Supplementary Note 1

Protein expression and purification

Viruses of STRIPAK core, PP2AA-C, and SLMAP–SIKE1 were amplified in Sf9 insect cells at 27°C using Sf-900 III SFM (Gibco) supplemented with 10% FBS, respectively. High five insect cells for the expression of STRIPAK core were cultured in EX-CELL 405 serum-free medium (Millipore-Sigma) at 27°C. Cells were harvested 1.5 days after viral infection and resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 2 mM $MgCl_2$, and 1 mM DTT). STRIPAK core was purified using amylose beads (New England Biolabs) pre-equilibrated with buffer A, followed by anion exchange chromatography with a Mono Q 5/50 GL column (GE Healthcare). For the activity assay, the fractions containing STRIPAK core were pooled and further purified by size exclusion chromatography (SEC) with a 10/300 Superose 6 column (GE Healthcare) equilibrated with buffer B (20 mM HEPES-Na, pH 7.5, 300 mM NaCl, 2 mM $MgCl_2$, and 0.5 mM TCEP). STRIPAK core was concentrated to 10 μM using a 100 kDa cut-off filter (Amicon Ultra) and stored at -80°C. For EM analysis, the peak fraction with the highest absorbance at 280 nm from Mono Q was collected and further purified by SEC with a 3.2/300 Superose 6 column (GE Healthcare) equilibrated with buffer C (20 mM HEPES-Na, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 0.5 mM TCEP, and 0.005% NP-40). The peak fraction with the highest absorbance at 280 nm (about 2 mg/ml) was used for EM analysis without further concentrating.

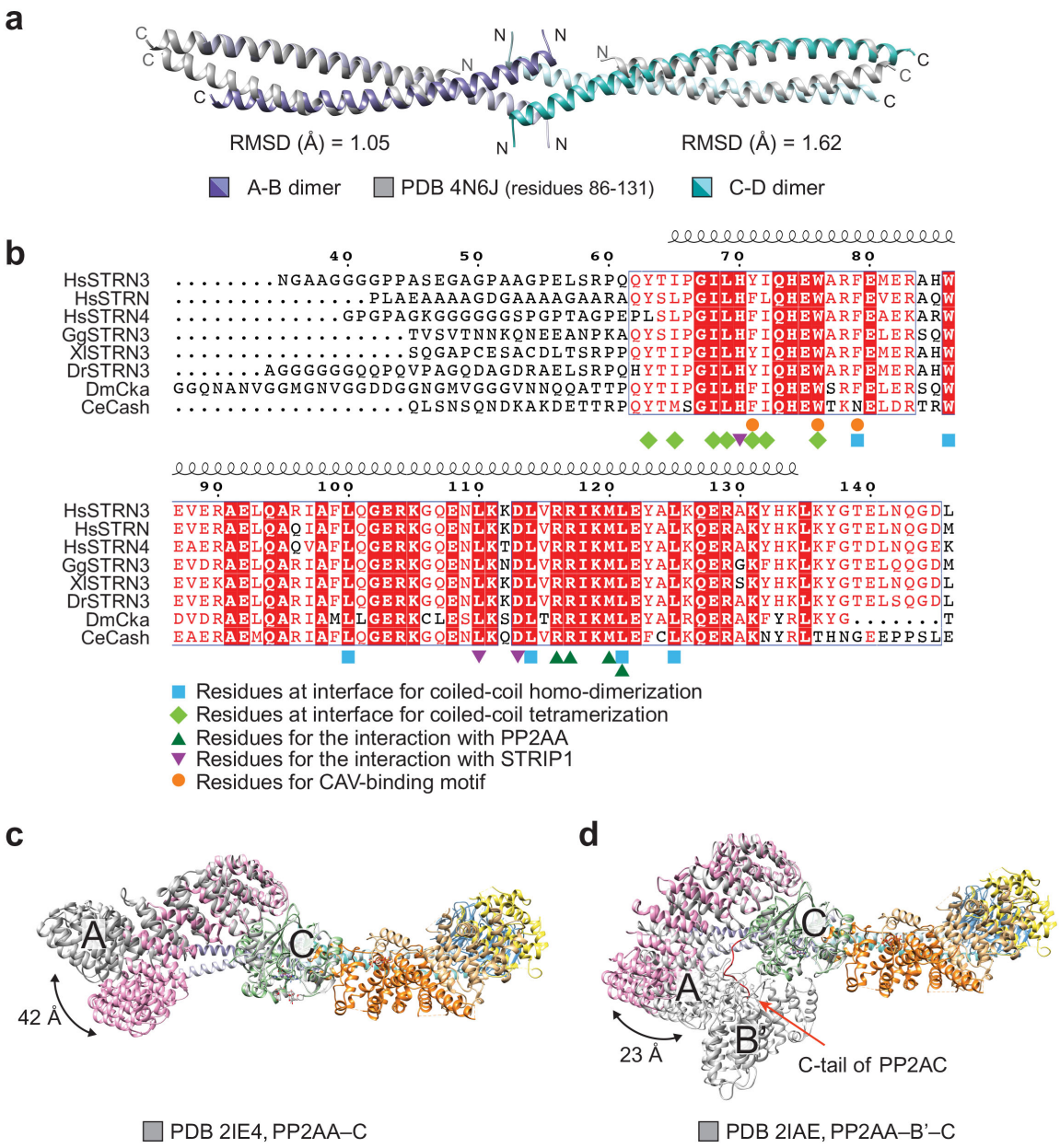
ESf9 insect cells for the expression of PP2AA-C and SLMAP–SIKE1 were cultured in ESF921 serum-free medium (Expression Systems) at 27°C, respectively. For PP2AA-C purification, cells were harvested 3 days after infection and resuspended in buffer D (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$, 20 mM imidazole, and 5 mM β-mercaptoethanol). PP2AA-C was purified using Ni-NTA beads (Qiagen) pre-equilibrated with buffer D, followed by

anion exchange chromatography with a RESOURCE Q column (1 ml, GE Healthcare). The fractions containing PP2AA-C were pooled and further purified by SEC with a 10/300 Superdex 200 (GE Healthcare) equilibrated with buffer B. The PP2AA-C-containing fractions were pooled and concentrated to 10 μM using a 30 kDa cut-off filter (Amicon Ultra) and stored at -80°C.

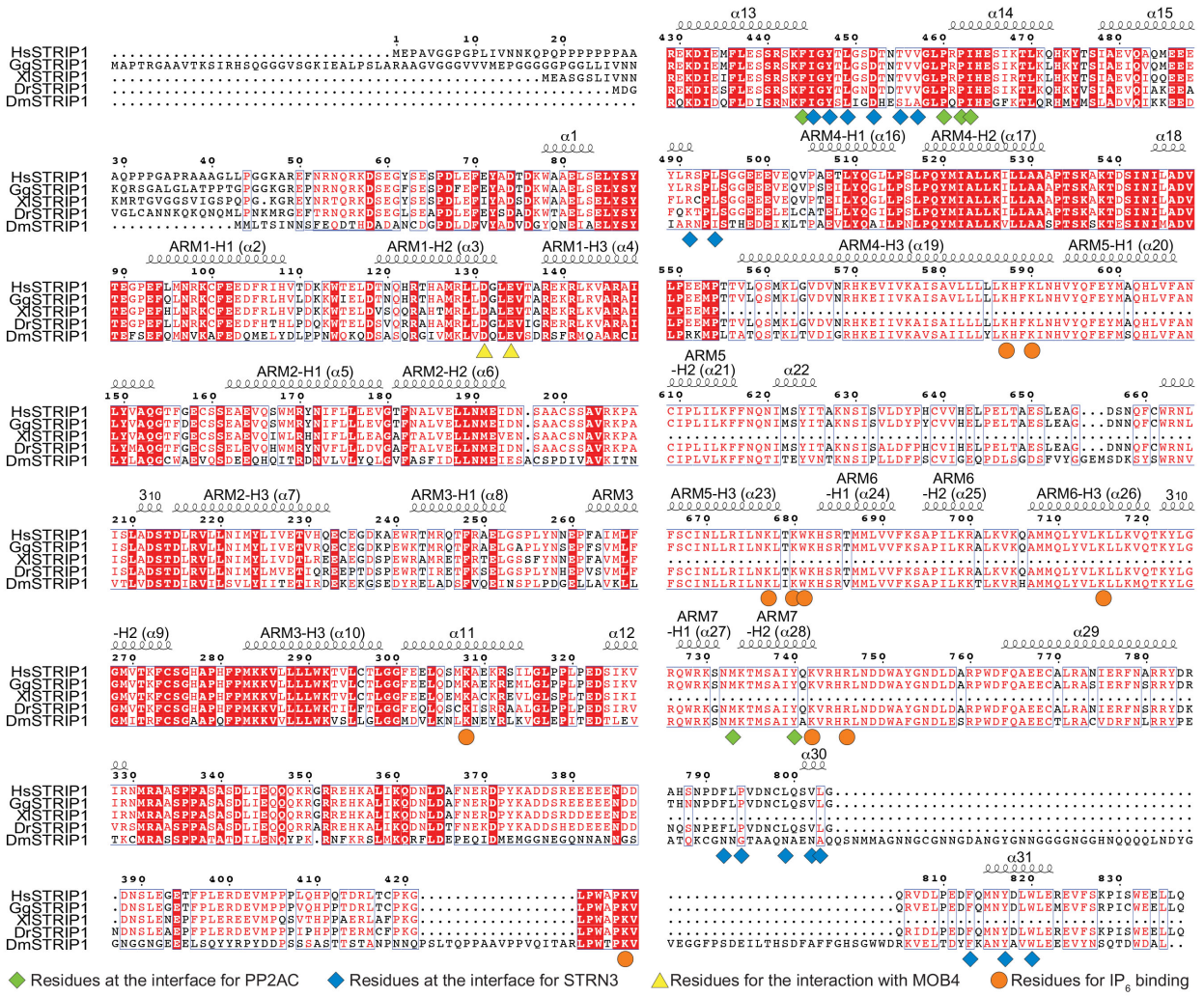
To purify SLMAP–SIKE1, cells were harvested 2.5 days after infection and resuspended in buffer A. SLMAP–SIKE1 was purified using amylose beads pre-equilibrated with buffer A, and further purified by anion exchange chromatography with a RESOURCE Q column (6 ml). The fractions containing SLMAP–SIKE1 were pooled and cleaved with 3C protease overnight at 4°C. The cleaved SLMAP–SIKE1 was re-loaded onto the RESOURCE Q column to remove the MBP moiety. The fractions containing SLMAP–SIKE1 were then pooled and further purified by SEC with a 10/300 Superose 6 equilibrated with buffer B. The fractions containing SLMAP–SIKE1 were pooled and concentrated to 10 μM using a 50 kDa cut-off filter (Amicon Ultra) and stored at -80°C.

The pET-28-STK25 and pET-28-CCM3 plasmids were transformed into in the bacterial strain BL21 (DE3)-T1^{R} cells (Sigma), respectively. STK25 was purified with Ni-NTA beads pre-equilibrated with buffer D, then cleaved with TEV protease overnight at 4°C. CCM3 was purified with Ni-NTA beads pre-equilibrated with buffer D. The cleaved STK25 mixture and CCM3 elution were purified by a RESOURCE Q column (6 ml), respectively. Fractions containing STK25 or CCM3 were pooled separately, STK25 was then mixed with 1.2 mol equivalent of CCM3. The STK25–CCM3 mixture was further purified by SEC with a 10/300 Superdex 200 column equilibrated with buffer E (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$, and 0.5 mM TCEP), and fractions containing STRK25–CCM3 were pooled and concentrated to 100 μM using a 50 kDa cut-off filter and stored at -80°C.

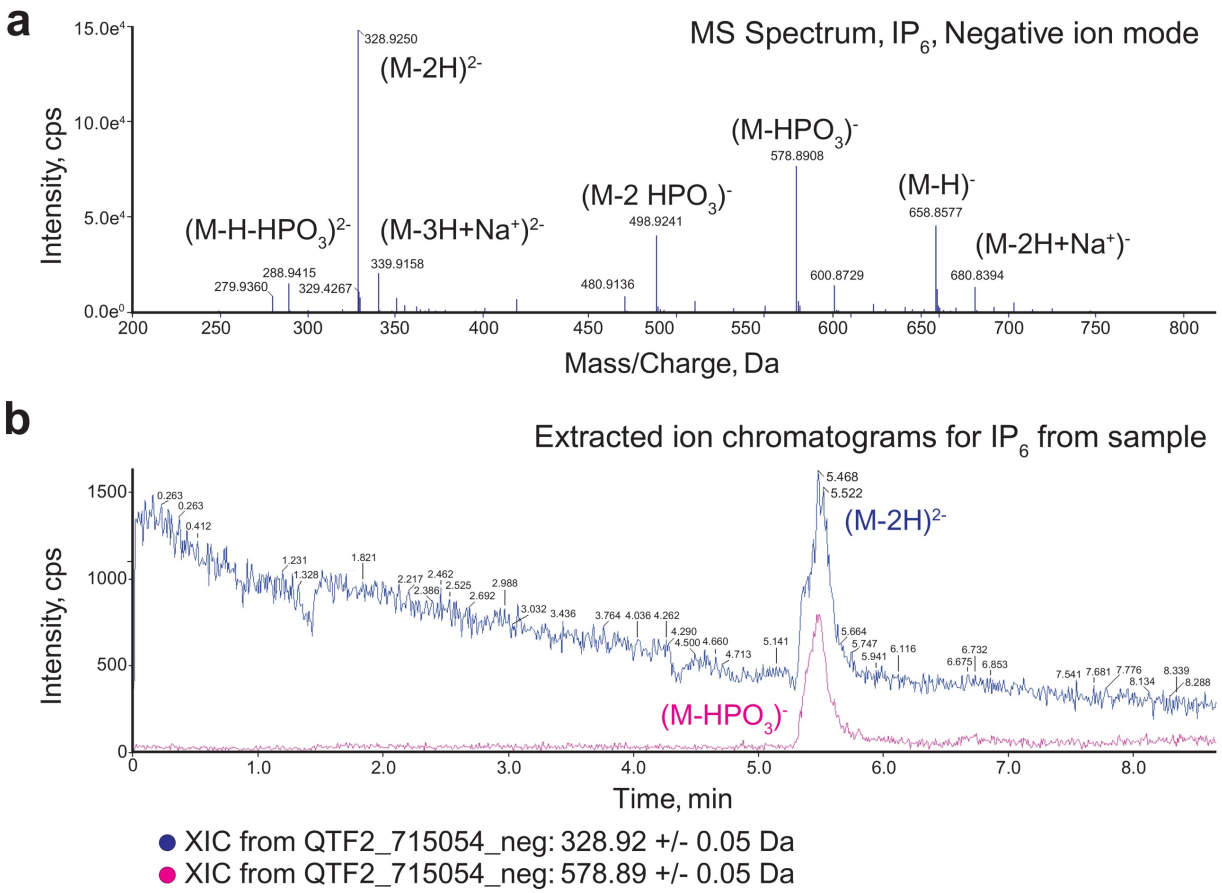
To purify the STRIPAK complex containing STK25–CCM3 (STRIPAK), purified STRIPAK core was mixed with 5 mol equivalent of purified STK25–CCM3. The mixture was purified by SEC with a 3.2/300 Superose 6 column equilibrated with buffer C. The peak fraction with the highest 280 nm absorbance (about 2 mg/ml) was used for EM analysis without additional concentrating step.



Supplementary Fig. 1. Structural analysis of STRN3 CC and PP2AA. **a**, Superposition of the A-B CC dimer (colored in light blue and slate) and the C-D CC dimer (colored in teal and cyan) with the crystal structure of the STRN3 CC dimer (colored in grey; PDB 4N6J), respectively. **b**, Sequence alignment of STRN3 CC from human, chicken, frog, zebrafish, fly and yeast (denoted by Hs-, Gg-, Xl-, Dr-, Dm-, and Ce- prefixes, respectively). The secondary structure elements and residue numbers of human STRN3 are shown above the aligned sequences. Residues with functional defects based on mutagenesis are indicated, and the scheme is shown below the sequence. CAV: caveolin. **c**, Superposition of STRIPAK with the PP2AA–C heterodimer (PDB 2IE4). **d**, Superposition of STRIPAK with the PP2AA–B′–C holoenzyme (PDB 2IAE). The C-terminal tail (C-tail) of PP2AC from PP2A holoenzyme is colored in red.



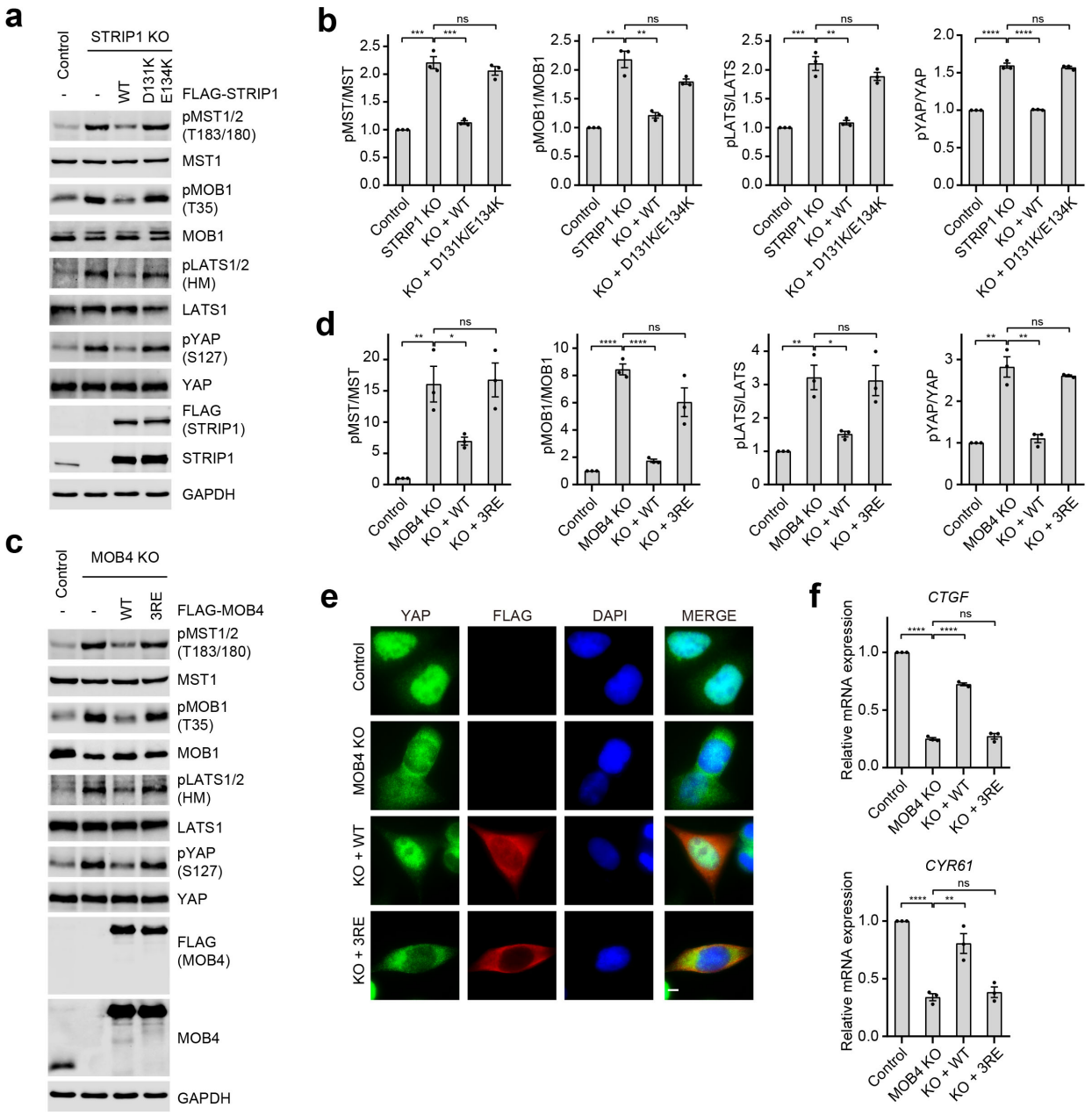
Supplementary Fig. 2. Sequence alignment of STRIP1. STRIP1 sequences are from human, chicken, frog, zebrafish, and fly. Secondary structures and residue numbers of human STRIP1 are shown above the aligned sequences. Residues with functional defects based on mutagenesis are indicated, and the scheme is shown below the sequence.



c

Peak	Formula	Composition	Charge	Expected mass	Observed mass	Error
$(M-H)^-$	$C_6H_6O_6(PO_3H_2)_6$,-H	$C_6H_{17}O_{24}P_6$	-1	658.854	658.858	0.004
$(M-HPO_3)^-$	$C_6H_7O_6(PO_3H_2)_5$,-H	$C_6H_{16}O_{21}P_5$	-1	578.887	578.891	0.004
$(M-2HPO_3)^-$	$C_6H_8O_6(PO_3H_2)_4$,-H	$C_6H_{15}O_{18}P_4$	-1	498.921	498.924	0.003
$(M-2H)^{2-}$	$C_6H_6O_6(PO_3H_2)_6$,-2H	$C_6H_{16}O_{24}P_6$	-2	328.923	328.925	0.002

Supplementary Fig. 3. IP_6 as a structural co-factor of STRIP1. **a**, MALDI-TOF mass spectrometry spectrum of the IP_6 standard. **b**, MALDI-TOF mass spectrometry spectrum of IP_6 isolated from recombinant human STRIP1. The 328.92 peak (IP_6, -2 charge, blue) and the 578.89 peak (IP_6, loss of phosphate group, -1 charge, magenta) are present in both the standard and STRIP1. **c**, The peak label, chemical formula, composition, charge, the monoisotopic molecular mass (expected and observed) and error of each daughter ion.



Supplementary Fig. 4. Functional validation of the MOB4–STRIP1 interface. **a**, Immunoblot of lysates of control or STRIP1 KO 293A cells transfected with mock vector or indicated plasmids. **b**, Quantification of the ratios of phospho- and total proteins in **a**. **c**, Immunoblot of lysates of control or MOB4 KO 293A cells transfected with mock vector or indicated plasmids. **d**, Quantification of the ratios of phospho- and total proteins in **c**. **e**, Immunofluorescence staining of YAP localization in control or MOB4 KO 293A cells transfected with mock vector or indicated plasmids. Scale bar, 5 μm. **f**, Relative expression of YAP target genes *CTGF* and *CYR61* in control or MOB4 KO 293A cells transfected with mock vector or indicated plasmids. Data in **b,d,f** are mean ± SEM of three independent experiments. Results were evaluated by Two-tailed unpaired t tests (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; ns, non-significant).