Supplemental Figures

Supplemental Figure 1. Loss of Smad2 and Smad4, but not Smad3 protein in human skin SCCs. Immunohistochemistry (IHC) staining in 83 human skin SCCs were performed. Staining of Smad2, Smad3 and Smad4 was predominantly localized to the epidermis in normal skin. IHC for Smad2 was preserved in well differentiated tumors, but lost in poorly differentiated SCCs. The intensity of Smad4 staining was reduced in well-differentiated SCCs, and completely lost in poorly differentiated SCCs. In contrast, Smad3 was largely retained. Scale bar represents 100μm.

Supplemental Figure 2. Examples of loss of heterozygosity peaks used for analysis at markers. Sample profiles for cases exhibiting LOH at microsatellite markers used for *Smad2* A: D18S1137 and B: D18S555 and for *Smad4* C: D18S46 and D: D18S1110. N: denotes normal adjacent dissected tissue, T: denotes dissected tumor tissue. Arrow denotes allele peak lost for LOH.

Supplemental Figure 3. Generation of keratinocyte-specific *Smad2* knockout mice. Monogenic mice (*K5.Cre*PR1* or $Smad2^{f/f}$) treated with RU486 were used as wildtype controls. Heterozygous and homozygous bigenic mice ($K5.Cre*PR1/Smad2^{f/wt}$ and $K5.Cre*PR1/Smad2^{f/f}$, respectively) were used to generate heterozygous and homozygous *Smad2* deletion in keratinocytes (designated as *K5.Smad2+/-* and *K5.Smad2-/-*). A: PCR confirmation of *Smad2* genotype. All mice have the Cre band, but control mice lack a *Smad2* floxed allele, heterozygotes contain one *Smad2* floxed allele, and knockouts lack a wildtype allele. B: Schematic representation of expected bands. C: qRT-PCR of Smad2

mRNA normalized to GAPDH. *K5.Smad2+/-* and *K5.Smad2-/-* displayed a significant reduction in Smad2 expression. Residual Smad2 expression in knockouts is due to non-keratinocyte populations in the stroma of the sample. D: Newborn pups were treated daily with RU486 (20µg in 100µl ethanol) for 3 days and sacrificed on the third day. IHC of Smad2 protein in RU486 treated neonatal back skin. Note Smad2 loss in knockout epidermis, but Smad2 staining remained in the stroma. Scale bar represents 100µm. *: $p < 0.05$.

Supplemental Figure 4. Immunohistochemistry of Smad2 in mouse tumors. Smad2 expression was retained in wildtype and to a lesser degree in heterozygotes, at the papilloma stage indicating haploid insufficiency. At later stages, Smad2 was lost with equal frequency in wildtype and heterozygotes. Scale bar represents 100µm.

Supplemental Figure 5. Increased proliferation in TPA-treated *K5.Smad2-/-* skin. Six-week-old monogenic and bigenic mice were treated with RU486 (20µg in 100µl ethanol for 5 days) to induce *Smad2* deletion in bigenic mice. Two weeks later, mice were treated with TPA (5µg per mouse), and sacrificed forty-eight hours later. Two hours prior to sacrifice, mice were injected with BrdU. Ten-week-old RU486 treated mice, not treated with TPA were used as control (top panel). BrdU (green) staining in TPA treated skin showed increased proliferation amongst *K5.Smad2-/-* mice compared to *K5.Smad2+/+* mice. Keratinocytes are highlighted with K14 (red). No difference in proliferation was noted among non-TPA treated skin. Scale bar represents 100µm.

Supplemental Figure 6. Epithelial expression of mesenchymal markers in *K5.Smad2-/-* tissues. A: *K5.Smad2-/-* papillomas (pap) showed increased presence of mesenchymal marker αSMA in green (top panel) and vimentin in green (bottom panel). Keratinocytes are highlighted with K14 (red). Wildtype papillomas and *K5.Smad4-/-* spontaneous SCCs showed exclusive stromal staining for αSMA and vimentin. B: Mesenchymal marker staining in the hyperplastic skin of *K5.Smad2-/-* animals adjacent to SCC formation. αSMA (green, top panel) and vimentin (green, bottom panel) were stained in *K5.Smad2-/-* epidermis, but were stained exclusively in the stroma of *K5.Smad2+/+* skin. Keratinocytes are highlighted with K14 (red). Scale bar represents 100μm.

Supplemental Figure 7. TGFβ1 protein levels and Smad expression patters in *K5.Smad2+/+* and *K5.Smad2-/-* SCCs. Protein extraction was performed by homogenizing tissue in Complete Lysis Buffer M (Roche). Total protein was determined using detergent compatible to Bradford Assay reagents (BioRad). ELISA kit for TGFβ1 (R&D Systems) was used to determine the concentration of TGFβ1, as per the manufacturer's instructions. A: TGFβ1 level was comparable between *K5.Smad2+/+* and *K5.Smad2-/-* SCCs. *$p<0.05$ compared to K5.Smad2+/+ skin. B: Smad3 and Smad4 staining (brown) showed patterns in *K5.Smad2-/-* SCCs similar to *K5.Smad2+/+* SCCs. Hematoxylin was used as counterstain. Scale bar represents 100μm.

Supplemental Figure 8. Knockdown of Smads or Snail by siRNA. RNA extraction followed by qRT-PCR was performed as described previously (1). Protein was extracted from cells harvested in Complete Lysis Buffer M (Roche). Equal amounts of protein

were separated on a 10% SDS-PAGE resolving gel with a 4% SDS-PAGE stacking gel. Protein was transferred to a nitrocellulose membrane and blocked using 5% non-fat milk in 0.1% Tween in TBS for 1 h at room temperature. Blots were stained with donkey IRDye-labeled secondary antibodies (Rockland, 1:5,000) against Rabbit anti-mouse-Smad2 antibody (Zymed,1:1,000), Rabbit anti-mouse-Smad3 (Santa Cruz, 1:1,000), Mouse monoclonal anti-mouse-Smad4 (Santa Cruz, 1:1,000), or Rabbit anti-Snail (Zymed, 1:1,000). Antibody against mouse GAPDH (Santa Cruz, 1:5,000) or mouse Actin (Santa Cruz, 1:2,500) was used as a loading control. Gels were scanned and analyzed using LiCor Odyssey scanner (LiCor Biotechnology). Smad2 siRNA specifically knocked down Smad2 expression at the mRNA (A) and protein (B) levels. Smad3 siRNA specifically knocked down Smad3 expression at the mRNA (C) and protein (D) levels. Smad4 siRNA specifically knocked down Smad4 expression at the mRNA (E) and protein (F) levels. G: Snail siRNA significantly reduced Snail mRNA expression after 72 h of knockdown. H: Western analysis for Snail protein demonstrated ~50% reduction in signal in Snail siRNA transfected cells shown in G. However, the cross-reaction of the Snail antibody with Slug could also account for remaining signal. I: Snail siRNA transfected cells in G did not show alterations in Slug mRNA expression after 72 h of knockdown. *$p<0.05$ compared to mock transfection. **$p<0.001$ compared to mock transfection.

Supplemental Table 1. Snail ChIP primers.

Primer Name	Tm	%GC	Sequence	Ampllicon Length (bp)	SBE Flanked
Snail1 ChIP 2F	60	60	GGACTCAGGGAGACTCATGG	197	-1076
Snail1 ChIP 2R	60.87	60	GGGTCTACGGAAACCTCTGG		
Snail1 ChIP 3F	59.99	55	CGGTGCTTCTTCACTTCCTC	200	-437
Snail1 ChIP 3R	60.21	60	ACTACCCAGGGATGCCCTAC		

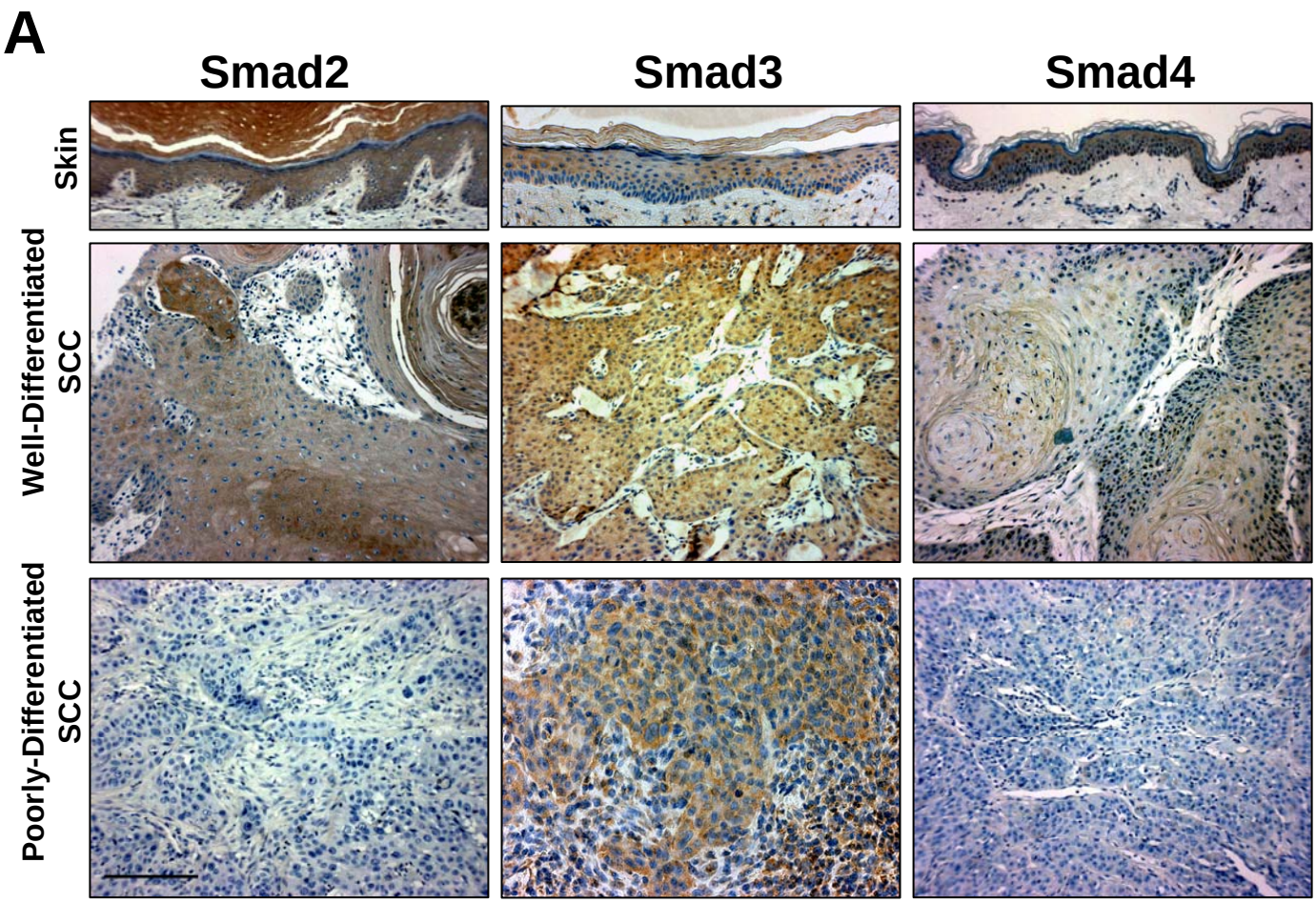
Supplemental Table 2. siRNA Sequence Information.

siRNA	Sequence	Vendor
Smad2	UUCUCAAGCUCAUCUAACCGUCCUG	Invitrogen
Smad3	CCUGCUGGAUUGAGCUACACCUGAA	Invitrogen
Smad4	GGUGAUGUUUGGGUCAGGUGCCUUA	Invitrogen
Snail	GAGUAAUGGCUGUCACUUGUU	Dharmacon
	GCGAGCUGCAGGACUCUAAUU	
	AAUCGGAAGCCUAACUACAUU	
	GUGACUAACUAUGCAAUAAUU	

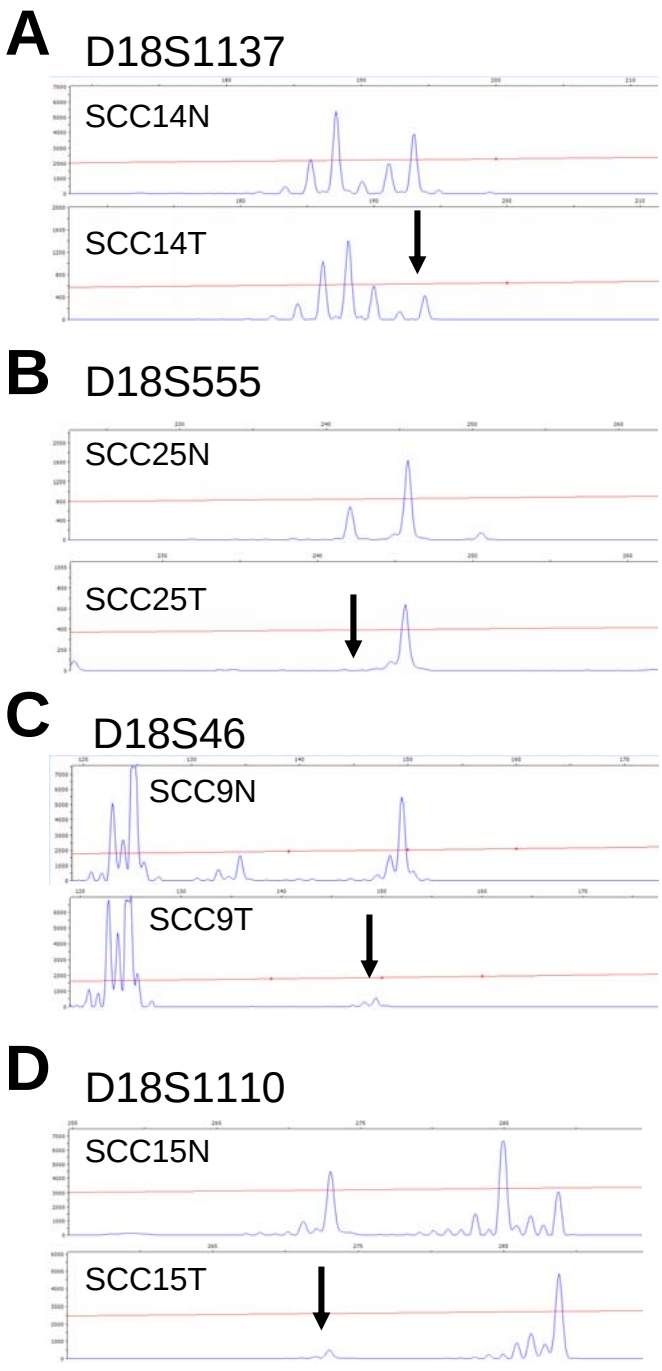
References

1. Wang XJ, Greenhalgh DA, Lu XR, Bickenbach JR, Roop DR. 1995. TGF alpha and v-fos cooperation in transgenic mouse epidermis induces aberrant keratinocyte differentiation and stable, autonomous papillomas. *Oncogene* 10:279-289.

Supplemental Figure 1



Supplemental Figure 2



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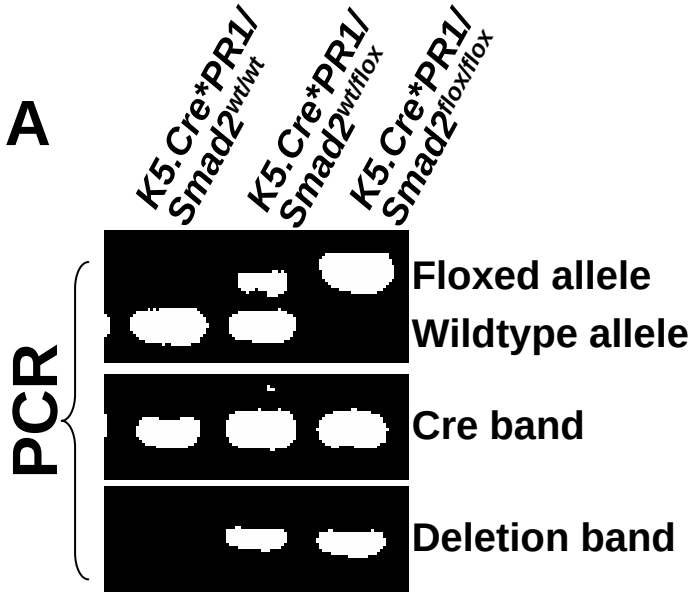

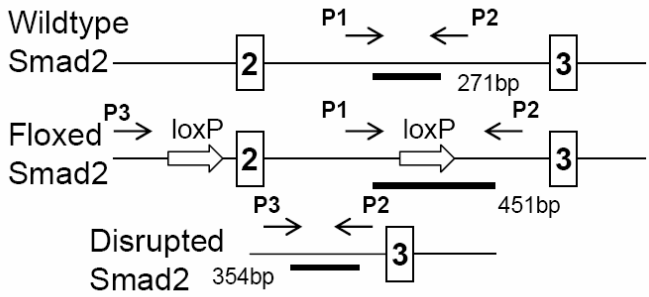

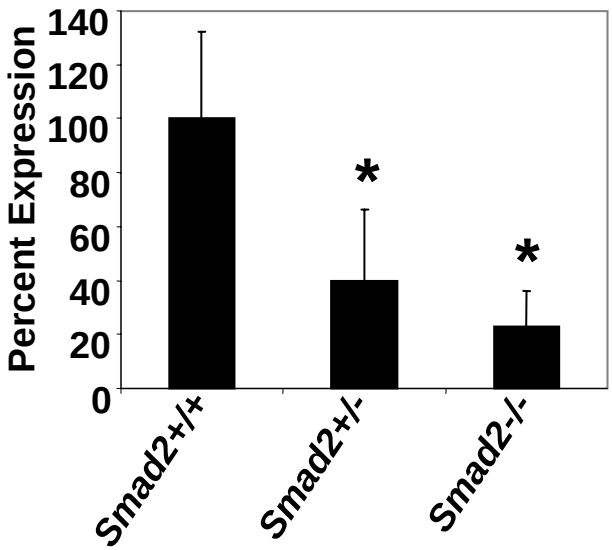

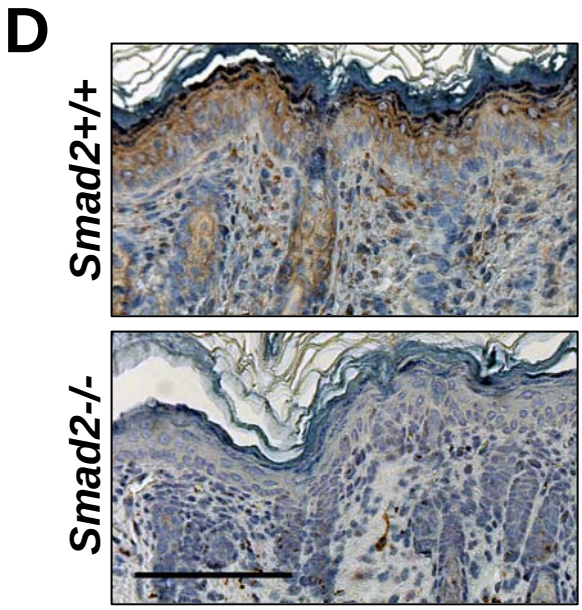

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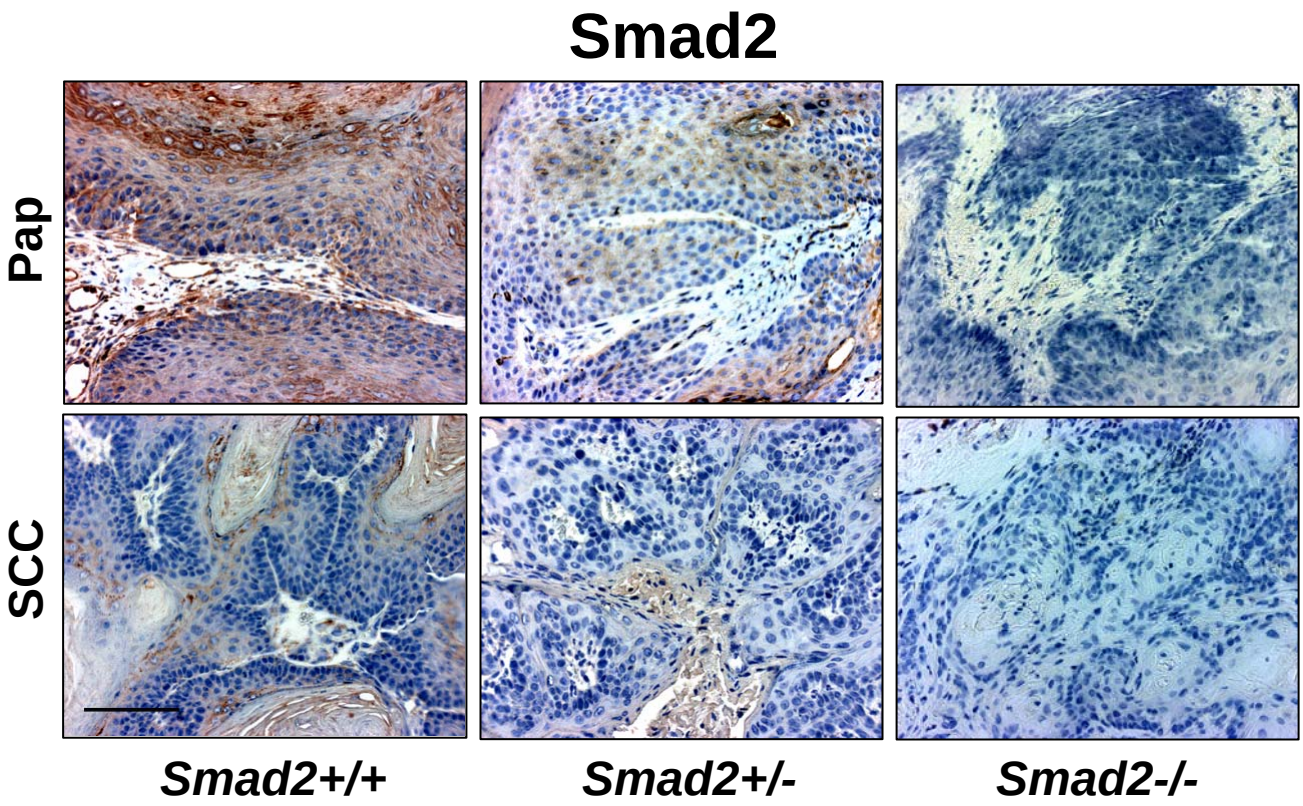

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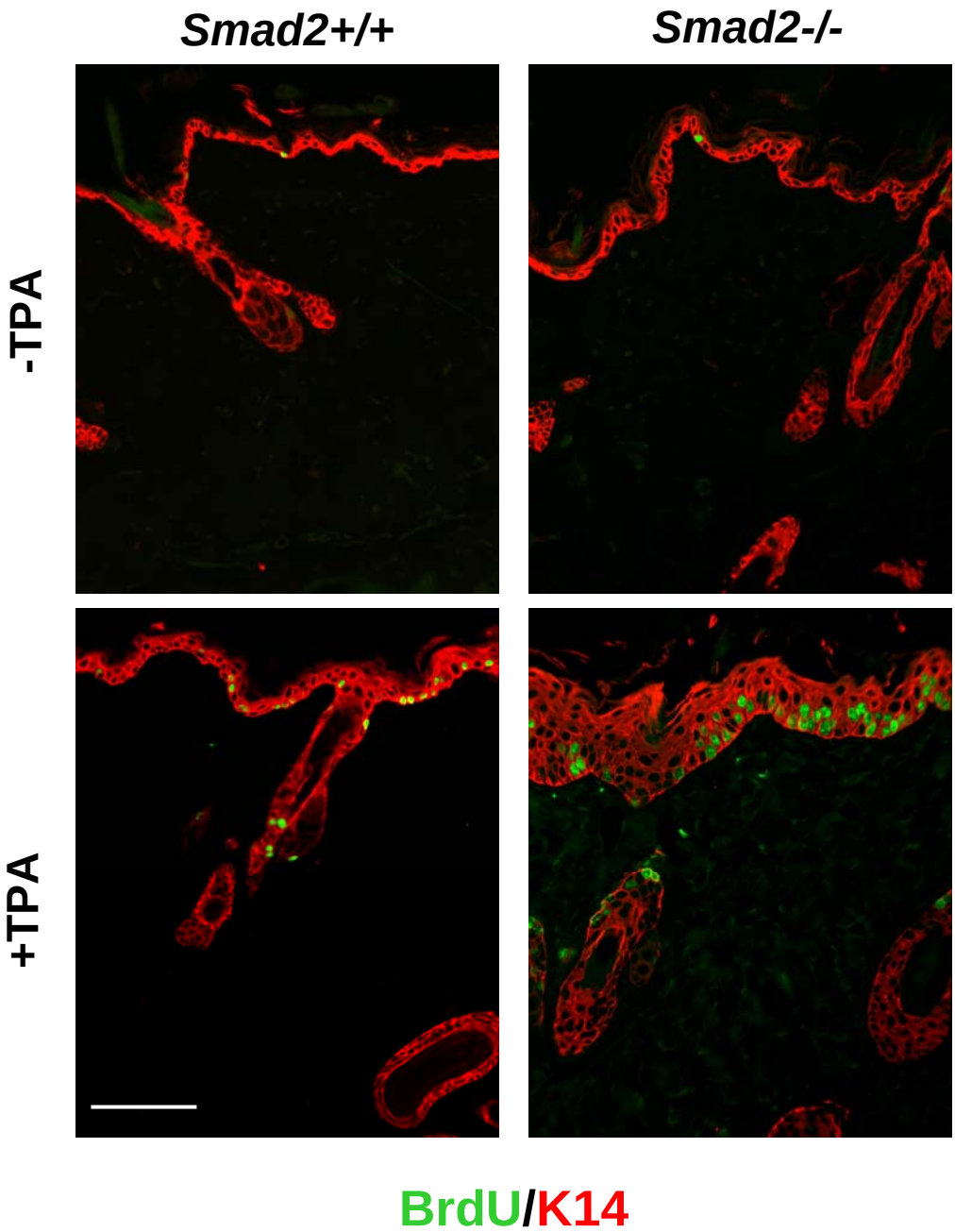

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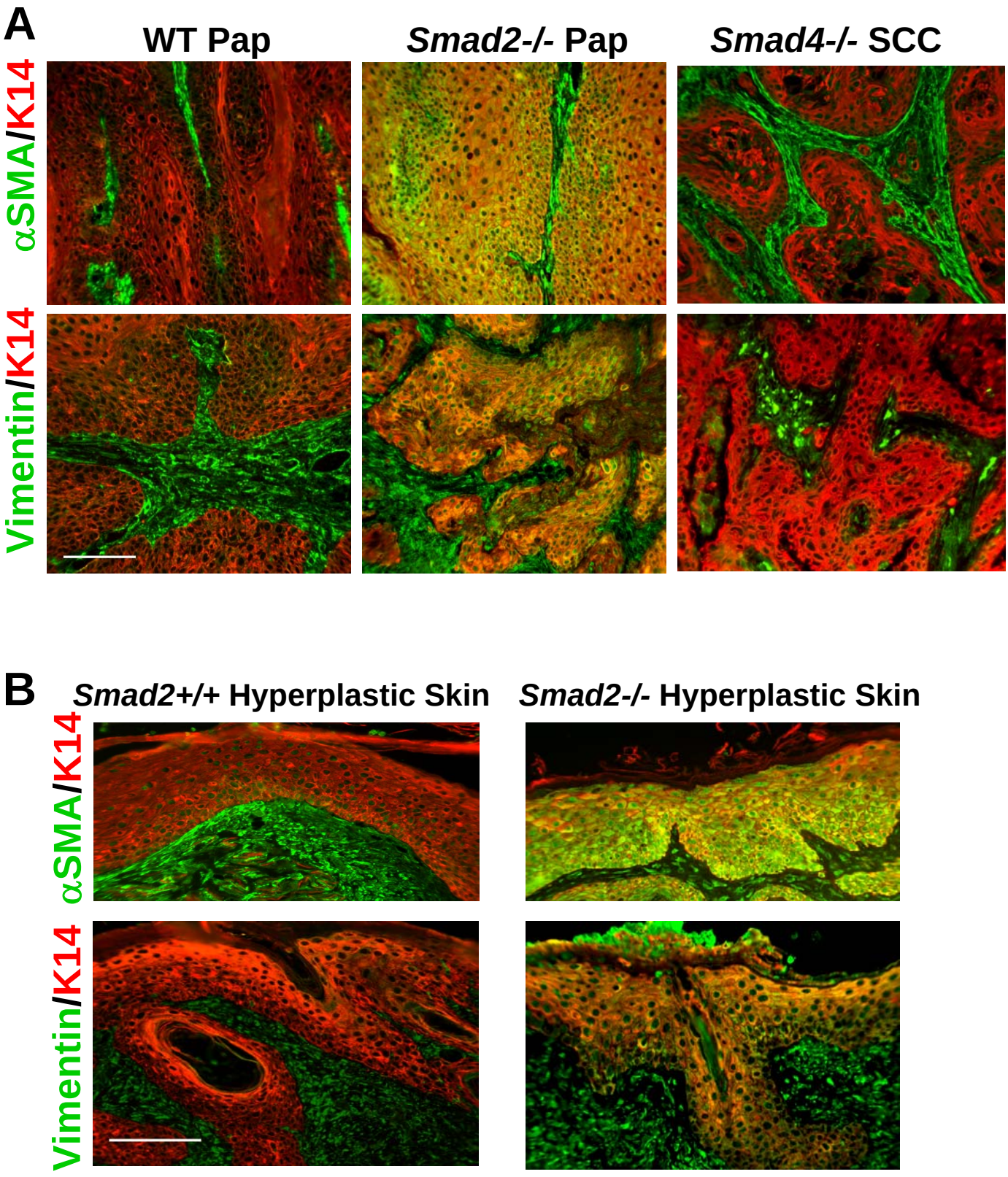

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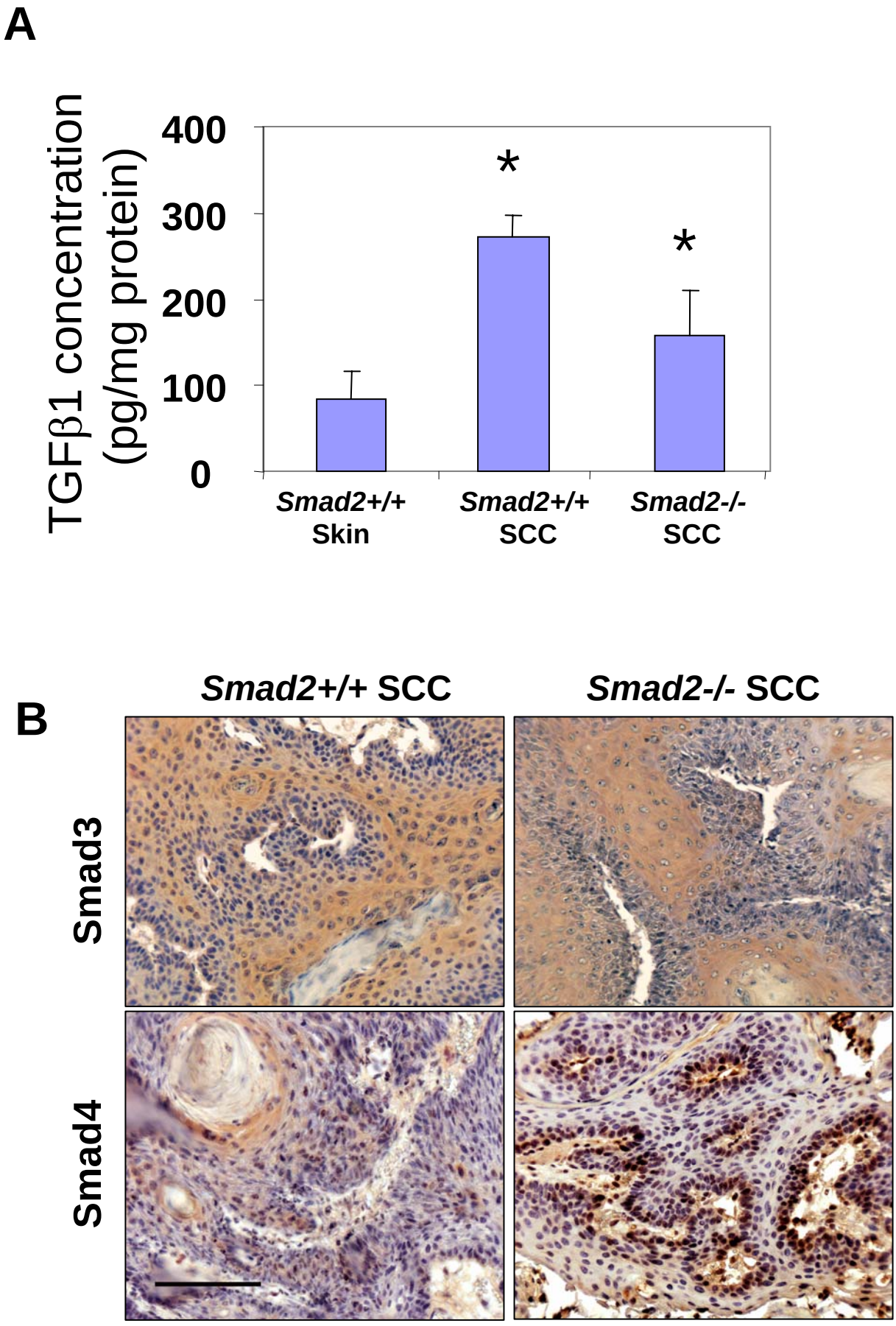

Supplemental Figure 8