Supplemental Materials and Methods

Immunohistochemistry and image analysis. Paraffin-embedded sections were cut into 6μm sections and antigen retrieval was performed by incubating the slides in 0.01M citric acid buffer (pH6.0) at 95ºC for 20 min. Sections were then blocked with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) at room temperature (RT) for 30 min and incubated with the primary antibodies overnight at 4ºC. Cultured cells in dishes were fixed in 10% buffered formalin for 10 min and permeabilized in 0.1% Triton X-100 in PBS for 5 min, followed by blocking with 10% FBS at RT for 30 min. The primary antibodies used were mouse anti-p63 (clone 4A4, 1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-p63 (clone H137, 1:500 dilution, Santa Cruz Biotechnology), rabbit anti-phosphorylated p63 (#4981-corresponding to Ser66/68 of ΔNp63α, 1:100 dilution, Cell Signaling Technology, Danvers, MA), biotin-labeled rat anti-integrin β4 (clone 439-9B, 1:25 dilution, eBiosciences, San Diego, CA), rat anti-cytokeratin (clone Troma I, 1:100 dilution, Developmental Hybridoma Bank, University of Iowa) and mouse anti-cytokeratin 14 (clone CBL197, 1:100 dilution, Santa Cruz Biotechnology) antibodies. Following three washes with 0.1% Tween-20 in phosphate buffered saline (PBS), the antigens were visualized using the fluorescence labeled secondary antibodies for 1 hr at RT. Secondary antibodies used were Alexa 488-goat anti-mouse IgG, Alexa 488-goat anti-rabbit IgG, Alexa 594-goat anti-mouse IgG, Alexa 594-goat anti-rabbit IgG, Alexa 594-goat anti-rat IgG (all 1:1,000 dilution, Molecular Probes, Grand Island, NY), pre-adsorbed DyLight 594-goat anti-mouse IgG (1:1,000 dilution, Abcam, Cambridge, MA) and Alexa 647-conjugated Streptavidin (1:500 dilution, Jackson Immuno Research Laboratories, West Grove, PA). All fluorescence imaging was done on a Nikon Eclipse 80i microscope with a Nikon DS-Qi1Mc digital camera using the NIS Elements software (Nikon, Tokyo, Japan). To quantify p63 and pp63 signals in the epidermis, approximately 350 epidermal cells per field were captured with a x10 lens. Fluorescence intensities for the p63 and pp63 signals were normalized by nuclear counterstaining with Hoechst 33342 dye (Molecular Probes).

HPEK differentiation assays. Human primary epidermal keratinocytes (HPEKs) (Cellntec, Bern, Switzerland) were grown in CnT-57 basal medium (Cellntec) that contains 0.07mM Ca^{2+} and human keratinocyte growth supplement CnT-57.S (Cellntec) at 37°C with 5% CO_2. Sub-confluent cultures were washed twice with PBS and induced to differentiate in the growth factor-deficient Ham's F12 medium (Mediatech, Manassas, VA) in the presence of either 0.3mM or 1.3mM $CaCl_2$ (Sigma-Aldrich, St. Louis, MO). Culture medium was changed every 24 hrs and cells were harvested at day 0, 1, 3 and 5 after the induction of differentiation.

Clonogenic culture and cell isolation. Human primary epidermal keratinocytes were cultured according to the Green method (Barrandon and Green, 1987) and used at passage 4-5. Briefly, the cells were grown on lethally irradiated 3T3-J2 cells in complete FAD medium (DMEM/Ham's F12 = 3:1 medium) containing

10ng/ml epidermal growth factor (R&D Systems, Minneapolis, MN), 5μg/ml insulin, 2x10^{-9} M 3,3',5-triiodo-L-thyronine, 0.4μg/ml hydrocortisone, 24μg/ml adenine, 1x10^{-10} M cholera toxin (all from Sigma-Aldrich), supplemented with 10% FBS, 10U/ml penicillin and 100μg/ml streptomycin (Invitrogen, Grand Island, NY). To isolate peripheral and central cells from holoclones, day 12-14 clones were gently trypsinized for 5 min with 0.05% trypsin followed by neutralization with complete FAD medium to stop enzymatic digestion. While semi-attached to the culture plates, cells in the periphery and the center of holoclones were harvested by gentle pipetting under a Nikon Eclipse TS100 inverted microscope. We routinely collected 0.3-1x10^4 cells from the periphery and the center of holoclones. Using a small portion of the isolated cells (approximately 1,000 cells), the purity of the isolated cell populations was determined by cytospin immunofluorescence (Cytospin 4, Thermo Scientific, Asheville, NC) using anti-p63 and anti-phosphorylated p63 antibodies. Although cells with intermediate levels of p63 phosphorylation were found in samples from both the periphery and the center of holoclones, cells with low p63 phosphorylation were almost exclusively found in the peripheral samples (see Figure 2b). The isolated cells were expanded for 9 days to obtain cells for subsequent cultures. Equal numbers of epidermal cells (200 cells/well) were then seeded in 6-well plates and allowed to grow for two weeks. Subsequently, equal percentages of cells (1, 3 and 10% of culture) were grown with two-week intervals. Shown in data are from 10% subcultures. At each passage, the clones were fixed in 10% buffered formalin and stained with 1% rhodamine B (Sigma-Aldrich) to visualize epithelial cell colonies. Holoclones were determined based on cellular morphology within the clones (see Figure S2) that showed greater than 2mm in diameter.

Western blot analysis. Cells were suspended in RIPA buffer containing 50mM Tris-HCl (pH6.8), 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate, 10% glycerol, 1mM phenylmethanesulfonyl fluoride and 1mM sodium orthovanadate. Equal amounts of protein were subjected to SDS-PAGE followed by Western blot analysis. The primary antibodies used were mouse anti-p63 (1:1,000 dilution), rabbit anti-phosphorylated p63 (1:250 dilution), mouse anti-cytokeratin 14 (1:1,000 dilution), rabbit anti-integrin β1 (clone EP1041Y, 1:2,000 dilution, Abcam), mouse anti-involucrin (clone SY5, 1:2,000 dilution, NeoMarkers, Kalamazoo, MI) and mouse anti-tubulin α (clone 12G10, 1:500 dilution, Developmental Hybridoma Bank, University of Iowa) antibodies. Secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5,000 dilution, Vector Laboratories, Burlingame, CA) and HRP-conjugated goat anti-rabbit IgG (1:2,500 dilution, Cell Signaling Technology) antibodies.

Animals. Mice used in this study were on a mixture of C57BL/6 and 129Sv background and were handled in accordance with the Guidelines for Animal Experiments at University of Pennsylvania. p63-null animals were generated by homologous recombination in our lab and they show identical phenotypes as reported (Mills et al., 1999; Yang et al., 1999). The details of the generation of

these mice will be reported elsewhere. For staged embryos, the day of the vaginal plug was designated E0.5.

Mutagenesis. Site-directed mutagenesis of the p63 phosphorylation sites was performed using Phusion Site-Directed Mutagenesis kit (Finnzymes, Vantaa, Finland) with full-length human ΔNp63α cDNA as a template and mutated gene-specific primers: 5'-GAT GCT CTC GCT CCA GCA CCC GCC-3' and 5'-GAA GGT GGG GCT GGG CTG TGC GTA-3'. Correct mutations were confirmed by direct DNA sequencing and both wild type and mutated (S66A/S68A) cDNAs were subcloned into the pcDNA3 vector (invitrogen).

Cell culture and transient transfection. Baby hamster kidney (BHK) cells and human embryonic kidney 293T (HEK293T) cells were grown in DMEM medium containing 10% FBS, 10U/ml penicillin and 100μg/ml streptomycin in a humidified chamber at 37ºC with 5% CO_2. Cells were transiently transfected with 1μg pcDNA3 constructs (see above) by calcium-phosphate transfection. Twenty-four hours after transfection, cells were either fixed in 10% buffered formalin for immunohistochemistry or lysed in RIPA buffer for Western blot analysis.

Box-and-whisker plots. The bottom and top of the box represent the 25th and 75th percentiles, respectively, and the band within the box shows the 50th percentile. The ends of whiskers represent the lowest value within 1.5 interquartile range (IQR) of the lower quartile and the highest value within 1.5 IQR of the upper quartile while dots outside the whiskers indicate outliers.

Statistical analysis. Values are reported as mean ± standard error of the mean (SEM). Student's *t*-tests were performed with a *P* value where *p* < 0.05 was considered statistically significant.

Supplemental References

Mills AA, Zheng B, Wang XJ, *et al* (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* 398: 708-13.

Yang A, Schweitzer R, Sun D, *et al* (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 398: 714-8.
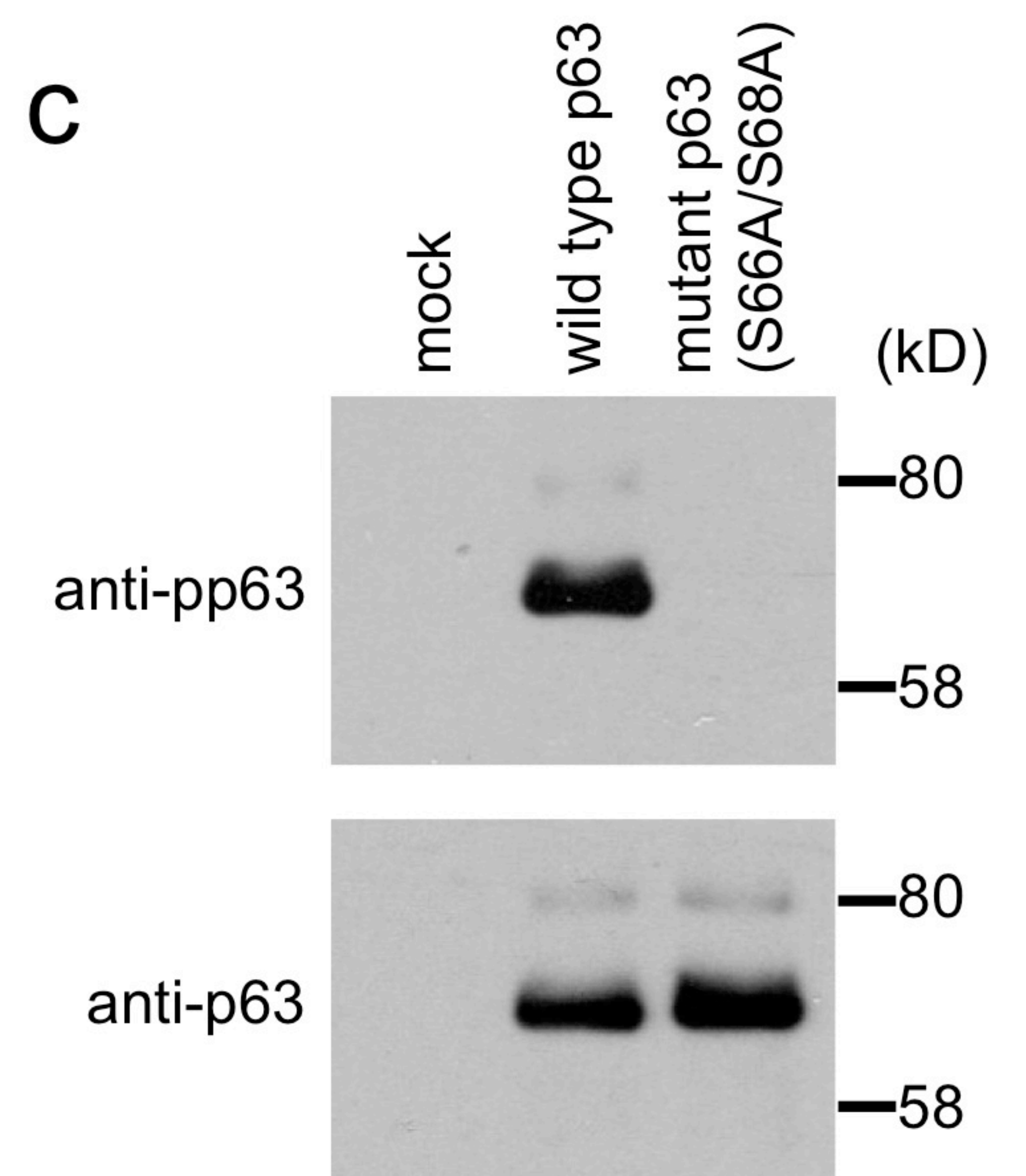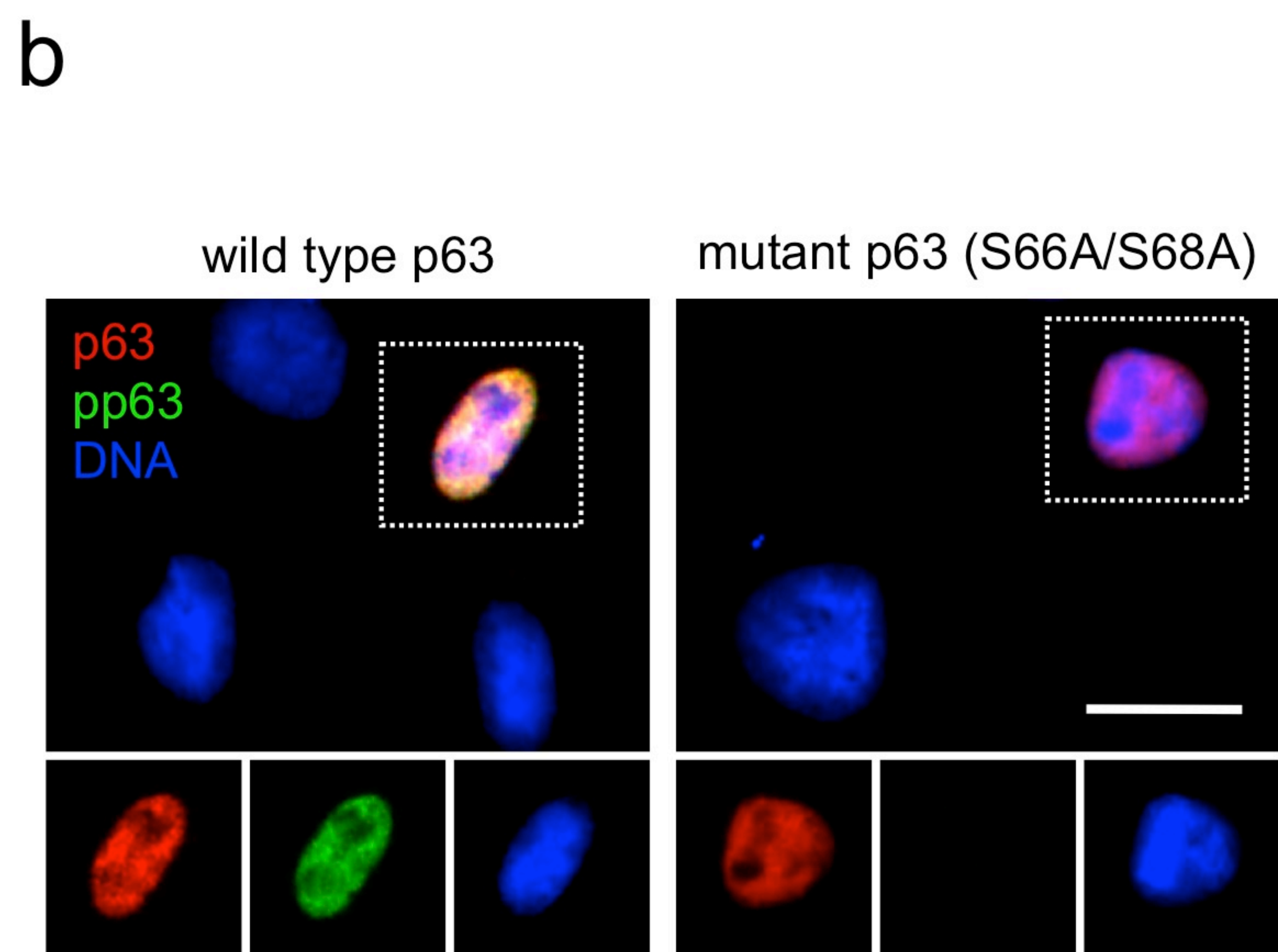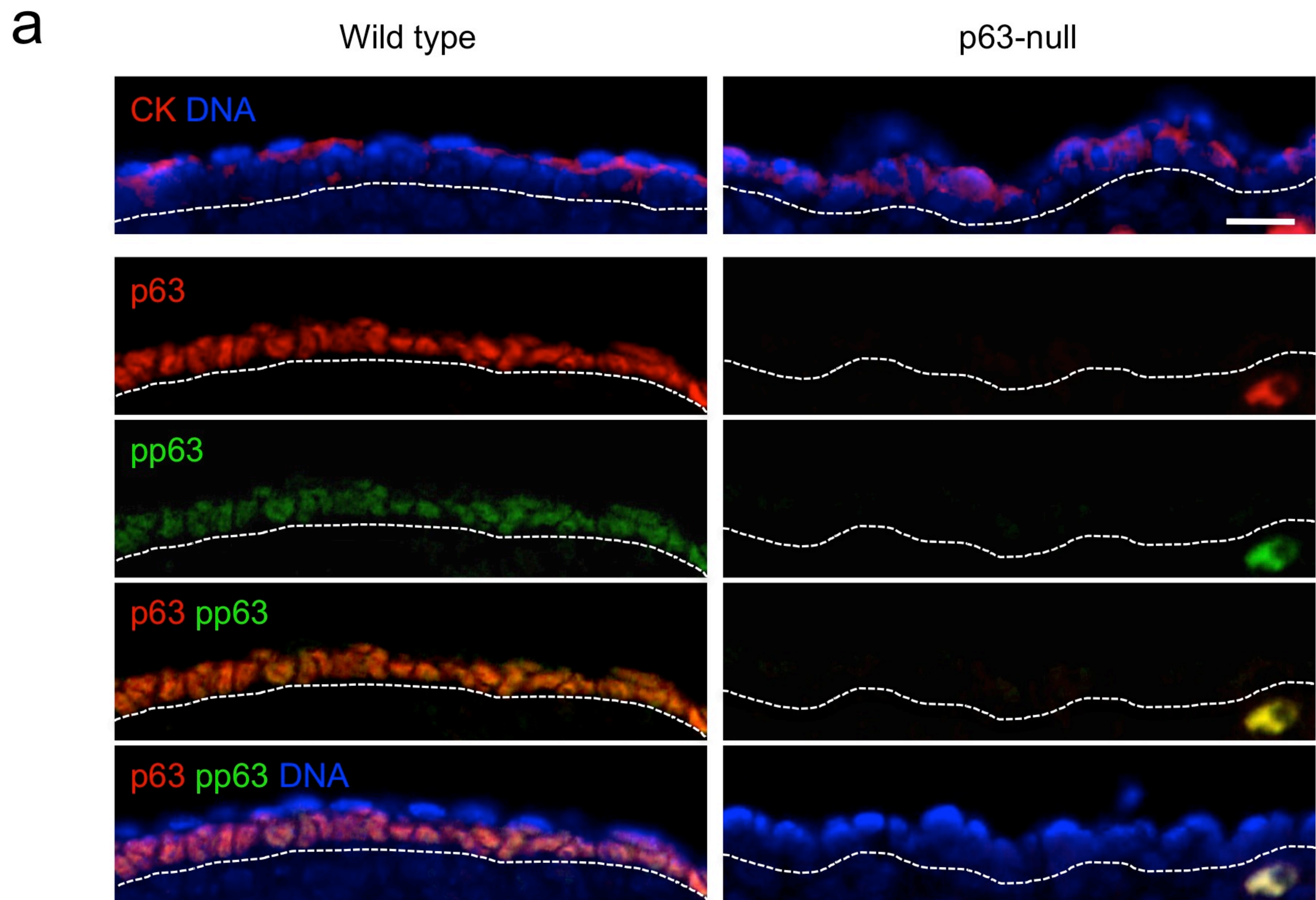
Supplemental Figure Legends

Figure S1. Validation of the specificity of anti-pp63 antibodies.

In (**a**)-(**c**), mouse anti-p63 (pan-p63, clone 4A4) and rabbit anti-phosphorylated p63 (anti-pp63, Cell Signaling Technology) antibodies were used.
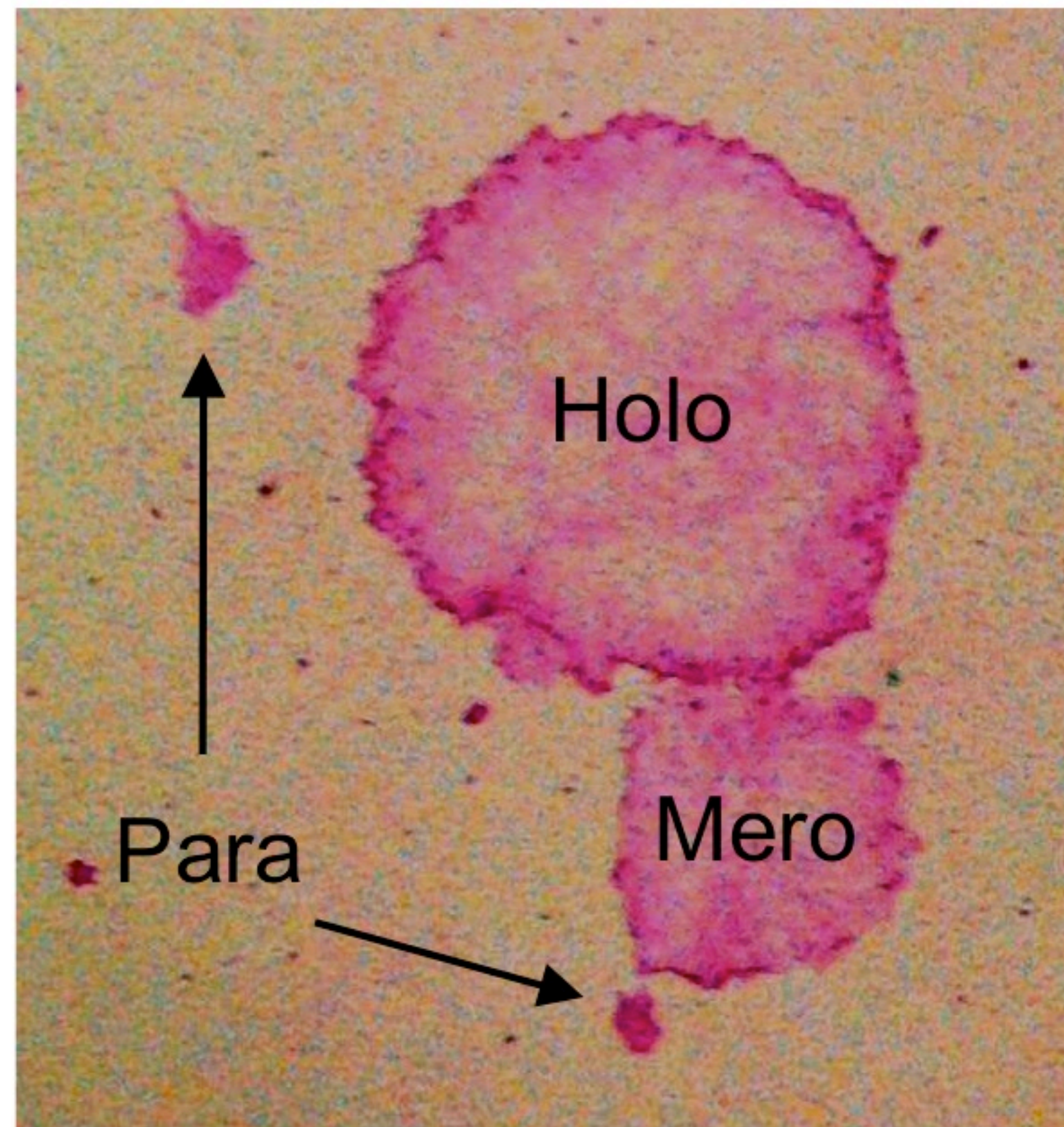(**a**) Immunofluorescence staining of E12.5 mouse epidermis for pp63 and p63. Note that p63-null embryos have cytokeratin (CK)-positive epidermal progenitor cells (top panels) but do not stain for pp63 due to the absence of p63 expression (lower panels). Dotted lines indicate the epidermal-dermal border. *DNA*, nuclear counterstaining; Bar=20μm. (**b**) Immunofluorescence staining of BHK cells transiently transfected with either wild type p63 (left) or unphosphorylatable mutant p63 (right) constructs. Lower panels show single channel images of the boxed area in upper panels. Note that cells expressing unphosphorylatable mutant p63 lack detectable pp63 signals (green). Bar=10μm. (**c**) Western blot analysis of 293T cells transiently transfected with either wild type or unphosphorylatable mutant p63 constructs. The membrane was first probed with anti-pp63 antibody (upper panel), stripped and re-probed with anti-p63 antibody (lower panel). *Mock*, non-transfected cell lysate.

Figure S2. Clonogenic culture of epidermal SCs.

(**a**) Gross appearance of holoclones, meroclone and paraclones stained with Rhodamine-B. (**b**) Evaluation of holoclones, meroclones and paraclones based on their cellular morphology and expression of p63 and CK14. *Upper*, Representative images of holoclones, meroclones and paraclones at a higher magnification. *Middle and lower*, Immunofluorescence staining of holoclones, meroclones and paraclones for p63 and CK14. Note that while holoclones are composed of small, morphologically immature cells with high p63 and CK14 expression throughout the clone, meroclones are morphologically more mature and differentiated toward the clone center with decreased levels of p63 and CK14. Paraclones are composed of large, morphologically mature cells with low-to-negative levels for p63 and CK14. Dotted lines indicate the clone border. Bars=50μm.

Suzuki and Senoo, Supplemental Figure S1