. RPE cells were transfected with two independent NF2 siRNAs. Forty-eight hours after transfection, cells were treated with 5 μM latrunculin B.

Supplementary Figure S16. Generation of *Lats2*-floxed mice. (A) Schematic representation of the *Lats2* locus, the targeting vector, and the targeted, floxed locus is shown. Exons 3–6, Southern blot probe, and restriction sites (H3, *Hind*III; Xa, *Xba*I; Sc, *Sac*I; Nd, *Nde*I) are shown. The targeting vector contained a diphtheria toxin A-chain (*DT-A*) gene and a neomycin resistance gene (*Neo*) flanked by *FRT* (squares) sites. Exon 4 of *Lats2* was flanked by *loxP* (triangles) sites. Mice carrying the $Lats2^{neo}$ allele were mated with a Flp recombinase strain to generate $Lats2^{flox}$ alleles. (B) Southern blot analysis of recombined ES cell clones. Genomic DNAs were digested with *Hind*III and then probed as indicated in (A). The wild-type allele is detected as 11.3 kb and the targeted allele is detected as 8.2 kb. Lanes 2 and 5 are targeted ES clones.

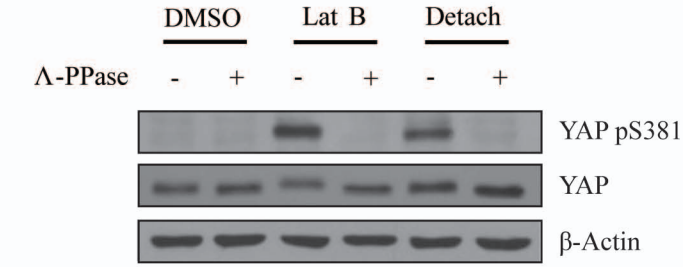
Supplementary Figure S1

A



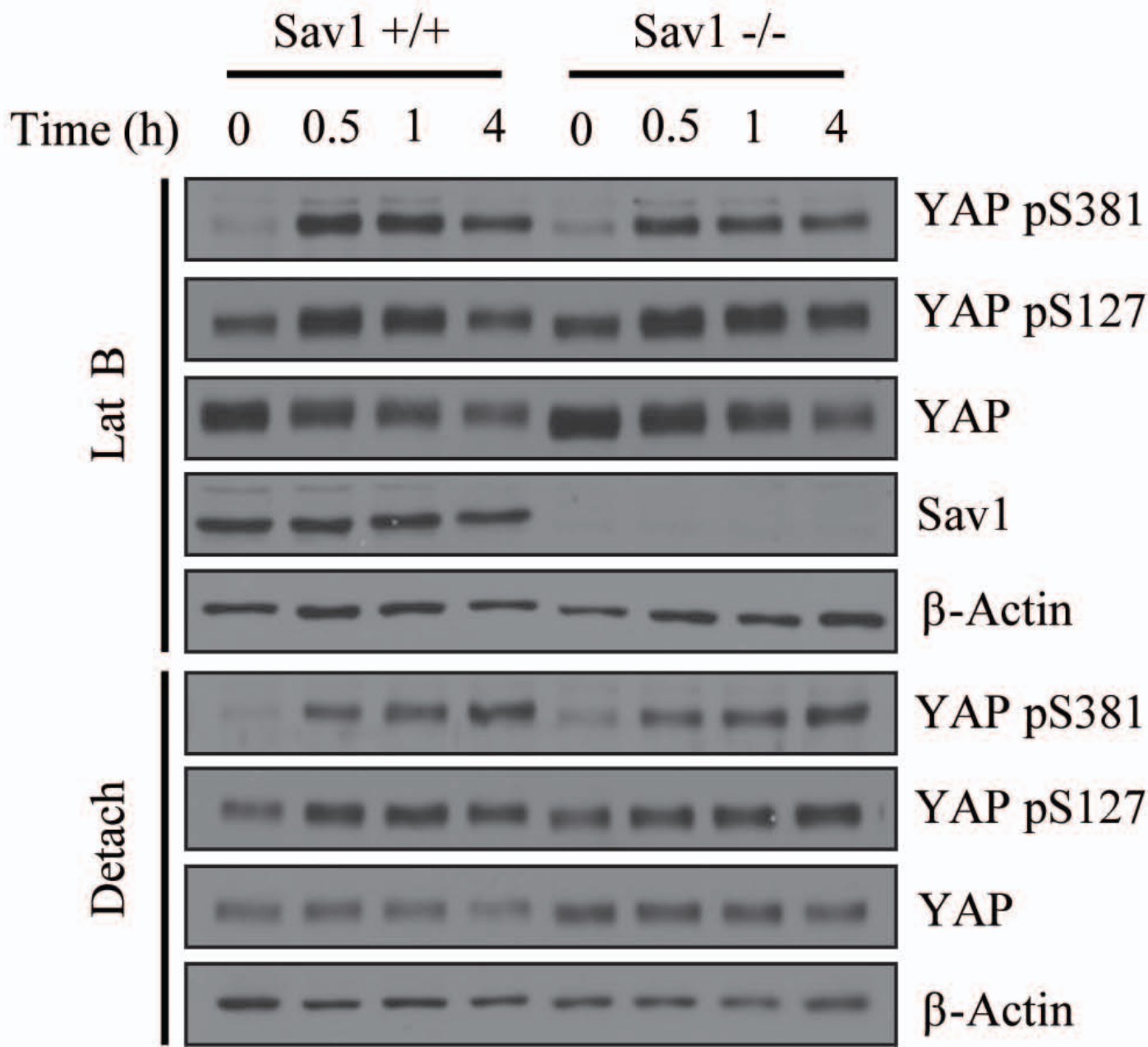
Lambda PPase treated on transferred membrane

B



Lambda PPase treated in crude lysate

Supplementary Figure S2



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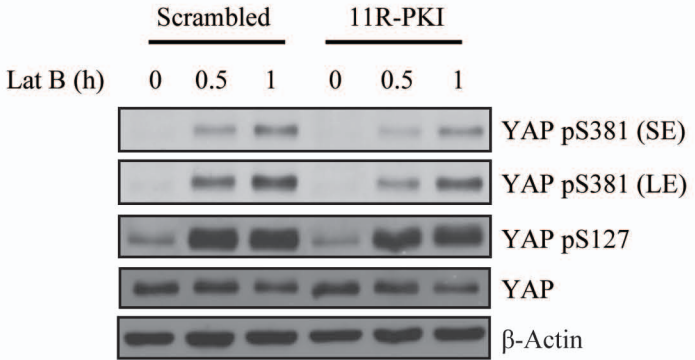

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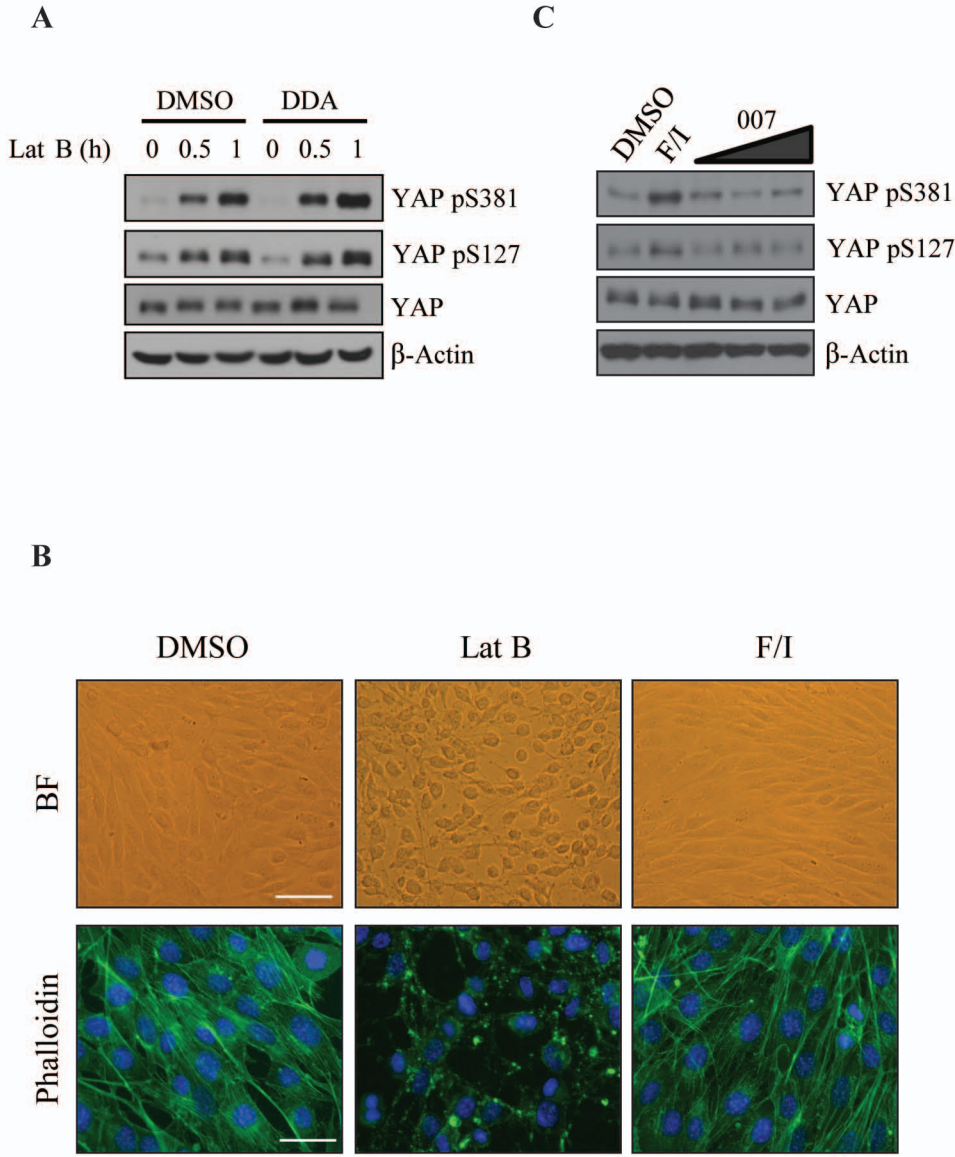

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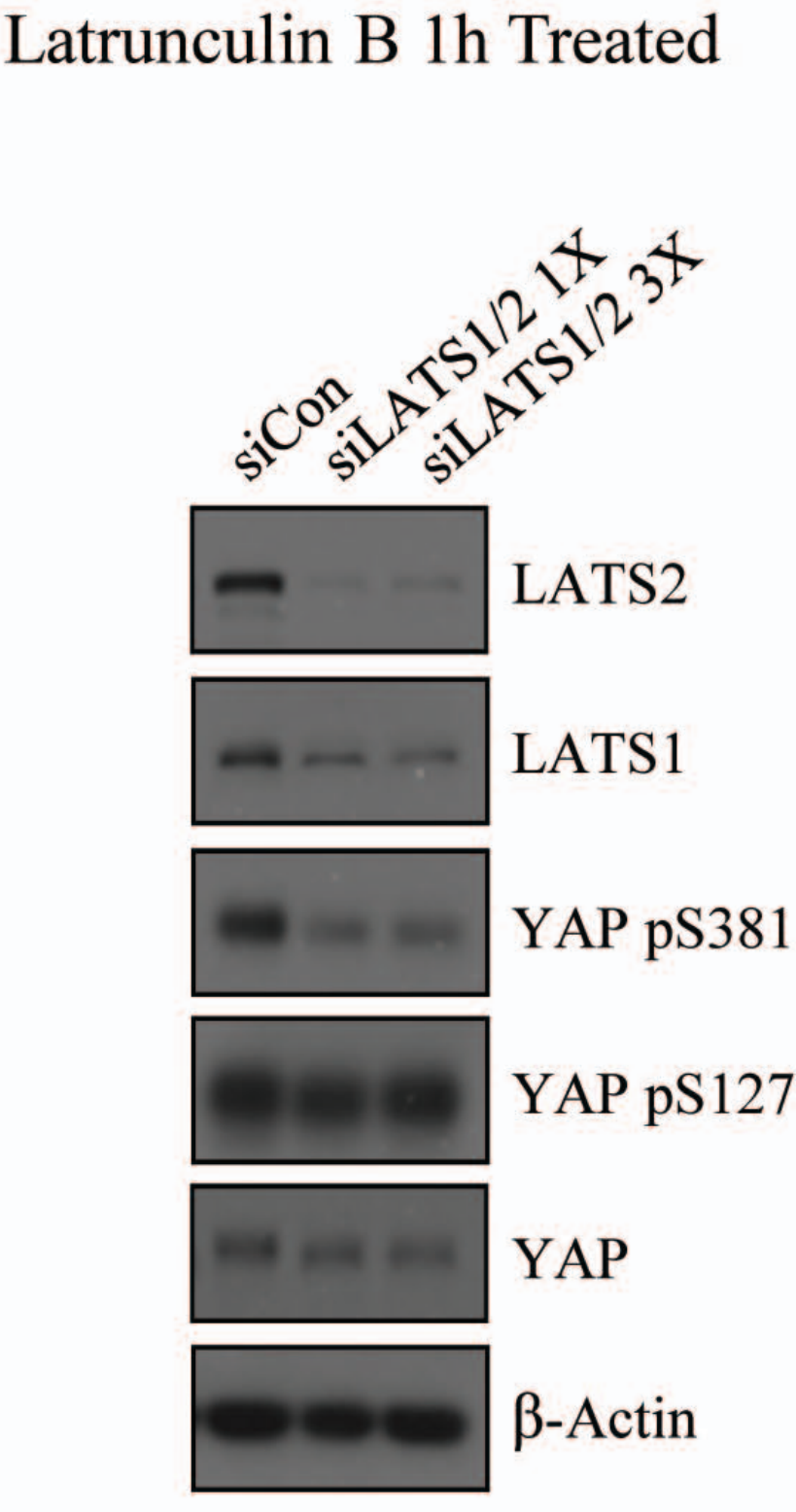

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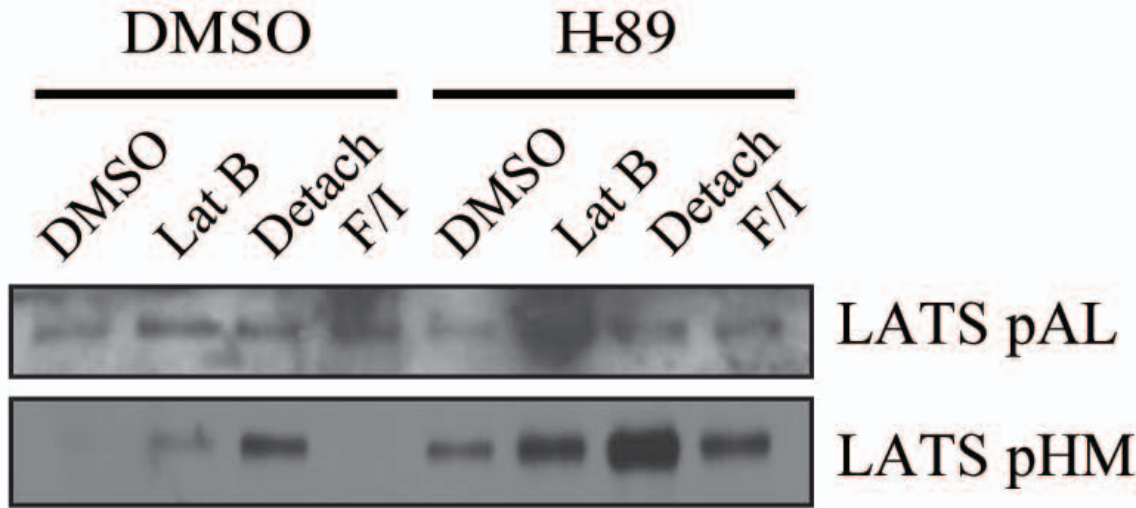

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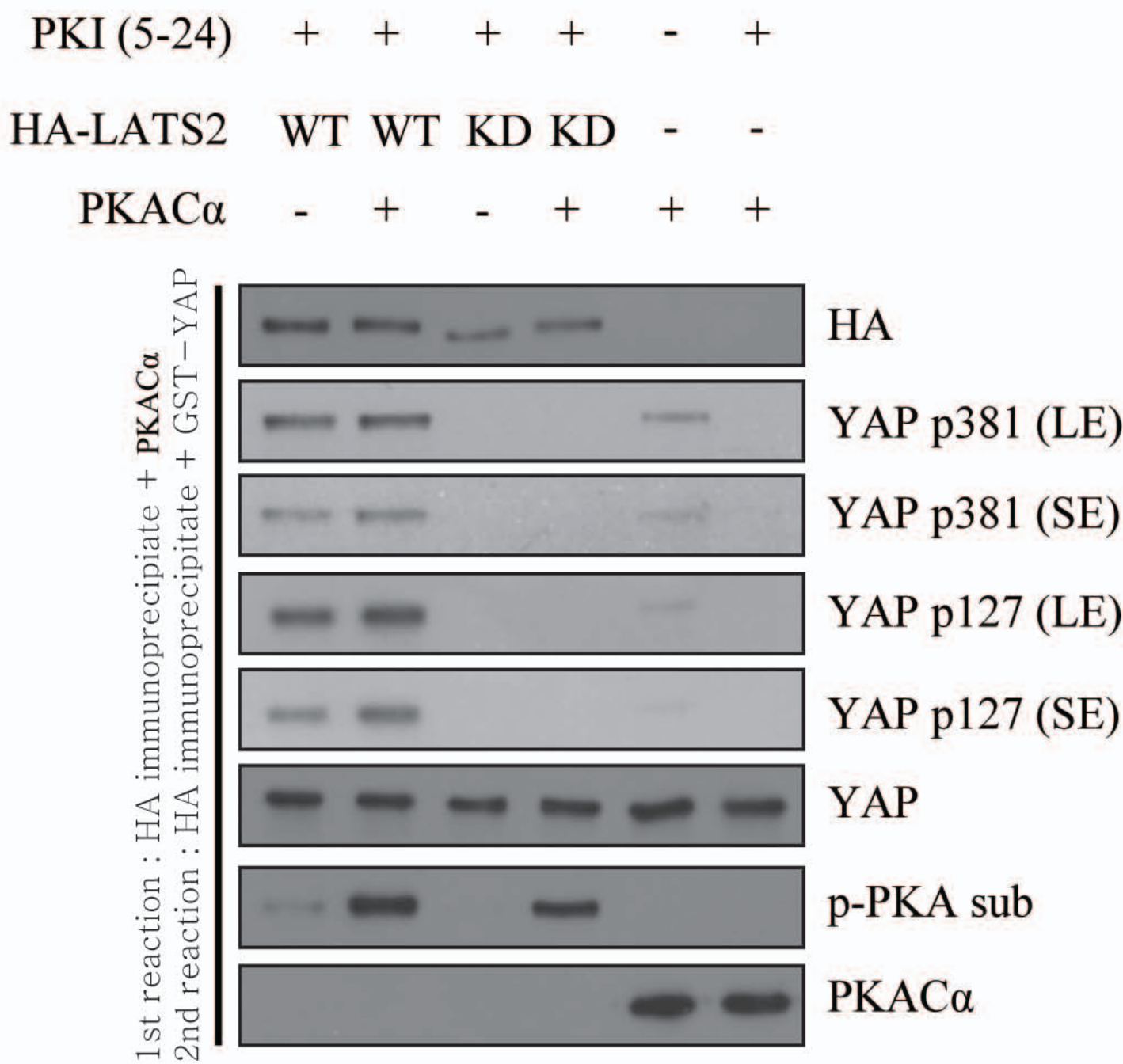

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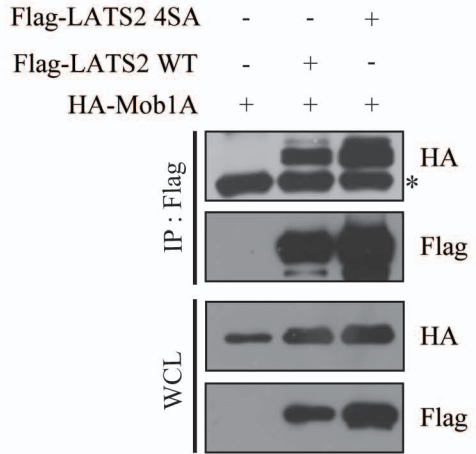

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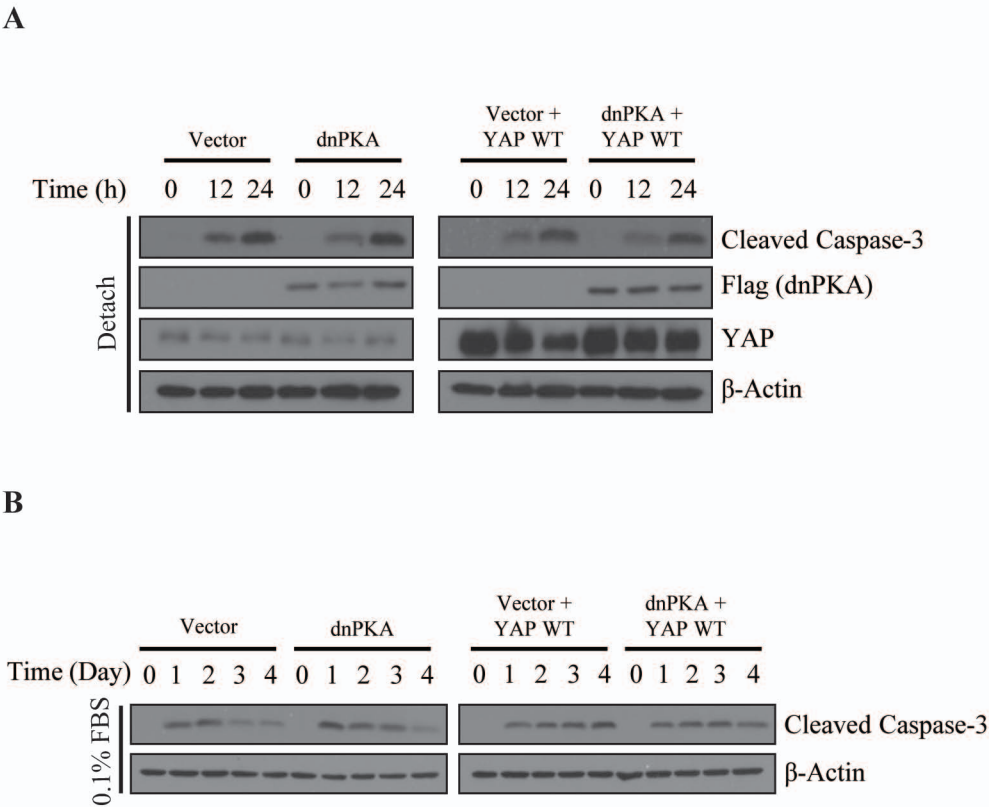

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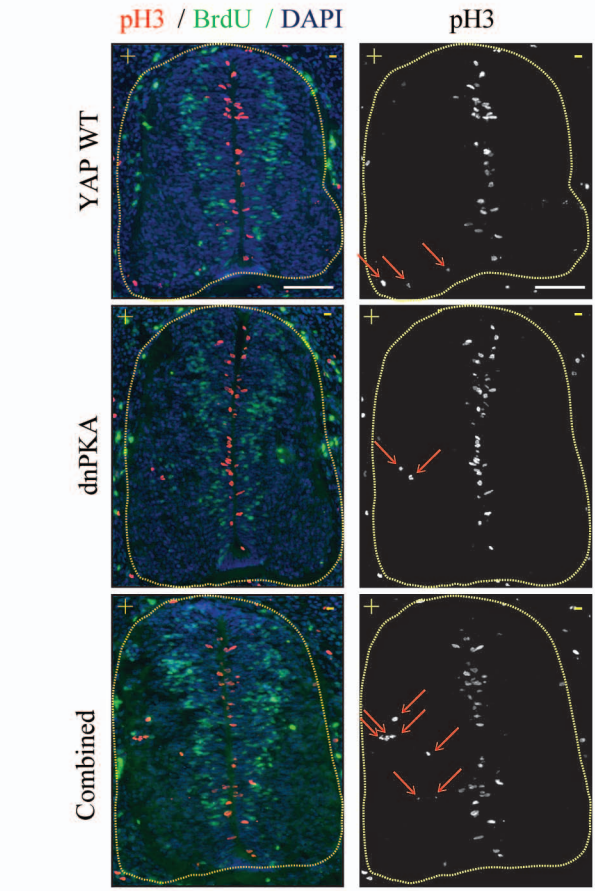

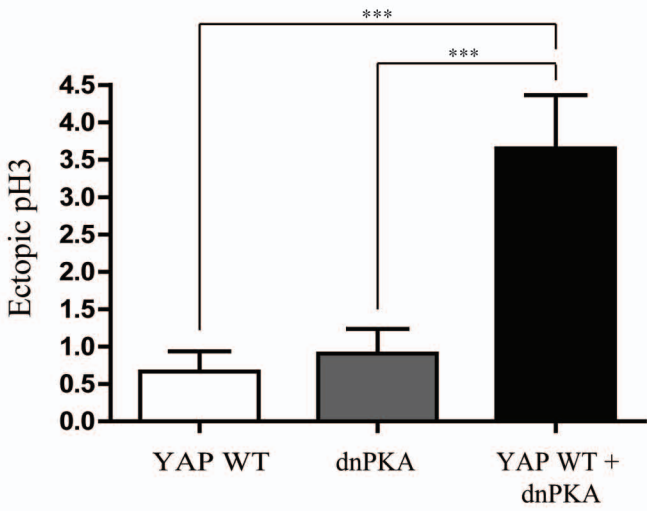

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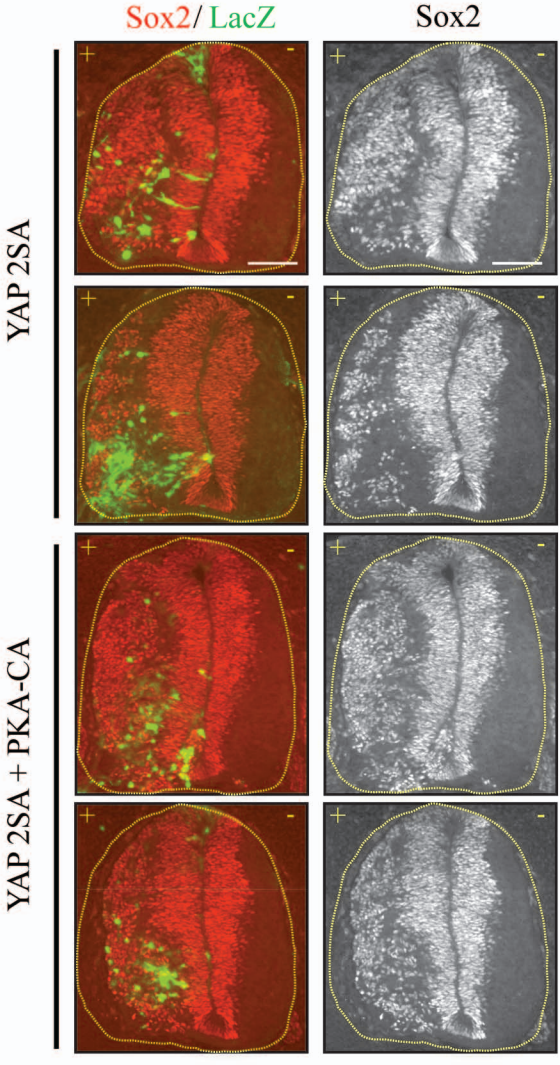

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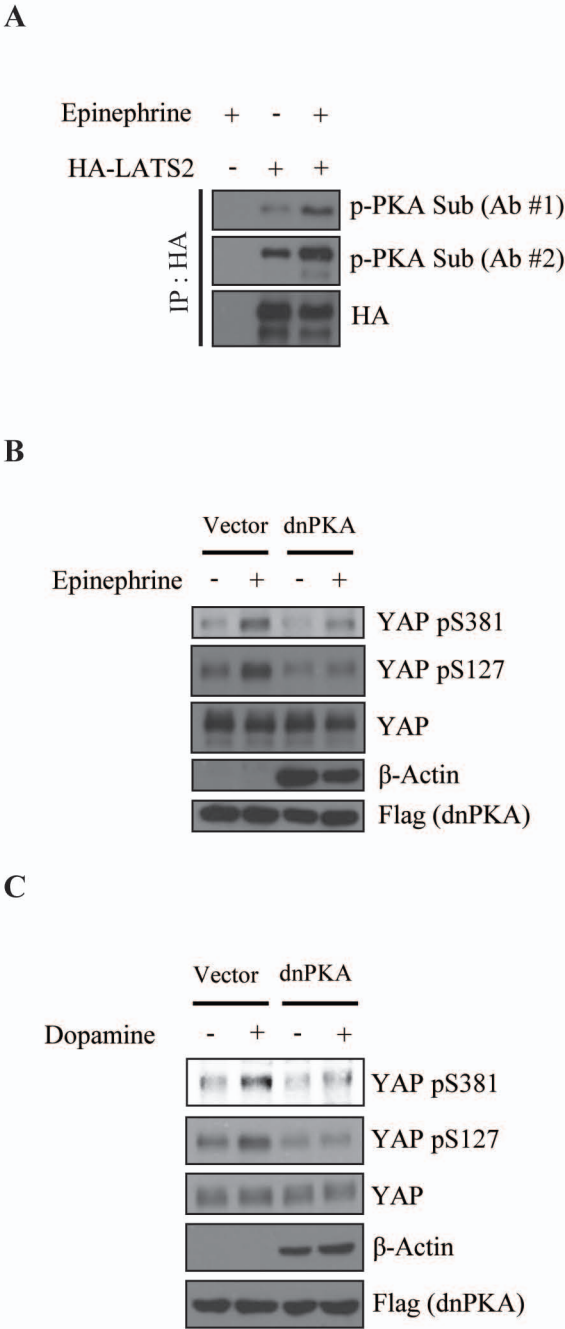

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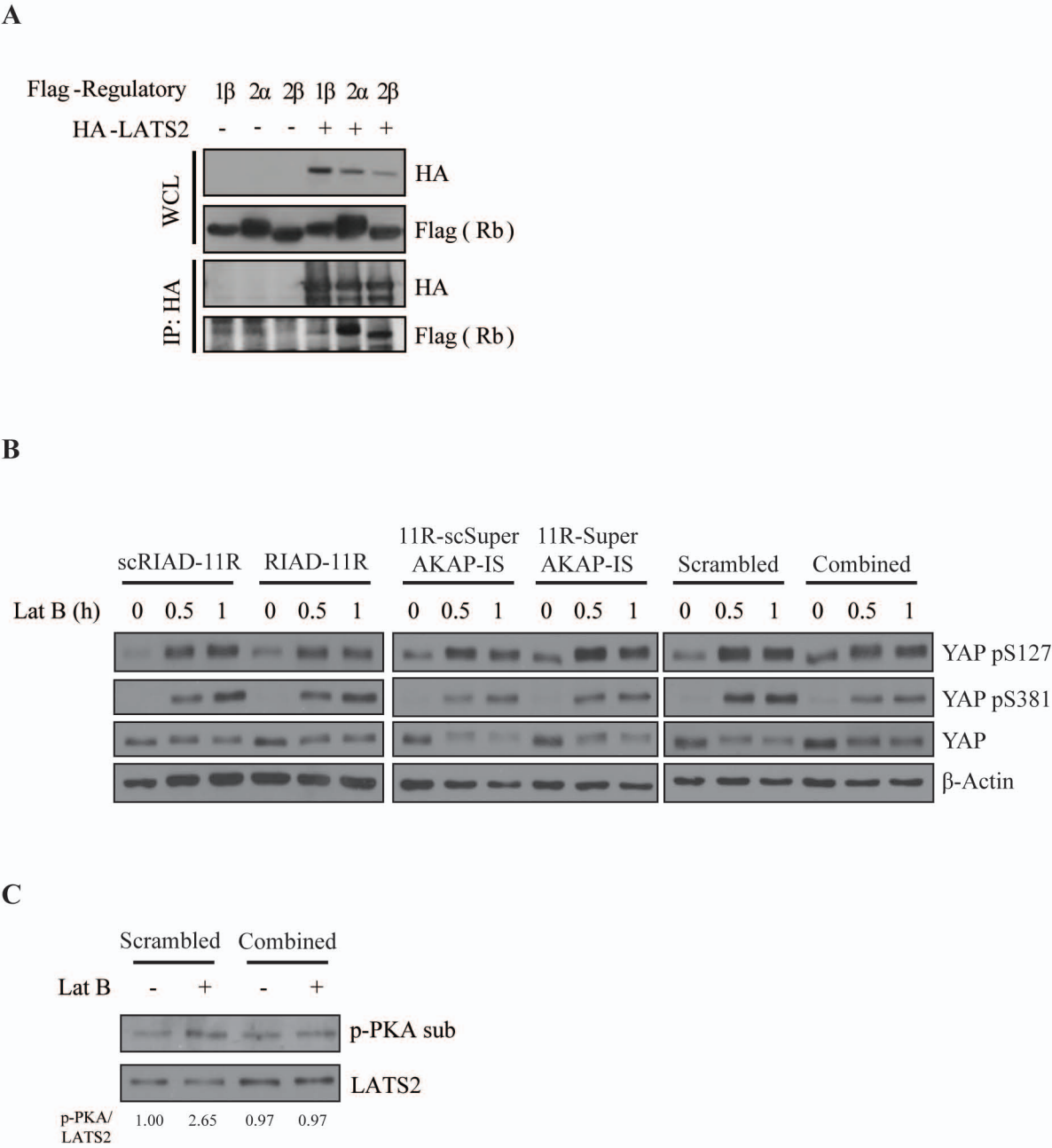

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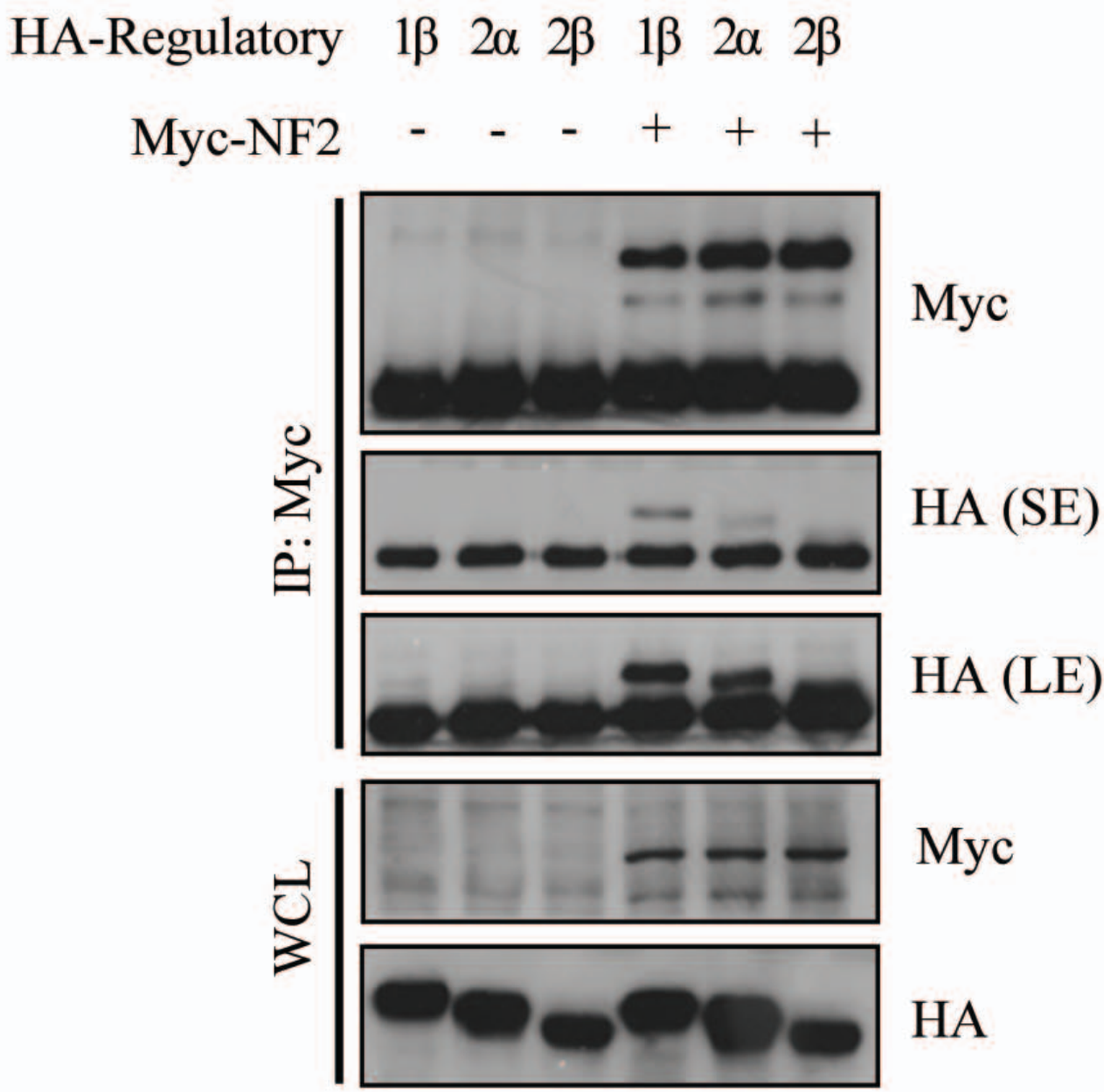

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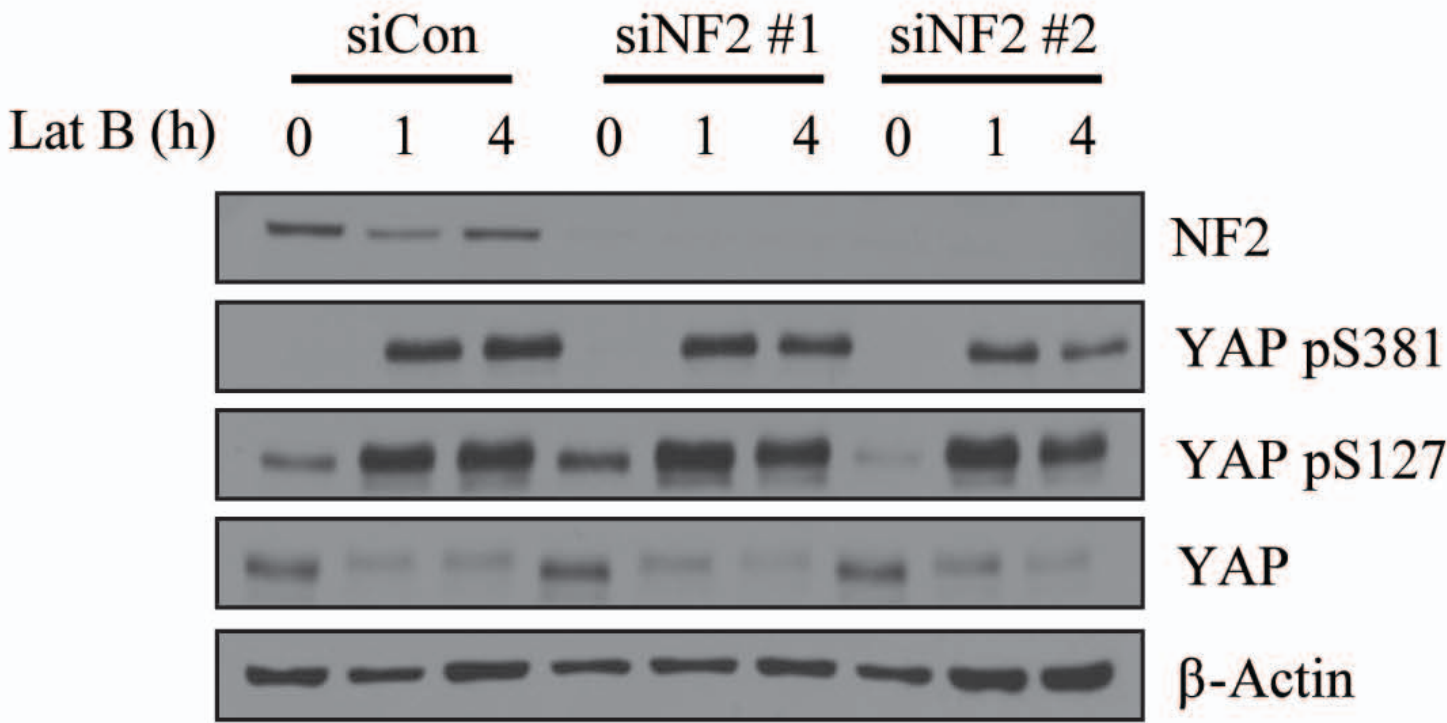

Supplementary Figure S16