Supporting Information

Marinari *et al.* 10.1073/pnas.0809288105

SI Materials and Methods

Cell Culture. HaCaT cells were grown in DMEM supplemented with 10% FBS and antibiotics. Primary mouse keratinocytes were isolated from newborn mice and cultured at 34 °C in low-calcium keratinocyte basal medium (Clonetics) and EGF (10 ng/mL). TE13 (*TP*53 null) and TE1 (harboring a temperature-sensitive mutated *TP*53) cells (gift from Pierre Hainaut) were cultured in RPMI medium supplemented with 10% FBS, whereas SCC25 (harboring a mutated *TP*53 allele) cells were cultured in 1:1 DMEM/Ham's F12 medium supplemented with 10% FBS. Unless otherwise indicated, treatment of cells with TGF-β (Calbiochem) was at 5 ng/mL for 30 min. To measure clonogenic growth, SCC cell lines transfected with Lipofectamine (Invitrogen) were selected in G418 (600 μg/mL; Invitrogen) for 15 days before fixation in 4% paraformaldehyde and staining with crystal violet. Where indicated, TGF-β was added together with selection medium every other day until fixation. Colonies with a diameter >1 mm were scored using National Institutes of Health Image software.

Plasmids, Retroviruses, and RNAi. Expression constructs for human IKKα and Gal4-IKKα were derived by PCR amplification from pCR-bAct-3xHAIKKα plasmid (1) and cloning into pCDNA3Flag and pCDNA-Gal4 vectors, respectively. The pCDNAFlag-IKKα-NLS expression plasmid (carrying 3 Lys-to-Ala substitutions in amino acids 235-236-237) was generated by site-directed mutagenesis and the following primers:

IKKαNLS for 5′-GGCATGAGAAGATTGCGGCAGCG-GATCCAAAGTG-3′

IKKαNLS rev 5′CACTTTGGATCCGCTGCCGCAATCT-TCTCATGCC-3′.

Mad1-Luc and mutMad1-luc reporter plasmids have been described previously (2) and contain the promoter plus intron 1 region of the Mad1 gene (Mad1-luc) or the same region carrying mutation in the Smad-responsive element (mutMad1-luc). siRNA duplexes targeting GFP were obtained from MWG-Biotech, the sense strand being 5′-GTTCAGCGTGTCCGGC-GAG-3′. siRNA duplexes targeting mouse IKKα (M-041014), human IKKα (M-003473), human Mad1 (Mxd1; M-009325), and human Ovol1 (M-006534) were purchased as SMART-Pools (Dharmacon). Primary mouse keratinocytes, HaCaT, and SCC cells, plated on 35-mm dishes, were transfected with 25 nM siRNAs using Lipofectamine 2000 (Invitrogen).

Retroviral vectors based on pBABE-puro-expressing WT-IKKα and mutNLS-IKKα were derived by PCR amplification from the pCDNA expression plasmids. Retroviruses were obtained by cotransfecting retroviral vectors and retroviral packaging plasmids (pVSV-G and pGAG) in subconfluent 293 cells. Stable WT-IKKα and mutNLS-IKKα-expressing TE13 cell lines were generated by infection of TE13 cells at high moi. in the presence of polybrene and selection in puromycin (1 μg/mL).

Immunochemistry. A semiquantitative digital image analysis to determine the intensity of IKKα expression in each sample was carried out. Briefly, stained tissue sections were viewed under a light microscope, and images were captured digitally (Nikon digital sight). Digital images were amplified to 1280 × 960 pixels. A total of 10 representative areas containing 100 SCC cells were analyzed using ImageJ software (National Institutes of Health). By using this system, staining intensity was quantifiable between 0 (white) and 225 (black). To adjust for day-to-day and section-to-section variations, cytoplasmic regions of 100 normal epithelial cells were used as an internal control. The expression intensity in tumor cells was standardized by subtraction of the mean gray level of internal control from that of 10 tumor fields. For quantification of nuclear staining, the mean cytoplasmic intensity in each microscopic field was set as background.

Immunofluorescence Analysis. Immunofluorescence analysis was performed on 5×10^4 to 10×10^4 cells seeded on Chamber Slides (Nalge Nunc International), fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. TE13 staining was performed using a monoclonal anti-Flag (M3, 1:50 dilution; Sigma–Aldrich) and a rabbit polyclonal anti-GAL-4 (1:50; Santa Cruz Biotechnology), followed by incubation with FITC-conjugated anti-mouse IgG or an FITC-conjugated anti-rabbit IgG. Nuclei were counterstained with DAPI (Sigma–Aldrich).

Flow Cytometry. After trypsinization, cells were pelleted in a clinical centrifuge at 200 g, resuspended in 300 μl PBS (PBS), and fixed in 700 μl cold ethanol while vortexing the mixture. Fixed cells were repelleted, resuspended in 1 mL PBS, and pelleted again. Cells were resuspended in 1 mL PBS containing 10 μg propidium iodide and 100 μg RNase-free DNase. Cell cycle profiles were determined using the CellQuest program on FACScan flow cytometer (Beckton-Dickinson).

Immunoblotting and Cell Fractionation. Polyclonal antibodies against actin (C-11), IKKα (H-744), p21 (C-19), p15INK4B (C-20), Gal4DBD (N-19), and HA (F-7) were from Santa Cruz Biotechnology; monoclonal anti-IKKα antibody was from BD–PharMingen. For immunoblotting, cells were lysed in 50 mM Tris (pH 8.0), 120 mM NaCl, and 0.5% Nonidet P-40, and protein concentration was determined by the Bio-Rad dye-binding assay. Total cell extract (50 μg) was separated by SDS/PAGE and blotted onto a poly(vinylidene difluoride) membrane. Western blot analysis was performed with the aid of the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

For cell fractionation studies, cells were washed twice in ice-cold PBS, pelleted by centrifugation at 1,200 g for 5 min, washed once in buffer A [10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, and 1 mM DTT] and centrifuged at 10,000 rpm for 10 min. Cell pellets were resuspended in buffer A also containing 0.1% Nonidet P-40 for 10 min on ice, and lysed cells were centrifuged at 9,200 g for 10 min. The nuclear pellet was extracted with buffer C [20 mM Hepes (pH 7.9), 400 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20% glycerol, and 1 mM PMSF] for 15 min on ice. After incubation, nuclei were centrifuged at 9,200 g for 10 min, and the supernatant was diluted with 4 volumes of buffer D [10 mM Hepes (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, and 1 mM DTT].

Luciferase Reporter Assay. For reporter gene assays, cells were seeded on 12-well plates and transfected with 0.1 μg of the indicated reporter plasmid, 0.02 μg pRL-null plasmid, and 1.5 μg IKKα expression plasmids using Lipofectamine. At 24 h after transfection, cells were treated with TGF-β. After 16 h, cell extracts were assayed for luciferase activity using the Dual-Luciferase Kit (Promega) on a Triathler luminometer (Hidex). Results from experiments performed 3 times in triplicate wells are expressed as relative luciferase activity after normalization with pRL-null plasmid as internal control.

mRNA Expression Analysis. For RT-qPCR, total RNA was extracted using TRIzol Reagent (Invitrogen). Total RNA (1 mg) was reverse transcribed with GeneAmp RNA PCR (Applied Biosystems). The TaqMan Universal PCR Master Mix (Applied Biosystems) was used for PCR amplification using Applied Biosystems' 7300 real-time PCR system. Each mRNA was normalized to the levels of cyclophilin mRNA for each reaction, and the relative amount of each sequence was determined by using the comparative C_T method. Primers and probes used in this study were as follows:

hIKKα sense: 5′-TGACAGCACAGAGATGGTGA-3′
hIKKα antisense: 5′-CTTCTGCTTACAGCCCAACA-3′
hIKKα Probe (5′FAM-3′TAMRA): 5′-CAGCTCCTTGAGCACACGGTCC-3′
hMad1 sense: 5′-ACAGGGAAGAAATCGACGTT-3′
hMad1 antisense: 5′-GTGCTGGAATAGCCCTCATC-3′
hMad1 Probe (5′FAM-3′TAMRA): 5′-CTGCCGAGGCTCTGCATGCT-3′
hOvol1 sense: 5′-TACAAGTGCAGCCTGTGTGA-3′
hOvol1 antisense: 5′-TGCACACCATGGATCTTCTT-3′
hOvol1 Probe (5′FAM-3′TAMRA): 5′-TCACGCAGCGCTGCTCTCTG-3′
hSMAD7 sense: 5′-CTCCTGCTGTGCAAAGTGTT-3′
hSMAD7 antisense: 5′-GATTCACAGCAACACAGCCT-3′
hSMAD7 Probe (5′FAM-3′TAMRA): 5′-ATGCCTGAGATCCGGCCACC-3′
E-Cadherin sense: 5′-CATCTCCCTTCACAGCAGAA-3′
E-Cadherin antisense: 5′-CTAAGGCCATCTTTGGCTTC-3′
E-Cadherin Probe (5′FAM-3′TAMRA): 5′-TGGTCCAGTTGGCACTCGCC-3′
c-Myc sense: 5′-CACAGCAAACCTCCTCACAG-3′
c-Myc antisense: 5′-GGATAGTCCTTCCGAGTGGA-3′
c-Myc Probe (5′FAM-3′TAMRA): 5′-TCAGCACAACTACGCAGCGCC-3′.

Assay-on-Demand TaqMan probes for mouse Ovol1, Ovol2, Mad1, Mad2, Mad3, Mad4, and IKKα were obtained from Applied Biosystems.

GeneChip and Gene Expression Analysis. Following RNase H-mediated second-strand cDNA synthesis, the cDNA was transcribed in the presence of T7 RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mix for cRNA amplification and biotin labeling. All reactions were carried out using the GeneChip One-Cycle Target Labeling and Control Reagents kit (Affymetrix). Fragmented cRNA (15 μg) were used for hybridization to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix) for analysis of more than 39,000 transcripts. Hybridized microarrays were scanned at high resolution by GeneChip Scanner 3000 (Affymetrix) using the GeneChip Operating Software (GCOS) v1.1.1, and the resulting data were analyzed with Microarray Suite 5.0 software (Affymetrix). Array data were normalized and statistically analyzed by DNA-Chip Analyzer (dChip) software (www.dchip.org). After normalization, principle component analysis was carried out as a quality control with the normalized raw data, and samples were classified by linear discriminant analysis. Arrays then were compared, and resulting up- or down-regulated genes were filtered. Statistical comparison of gene expression levels between siCtr and siIKKα cells were performed by dChip. Analysis criteria were a fold-change of at least 1.2 with a lower 90% confidence bound of fold-change, a probe call in all samples >20%, and a false discovery rate, estimated by permutation as ≤5%. Based on the full data set, paired Welch tests (*t* test for unequal variance) were performed to assess the interesting genes for the distinction: a *P* value for paired *t* test ≤0.05 was considered significant. Expression value of each replicate was pooled considering measurement error, and absolute calls for the pooled sample were determined by the "majority vote" scheme of DNA-chip analyzer dChip.

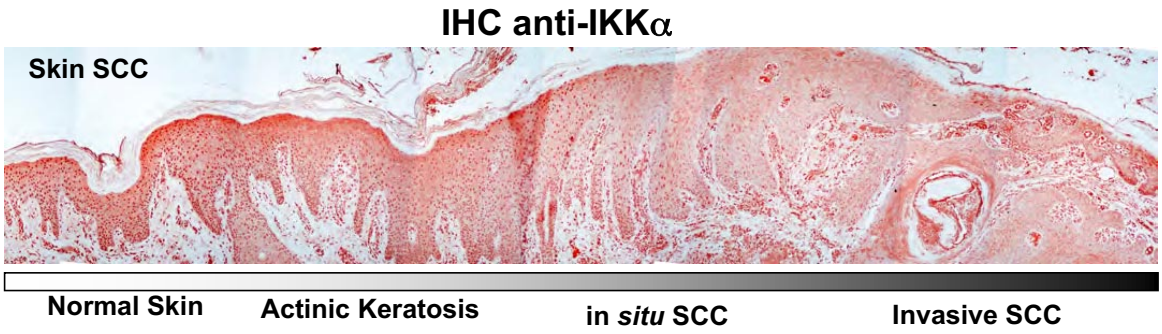
ChIP. The Primers Used for PCR Amplification Were as Follows: hOvol1 sense: 5′-cgcagttgaagccacttcc-3′
hOvol1 antisense: 5′-aggcttggctaagcaactcg-3′;
spanning positions −747 to −555 of the *Ovol1* gene
hMad1 sense: 5′-CACCTGAGGTCAAGAGTTCG-3′
hMad1 antisense: 5′-TCACTGCAACCTCCTTCTCC-3′
spanning positions −1337 to −1170 of the *Mad1* gene.
hIkBa sense: 5′-ACTTGCAGAGGGACAGGATTACAG-3′
hIkBa antisense: 5′-AGGCTCGGGGAATTTCCAAG-3′
spanning positions −324 to −77 of the *IkBa* gene.

Reactions were performed for 25–35 cycles of denaturation at 95 °C for 45 s, annealing at 55–57 °C for 45 s, and extension at 72 °C for 45 s.

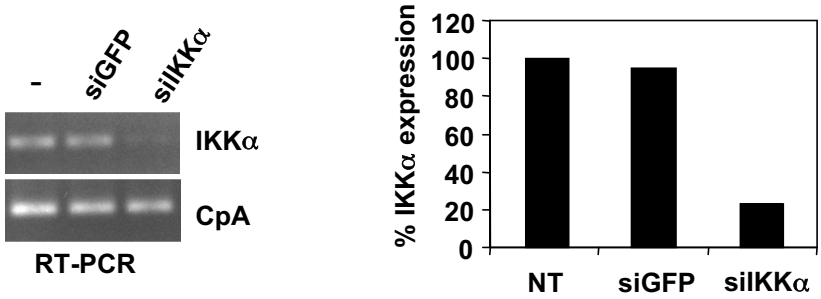
1. Han G, *et al*. (2005) Distinct mechanisms of TGFβ1-mediated epithelial-to-mesenchymal transition and metastasis during skin carcinogenesis. *J Clin Invest* 115:1714–1723.

2. Descargues P, *et al*. (2008) IKKα is a critical coregulator of a Smad4-independent TGFβ-Smad2/3 signaling pathway that controls keratinocyte differentiation. *Proc Natl Acad Sci USA* 105:2487–2492.

A



B



C

Gene	fold change	paired P value
keratin complex 2, basic, gene 5	1.42	0.037285
cyclin D2	1.47	0.009730
retinoblastoma binding protein 4	1.49	0.005412

Gene	fold change	paired P value
mitogen activated protein kinase kinase 1	-1.40	0.012873
scotin gene	-1.43	0.016940
septin 7	-1.43	0.049920
small proline-rich protein 2A	-1.48	0.032770
RAN, member RAS oncogene family	-1.54	0.013433
stratifin	-1.60	0.009031
hypoxia inducible factor 1, alpha subunit	-1.70	0.026485
transforming growth factor, beta receptor II	-1.79	0.005872
small proline-rich protein 1B	-1.83	0.040920
tumor necrosis factor receptor superfamily, member 12a	-1.96	0.016281
calmodulin 1	-2.07	0.004077
periplakin	-2.25	0.012492
tissue inhibitor of metalloproteinase 2	-2.29	0.004776
PERP, TP53 apoptosis effector	-2.31	0.003231
protein phosphatase 1, catalytic subunit, alpha isoform	-2.40	0.021159
integrin beta 1 (fibronectin receptor beta)	-2.42	0.006987
catenin (cadherin associated protein), beta 1, 88kDa	-2.62	0.006772
mitogen activated protein kinase 1	-2.86	0.005491
calmodulin 2	-2.94	0.001347
small proline-rich protein 2D	-3.14	0.006533
small proline-rich protein 1A	-3.19	0.001277
par-6 (partitioning defective 6) homolog beta (C. elegans)	-3.30	0.000654
proteasome (prosome, macropain) 26S subunit, non-ATPase, 8	-4.63	0.003600
ubiquitin-activating enzyme E1C	-5.01	0.010535
cyclin G2	-5.74	0.004589
Tumor differentially expressed 1	-10.83	0.000853

Fig. S1. IKKα down-regulation in primary keratinocytes affects genes belonging to the keratinocyte differentiation pathway. (*A*) A biopsy of skin SCC with adjacent normal skin was subjected to anti-IKKα IHC. The panel is a compound picture where both the normal skin (left) and the invasive SCC (right) are evident. (Magnification: 10×.) (*B*) Primary mouse keratinocytes were left untreated or were transiently transfected with the indicated siRNAs. After 24 h, total RNA was extracted and subjected to semiquantitative RT-PCR to determine expression of IKKα and cyclophilin A (CpA). Band densitometry was performed using Kodak software (MI v.4.0.1; *right*). IKKα band intensity was normalized to that of CpA. (*C*) A list of genes related to keratinocyte proliferation and differentiation that were found to be significantly affected by IKKα knockdown.

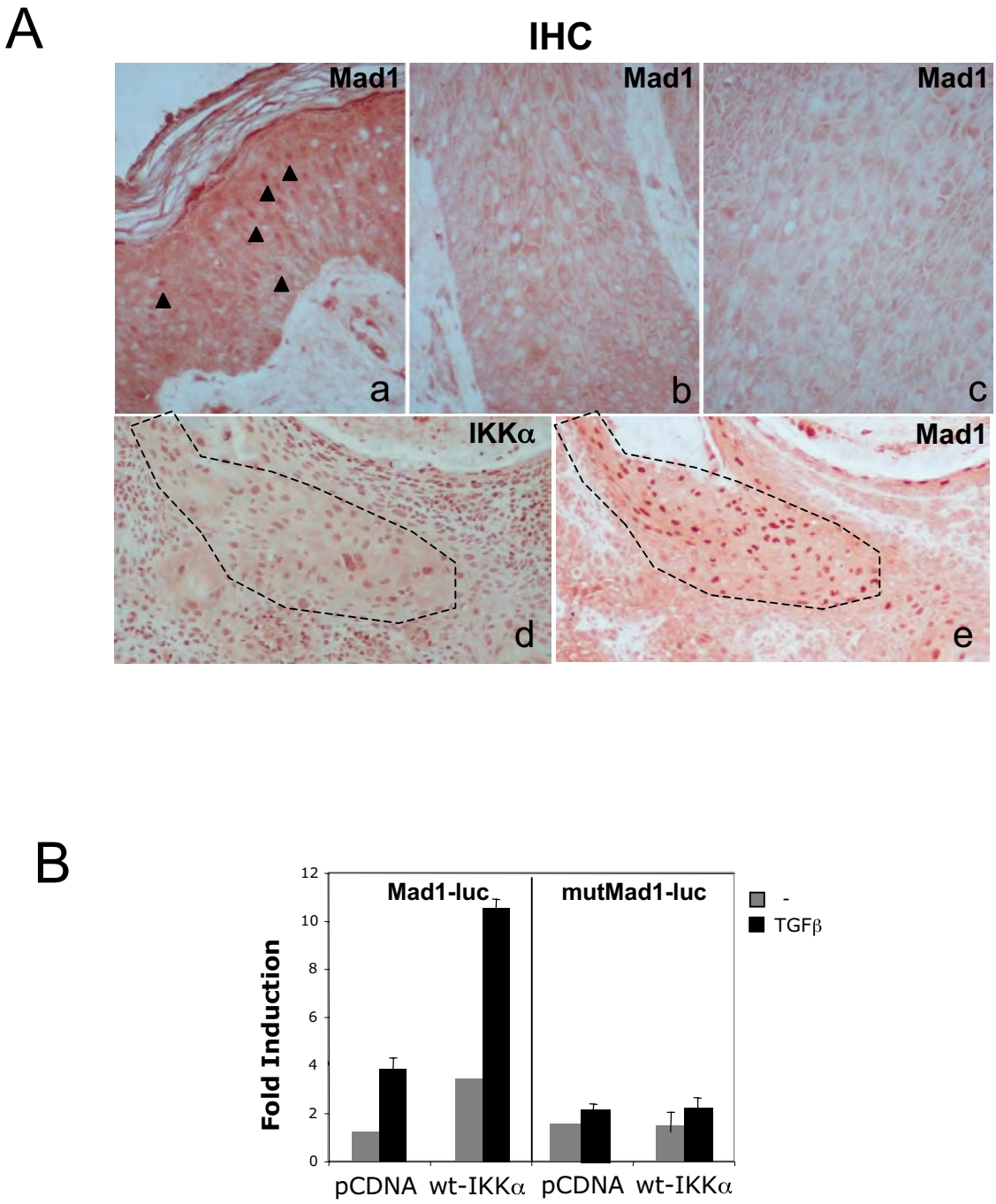


Fig. S2. Mad1 and IKKα are coexpressed in SCC. (*A*) IHC staining with a Mad1-specific antibody of normal skin (*a*), dysplastic region (*b*), and invasive portion (*c*) of the SCC shown in Fig. S1*A*. (*d* and *e*) IKKα and Mad1 stainings of the same region in a well-differentiated SCC. Arrowheads indicate nuclei positive for Mad1 in suprabasal layers of epidermis. The dashed line delimits the same region of consecutive sections of the same well-differentiated SCC, where nuclear IKKα (*d*) and Mad1 (*e*) are evident. Nuclear images are representative of 5 poorly differentiated SCCs with adjacent normal skin and 15 well-differentiated SCCs. Note that IKKα nuclear localization correlates with Mad1 nuclear expression. (Magnification 40×.) (*B*) HaCaT cells were transfected with Mad1-luc reporter or a mutant thereof lacking the SMAD-responsive element in the presence or absence of WT-IKKα expression plasmid. After 24 h, cells were treated with TGF-β for 16 more h, and luciferase activity was determined and normalized to the activity of cotransfected pRL plasmid. Histograms represent mean + 1 SD and are representative of 3 independent experiments performed in triplicate.

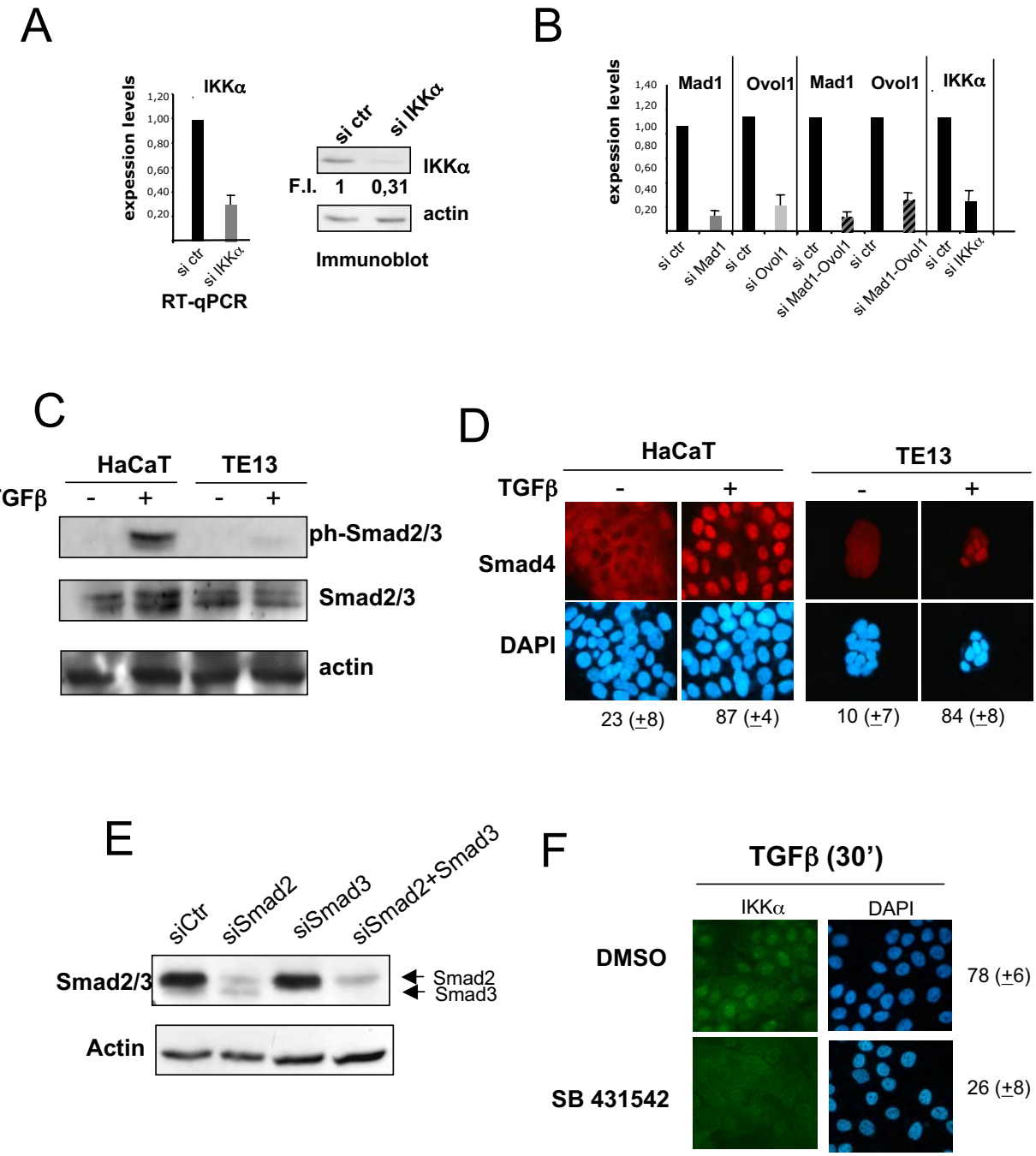
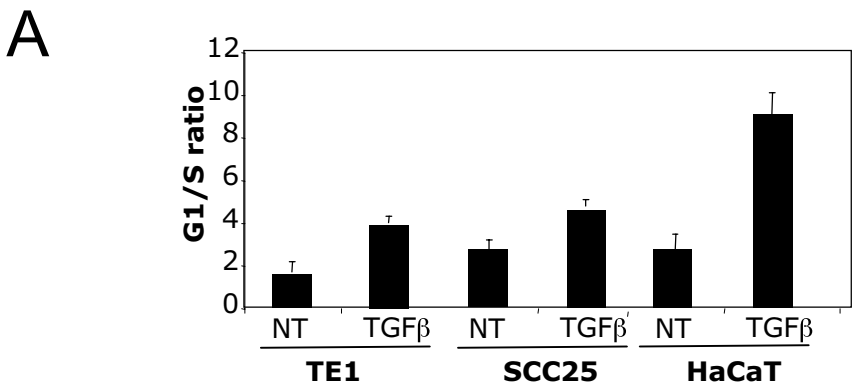


Fig. S3. (*A*) Detection of IKKα expression by RT-qPCR (*Left*) and immunoblotting (*Right*) in HaCaT cells transfected with either control or IKKα-specific siRNA. The IKKα bands were quantified and normalized relative to actin by Kodak software. (*B*) RT-qPCR was performed on HaCaT cells transiently transfected with the indicated siRNAs. Expression levels in cells transfected with control siRNA were set as 1. Results represent means of 3 independent experiments. Error bars represent standard deviations. (*C*) HaCaT or TE13 cells were left untreated or treated with TGF-β for 30 min, lysed, and analyzed by immunoblotting for phosphorylated SMAD2/3, total SMAD2/3, or actin expression. (*D*) HaCaT or TE13 cells were left untreated or treated with TGF-β for 30 min and subjected to indirect immunofluorescence to detect subcellular localization of SMAD4. Nuclei were counterstained with DAPI. The percentage of cells displaying a strong nuclear signal was determined by counting at least 200 cells per field in 3 separate experiments performed in duplicate. (*E*) HaCaT cells were transfected with either control or the indicated siRNAs. After 24 h, cell lysates were analyzed by immunoblotting to detect expression of SMAD2/3 or actin. (*F*) HaCaT cells were treated with DMSO or SB 431542 for 30 min and then with TGF-β for 30 more min. Cells were examined by indirect immunofluorescence for subcellular localization of IKKα. Nuclei were counterstained with DAPI. The percentage of cells displaying a strong nuclear signal was determined by counting at least 200 cells per field in 3 separate experiments done in duplicate.



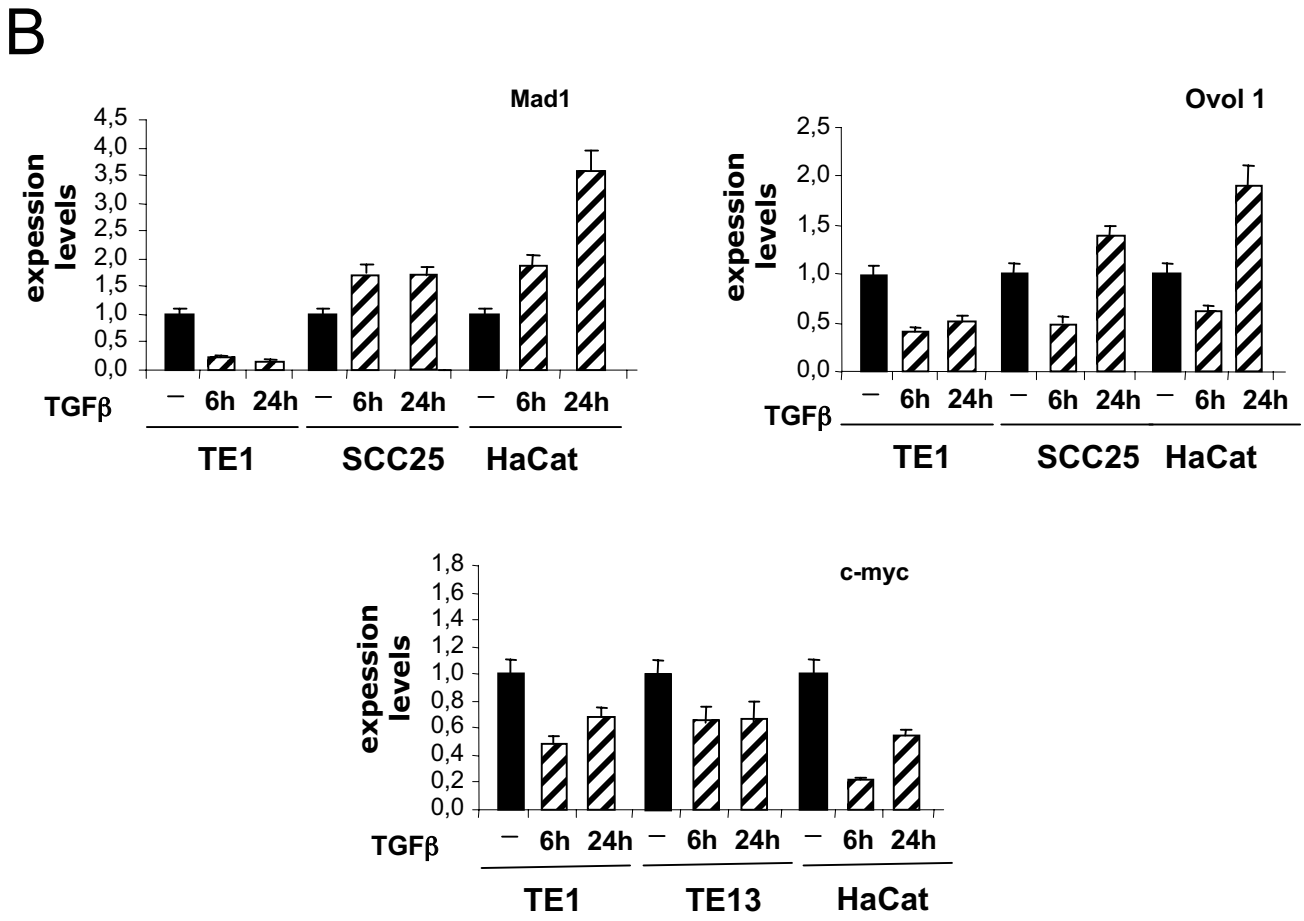


Fig. S4. SCC cell lines display reduced TGF-β sensitivity. (*A*) TE1 (esophageal SCC cell line), SCC25 (oral SCC cell line), and HaCaT cells were left untreated or exposed to TGF-β (5 ng/mL) for 24 h. Cells then were stained with propidium iodide, and the DNA content was analyzed by flow cytometry to determine cell cycle distribution. The histogram shows G_1/S ratios determined in triplicate and is representative of 3 independent experiments. (*B*) TE1, SCC25, or HaCaT cells were treated with TGF-β as described above. Total RNA was analyzed by quantitative RT-PCR to determine expression of Mad1, Ovol1, and c-Myc.

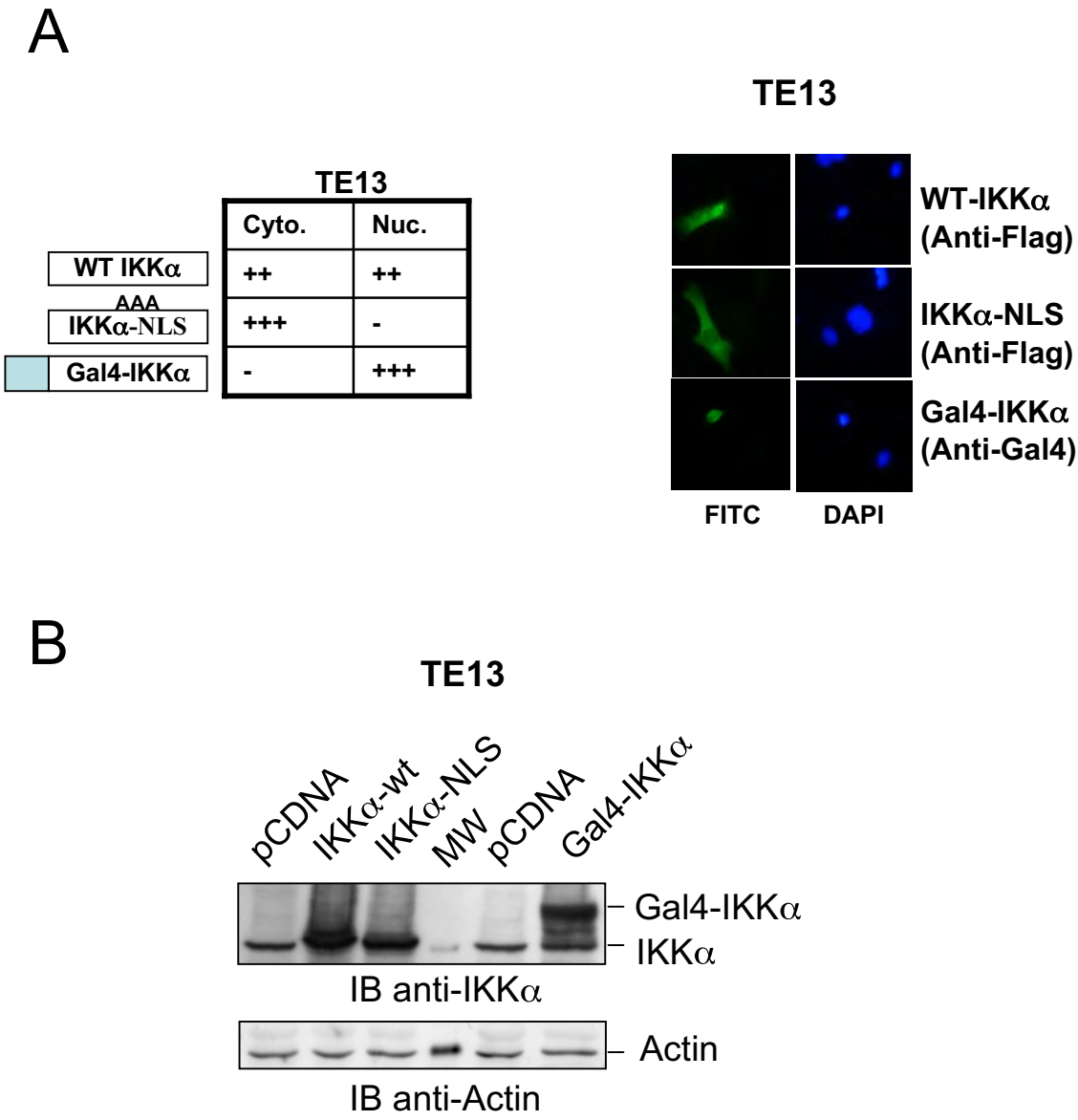


Fig. S5. Gal4-IKKα accumulates in the nuclei of transfected TE13 cells. (*A*) TE13 cells were transiently transfected with plasmids bearing Flag-tagged WT IKKα, Gal4-DNA-binding domain-IKKα fusion protein (Gal4-IKKα), or Flag-tagged IKKα protein carrying mutations in nuclear localization signal (IKKα-NLS). The subcellular distribution of exogenously expressed proteins was determined by immunofluorescence using Flag- or Gal4-specific antibodies (*Right*). Nuclear and cytoplasmic staining was scored in at least 50 positive cells for each experimental point (*Left*; $-$: less than 25% positive cells; $+$: 25–50% positive cells; $++$: 50–75% positive cells; and $+++$: 75–100% positive cells). (*B*) Expression of IKKα constructs in the TE13 cells used for the experiments described in Fig. 5*C*.

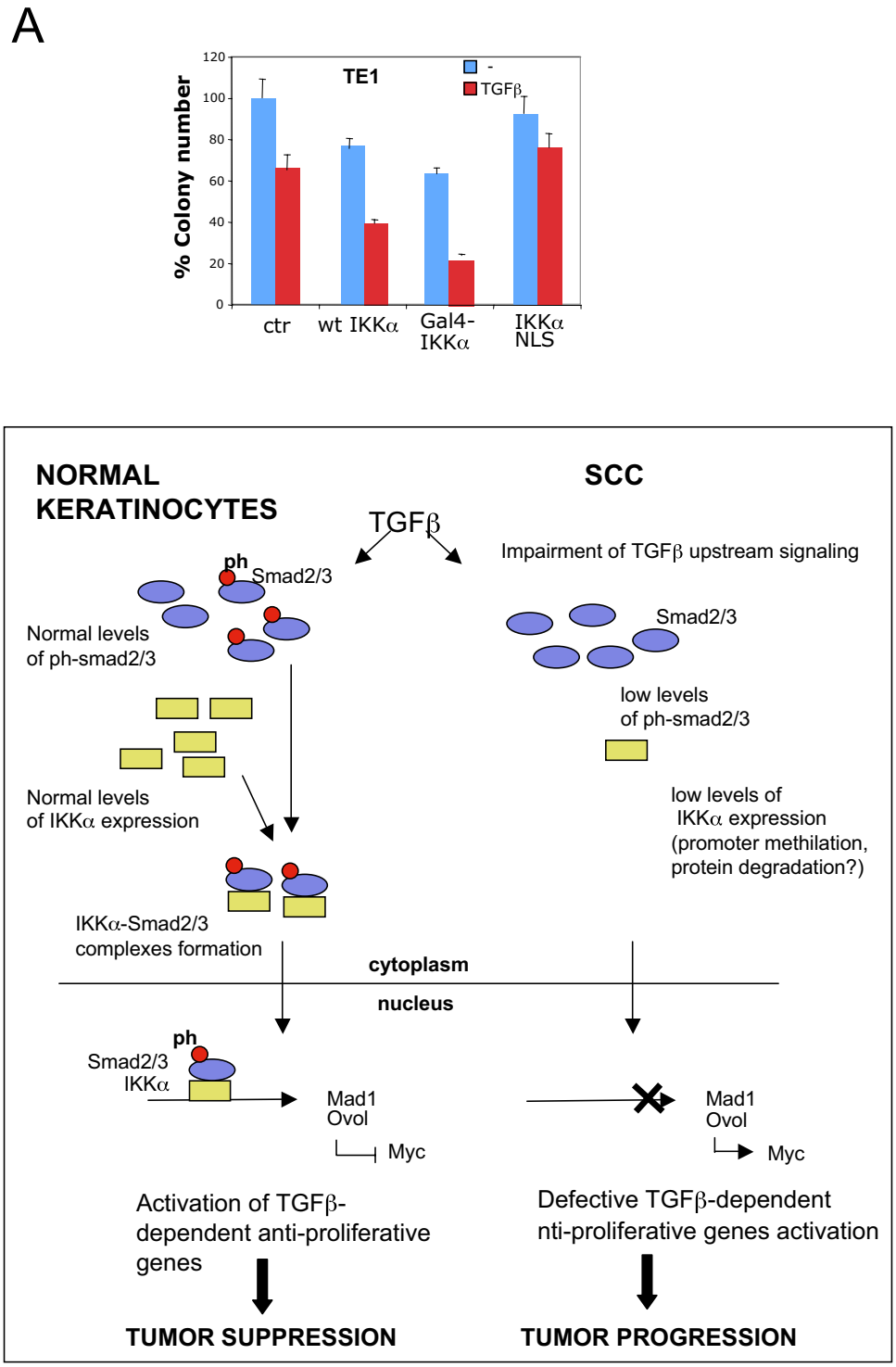


Fig. S6. Nuclear IKKα confers sensitivity to TGF-β-induced growth arrest. (*A*) TE1 cells were transfected with WT-IKKα, Gal4-IKKα, and IKKα-NLS constructs and selected in G418 (600 μg/mL) for 15 days. Cultures were stained with crystal violet dye, and colonies $>$1 mm in diameter were counted using ImageJ software. The histogram represents the mean + SD of 3 independent experiments performed in duplicate. (*B*) Schematic diagram representing the cross-talk between IKKα and TGF-β signaling in SCC development. In normal keratinocytes, TGF-β activates R-SMADs, which bind to IKKα (2) and translocate to the nucleus. IKKα–R-SMAD complexes activate antiproliferative genes, such as Ovol1 and Mad1, which in turn down-regulate Myc expression and activity. During SCC formation, TGF-β-induced R-SMAD activation is defective, and IKKα levels are reduced. The defective nuclear accumulation of R-SMAD–IKKα complexes correlates with Mad1 and Ovol1 expression and increased Myc activity or expression, leading to tumor progression.