SUPPLEMENTAL DATA

Supplemental Materials and Methods

Co-Immunoprecipitations and Western Blotting

Co-Immunoprecipitations were performed as described (Lien et al., 2008). Briefly, keratinocytes were lysed in 50mM Tris-HCl, pH7.5, 100mM NaCl, 1% Triton X-100, 0.1mM EDTA, 0.5mM $MgCl_2$, 10% glycerol, protease inhibitor cocktail (Roche), phosphatase inhibitor cocktail (Roche), and 10μM pervanadate (for co-IPs examining tyrosine phosphorylated proteins). Lysates were pre-cleared with 30μL of a 50% Protein A/G sepharose beads (Thermo Fisher Scientific) for one hour, then incubated with antibody overnight at 4°C. Antibody/protein complexes were precipitated with 50μL Protein A/G (blocked in 1% IgG-free BSA) for 1 hour, washed in lysis buffer four times and analyzed by Western blot. For the FLAG-M2 IP assay, we used the Anti-FLAG M2 Affinity Gel (A2220) from Sigma.

DNA constructs:

Retroviral pBABE-FLAG-hYAP1 expression construct was generated by J. Brugge and was obtained from Addgene (Plasmid #: 15682). Mutagenesis was performed using a site-directed mutagenesis kit (Stratagene) with the following primers: hYAP Y247/8A: 5`-gccatgactcaggatggagaaattttctttataaaccataagaacaagac-3` and 5`-gtcttgttcttatggtttataaagaaaatttctccatcctgagtcatggc-3`. hYAP Y341F: 5`-tccttaacagtggcacctttcactctcgagatgag-3` and 5`-ctcatctcgagagtgaaaggtgccactgttaagga-3`. hYAP Y341E: 5`-tttccttaacagtggcaccgagcactctcgagatgagagta-3` and 5`-tactctcatctcgagagtgctcggtgccactgttaaggaaa-3`. hYAP Y357F: 5`-ctaagcatgagcagcttcagtgtccctcgaacc-3` and 5`-ggttcgagggacactgaagctgctcatgcttag-3`. hYAP Y357E: 5`-gggttcgagggacactctcgctgctcatgcttagt-3` and 5`-actaagcatgagcagcgagagtgtccctcgaaccc-3`. hYAP Y394F: 5`-agcagaaccgtttcccagacttccttgaagcca-3` and 5`-tggcttca aggaagtctgggaaacggttctgct-3`. hYAP Y394E: 5`-gaaccgtttcccagacgagcttgaagccattcctg-3` and 5`-caggaatggcttcaagctcgtctgggaaacggttc-3`. hYAP S127A: 5`-gttcgagctcatgcctctccagcttct-3` and 5`-agaagctggagaggcatgagctcgaac-3`. hYAP S397A: 5`-ctatcactctcgagatgaggctacagacagtggactaagc-3` and 5`-gcttagtccactgtctgtagcctcatctcgagagtgatag-3`. hYAP S94A: 5`-ggaagctgcccgacgccttcttcaagccg-3` and 5`-cggcttgaagaaggcgtcgggcagcttcc-3`. Retroviral vectors pCLXSN with wild-type and 3YF mutant human Integrin beta 4 were received from Dr. L. Trusolino (Bertotti et al., 2006). Retroviral vectors pLXSH and pLXSH mSrc Y529F were a gift from Dr. J. Cooper (FHCRC). 8xGTIIC plasmid was generated by Dr. S. Piccolo(Dupont et al., 2011) and was obtained from Addgene (plasmid

#34615). pUAS-LUC2 was generated by L. Luo (Potter et al., 2010) and obtained from Addgene (Plasmid #: 24343). pCMX-GAL4-TEAD4 was generated by Dr. K. Guan and obtained from Addgene (Plasmid #: 24640). CMV-*Renilla*-LUC was obtained from Promega. pFN19A-YAP2 was generated by PCR-amplifying YAP2 from GST-YAP2 (generated by K. Guan and obtained from Addgene (Plasmid #:24637)) with 5`-gccggcgatcg ccatggatcccgggcagcagccgccgcct-3` and 5`-aggggtttaaacctataaccatgtaagaaagctttctttatcta-3` and cloning using SgfI and PmeI. The retroviral pTURN-shLUC was a gift from Dr. P. Paddison (FHCRC). pTURN-shYAP was made by cloning shYAP from (Open Biosystems, Clone ID: V3LMM_415944) into XhoI and MluI sites of pTURN-6. The lentiviral pGIPZ control plasmid was obtained from Open Biosystems (RHS4346). pGIPZ Src#1 and Src#2 were obtained from Open Biosystems (Clone ID:V2LMM_39817 and Clone ID:V2LMM_216182, respectively).

Antibodies

The antibodies used were anti-α-catenin (Epitomics, 2028-1, or Sigma, C2081), anti-β-catenin (Sigma, C2206), anti-E-cadherin (Invitrogen, 13-1900), anti-β-actin (Sigma, A5441), anti-YAP (Santa Cruz, sc-101199), anti-pS127-YAP (Cell Signaling, C4911), anti-pS397-YAP (Cell Signaling, 13619), anti-Src (Cell Signaling, C2109), anti-pY416-SFK (Cell Signaling, C2101), anti-Integrin β4 chain (BD Transduction Laboratories, 553745), anti-pTyr-HRP (BD Transduction Laboratories, 610011), anti-pY357-YAP (Abcam, ab62751), anti-β-tubulin (University of Iowa Hybridoma Bank), anti-LaminB1 (Abcam, ab16048), anti-LATS1 (Cell Signaling, 3477), anti-LATS2 (Bethyl Laboratories, A300-479A), anti-TEAD4 (Aviva Systems Biology, ARP38276_P050), anti-pY341-YAP (generated against SGTY(PO3)-HSRDESTDS-Cys and anti-pY394-YAP (generated against Cys-QNRFPD-pY-LEAIPGT) were produced and affinity purified by Pacific Immunology, Ramona, CA.

***In vitro* kinase assays**

HALO-YAP (wild-type, Y341F, Y357F, Y394F or 3YF) used for *in vitro* kinase phosphorylation was translated *in vitro* using the TNT SP6 High-Yield Wheat Germ Protein Expression System (Promega), according to the manufacturer's directions. Translated HALO-YAP was bound to HALO-Link beads (Promega) and the translation reaction was washed away with kinase reaction buffer (KRB) containing 100mM Tris-HCl, pH 7.2, 125mM $MgCl_2$, 25mM $MnCl_2$, 2mM EGTA, 250μM sodium orthovanadate, 2mM DTT. The kinase reaction was performed at 30°C for 30 minutes with active Src (Millipore) and ATP (Sigma) in KRB. The kinase reaction was stopped with the addition of EDTA and

phosphorylated YAP was cleaved from the HALO-tag using TEV protease (Promega) as per the manufacturer's directions.

Human skin samples

Human samples of cutaneous SCCs and normal skin were obtained and analyzed following the UCSF and Fred Hutchinson Cancer Center IRB approved protocols. The diagnosis of cutaneous squamous cell carcinoma was confirmed for all tumors via histological examination of a standard biopsy specimen by a board-certified dermatopathologist at UCSF. Tumor samples were obtained by gross debulking during surgical treatment directed at these cancers and paired with control samples that were obtained from peritumoral normal skin removed during reconstruction. All samples were flash-frozen in liquid nitrogen and preserved at -80˚C until total protein extraction.

Supplemental Figures

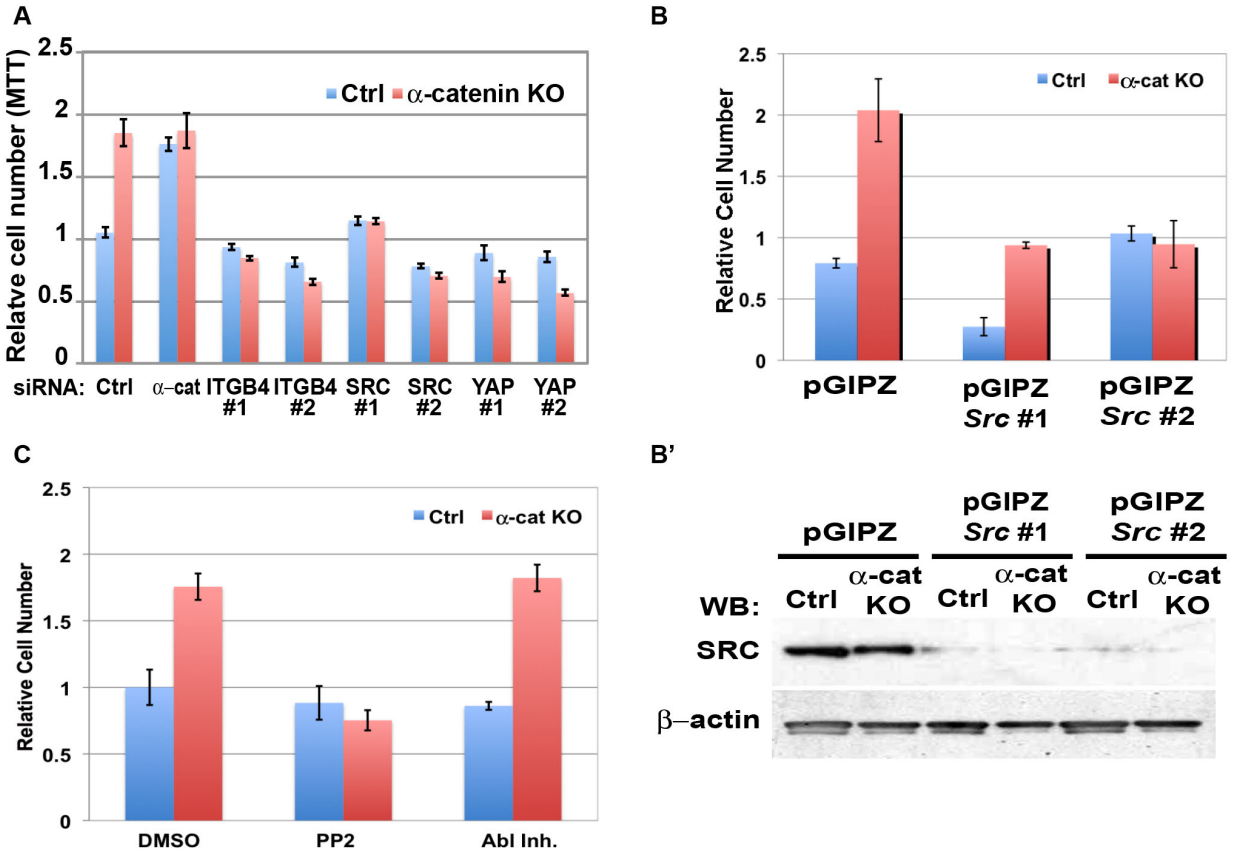

Figure S1. *Src*, *Itgb4* and *Yap1* are necessary for hyperproliferation of *αE-catenin*$^{-/-}$ keratinocytes.
(A) *αE-catenin*$^{fl/fl}$ (Ctrl) or *αE-catenin*$^{-/-}$ (α-cat KO) keratinocytes were plated at high density in indicated siRNA-Lipofectamine mixtures in triplicates and cultured for 5 days. Relative cell numbers were determined by MTT assay. Bar graphs show mean values ±SD. (B-B') shRNA-mediated knockdown reveals requirement for endogenous Src for hyperproliferation phenotype of *αE-catenin*$^{-/-}$ keratinocytes. *αE-catenin*$^{fl/fl}$ (Ctrl) or *αE-catenin*$^{-/-}$ (α-cat KO) mouse keratinocytes stably transduced with pGIPZ control, pGIPZ-Src#1 or pGIPZ-Src#2 shRNA lentiviruses were FACS sorted for EGFP, plated at high density and cultured for 5 days. Relative cell numbers were determined by MTT assay. Bar graphs show mean values ±SD. Western blot (WB) analyses of proteins from virus-transduced cells confirm Src knockdown. (C) Kinase inhibitor treatment reveals requirement for Src family kinases (SFK) for hyperproliferation phenotype of *αE-catenin*$^{-/-}$ mouse keratinocytes. *αE-catenin*$^{fl/fl}$ (Ctrl) or *αE-catenin*$^{-/-}$ (α-cat KO) keratinocytes were plated at high density and cultured for 5 days with DMSO, SFK inhibitor PP2, or Abl inhibitor Imatinib. Relative cell number was determined using MTT assay. Bar graphs show mean values ±SD.

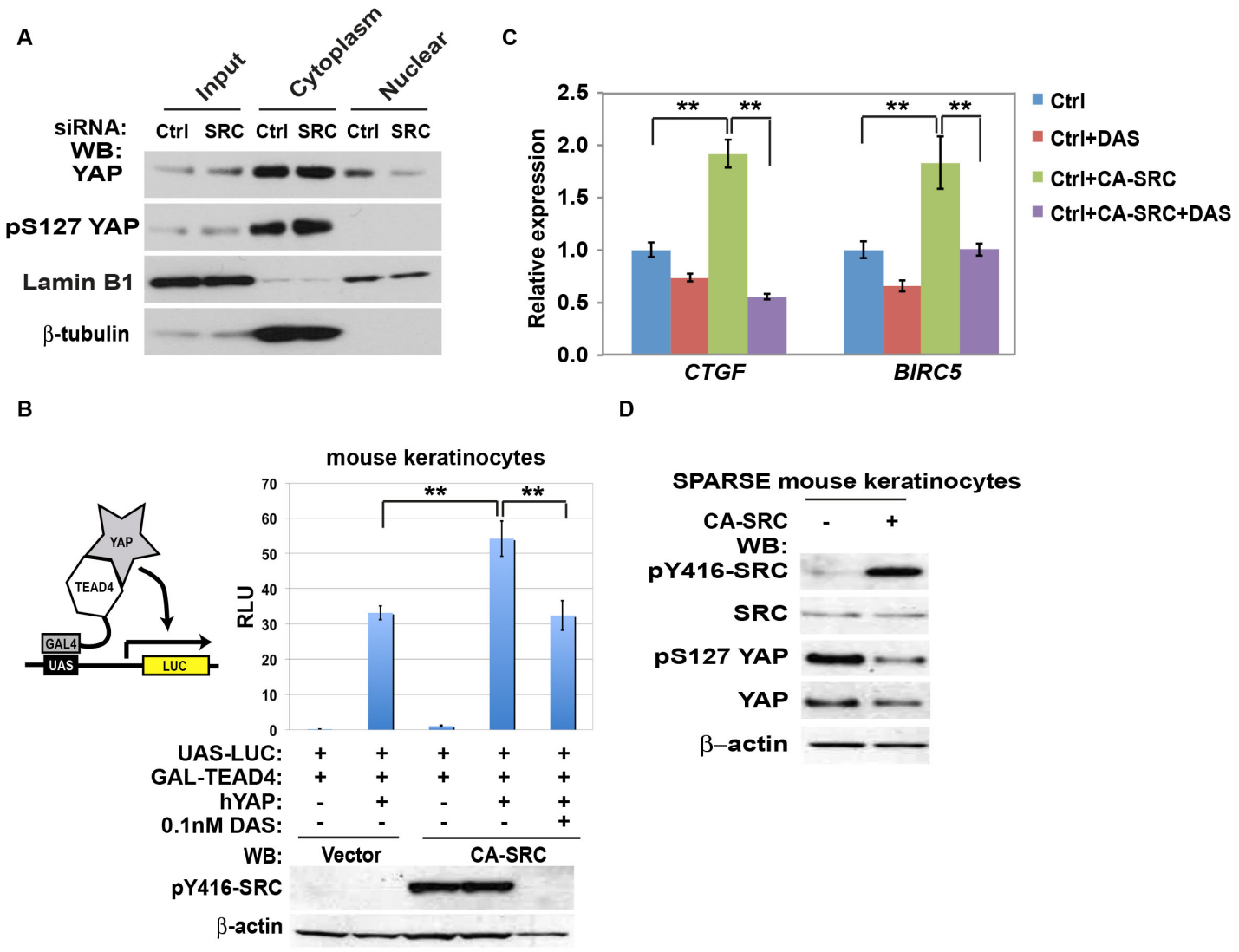

Figure S2. Constitutively active SRC (CA-SRC) increases transcriptional activity of YAP1.

(A) Endogenous SRC is necessary for nuclear localization of YAP1 in α*E-catenin*$^{-/-}$ keratinocytes. Western blot (WB) analyses of proteins extracted from total (Input), cytoplasmic or nuclear fractions of α*E-catenin*$^{-/-}$ keratinocytes with indicated antibodies. (B) Luciferase assay using confluent α*E-catenin*$^{fl/fl}$ keratinocytes stably transduced with empty vector or CA-SRC retroviruses and transfected with UAS-Luc, GAL4-TEAD4 and hYAP plasmids. The GAL4-TEAD4 fusion recruits YAP1 to the UAS element to induce expression of luciferase. *Renilla* luciferase was used for data normalization. DAS=0.1nM Dasatinib. Bars represent means ±SE. Student's t-test. n=4. (C) CA-SRC stimulates expression of endogenous YAP1 target genes. qRT-PCR analyses of *Ctgf* and *Birc5* in α*E-catenin*$^{fl/fl}$ keratinocytes stably transduced with vector (Ctrl) or CA-SRC, untreated or treated with Dasatinib (DAS). Bars represent means ±SD. Student's t-test. n= 3. (D) CA-SRC negatively regulates S127 YAP1 phosphorylation in sparse keratinocytes. Western blot (WB) analyses of total proteins from sparse α*E-catenin*$^{fl/fl}$ keratinocytes transduced with empty vector control (-) or CA-SRC (+) using indicated antibodies.

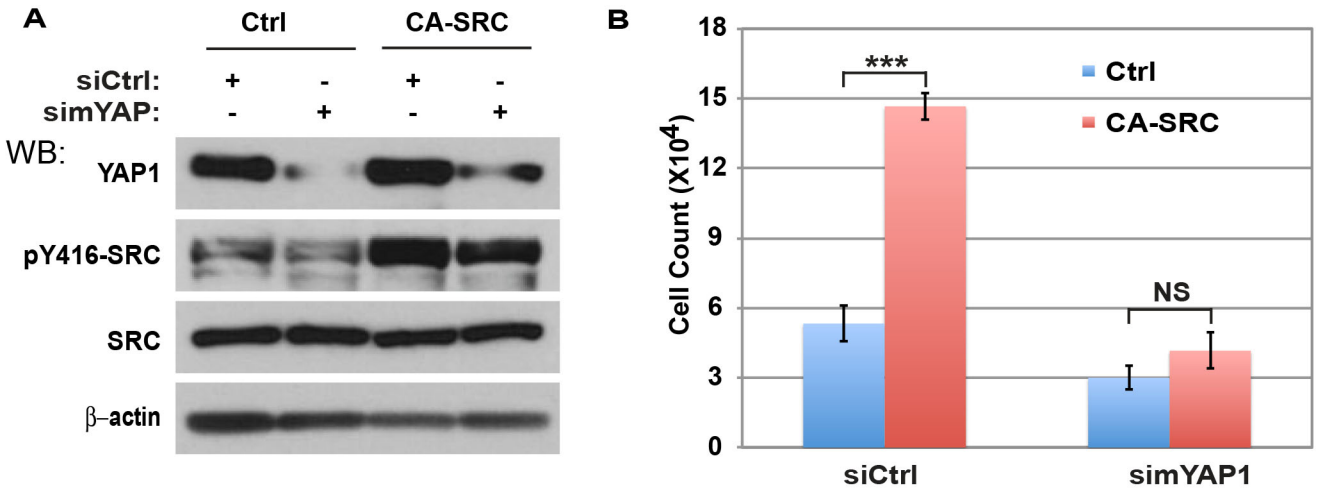

Figure S3. Endogenous YAP1 is necessary for CA-SRC-mediated hyperproliferation of *αE-catenin*$^{fl/fl}$ keratinocytes. (A) Western blot (WB) analyses of total proteins from *αE-catenin*$^{fl/fl}$ keratinocytes stably transduced with empty vector control (Ctrl) or CA-SRC and transfected with control (siCtrl) or mouse-*Yap1 (simYAP)* siRNA oligos using indicated antibodies. (B) *αE-catenin*$^{fl/fl}$ keratinocytes stably transduced with empty vector control (Ctrl) or CA-SRC were plated at high density in indicated control (siCtrl) or mouse-*Yap1 (simYAP1)* siRNA-Lipofectamine mixtures in triplicates and cultured for 5 days. Relative cell numbers were determined by cell counting. Bars represent means ±SD. Student's t-test.

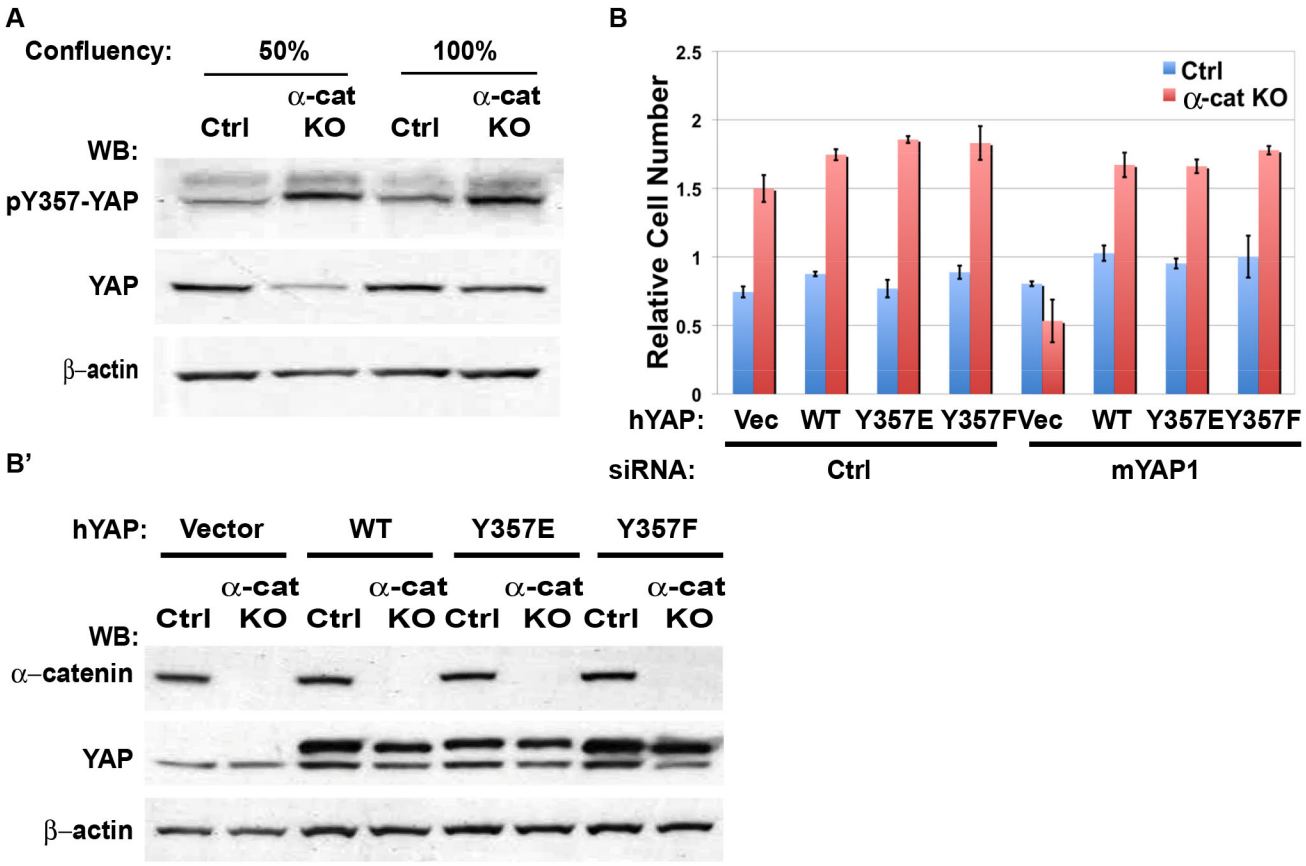

Figure S4. Tyrosine phosphorylation of YAP1 at Y357 and lack of functional significance of this phosphorylation in *αE-catenin*$^{-/-}$ keratinocytes.

(A) Y357 of YAP1 is hyperphosphorylated in *αE-catenin*$^{-/-}$ keratinocytes. WB analyses of proteins from *αE-catenin*$^{fl/fl}$ (Ctrl) and *αE-catenin*$^{-/-}$ (α-cat KO) mouse keratinocytes cultured at 50% or 100% confluency with anti-phospho-Y357-YAP (pY357-YAP), anti-total YAP (YAP) and anti-β-actin antibodies. (B-B') Phosphorylation of Y357 in YAP1 is not required for hyperproliferation of *αE-catenin*$^{-/-}$ mouse keratinocytes. Relative proliferation of *αE-catenin*$^{fl/fl}$ (Ctrl) or *αE-catenin*$^{-/-}$ (α-cat KO) mouse keratinocytes stably transduced with empty vector, wild-type (WT) or mutant (Y357E or Y357F) human YAP1 and transiently transfected with scrambled (Ctrl) or mouse YAP1-targeting (mYAP) siRNA oligos. Cell were plated at high density and cultured for 5 days. Relative cell numbers were determined by MTT assay. Bar graphs show mean values ±SD. WB analyses confirm expression of wild-type and mutant human YAP1 proteins, B'.

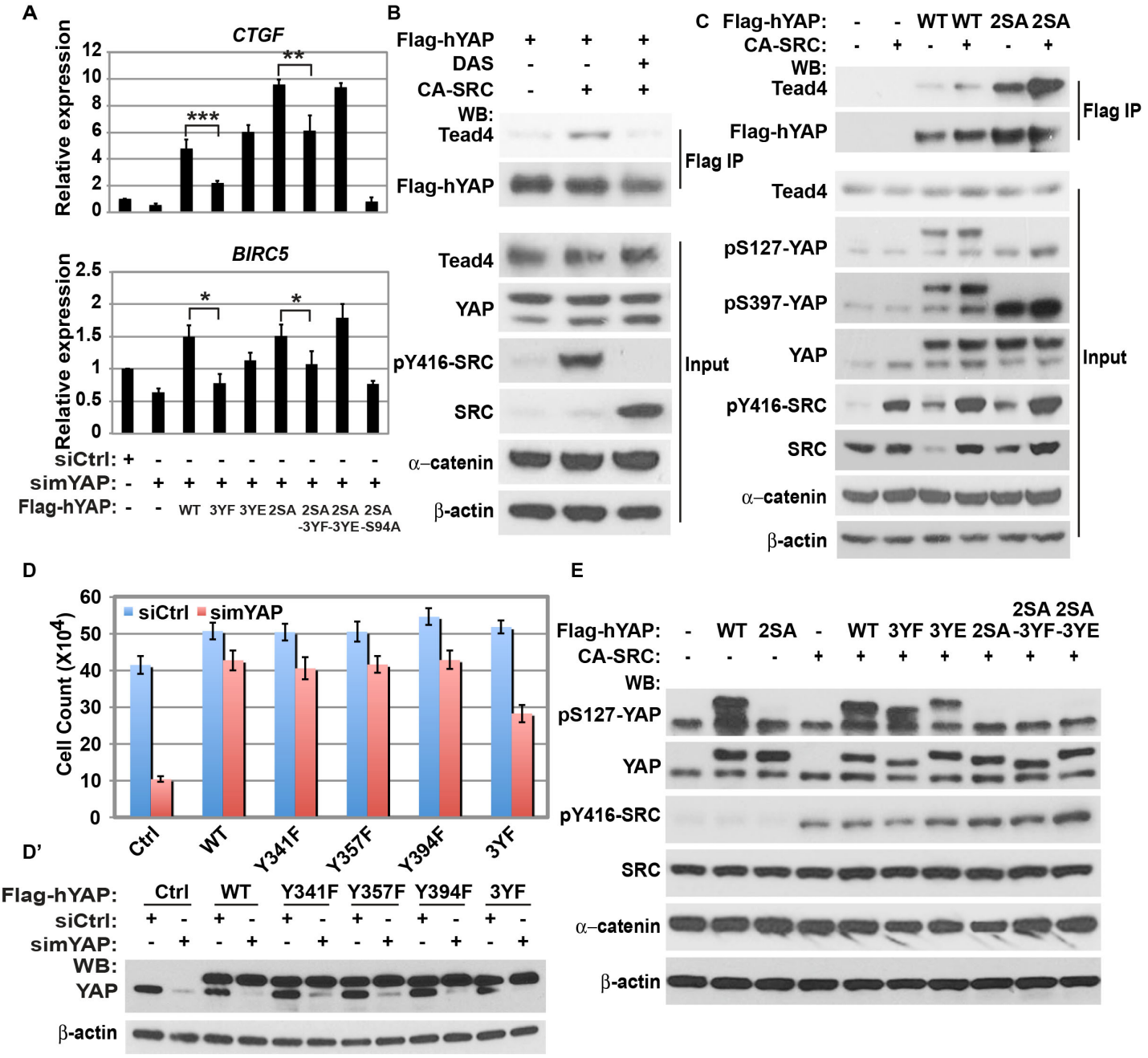

Figure S5. Critical role of SRC-mediated tyrosine phosphorylation of YAP1 for its transcriptional activity, interaction with TEAD and hyperproliferation of *αE-catenin*$^{-/-}$ keratinocytes.

(A) Y341/357/394 (3Y) YAP1 phosphorylation is necessary for YAP1 transcriptional activity. qRT-PCR analyses of *Ctgf* and *Birc5* in *αE-catenin*$^{-/-}$ keratinocytes stably transduced with empty vector (Ctrl) or indicated human YAP1 (Flag-hYAP) constructs and also transfected with control (siCtrl) or mouse-YAP1 (simYAP1) siRNA oligos. Graph shows mean values ±SD. Student's t-test. n=3. (B-C) CA-SRC promotes interaction between YAP1 and TEAD. WB analyses of total (Input) or anti-FLAG antibody-immunoprecipitated (Flag IP) proteins from *αE-catenin*$^{fl/fl}$ keratinocytes stably expressing CA-SRC, wild-type YAP1 (Flag-hYAP in B, WT in C), and S127/397A YAP1 (2SA in C) using indicated antibodies. DAS, 0.1nM Dasatinib. (D-D') 3Y YAP1

phosphorylation is necessary for hyperproliferation of α*E-catenin*$^{-/-}$ keratinocytes. Cell counts of α*E-catenin*$^{-/-}$ keratinocytes stably expressing vector control (Ctrl), wild-type (WT) or indicated Y mutant human YAP1 proteins, transfected with control (siCtrl) or mouse YAP1-specific (siYAP1) oligos, plated at high confluency and cultured for 5 days. Bar graph shows the mean values ±SD. WB analyses of total proteins from cells in D using indicated antibodies. (E) WB analyses of total proteins from cells described in Figure 6F-G'.

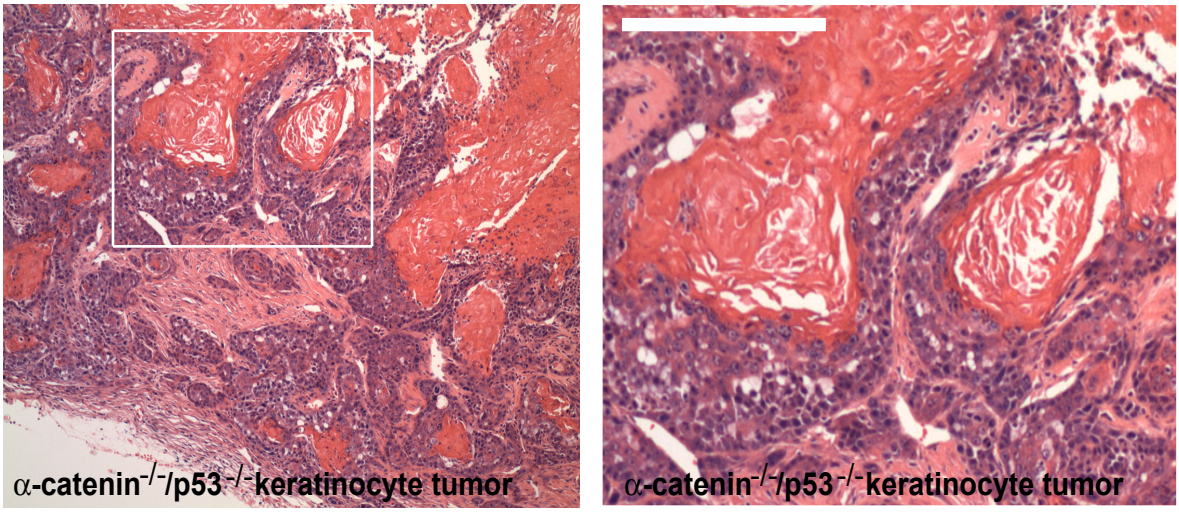

Figure S6. Orthotopic allograft tumors generated by intradermal injection of *αE-catenin$^{-/-}$/p53$^{-/-}$* mouse SCC cells are histologically identical to primary SCC tumors from *GFAP/αE-catenin$^{-/-}$/p53$^{-/-}$* mice (Silvis et al., 2011). Hematoxylin & eosin staining of paraffin sections from skin tumors generated by intradermal injection of *αE-catenin$^{-/-}$/p53$^{-/-}$* mouse SCC cells. Area highlighted in white box (left frame) is shown at higher magnification on the right. Size bar, 150μm.

SUPPLEMENTARY DATA REFERENCES

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