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(Article begins on next page)



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## Constitutive STAT3 activation in epidermal keratinocytes enhances cell clonogenicity and favors spontaneous immortalization by opposing differentiation and senescence checkpoints

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Complete List of Authors:	Orecchia, Valeria; Molecular Biotechnology Center, University of Turin, Dept. of Molecular Biotechnology and Health Sciences Regis, Gabriella; Molecular Biotechnology Center, University of Turin, Dept. of Molecular Biotechnology and Health Sciences Tassone, Beatrice; Molecular Biotechnology Center, University of Turin, Dept. of Molecular Biotechnology and Health Sciences Valenti, Chiara; Molecular Biotechnology Center, University of Turin, Dept. of Molecular Biotechnology and Health Sciences Avalle, Lidia; Molecular Biotechnology Center, University of Turin, Dept. of Molecular Biotechnology and Health Sciences Saoncella, Stefania; Molecular Biotechnology Center, University of Turin, Dept. of Molecular Biotechnology and Health Sciences Calautti, Enzo; Molecular Biotechnology Center, University of Turin, Dept. of Molecular Biotechnology and Health Sciences; Poli, Valeria; Molecular Biotechnology Center, University of Turin, Dept. of Molecular Biotechnology and Health Sciences
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5 **Constitutive STAT3 activation in epidermal keratinocytes enhances cell clonogenicity and**  
6 **favors spontaneous immortalization by opposing differentiation and senescence checkpoints**  
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11 **Valeria Orecchia\*, Gabriella Regis\*, Beatrice Tassone, Chiara Valenti, Lidia Avalue, Stefania**  
12 **Saoncella, Enzo Calautti\*\*, Valeria Poli\*\***  
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18 Department of Molecular Biotechnology and Health Sciences, Molecular Biotechnology Center,  
19 University of Torino, Italy.  
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22  
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25 \* These authors equally contributed to this work  
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27

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30 \*\* Equal contribution and co-corresponding authors:  
31

32 [vincenzo.calautti@unito.it](mailto:vincenzo.calautti@unito.it); [valeria.poli@unito.it](mailto:valeria.poli@unito.it)  
33  
34  
35

36 Department of Molecular Biotechnology and Health Sciences  
37

38 Molecular Biotechnology Center  
39

40 University of Torino  
41

42 Via Nizza 52, 10126 Torino  
43

44 Italy  
45

46  
47 Phone: +39-011-6706417  
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49 FAX: +39-011-6706432  
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**Abstract**

STAT3, a pleiotropic transcription factor acting downstream of cytokines and growth factors, is known to enhance proliferation, migration, invasion and aerobic glycolysis in tumors upon aberrant activation. In the murine epidermis, STAT3 is necessary for experimentally induced carcinogenesis, which is enhanced by the overexpression of the constitutively active STAT3C mutant that also induces robust, psoriasis-like epidermal hyperplasia. We show that STAT3C expression at physiological levels in knock-in mice leads to mild epidermal hyperplasia and attenuated expression of terminal differentiation markers. This delayed differentiation is confirmed in isolated primary epidermal keratinocytes *in vitro*, correlating with enhanced proliferative and clonogenic potential, attenuated senescence and, strikingly, spontaneous immortalization at high frequency. These results suggest that moderate levels of continuous STAT3 activation, which more closely resemble those triggered by chronic inflammation or persistent growth factor stimulation, may favor epidermal carcinogenesis in part by promoting the escape of epidermal progenitor cells from differentiation and senescence checkpoints.

## Introduction

Signal Transducers and Activators of Transcription (STAT) factors become activated via tyrosine-phosphorylation (Y-P) and concentrate into the nucleus to regulate the expression of target genes (1). STAT3 can be activated by a wide variety of cytokines, growth factors and oncogenes (2), and plays pleiotropic roles in cell growth and survival including liver regeneration, B lymphocytes proliferation, terminal differentiation and growth arrest in monocytes, lysosome-mediated apoptosis during mammary gland involution, and maintenance of pluripotency in embryonic stem cells (3, 4). STAT3 is considered as an oncogene, being constitutively Y-P in many tumors that often become addicted to its activity (5-7), and required for cell transformation downstream of v-Src and other oncogenes that trigger its Y-P (8, 9). Additionally, overexpression of the constitutively active mutant STAT3C can transform immortalized fibroblasts and epithelial cells (6, 10, 11). In tumors, STAT3 can enhance cell survival and proliferation, promote immune escape, angiogenesis, invasion and metastasis, and modify energy metabolism (12, 13). Moreover, STAT3 is a key player in mediating inflammation-driven tumorigenesis, downstream of chronically high levels of the pro-inflammatory cytokine IL-6 (14), and its activity has been implicated in the maintenance of both normal and cancer stem cells (SC) (13).

The murine skin provides an excellent model to investigate epithelial SC biology and carcinogenesis (15-17). Conditional gene targeting in the epidermis and hair follicles has shown that STAT3 is required both for the initiation and promotion phases in DMBA-TPA skin tumorigenesis and for UVA-induced papilloma development (18, 19). Conversely, keratinocyte-specific STAT3C overexpression (keratin 5-STAT3C TG mice) enhanced both DMBA-TPA and UVA-induced tumorigenesis and elicited the appearance of psoriatic lesions in aging mice (20-22). Moreover, in the aging mouse skin, gains in endogenous JAK/STAT3 signaling downstream of inflammatory cytokines is responsible for impaired functionality of hair follicle SCs (23).

In an effort to model STAT3 activity in tumors, which is usually characterized by low-level, continuous activity rather than by overexpression, we have recently generated knock-in mice

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3 expressing physiological levels of STAT3C and shown its *in vivo* oncogenic potential (24).  
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5 STAT3<sup>C/C</sup> mice die between four and six weeks of age due to the development of immune-mediated  
6  
7 myocarditis (25), and could therefore not be used for studies on adult mice. Making use of  
8  
9 STAT3<sup>C/C</sup> mouse embryonal fibroblasts (MEFs), we could show that constitutively active STAT3  
10  
11 enhances proliferation and promotes resistance to apoptosis and senescence, correlating with a  
12  
13 switch towards aerobic glycolysis and with tumor transformation upon spontaneous  
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15 immortalization, thus acting as a first hit in malignant transformation (26, 27).  
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18 Here, we analyze the outcome of STAT3C expression in the skin and show that continuous aberrant  
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20 STAT3 activity, which can be observed under chronic inflammatory conditions, alters the pool of  
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22 clonogenic and proliferative precursors in the epidermis promoting, like in fibroblasts, pre-  
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24 oncogenic features.  
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## Materials and methods

### *Mice and treatments*

STAT3<sup>C/C</sup> mice were generated as described (28) and maintained in the transgenic unit of the MBC with water and food *ad libitum*. Genetic screening was performed by PCR as previously described (28). Procedures were conducted in conformity with national and international laws and policies as approved by the Faculty Ethical Committee.

### *Cell culture, treatment and viral transduction*

Primary mKCs were isolated from 3-day-old mice and cultured in low calcium medium (50  $\mu$ M CaCl<sub>2</sub>) as described (29, 30). 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in DMSO was used at 100ng/ml (31, 32). Cells were passaged at subconfluency for serial propagation and immortalization analysis. For morphological analyses of cultured cells Axiovert 200M Zeiss microscope was used. Colony forming efficiency assay (CFE) were performed as described (33) with minor modifications. Briefly, 10<sup>4</sup> cells were plated on a feeder layer of lethally irradiated 3T3-J2 and after 12 days colonies were fixed and stained with Rhodamine-B, and scored under a dissecting microscope. Total colonies were calculated as a percentage of total plated cells (number of colonies x 100/number of cells plated).

pLKO.1 lentiviral vectors (Open Biosystems, Huntsville AL, USA) contained the following sequences:

scr, CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG)

shST3-H3: CGACTTTGATTTCAACTACAACCTCGAGTTGTAGTTGAAATCAAAGTCG

shST3-H4: CACCATTGATTGATGCAGTTTCTCGAGAACTGCATCAATGAATGGTG;

lentiviral particles produced by co-transfecting 293T cells with packaging plasmids pCMV-dR8.74 and pMD2.G, harvested and concentrated as described (33), were used to transduce immortalized mKCs for 12 hours, followed by puromycin selection for 48 hours.

### *IF, immunohistochemistry and image analysis*



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3 Skin sections obtained from 3-day-old mice were embedded in OCT (Tissue Tek; American Master  
4 Tek Scientific) and fixed in methanol-acetone (1:1) at -20 °C. Cryosections were then processed for  
5 IF and IHC staining as described (29) with the following primary antibodies: Loricrin, Filaggrin and  
6 Keratin 14 (Covance); p-STAT3 (Cell Signaling and Technology); PCNA (Santa Cruz). For IF  
7 samples were counterstained with DAPI and mounted in Vectashield aqueous medium (Vector  
8 Labs, Burlingame, CA), then analyzed using a Leica TSCII SP5 confocal microscope (multi-track  
9 analysis was used for image acquisition) or a Zeiss Axiobserver microscope with Apotome module.  
10 H&E and IHC staining samples were analyzed using Olympus BH2 microscope. For the  
11 quantitative analysis of H&E, IF and IHC performed on skin sections, the ImageJ software (NIH)  
12 was used.  
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#### 25 *Real-time PCR*

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27 Total RNA was extracted from primary mKCs and reverse-transcribed as previously described (34).  
28 Taqman PCR reactions were performed using the Universal Probe Library system (Roche Italia,  
29 Monza, Italy) as previously described (34), on an AB 7300 Real Time PCR System (Applied  
30 Biosystem, Foster city, CA, USA). The 18S rRNA predeveloped TaqMan assay (Applied  
31 Biosystems, Foster city, CA, USA) was used as an internal control. Specific primers and UPL  
32 probes used are listed in Supplementary informations.  
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#### 41 *Western blot analysis*

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43 Total protein extracts were obtained in 2%SDS, 50 mM Tris/HCl (pH 7.4) lysis buffer  
44 supplemented with 1mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF and 40mg/ml protease inhibitor  
45 cocktail (Complete, Roche). Proteins were boiled at 95°C for 5min, cleared by centrifugation  
46 (12000 g for 10 min at 4°C) and the concentration was measured using the Bradford assay (Bio-  
47 Rad). Samples were fractionated on SDS/PAGE and transferred on to PVDF membrane (Millipore)  
48 for immunoblotting with the following antibodies: total STAT3 (K15, Santa Cruz Biotechnology),  
49 actin (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary antibodies  
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3 (Sigma-Aldrich). Immunoblots were acquired with the molecular imager ChemiDoc XRS, and  
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5 densitometric analysis was performed with Quantity One software (Bio-Rad).  
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8 *Statistical analysis*

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10 Data obtained from analysis of cellular growth, CFE assay, IF, IHC and RT-PCR were plotted as  
11  
12 mean  $\pm$  SEM. Results were assessed for statistical significance by a standard two-tailed Student's t  
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14 test as indicated. p values \*P  $\leq$  0.05, \*\* P  $\leq$  0.01, \*\*\* P  $\leq$  0.001.  
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For Review Only

## Results

### **STAT3<sup>C/C</sup> newborn mice display epidermal hyperplasia and differentiation abnormalities.**

Histological analysis of STAT3<sup>C/C</sup> newborn mice revealed significantly increased epidermal thickness, suggestive of a mild tissue hyperplasia (Fig. 1a). Consistently, STAT3<sup>C/C</sup> epidermis displayed ectopic expression of keratin14 in suprabasal cell layers, with a concomitant decreased expression of the granular layer markers filaggrin (FLG) and loricrin (LOR) (15) (Fig. 1b). STAT3 Y-P in the epidermis was increased, confirming enhanced activation, and correlated with higher expression of the proliferative marker PCNA (Fig. 1c). A similar unbalance between proliferation and differentiation markers was confirmed by quantitative RT-PCR analysis of freshly isolated mouse keratinocytes (mKCs) of the two genotypes (Fig. 1d). Thus, constitutively active STAT3 appears not only to enhance the proliferative rate of epidermal mKCs, but also to interfere with their differentiation program.

### **Delayed TPA-induced differentiation of primary STAT3<sup>C/C</sup> mKCs.**

To test this idea, we evaluated the *in vitro* differentiation ability of STAT3<sup>C/C</sup> mKCs in response to the *in vitro* differentiating agent TPA (31, 32, 36, 37). Freshly isolated KCs from either STAT3<sup>C/C</sup> or STAT3<sup>WT/WT</sup> newborn mice were seeded on collagen-coated dishes for 4 hours before TPA addition, followed by 7 or 24 hours incubation (Fig. 2a). TPA treatment triggered differentiation of STAT3<sup>WT/WT</sup> mKCs already by 7 hours, as indicated by the sharp reduction in the mRNAs levels for the KRT14 and p63 proliferative markers, paralleled by a strong increase in the levels of the mRNAs for the differentiation markers FLG and LOR. In contrast, downregulation of KRT14 and p63 mRNAs was partial at this time point in STAT3<sup>C/C</sup> mKCs and, even more strikingly, FLG and LOR mRNAs were unaffected. By 24 hours of treatment however, the levels of these markers became indistinguishable between the two genotypes, indicating that TPA-induced differentiation is only delayed in STAT3<sup>C/C</sup> mKCs. Taken together, these results are in agreement with the *in vivo*

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3 observations, and suggest that STAT3<sup>C/C</sup> mKCs possess an intrinsic defect in the response to  
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5 differentiating stimuli.  
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### 9 **Increased proliferative potential and delayed senescence in primary STAT3<sup>C/C</sup> mKCs.**

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11 To directly assess their proliferative potential, primary mKCs were isolated from 3-days old  
12 STAT3<sup>WT/WT</sup> and STAT3<sup>C/C</sup> mice and serially passaged in low calcium medium (30, 31). Under  
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14 these conditions, mKCs are unable to withstand prolonged subcultivation, owing to the depletion of  
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16 proliferating cells by senescence and/or differentiation (38). At early passages, both morphology  
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18 and proliferation rates of STAT3<sup>C/C</sup> mKCs were indistinguishable from those of their wild-type  
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20 counterparts (Fig. 2b, passage (p) 1 and data not shown). As expected, proliferation of STAT3<sup>WT/WT</sup>  
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22 mKCs progressively dropped in later passages, correlating with the appearance of morphological  
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24 features of differentiation and/or senescence, reaching complete proliferative arrest around p 9-10  
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26 (Fig. 2b, p7 and p9). In contrast, STAT3<sup>C/C</sup> cells maintained higher proliferation rates, displaying a  
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28 typical proliferating morphology with small, tightly packed cells until at least p 7 (compare images  
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30 at p7 of the two genotypes in Fig. 2b). Accordingly, quantification of senescence-associated  $\beta$ -  
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32 galactosidase (SA  $\beta$ -gal) activity showed a sharp increase in the numbers of  $\beta$ -gal-positive cells  
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34 starting at passage 4 in STAT3<sup>WT/WT</sup> mKCs, while this increase was substantially delayed in  
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36 STAT3<sup>C/C</sup> mKCs (Fig. 2c).  
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### 45 **Primary STAT3<sup>C/C</sup> mKCs display enhanced clonogenic potential.**

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47 We thus decided to assess the clonogenic potential of freshly isolated mKCs by means of a low  
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49 density colony forming efficiency (CFE) assay. In this setting, the number of total colonies  
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51 indicates the overall ability of cells to initiate a culture, whereas colony size and morphology  
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53 reflects the intrinsic proliferative potential of individual colony-initiating cells (39, 40). Cells were  
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55 isolated from newborn STAT3<sup>WT/WT</sup> and STAT3<sup>C/C</sup> mice, seeded on a layer of mitotically-  
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57 inactivated feeder cells and cultivated for 12 days (Fig. 3a, c). In order to assess colony size and cell  
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3 morphology, colonies were classified into three groups: small colonies (<2 mm); intermediate  
4 colonies (2-4 mm); and large colonies (> 4 mm). Small colonies are typically formed of large cells  
5 with senescent morphology, while intermediate and large colonies include areas of both senescent  
6 and proliferative cells (Fig. 3b). Although the total number of colonies did not differ between  
7 primary STAT3<sup>C/C</sup> and STAT3<sup>WT/WT</sup> mKCs (Fig. 3d, 1<sup>st</sup> generation), the two genotypes originated  
8 colonies of strikingly different size (Fig. 3a). Whereas in STAT3<sup>WT/WT</sup> mKCs the majority of  
9 colonies fell in the smallest category, STAT3<sup>C/C</sup> cells generated significantly higher numbers of  
10 large colonies with “holoclone-like” morphology, and lower numbers of small-intermediate  
11 colonies (Fig. 3a, c). Both intermediate and large STAT3<sup>C/C</sup> colonies presented large areas of small,  
12 tightly packed cells, typical of actively-proliferating mKCs (Fig. 3b). In contrast, STAT3<sup>WT/WT</sup>  
13 intermediate/large colonies were mainly composed of flattened cells with senescent/differentiated  
14 appearance. Moreover, despite similar numbers of total colonies in 1<sup>st</sup> generation assays, when cells  
15 where tested for their secondary CFE after one week in culture under standard conditions,  
16 STAT3<sup>C/C</sup> mKCs exhibited significantly higher numbers of total colonies (Fig. 3d, 2<sup>nd</sup> generation),  
17 in addition to maintaining in average a larger colony size (data not shown). Thus, STAT3<sup>C/C</sup> mKCs  
18 contain an enlarged pool of clonogenic cells endowed with high proliferative potential, reminiscent  
19 of cultured keratinocyte SCs (holoclones).  
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#### 43 **STAT3<sup>C/C</sup> primary mKCs become spontaneously immortalized at high frequency.**

44 Spontaneous immortalization of mKCs is a rare event, unless cells are propagated under specific  
45 conditions (38, 41). Accordingly, immortalization was never observed in wild type mKCs when  
46 serially passaged in low calcium medium (0 out of 8 individual cultures). In contrast, STAT3<sup>C/C</sup>  
47 cells became immortalized in 50% of the cases (4 out of 8 individual cultures). Immortalization was  
48 accompanied by progressively higher numbers of small, tightly-packed cells with undifferentiated  
49 morphology and by a remarkable increase in cell clonogenicity (Fig. 4a, b). Immortalized cells  
50 expressed high KRT14 and p63 mRNA levels, while FLG and LOR mRNAs were almost  
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3 undetectable as compared to STAT3<sup>C/C</sup> primary mKCs (Fig. 4c), indicating a differentiation  
4 roadblock. Consistently, in immortalized STAT3<sup>C/C</sup> lines, no differentiation morphological changes  
5 occurred in response to TPA (Fig. 4d). Of note, both wild type and STAT3<sup>C/C</sup> KCs similarly  
6 acquired the immortal phenotype when propagated under specific inducing conditions (41) (data not  
7 shown). These results suggest that constitutive STAT3 activation predisposes mKCs to escape  
8 replicative senescence, overcoming a block normally forbidding immortalization under standard  
9 culture conditions. Immortalized STAT3<sup>C/C</sup> mKCs did not exhibit features of transformed cells, as  
10 judged by failure to form colonies in soft agar (not shown).

11  
12 To determine whether STAT3 activity was required to maintain the immortalized cell phenotype,  
13 shRNA-mediated STAT3 silencing was performed in the STAT3<sup>C/C</sup> line 1. Supplementary Fig. 1a  
14 shows efficient lentiviral-mediated STAT3 downregulation. STAT3-silenced immortalized cells  
15 maintained a tightly packed morphology (Suppl. Fig. 1b), and their clonogenic potential was  
16 unaffected (Fig. 4e), suggesting that STAT3 activity favors the immortalization process to become  
17 then dispensable. However, STAT3 silencing decreased the expression of both KRT14 and p63  
18 mRNAs, while that of LOR was increased (Fig. 4f), suggesting that STAT3 participates to the  
19 regulation of these genes even in immortalized cells.

## 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 **Discussion**

42 In this study, we show that the expression of physiological levels of constitutively active STAT3 in  
43 Stat3C-knockin mouse model (24) alters epidermal homeostasis. Indeed, we observed mild  
44 epidermal hyperplasia coupled to impaired keratinocyte terminal differentiation in the suprabasal  
45 cell layers of the newborn skin *in vivo*. This is in partial agreement with the strong tissue  
46 hyperplasia, reminiscent of human psoriatic lesions, observed in a model of ectopic STAT3C  
47 overexpression via a Keratin-5 promoter (22). The less dramatic phenotype observed in our knockin  
48 mice is likely due to the lower STAT3C expression levels, which potentially allow the observation  
49 of more subtle effects. Indeed, moderate levels of continuous STAT3 activation with minor  
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3 increases in expression levels more closely resemble those observed during chronic inflammation or  
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5 persistent growth factor stimulation.  
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7 Keratinocyte differentiation was reduced, but not abrogated in our STAT3<sup>C/C</sup> mice *in vivo*,  
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9 suggesting that Stat3C expression may rather affect the choices of epidermal progenitor cells  
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11 between proliferation and differentiation. STAT3<sup>C/C</sup> knockin mice die within few weeks from birth  
12  
13 (25), thus precluding the analysis of long-term effects of Stat3C expression on epidermal  
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15 homeostasis or hair follicle cycling *in vivo*. However, by using primary epidermal cells isolated  
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17 from newborn STAT3<sup>C/C</sup> mice, we observed a remarkable correspondence with the *in vivo*  
18  
19 phenotype, suggesting epidermal cell-autonomous defects. For example, isolated STAT3<sup>C/C</sup> display  
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21 altered responses to TPA, a potent inducer of granular layer markers expression and of keratinocyte  
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23 terminal differentiation *in vitro* (31, 32, 36, 37), with a significant delay in the induction of the LOR  
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25 and FLG mRNAs, encoding for markers of terminal differentiation, and a symmetrically retarded  
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27 downregulation of mRNAs encoding for proliferative markers such as p63 and KRT14. Therefore, a  
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29 persistently elevated STAT3 signaling similar to that observed under chronic inflammatory  
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31 conditions appears to make keratinocytes intrinsically more resistant to differentiating stimuli by  
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33 enforcing their stay in the proliferative compartment. This could be due in part to STAT3-  
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35 dependent activation of proliferative genes such as Cyclin D1 and cMyc in epidermal cells (data not  
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37 shown), which may oppose the exit of keratinocytes from the cell cycle to engage terminal  
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39 differentiation. Increased proliferation does not appear however to represent an intrinsic cell-  
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41 autonomous feature of STAT3<sup>C/C</sup> mKCs, since their proliferation rates in culture are comparable to  
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43 those of the wild type controls. The effects of STAT3C on cell proliferation may thus depend on  
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45 specific tissue environmental contexts, which are likely not entirely recapitulated *in vitro*. On the  
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47 other hand, STAT3 signaling may enforce keratinocytes within the undifferentiated stem/progenitor  
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49 compartment by acting on cell fate choices independently of quantitative effects on mitotic rates,  
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51 similar to what reported in embryonic SCs, where STAT3 has a master role in the maintenance of  
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53 the “naïve” pluripotent state downstream of LIF (42, 43). This hypothesis is supported by the  
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3 finding in CFE assays of a selective increase in the number of cells originating large,  
4 undifferentiated colonies with holoclone-like morphology in STAT3<sup>C/C</sup> mKCs. Moreover,  
5 STAT3<sup>C/C</sup> cells show a significant increase in their long-term proliferative potential upon sustained  
6 subcultivation, feature typical of keratinocyte cultures enriched for stem/progenitor cells (44, 45).  
7  
8 Finally, STAT3<sup>C/C</sup> mKCs show a delay in the upregulation of SA  $\beta$ -Gal activity, and fail to acquire  
9 a senescent morphology even upon prolonged subcultivation. Protection from senescence and  
10 resistance to differentiation play key roles in SC maintenance in the majority of cellular contexts  
11 (46, 47). Our data suggest that mild gains in STAT3 activity, by opposing differentiation and/or  
12 senescence programs, may favor maintenance of self-renewal ability in cultured epidermal  
13 keratinocyte progenitors.  
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16 Resistance to both differentiation and senescence may also underlie the remarkable ability of  
17 STAT3<sup>C/C</sup> mKCs to become spontaneously immortalized under culture conditions that normally  
18 promote the exhaustion of proliferative potential in murine keratinocytes (38). Interestingly,  
19 immortalized STAT3<sup>C/C</sup> mKCs do not display features of malignant transformation, in contrast with  
20 what observed in MEFs, where the expression of STAT3C serves as a first oncogenic hit (27). This  
21 difference underscores the tissue and context-specificity of tumor transformation and may partly  
22 explain why, in contrast with other chronic inflammatory conditions characterized by high levels of  
23 IL-6 and STAT3 activity (14), psoriasis does not appear per se to significantly predispose to  
24 developing skin cancer (48).  
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27 Our data indicating a role of STAT3 in protection from cell senescence are in agreement with what  
28 observed in both STAT3<sup>C/C</sup> MEF cells and STAT3-addicted tumor cells (26), and consistent with  
29 previous work indicating that deletion of Stat3 in the epidermis leads to skin alterations that are  
30 reminiscent of anticipated aging (49), which could be attributed in part to a reduced ability of aging  
31 SCs to repair tissue damage. Consistently, specific deletion of STAT3 in KCs of the hair follicle  
32 bulge leads to significant reduction of DMBA-TPA-induced tumors, correlating with a decrease in  
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3 CD34 and  $\alpha 6$ -integrin double-positive cells in the bulge (50). Interestingly however, STAT3C  
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5 overexpression under the K5 promoter was also shown to decrease the number of CD34<sup>+</sup>,  $\alpha 6$ <sup>+</sup> cells,  
6  
7 in line with recent work indicating that increased JAK/STAT3 signaling generated by chronic  
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9 inflammatory conditions contributes to the loss of functional hair follicle SCs occurring in the aging  
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11 murine skin (23). Thus, like in many other systems, balanced STAT3 activity is required for tissue  
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13 homeostasis, and depending on the specific context, both gain and loss of activity may contribute to  
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15 skin aging and SC dysfunctions.  
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### Figure legends

#### Figure 1. STAT3<sup>C/C</sup> mice display epidermal hyperplasia and reduced differentiation. (a-c)

Representative images of skin sections obtained from 3 day-old STAT3<sup>C/C</sup> and STAT3<sup>WT/WT</sup> mice. Epi: epidermis; Der: dermis. Grey bars, STAT3<sup>WT/WT</sup> mice; black bars, STAT3<sup>C/C</sup> mice. **(a)** H&E staining of paraffin sections (magnification 10X). Bars represent mean  $\pm$  SEM of the epidermal thickness (n=10 fields/genotype). **(b)** Confocal analysis of cryo-sections subjected to immunofluorescence with the indicated antibodies (red), counterstained with DAPI (blue). Dotted lines indicate the border between epidermis and dermis. Bar: 50 $\mu$ m. Bars represent mean  $\pm$  SEM thickness of the epidermal layer stained by the corresponding antibody determined as in (a). **(c)** IHC analysis with anti-p-STAT3 and anti-PCNA antibodies (magnification 20X). Bars represent the mean positive area per epidermal area  $\pm$  SEM (n= 5 fields/genotype), measured using the Metamorph Image Software (Zeiss). **(d)** Taqman RT-PCR on total RNA from primary STAT3<sup>C/C</sup> and STAT3<sup>WT/WT</sup> mKCs, represented as mean  $\pm$  SEM of the values normalized to the 18SrRNA internal control. n=3 per genotype. KRT14, keratin 14; FLG, filaggrin; LOR, loricrine; INV, involucrin.

#### Figure 2. Cultured primary STAT3<sup>C/C</sup> mKCs display enhanced lifespan and delayed senescence. (a)

Freshly isolated mKCs from STAT3<sup>C/C</sup> (black bars) and STAT3<sup>WT/WT</sup> (grey bars) mice were seeded for 4 hours, then treated with DMSO (-) or TPA for 7 or 24 hours. Total RNA was analyzed by Taqman RT-PCR for the indicated markers of epidermal proliferation (KRT14, p63) and differentiation (LOR, FLG). Data are mean  $\pm$  SEM of 3 samples/genotype. **(b-c)** mKCs of the indicated genotypes were serially cultured for the indicated passages (p) and analyzed **(b)** by phase contrast imaging. All STAT3<sup>WT/WT</sup> cells were dead by p9 (+). Magnification 10X. **(c)** Percentage of SA  $\beta$ -gal<sup>+</sup> STAT3<sup>WT/WT</sup> and STAT3<sup>C/C</sup> cells at the indicated passages, calculated on at least 300 cells per genotype (n=3) from 2 independent experiments.

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6 **Figure 3. STAT3<sup>C/C</sup> mKCs display increased colony forming efficiency. (a-d)** CFE assay.  $4 \times 10^3$   
7 primary STAT3<sup>C/C</sup> and STAT3<sup>WT/WT</sup> mKCs were seeded under clonogenic conditions. After 12  
8 days colonies were fixed and stained with Rhodamine-B **(a)**. **(b)** Phase contrast images of  
9 representative colonies (Magnification 10X). **(c)** Bars represent the mean percentage of colonies of  
10 the indicated sizes  $\pm$  SEM of at least 100 colonies/genotype, from two independent experiments  
11 performed in duplicate (n=4 per genotype). **(d)** The total colony numbers of CFE assay were  
12 determined on 1<sup>st</sup> and 2<sup>nd</sup> generation (grey bars, STAT3<sup>WT/WT</sup>; black bars, STAT3<sup>C/C</sup>), and reported  
13 to the unitary value of 1<sup>st</sup> generation STAT3<sup>WT/WT</sup> cells.  
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26 **Figure 4. Spontaneous immortalization of STAT3<sup>C/C</sup> mKCs. (a,b)** Representative images of  
27 STAT3<sup>C/C</sup> KCs at passage 1 (P, left) and after spontaneous immortalization (I, right). **(a)** Phase  
28 contrast images of cultured cells; **(b)** Rhodamine-B stained CFE assay. **(c)** Total RNA from P  
29 (black bars) or I (striped bars) STAT3<sup>C/C</sup> mKCs was analyzed by Taqman RT-PCR for the indicated  
30 markers. Data are mean  $\pm$  SEM of 3 samples per condition. **(d-f)** Immortalized STAT3<sup>C/C</sup> mKCs  
31 were: **(d)** exposed to DMSO (-) or TPA for 24h (phase contrast images are shown); transduced  
32 with lentiviral vectors expressing scrambled (scr) or STAT3-directed shRNA (Stat3 H3) and **(e)**  
33 analyzed for colony forming efficiency (Rhodamine-B staining) or **(f)** for the expression of the  
34 indicated markers by Taqman RT-PCR. Data are mean  $\pm$  SEM of the values from at least 3  
35 independent experiments, normalized to the 18S rRNA internal control and reported to those  
36 obtained with the scr samples.  
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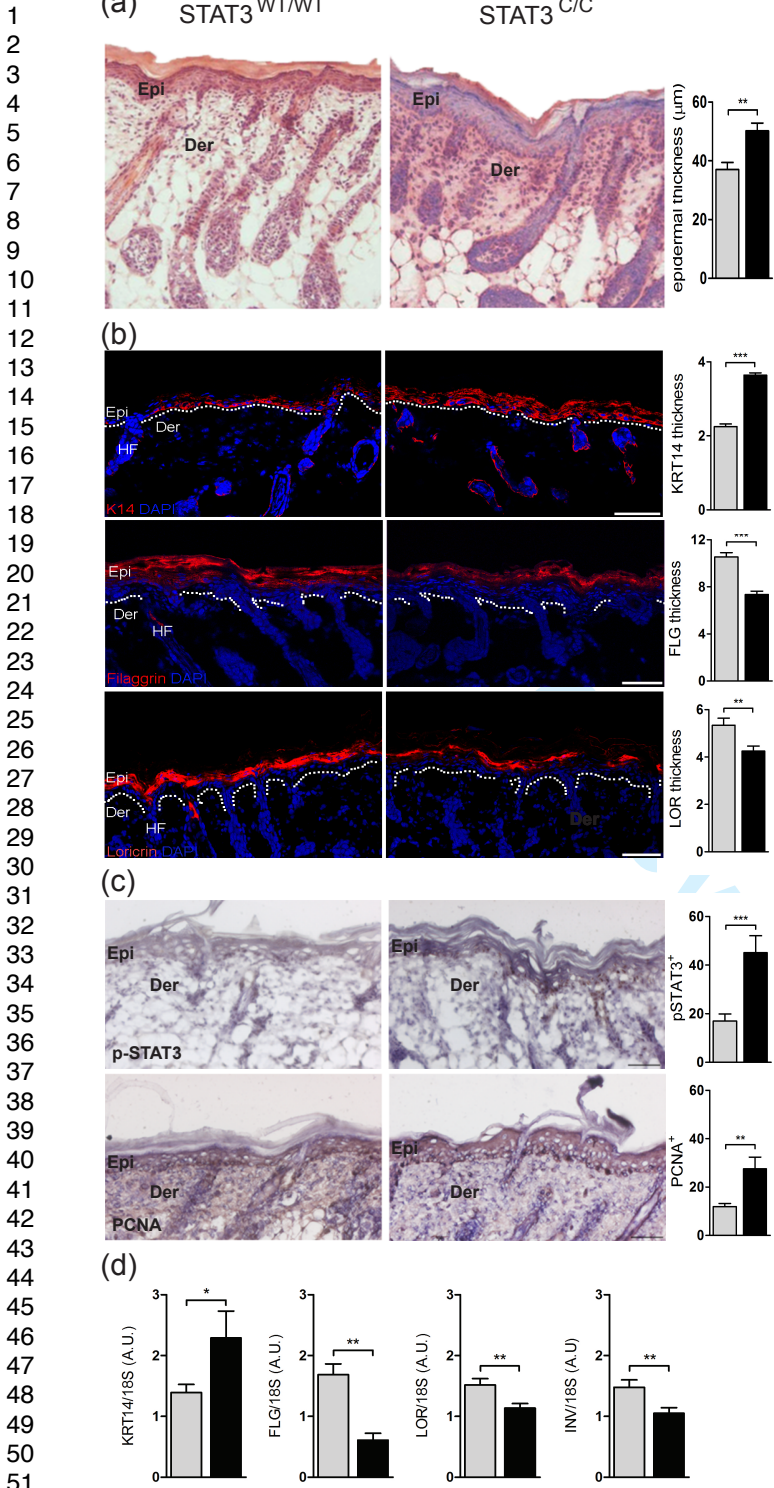


Fig.1



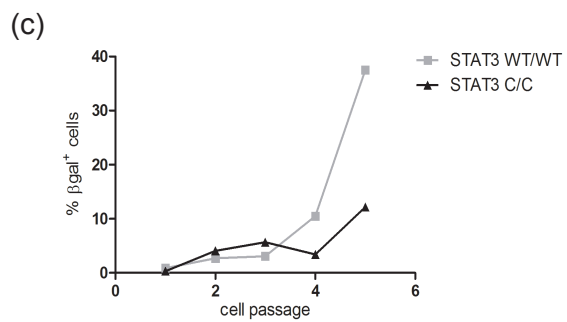
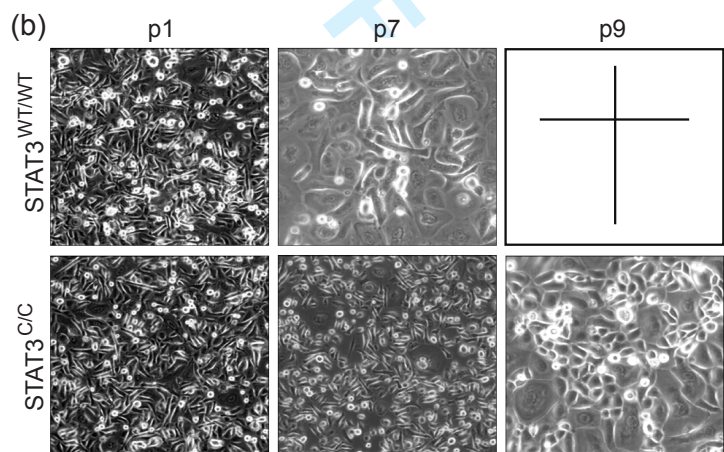
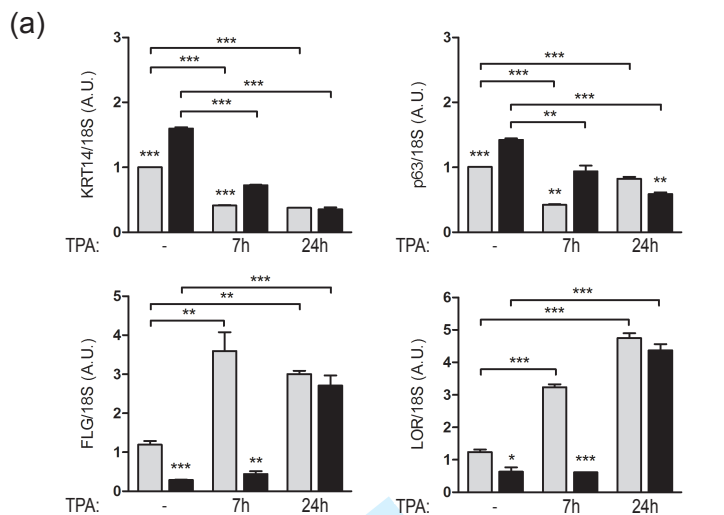


Fig.2

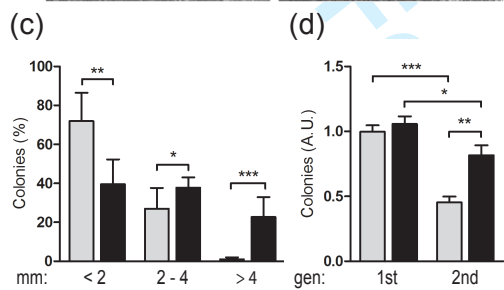
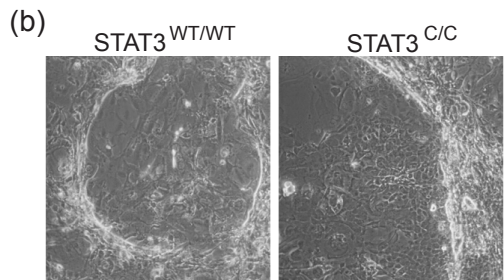
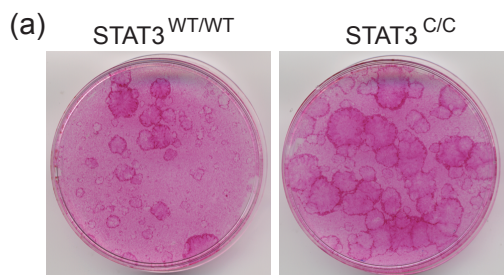


Fig.3

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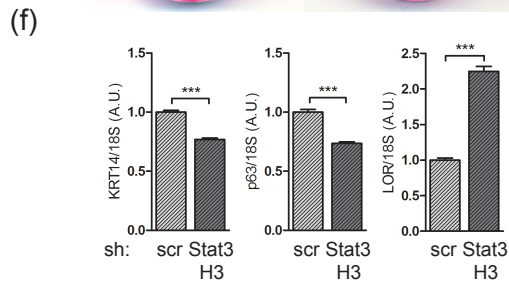
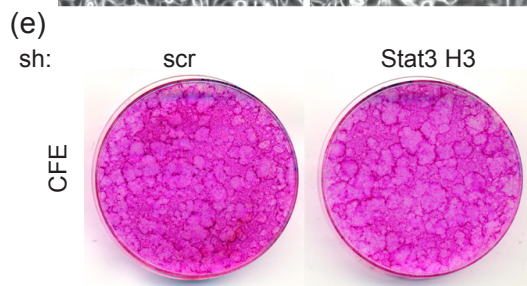
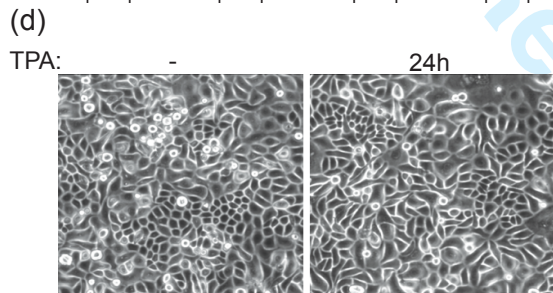
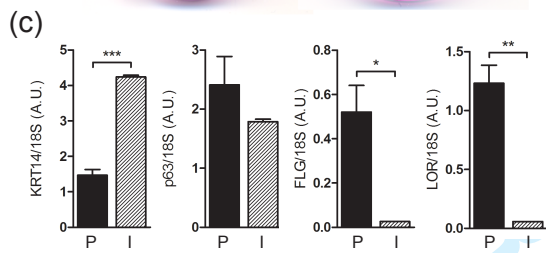
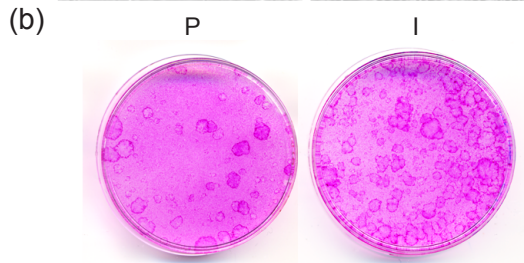
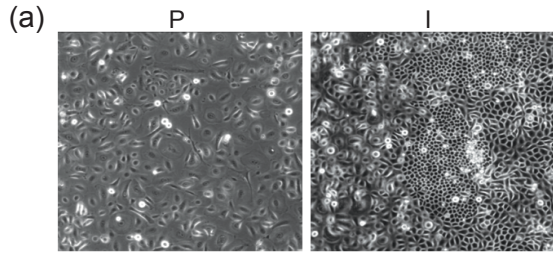
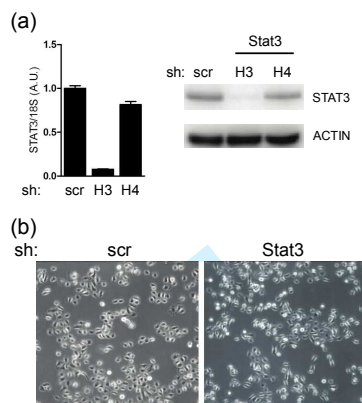


Fig.4

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### Supplementary informations



Supplementary Fig.1

**Figure S1. STAT3 silencing in immortalized STAT3<sup>C/C</sup> mKCs.** Immortalized STAT3<sup>C/C</sup> mKCs at p13 were transduced with lentiviral vectors expressing two independent shRNA constructs targeted to STAT3 (shST3-H3, shST3-H4) and a scrambled control shRNA (scr). **(a)** The histograms represent Taqman RT-PCR analysis of STAT3 mRNA levels in the transduced cells (mean values  $\pm$  SEM) of 3 independent experiments as normalized to the 18S internal control and reported to those obtained with the scr controls. The right panel shows Western blot analysis with antibodies against STAT3 and actin as a loading control. The shST3-H3 induces a nearly 90% STAT3 downregulation and was used for the experiments shown in Figure 4 (e-f). **(b)** Phase contrast images of transduced cells grown at subconfluency.

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**Specific primers and UPL probes:**

KRT14, Fw-atcgaggacctgaagagcaa, Rv-tcgatctgcaggaggacatt, UPL #83;

FLG, Fw-gcctctgcaggtggactg, Rv-gaatggactggctgtcactg, UPL #20;

LOR, Fw-ggttgaacggagacaaca, Rv-catgagaaagtaagcccatcg, UPL #11;

INV, Fw-ggatctgcctgatcaaaagtg, Rv-cagctgctgttttgtgg, UPL#71;

p63, Fw-ggaaaacaatgccagactc, Rv-aatctgctggccatgctgt, UPL #45;

SOCS3, Fw-atttcgcttcgggactagc, Rv-aacttgctgtgggtgacat, UPL #83;

STAT3, Fw-tggcaccttggattgagag, Rv-caacgtggcatgtgactctt, UPL #71;

For Review Only