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Supplemental Data

Loss of TGFβ Signaling Destabilizes

Homeostasis and Promotes Squamous Cell

Carcinomas in Stratified Epithelia

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Supplemental Experimental Procedures

Cell culture, virus infection and graft experiments

Primary mouse keratinocytes (MK) were isolated from the skin of newborn mice using trypsin without EDTA overnight at 4^{o}C. The epidermal cells were separated from dermal cells and plated in 0.2mM calcium media for one day. The next day, keratinocytes were fed with low calcium media (0.05mM), a condition that selects for basal cells. At day 3 of culture, keratinocytes reach 60% confluency and were infected with a replication defective transforming retrovirus carrying the v-*Ras*^{H} gene of Harvey murine sarcoma virus (kind gift from Dr Yuspa) in the presence of 4µg/ml polybrene. 3d after infection, 2 X10^{6} infected keratinocytes were mixed with 6 X10^{6} freshly isolated dermal cells and 200µl were injected into a silicon chamber implanted onto the back of an anesthetized athymic nude mouse as described. (Weinberg et al., 1993; Yuspa et al., 1970). After one week, wounds had healed, and chambers were removed.

Immunofluorescence, antibodies, inhibitors, and in situ hybridization

OCT sections were fixed for 10 min in 4% paraformaldehyde in PBS and washed 3X for 5 min in PBS at room temperature. Paraffin was removed from sections which were then permeabilized in 0.1% Triton X100 for 5 minutes and blocked in the following solution: 2.5% normal goat serum, 2.5% normal donkey serum, 2% gelatin, 0.1% Triton X100 and 1% bovine serum albumin in PBS. Whenever mouse antibodies were required, the MOM kit (vector Lab) was used.

Primary antibodies against the following proteins were used (if not otherwise noted, antibodies were developed in the Fuchs Laboratory): TβRII (Santa-Cruz; sc-220, 1/200), p-Smad2 (Cell Signaling, 1/100), Smad2/3 (BD Biosciences, 1/500), H-Ras (Santa-Cruz, sc-520, 1/200), Pan-Ras (Upstate Biotechnology, 1/200), TGFβ1 (Santa-Cruz, sc-146, 1/100), Tenascin C (Abcam, 1/500), Mac-1 (BD Biosciences 1/20), p15INK4B (Cell signaling, 1/200), p27 (Santa-Cruz, sc-528, 1/200), c-Myc (Calbiochem, 1/1000), β-actin (Sigma, 1/1000), bromodeoxyuridine (Abcam, 1/200), Actin Smooth Muscle Ab-1 (Neomarkers, 1/50), Active Caspase 3 (R&D, 1/500), Phospho-MAPK Thr202/Tyr204 (Cell signaling 1/100), Total MAPK p44/42 (Cell signaling 1/1000), Phospho-FAK Tyr397 (Biosource, 1/1000), FAK (Upstate Biotechnology, 1/1000),

Phospho-AKT Ser473 (Cell signaling, 1/1000), AKT (Cell signaling, 1/1000) , Src (Upstate Biotechnology, 1/100), Active Src [pY418] (Biosource, 1/1000), K5 (1/300), K14 (1/1000), K17 (Dr Coulombe gift 1/5000), active β1 integrin CD29 (BD Biosciences, 1/100), β4 integrin (CD104 BD Biosciences, 1/100), β1 integrin subunit (Chemicon, 1/100), β6 integrin (Dr Sheppard gift 1/20), and αvβ6 integrin (Chemicon, 1/100). Additional reagent was DAPI (1:5000; Sigma).

Cell proliferation and cell cycle analysis

For growth comparisons, $1X10^4$ of freshly isolated cells were plated in 24-well dishes in low calcium condition media. Keratinocyte numbers were determined after trypsinization and counting using a coulter counter (Beckman). For conditions using TGFβ1, 5ng/ml was added at day 2 and refreshed every day. Cell cycle analyses were performed as described(Tumbar et al., 2004). BrdU detection was performed using BD Pharmingen BrdU Flow.

Immunoblots

Proteins were separated by electrophoresis on 4–12% gradient gels (Invitrogen), transferred to nitrocellulose membranes and subjected to immunoblotting. Membranes were blocked for 30 minutes with 5% non-fat milk in PBS containing 0.1% Tween-20. Primary antibodies were generally used at a concentration of 1:1000, HRP-coupled secondary antibodies were used at 1:2000. Immunoblots were developed using standard ECL (Amersham), Super Signal West Pico substrate (Pierce) or Licor Odyssey system.

BrdU labeling

For 5-bromo-2'-deoxyuridine (BrdU), (Sigma-Aldrich) incorporation studies in backskin, 7 to 9 wk old mice were injected intraperitoneally 2X per day with 50 μg/g BrdU and analyzed 2 days later by fluorescence activated cell sorting (FACS). For BrdU incorporation in tumor areas or in anal regions, 50 μg/g BrdU was injected in mice and analyzed 4 hr later by anti-BrdU immunofluorescence of OCT frozen tissue sections.

Real-time PCR

Total RNAs were isolated using Trizol (Invitrogen) and further purified using a Qiagen Rneasy Mini Kit and reverse transcribed using oligo(dT) primers according to the manufacturer's instruction (Superscript III First-Strand Synthesis System, Invitrogen). cDNAs were adjusted to equal levels by PCR amplification using primers to GAPDH. The LightCycler System, LightCycler 3.5 software and the LightCycler DNA Master SYBR Green I reagents were used for real-time PCR. Differences between samples and controls were calculated based on the $2^{-\Delta\Delta CP}$ method.

Quantifications for data in Figure 3 and 4.

For Figure 3 (Bb, Fb), the number of fields counted represents the sum of 20 sections in total from two animals from two different experimental group.

The % of BrdU-positive cells (Figure 3Cb) was determined by counting the total keratin 14 (K14) positive nuclei and the number of double positive Brdu and K14 cells. Apoptosis, as measured by active caspase 3 or TUNEL, is an extremely rare in normal adult skin epidermis. For Fb (7wk), Caspase 3: WT: 22 fields, 7 positive cells. cKO: 27 fields, 37 positive. TUNEL: WT: 86 fields, 16 positive. cKO: 82 fields, 137 positive. For Fb (7mo.), Caspase 3: WT: 64 fields: 6 positive. cKO: 70 fields: 5 positive.

For Figure 4A**.** Since backskins contain two K14(+) proliferative compartments, follicle stem cells (α6+CD34+) and basal epidermal cells (α6+CD34-) were isolated by FACS prior to analyses. Quantifications of >200 fields from 3 different litters of matched WT and cKO mice. WT: 206 fields, 34 Caspase3-positive cells cKO: 226 fields, 236 Caspase3-positive cells. **F,** the necrotic area in the cKO-Ras SQCC was avoided for Caspase 3 analyses: WT-Ras: 75 fields, 31 positive; cKO-Ras: 73 fields, 30 positive.

Figure S1. Conditional targeting of *TGFβ receptor type II* (*TβRII*) gene in skin epithelium.

A: Exon 4 (Ex 4) of the *TβRII* locus, encoding the transmembrane domain (TM) and the intracellular phosphorylation sites (P) of the TβRII protein, is flanked by loxP-sites and deleted in skin epidermis upon breeding with *K14-Cre* mice. Identification of the 575 bp floxed *TβRII* allele in heterozygous (het) and conditional knockout (cKO) mice and of the 422 bp wild type (WT) allele in heterozygous animals by PCR with indicated primers P3, P4 (Leveen et al., 2002). **B**. mRNAs were isolated and real time PCR was conducted using a probe that corresponded to the floxed exon 4 of *TβRII*. Additional controls were conducted with PCR primers against TGFβ1, GAPDH, Dach1 and TGFα. Of these, only a slight (1.5X) increase in the expression of the EGF receptor ligand TGFα was observed in keratinocytes lacking TβRII expression.**C.** Presence and absence of *TβRII* mRNA detected by in situ hybridization using exon 4 as a probe. Shh was using as an internal positive control. Mx: Matrix. ORS: Outer Root Sheath. **D.** Detection of the protein level by immunoblot analysis. An expression vector encompassing full length *TβRII* cDNA was transfected in 293T cells as a positive control. MK: Keratinocytes.

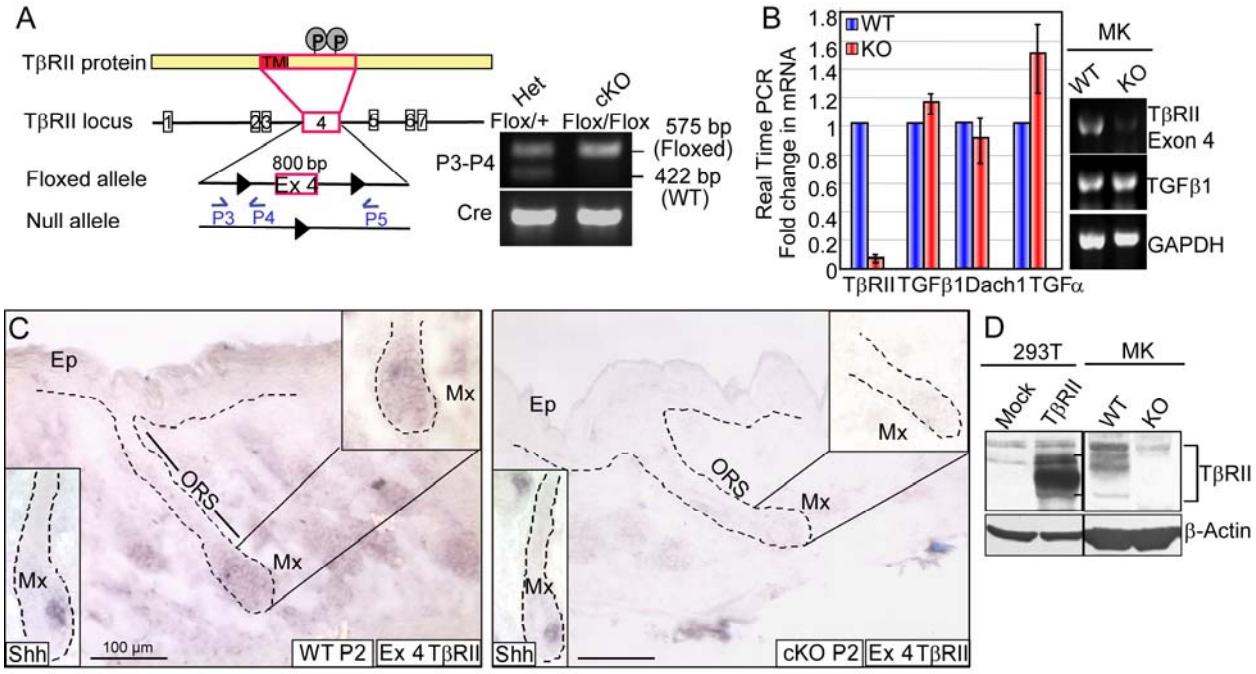


Figure S2. Tumor pathology of spontaneous anogenital SQCCs. All tumor samples were fixed and embedded into paraffin prior to sectioning and staining with hematoxylin and eosin. **A.** Transverse section of prepuce with preputial glands ducts of 7 mo. old WT and cKO male littermates. Note abnormal epithelial tumor masses in cKO but not WT tissue. Keratinization and morphology of the tumors were characteristic of SQCC. **B.** Section of vagina and rectum of 8 mo. old WT and cKO female littermates. Multicentric SQCC involving mucosal epithelium and follicular/adnexal epithelium adjacent to the vagina and rectum are presented. Note prevalence of keratinized cells within the tumor masses. Boxed areas are regions that were magnified 10X for Fig S2A, S2B lower panels. Boxed areas from Fig S2B upper panels are regions that were magnified 2X.

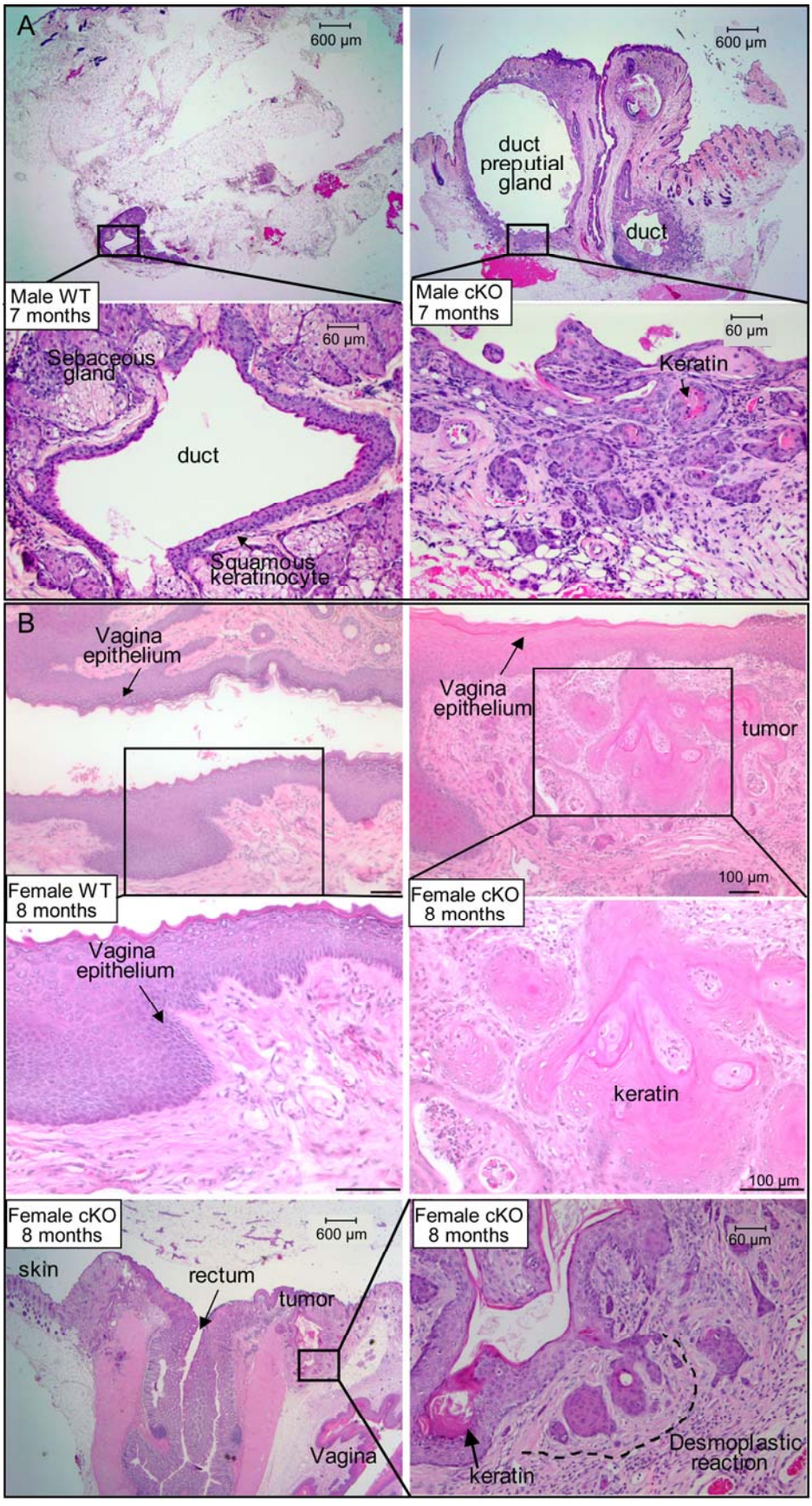

A
600 µm
600 µm
duct
preputial
gland
duct
Male WT
7 months
Male cKO
7 months
60 µm
60 µm
Sebaceous
gland
duct
Keratin
Squamous
keratinocyte
B
Vagina
epithelium
Vagina
epithelium
tumor
100 µm
Female WT
8 months
Female cKO
8 months
Vagina
epithelium
keratin
100 µm
Female cKO
8 months
600 µm
Female cKO
8 months
60 µm
skin
rectum
tumor
Vagina
keratin
Desmoplastic
reaction

Figure S3. Loss of TβRII protein expression (A) and loss of nuclear p-Smad2 in the epithelial tumors of *TβRII* cKO mice (C). **A.** Anti-TβRII immunohistochemistry of paraformaldehyde fixed, paraffin embedded sections of anal tissues from WT and cKO mice. The dashed lines denote the epithelial/stromal border in the WT section. Note presence of TβRII staining in WT (arrows) but not cKO anal tumor (staining is in stroma). **B-D.** Immunofluorescence microscopy of frozen sections from WT and cKO anal tissue taken at 7 months of age. Antibodies are color-coded as indicated, according to the secondary antibodies used. DAPI, in blue. Note the increase in K14-positive layers in the cKO anal canal epithelium (B). Note increase of anti-p-Smad2 positive in tumor stroma (K5-negative) but absence of anti-p-Smad2 positive keratinocytes in tumor (C). Antibodies against smooth actin muscle (Sma in D) reveal presence of smooth muscle myofibroblasts in stroma (arrows) surrounding the cKO tumor, but not in WT stroma.

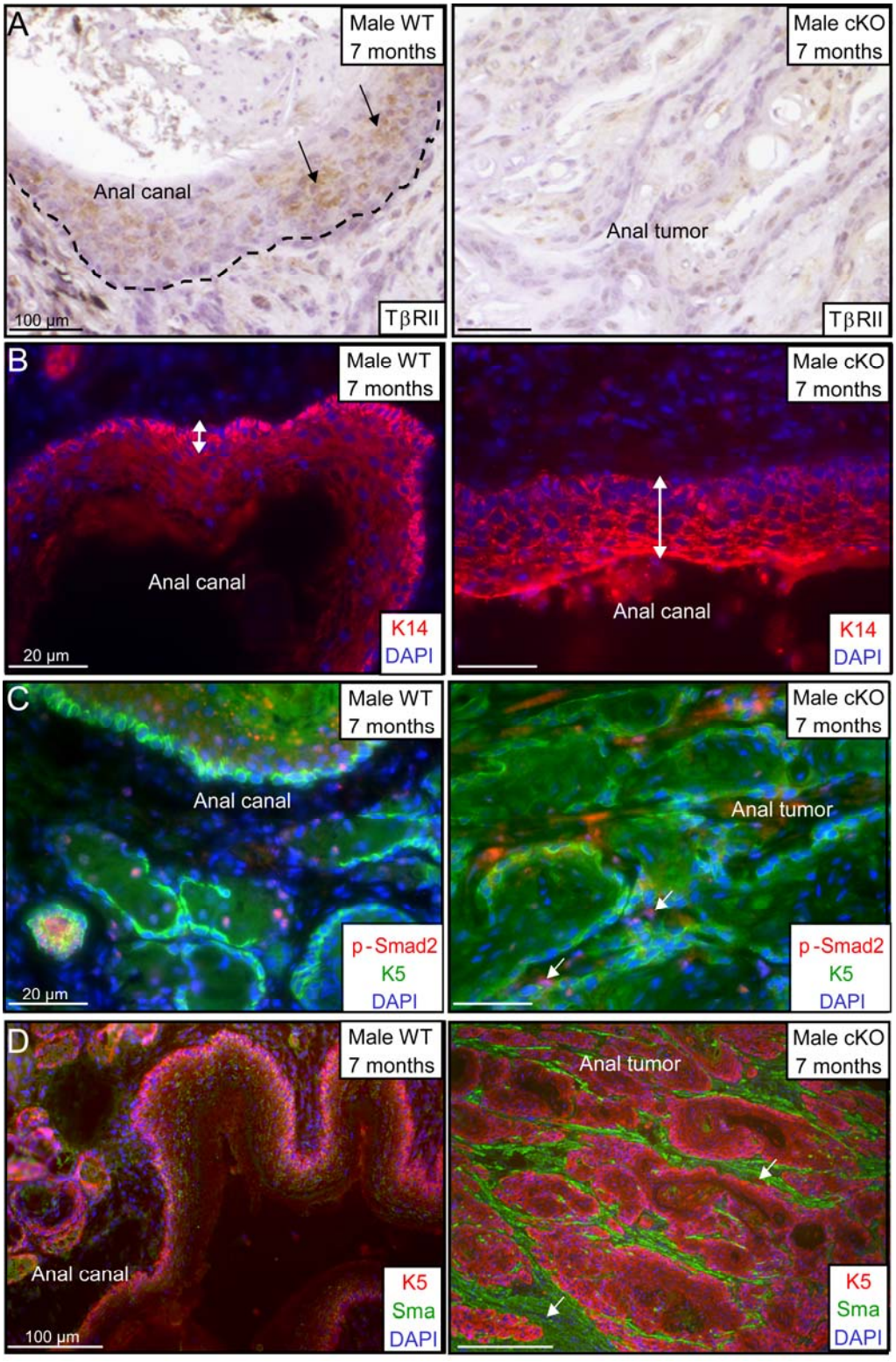
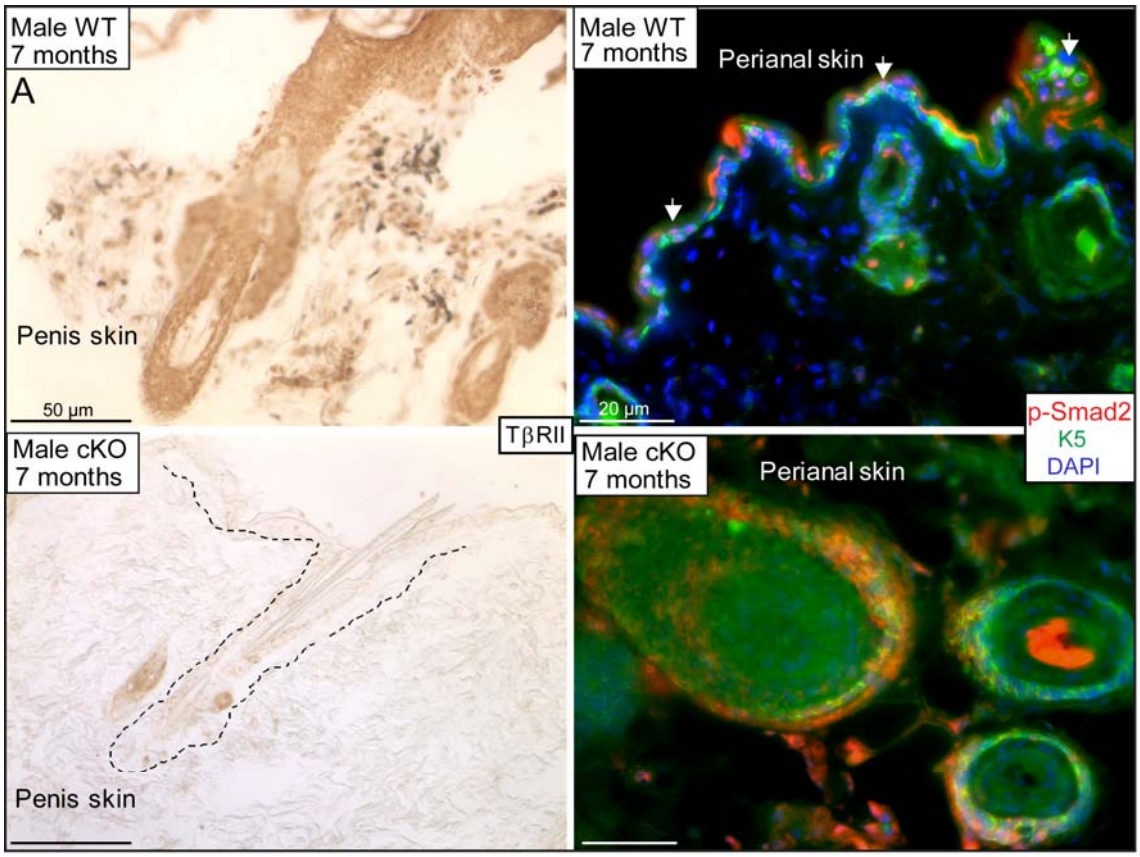

A
Male WT
7 months
Anal canal
100 μm
TβRII
Male cKO
7 months
Anal tumor
TβRII
B
Male WT
7 months
Anal canal
20 μm
K14
DAPI
Male cKO
7 months
Anal canal
K14
DAPI
C
Male WT
7 months
Anal canal
20 μm
p-Smad2
K5
DAPI
Male cKO
7 months
Anal tumor
p-Smad2
K5
DAPI
D
Male WT
7 months
Anal canal
100 μm
K5
Sma
DAPI
Male cKO
7 months
Anal tumor
K5
Sma
DAPI

Figure S4. Biochemical alterations in anogenital tissues targeted for loss of TβRII signaling. **A.** Immunohistochemistry and immunofluorescence of penal and perianal tissue sections from 7 month old male mice. Antibodies are color-coded as shown according to the secondary Abs used for detection. Note loss of TβRII protein expression and loss of nuclear p-Smad2 in skin surrounding the male genital tumors of *TβRII* cKO mice. Note p-Smad2 only in WT epithelium (arrows) and in surrounding stroma but not epithelium of cKO samples. **B.** Same as in (A) except samples are from 8 month old female mice. See Supplemental Experimental Procedures section for details of BrdU injections prior to analyses.


Male WT
7 months
A
Penis skin
50 μm
TβRII
Male WT
7 months
Perianal skin
20 μm
p-Smad2
K5
DAPI
Male cKO
7 months
Penis skin
Male cKO
7 months
Perianal skin

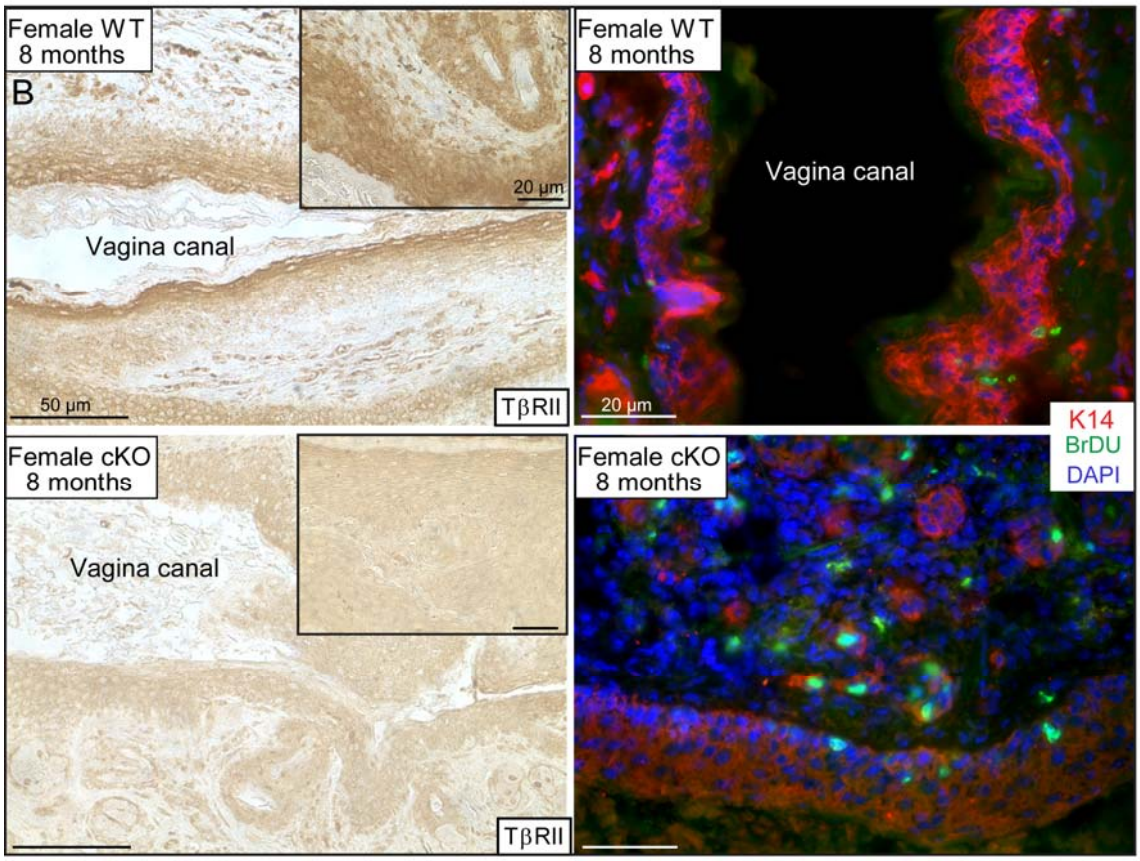

Female WT
8 months
B
20 μm
Vagina canal
50 μm
TβRII
Female WT
8 months
Vagina canal
20 μm
K14
BrDU
DAPI
Female cKO
8 months
Vagina canal
TβRII
Female cKO
8 months

Figure S5. Backskins of WT and cKO mice maintain homeostasis and do not show morphological or biochemical features of a hyperproliferative state. Immunofluorescence microscopy was performed on frozen sections of backskins from adult WT and cKO mice. Antibodies are color-coded as shown according to the secondary Abs used for detection. Note that keratin 17 (K17) and tenascin C (TnC), were only expressed in the WT and cKO hair follicles, which is normal. These markers were not detected in WT or cKO epidermis. Expression of these markers has typically been seen only when associated with a hyperproliferative, wounded, proinflammatory or otherwise altered state of mouse and human epidermis. Similarly note that Macrophages (Mac1) are rare in backskin epidermis of WT and cKO mice. If the skin was in a proinflammatory, cancerous or wounded state, immune infiltration would be prevalent. Finally, not that the adult backskin epidermis is very thin, both in WT and cKO, again a sign that homeostasis is maintained and proliferative levels are low. Epi: Epidermis; Der: Dermis; HF: Hair Follicle.

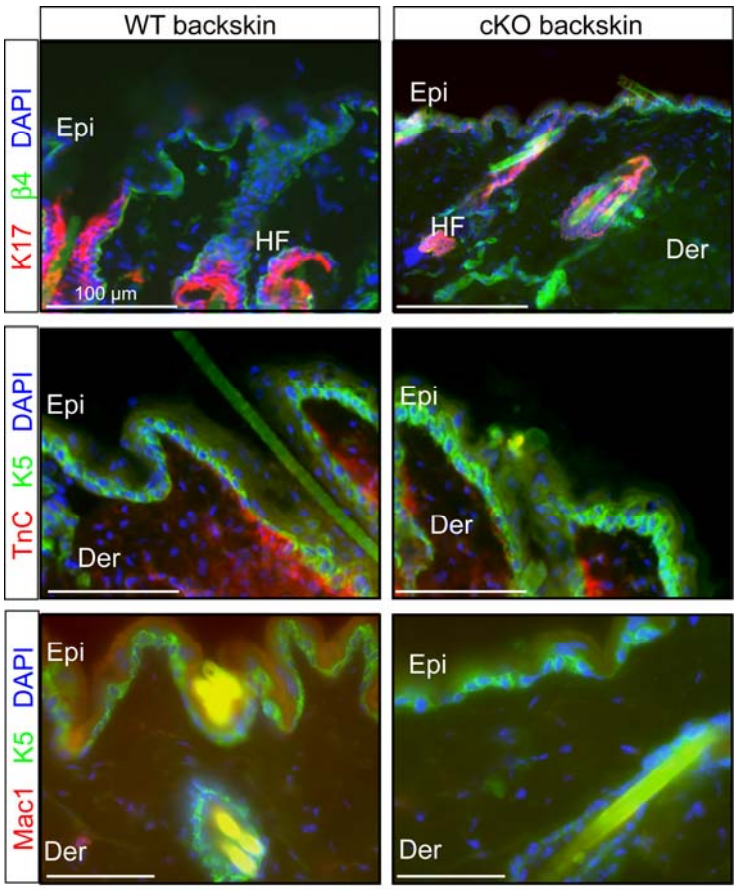


Figure S6. Oncogenic Ha-Ras transformation of *TβRII*-deficient keratinocytes leads to rapid progression to SQCC. MK were infected with a retroviral expression vector for the oncogenic form of Ha-Ras, as described in the text. **A**. Immunoblot showing comparable expression of oncogenic Ha-Ras protein in the WT and KO MKs used for the grafting experiments. **B.** Real-time PCR showing the expression of Ras mRNA in keratinocytes used for the infection and the loss of TβRII mRNA in KO keratinocytes. Graph indicates the mean value of three independent experiments (+/- SD). **C**. Low magnification of hematoxylin and eosin stained WT-Ras papilloma and cKO-Ras SQCC tumors. Higher magnification views can be found in Figure 4. **D.** Gross appearance of the grafts at 9 days after transplanting Ha-*Ras*-infected WT and *TβRII*-null keratinocytes. Even during the wound-repair process, abnormalities have begun to emerge. For later days after grafting, see Figure 4. **E.** TβRII protein detected in the WT papilloma (arrow) but not the *TβRII*-null SQCC . As an internal control, *Nude* mouse skin far from the tumor in the *TβRII*-null/Ha-Ras grafted mouse shows TβRII expression in the epidermis and hair follicle (far right panel). Epi: Epidermis. HF: Hair follicle. Der: Dermis. **F,** Presence of TGFβ signaling activity in the stroma surrounding the cKO-Ras induced tumor, as detected by p-Smad2 and TGFβ1 **G,** After 23 days, engrafted *Nude* mice were injected with 50 μg/g 5-bromo-2'-deoxyuridine (BrdU) for 4 hr. Following this labeling, grafts were harvested and frozen skin sections of the tumor regions were subjected to anti-BrdU immunofluorescence analyses. The insets show sections stained for K14 protein (red), BrDU (green) and DAPI (blue). **H.** Caspase 3 (Ac-casp3) activity is not changed in cKO-Ras SQCC relative to WT-Ras papilloma within the tumor area. Shown are representative tumor sections stained for active-caspase 3 within the keratin 14 (K14) positive cells. Control is from necrotic area of the tumor.

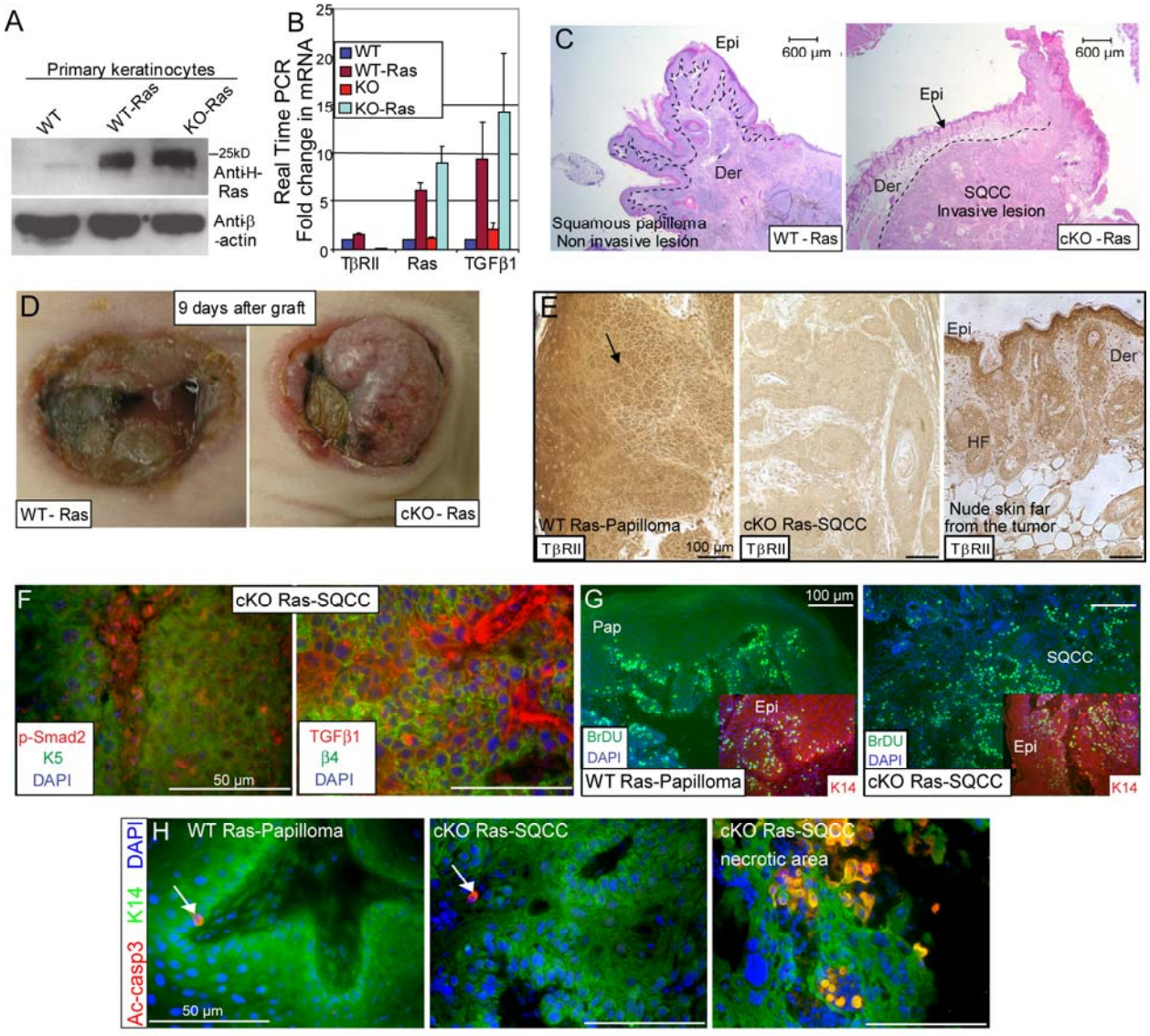
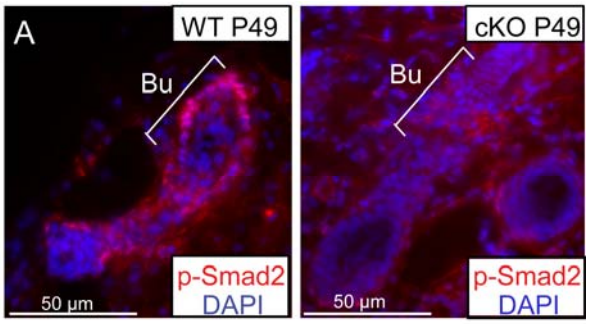
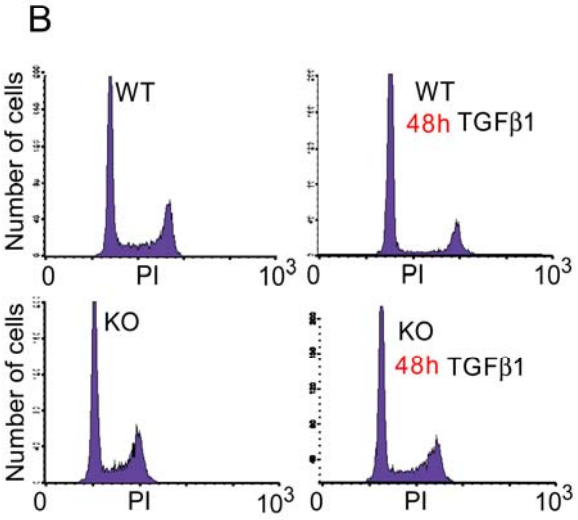
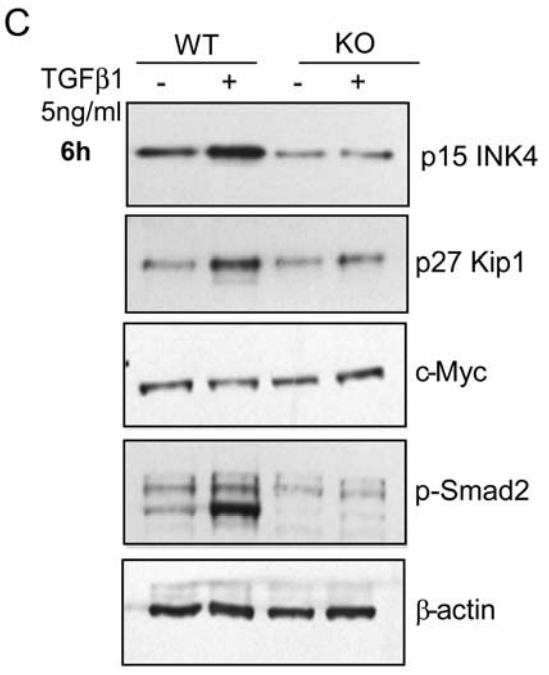

A
Primary keratinocytes
WT
WT-Ras
KO-Ras
25kD
Anti-H-Ras
Anti-β-actin
B
Real Time PCR
Fold change in mRNA
WT
WT-Ras
KO
KO-Ras
TβRII
Ras
TGFβ1
C
Epi
600 μm
Der
Squamous papilloma
Non invasive lesion
WT - Ras
Epi
600 μm
Der
SQCC
Invasive lesion
cKO - Ras
D
9 days after graft
WT- Ras
cKO- Ras
E
WT Ras-Papilloma
TβRII
100 μm
cKO Ras-SQCC
TβRII
Epi
Der
HF
Nude skin far from the tumor
TβRII
F
cKO Ras-SQCC
p-Smad2
K5
DAPI
50 μm
TGFβ1
β4
DAPI
G
100 μm
Pap
Epi
BrDU
DAPI
WT Ras-Papilloma
K14
SQCC
Epi
BrDU
DAPI
cKO Ras-SQCC
K14
H
Ac-casp3
K14
DAPI
WT Ras-Papilloma
50 μm
cKO Ras-SQCC
cKO Ras-SQCC
necrotic area

Figure S7. Loss of TGFβ. responsiveness in conditional *TβRII* knockout hair follicles and primary cultured keratinocytes. **A.** Previously, we noted that the stem cells of the normal adult hair follicle display an upregulation of genes that are typically associated with elevated TGFβ signaling (Tumbar et al., 2004). Here, we show by immunofluorescence that phosphorylated (active) Smad2 is found in the nuclei of the bulge (Bu) of WT but not *TβRII* cKO follicles. In Figure 4, we present data to show that in spite of a loss of TGFβ signaling, the bulge cells, which are positive for CD34, K14 and α6 integrin, do not show signs of elevated proliferation, and thus are not essential for maintaining the quiescent state of these stem cells. **B.** Cell cycle profile of WT and KO keratinocytes treated with TGFβ1 for 48h, and then subjected to propidium iodide staining and FACS analyses. Note that in the presence of TGFβ1, WT but not KO keratinocytes withdraw from the cell cycle and no longer show appreciable S-phase cells. For more information see Figure 4I. **C.** Expression of TGFβ/phospho-Smad2 target genes only in WT and not KO primary keratinocytes. Cells were stimulated with TGFβ1 at 5ng/ml for 6h and protein lysates were analyzed for the expression of known TGFβ signaling effectors, including phospho-Smad2, p15INK4, and p27Kip, known to be upregulated, and c-Myc, known to be downregulated. Note absence of TGFβ responsiveness in KO keratinocytes.


A
WT P49
Bu
p-Smad2
DAPI
50 μm
cKO P49
Bu
p-Smad2
DAPI
50 μm


B
Number of cells
WT
0
PI
10^3
WT
48h TGFβ1
0
PI
10^3
Number of cells
KO
0
PI
10^3
KO
48h TGFβ1
0
PI
10^3


C
WT
KO
TGFβ1
5ng/ml
6h
-
+
-
+
p15 INK4
p27 Kip1
c-Myc
p-Smad2
β-actin