METHODS SECTION

Cell culture, viruses and human samples

Culturing of primary human keratinocytes and SCC13 cells, and infection with retroviruses expressing the *H-ras*^{V12} [31], ATF3 [32] and constitutively active NFATc1 [19] genes, together with corresponding controls, were as previously reported [29]. CsA (Sigma) was dissolved in ethanol and stored in stock solution (20 mM) at -80°C. The VIVIT and VEET peptides were chemically synthesized by the peptide core facility of the University of Lausanne, HPLC purified (> 95% purity), dissolved in DMSO and stored in stock solution (20 mM) at -80°C. For knockdown experiments, cells were transfected as described [33] with validated stealth siRNAs for the human p53, calcineurin B1, NFATc1 genes (Invitrogen) and for ATF3 (GeneGlobe, Qiagen) in parallel with corresponding scrambled siRNA controls, and analyzed 72 hours after transfection. The sequence of all siRNAs is shown in Supplemental Table I.

HKCs were treated with cycloheximide (10 μg/ml) or ethanol vehicle alone, followed, 2 hours later, by treatment with either CsA or VIVIT. Cells were collected at subsequent times for mRNA analysis by real time RT-PCR. Preliminary experiments (based on immunoblot analysis) showed >90% protein synthesis inhibition in HKCs treated with cycloheximide at these concentrations.

Human skin samples were obtained from abdominoplasty procedures at Massachusetts General Hospital (Boston, MA) with patients' and institutional approvals and cultured as previously described [10] plus/minus treatment with CsA for 48 hours. For RNA collection, skin samples were placed in preheated PBS at 60 °C for 45 seconds, then chilled (on ice) in 0.1M PBS for 1 minute, followed by mechanical separation of epidermis and dermis. The epidermis was homogenized in TRI Reagent (Sigma) for RNA preparation.

Squamous cell carcinoma samples were obtained at the Department of Dermatology of the Zurich University Hospital Switzerland from clinical biopsies. Parts not needed for histological diagnosis were further processed with institutional review board approval.

Quantitative real time RT PCR, chromatin immunoprecipitation and immunodetection techniques

Conditions for real time RT-PCR analysis, immunoblotting, immunofluorescence, FACS analysis and Magnetic Cell Sorting (MACS) were as previously described [24,29,34]. The list of gene-specific primers is provided in Supplemental Table II. We used the following antibodies: rabbit antiserum against human keratin1 (PRB-149p) and loricrin (PRB-145p) (from Covance); mouse monoclonal against p53 (#2524), rabbit polyclonal against c-Fos (#4384) (from Cell Signaling); concentrated mouse monoclonal ((7A6)X, SC-7294) and rabbit polyclonal against NFATc1 ((H-110)X, sc-13033), rabbit polyclonal against ATF-3 (C-19, sc188), mouse monoclonal against p21 (sc-187), rabbit polyclonal against c-Jun (H-79, sc-1694) (from Santa Cruz Biotechnology); rabbit polyclonal anti human ATF3 (LS-B329)(Lifespan Bioscience); mouse monoclonal anti pankeratin (AE1+AE3, ab961-6), rabbit anti human vimentin (ab45939), rabbit polyclonal anti cytokeratin 5 (ab24647), rabbit monoclonal anti Ki67(ab16667) (from Abcam); mouse monoclonal anti Calcineurin B1 (CN-B1), mouse monoclonal anti γ-Tubulin (GTU88) (Sigma). For FACS analysis: mouse anti APC conjugated CD71 and rat anti FITC conjugated integrin α6 (CD49f) (from BD bioscience), and for MACS: mouse anti-CD133 and anti-mouse IgG beads (Miltenyi Biotec).

Senescence ß-galactosidase staining

Senescence ß-galactosidase (SA-ß-Gal) activity was assessed by use of a commercially available chromogenic assay kit (Cell Signalling, Cat. 9860), according to the manufacturer's recommendations. Assays for endogenous ß-galactosidase activity at a suboptimal pH (pH6), which is thought to reflect increased lysosomal activity in senescent cells 17 were performed in parallel with similar assays at optimal pH (pH 4), as positive control for ß-galactosidase activity in cells, irrespective of their growing or senescent state.

Tissue arrays

Biopsies of cutaneous SCCs from patients under CsA treatment and control patient population were collected for tissue array preparation, followed by immunohistochemical analysis of ATF3 protein expression using polyclonal rabbit anti-human ATF3 antibody (Atlas Antibodies, Stockholm, Sweden) and 10M citrate buffer at pH 6.0 as an antigen retrieval. Tumour specimens were distinguished into *in situ* and invasive squamous cell carcinoma (SCC) of the skin. The numbers of analyzed samples are: 126 invasive SCCs from immunocompetent patients and 127 from CsA treated patients; 18 in situ SCCs from immunocompetent patients, and 16 from CsA treated patients. Statistical analysis indicated that the differences in ATF3 nuclear staining between tumours from CsA-treated and untreated patients was highly significant for both in situ SCCs ($P < 0.05$) and invasive SCCs ($P < 0.01$), and for the two groups combined ($P < 0.001$).

ATF3 pull down assays

Conditions for these assays were as previously described [35]. Briefly, HKCs were treated with either CsA (5uM) or VIVIT (2uM) or corresponding controls for 24h, followed by lysis in HKMG buffer (10mM HEPES pH7.9/100mM KCl/5mM MgCl2/10% glycerol/1mM DTT/0.1% Nonidet P-40) with a mixture of protease inhibitors. Cell extracts were incubated with 1 µg of

biotinylated double-strand oligonucleotides for 16 h. Two biotinylated oligonucleotide sequences of the p53 promoter containing fully conserved ATF3 binding sites (p53-1 and p53-2, corresponding, respectively, to nucleotide positions -420 to -358 and -2105 to -2039 from the initiation codon) in parallel with a mutated oligonucleotide of the first sequence (mp53-1), with internal nucleotide substitutions disrupting the ATF3 binding site. The oligonucleotide sequences are provided in Supplemental Table III. DNA–protein complexes were collected by precipitation with streptavidin-agarose beads (Pierce) for 1 h, washed three times with HKMG buffer, followed by processing for standard immunoblot analysis with anti-ATF3 antibody.

Tumourigenicity assays

Mice with the CnB1 gene flanked by loxP sites and a Cre transgene driven by a keratinocyte-specific promoter ($CnB1^{loxP/loxP}$ x K5-CrePR1) [3] in parallel with transgene-negative controls ($CnB1^{loxP/loxP}$) were subjected to a multistep skin carcinogenesis protocol as previously described [36]. Briefly, mice (8 weeks old; 12–16 animals per group) were treated with 7,12-dimethylbenz [*a*] anthracene (DMBA) (20 μg in 200 μl acetone), followed by repeated treatments (twice a week) with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (10^{-4} M in acetone) for 2 months. Mice were examined throughout the duration of the experiments for papilloma-versus carcinoma formation by macroscopic examination and palpation of the tumour base, to determine the onset of an invasive pattern of growth as well as infiltration of the surrounding tissues.

For grafting assays, HKCs were infected with a H-ras^{V12} transducing retrovirus (LZRS-ras^{V12}) [31] and, 16 hours after infection, grafted onto the back of immunocompromised (Scid) mice ($2x10^6$ cells per graft) by use of dome-shaped transplantation chambers as previously described [29,37,38]. Mice were subsequently treated, three times a week, with CsA (via injection

into the transplantation chamber; 20 μg/g body weight, in ethanol), or ethanol vehicle alone (4 mice per group). Mice were sacrificed after 8 weeks for retrieval of the grafted tissue and histological analysis.

For intradermal tumourigenicity assays, HKCs, SCC12 and SCC13 cells were brought into suspension, and injected (10^6 cells per injection, two side flank injections per mouse) at the dermal-epidermal junction of 8 weeks old female mice (NOD/SCID or NOD/SCID with interleukin-2 receptor gamma chain null mutation (Il2rg(-/-) [22]), utilizing a procedure previously described for hair reconstitution assays[30]. Starting 24 hours later, mice were given I.P. injections, every other day, of CsA (20 μg/g body weight in ethanol) or FK506 (2 μg/g body weight in DMSO) in parallel with vehicles alone, or the VIVIT and VEET control peptides (10 μg/g body weight in ethanol), and sacrificed at the indicated times after cell injections.

As an alternative approach to drug treatment, HKCs or SCC cells were transfected with siRNAs against the indicated genes or scrambled siRNA controls (100nM siRNA in HiPerFect transfection reagent, Qiagen) for 12 hours prior to infection with the H-*ras*^{V12} transducing retrovirus. 20 hours later, cells were trypsinized and and injected into mice. In all cases, mice were sacrificed at 10 days after cell injections and the nodules found at the site of injections processed for histological analysis.

For serial dilution tumorigenicity assays, sorted H-*ras*^{V12} expressing HKCs or SCC cells were serially diluted, admixed in various numbers with a fixed amount of HKCs ($2x10^5$ cells / injection), mixed with matrigel (BD biosciences), and injected into dermal-epidermal junction of NOD/SCID or NOD/SCID(Il2rg(-/-) [22] mice. Mice were sacrificed 4 weeks later and nodules found at the site of injections processed for histological analysis.

Secondary tumorigenicity assays were performed as described[34]. Briefly, primary tumours formed by *Ha-ras*^{V12} expressing HKCs or SCC cells in mice plus/minus CsA treatment were isolated, 6 weeks after cell injection, and dissociated into single cells by collagenase digestion (0.35% solution in HBSS; Sigma) for 45 min at 37°C. After filtering of the cell suspension, recovered cells were washed with keratinocyte medium, counted and admixed in various numbers with a fixed amount of HKCs (2x10^5 cells) prior to injection at the dermal-epidermal junction of NOD/SCID mice. 4 weeks after injection, mice were sacrificed for histological analysis. Similar experiments were performed with freshly dissociated cells from primary tumors formed by *Ha-ras*^{V12} expressing HKCs or SCC13 cells plus/minus increased ATF3 expression.

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