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STAT3 can serve as a hit in the process of malignant transformation of primary cells.

Running title: STAT3 contributes to cell transformation

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ABSTRACT

The transcription factor STAT3 acts downstream of many pro-oncogenic signals, including cytokines, growth factors and oncogenes, and is accordingly constitutively active in a wide variety of tumors that often become addicted to it. Moreover, STAT3 is a key player in mediating inflammation-driven tumorigenesis, where its aberrant continuous activation is typically triggered by local or systemic production of the pro-inflammatory cytokine IL-6. We recently showed that MEFs derived from STAT3C k/in mice, which express physiological levels of the constitutively active mutant STAT3C, display features of transformed cells such as increased proliferation, resistance to apoptosis and senescence, and aerobic glycolysis. Here, we show that pre-existing constitutively active STAT3 is sufficient to prime primary MEFs for malignant transformation upon spontaneous immortalization. Transformation is strictly STAT3-dependent and correlates with high resistance to apoptosis and enhanced expression of anti-apoptotic/pro-survival genes. Additionally, HIF-1 α level is elevated by 2-fold and contributes to STAT3 oncogenic activity by supporting high rates of aerobic glycolysis. Thus, constitutively active STAT3, an accepted essential factor for tumor growth/progression, can also act as a first hit in multistep carcinogenesis; this ability to predispose cells to malignant transformation may be particularly relevant in the pro-oncogenic niche represented by chronically inflamed tissues.

KEYWORDS: STAT3, HIF-1 α , AEROBIC GLYCOLYSIS, TUMORIGENESIS, APOPTOSIS, 3T3 MEFs

INTRODUCTION

Signal Transducers and Activators of Transcription (STAT) factors mediate the signalling downstream of cytokine and growth factor receptors^{1,2}. Once activated by tyrosine-phosphorylation via receptor-associated JAK kinases, STATs concentrate into the nucleus and regulate the expression of target genes³. STAT3 can be activated by a wide variety of cytokines and growth factors and by a number of oncogenes⁴, and is accordingly constitutively phosphorylated on tyrosine in many tumors that often become addicted to its activity⁴⁻⁷. Indeed, STAT3 is required for cell transformation downstream of v-Src and many other oncogenes^{5,8,9}, and overexpression of a constitutively active mutant form can transform immortalized fibroblasts and epithelial cells^{10,11}. Even though STAT3 is often referred to as an oncogene, no naturally occurring activating mutations are known, and its aberrant activity in tumors mostly occurs downstream of multiple activated pro-oncogenic pathways. Importantly, STAT3 is a key player in mediating inflammation-driven tumorigenesis, being constitutively activated by chronically high levels of the pro-inflammatory cytokine IL-6¹². In tumors, STAT3 is known to enhance cell survival and proliferation and to promote immune escape and angiogenesis, invasion and metastasis^{13,14}.

In an effort to characterize pro-oncogenic functions of continuous, weak STAT3 activation, we have recently generated knock-in mice expressing physiological levels of the constitutively active STAT3C mutant form¹⁵. We have shown that constitutively active STAT3 acts as a master regulator of cell metabolism, promoting aerobic glycolysis and down-regulating mitochondrial activity via its canonical, nuclear functions both in STAT3^{C/C} MEFs and in STAT3-addicted tumoral cells¹⁶. On the other hand, mitochondrially localized STAT3 was recently demonstrated to play a role in preserving mitochondrial respiratory activity via its serine-phosphorylated form^{17,18}. Both activities contribute to malignant transformation downstream of distinct signals, which promote STAT3 phosphorylation on either tyrosine or serine thus activating nuclear or mitochondrial STAT3 (nSTAT3, mSTAT3) (Demaria et al., *Cell Cycle*, in press). While aerobic glycolysis induced by nuclear STAT3 is dependent on the transcriptional induction of the oxygen-

sensor HIF-1 α , down-regulation of mitochondrial function is HIF-1 α -independent¹⁶. HIF-1 is composed of a constitutively expressed β subunit and an O₂-regulated α subunit¹⁹, HIF-1 α , which undergoes O₂-dependent hydroxylation leading to its proteasomal degradation²⁰. Under hypoxic conditions, hydroxylation is inhibited and HIF-1 α rapidly accumulates and dimerizes with HIF-1 β , becoming transcriptionally active. Oncogenic pathways can lead to HIF-1 α accumulation even under normoxia¹⁹, and its enhanced activity correlates with poor prognosis in human cancer such as those of the breast, prostate and melanomas²¹⁻²³. HIF-1 α target genes control both oxygen delivery and usage via induction of angiogenesis and glycolysis, respectively, and this factor is one of the key players in inducing the Warburg effect, i.e. the ability of most cancer cells to undergo aerobic glycolysis^{24,25}.

Mouse embryonic cells (MEFs) can be spontaneously immortalized via regular passages during which, after undergoing a proliferative senescence crisis, a few immortalized cells emerge (the 3T3 protocol²⁶). 3T3-immortalized MEFs are not tumorigenic but, in contrast to their primary counterparts, can undergo full transformation when challenged with a single oncogene²⁷. Primary MEFs derived from STAT3C mice (Stat3^{C/C} MEFs) are not spontaneously transformed but show pre-oncogenic properties, such as increased proliferation, resistance to apoptosis and senescence and increased aerobic glycolysis with enhanced glucose dependence¹⁶. Here we show that spontaneous immortalization via the 3T3 protocol leads to complete transformation, demonstrating that continuous STAT3 activity can act as a first hit in tumorigenesis. This has important implications for understanding the mechanisms leading to inflammation-driven cancer, where chronically active STAT3 precedes tumor transformation.

RESULTS

Immortalization triggers tumorigenic transformation in Stat3^{C/C} MEFs. MEFs derived from 2 individual Stat3^{C/C} or Stat3^{WT/WT} embryos were subjected to the 3T3 spontaneous immortalization protocol²⁶. Compared to wild type cells, Stat3^{C/C} MEFs underwent a delayed and much shorter proliferative crisis, which peaked at passage (P) 16 as compared to P10 of their wild type counterparts (Figure 1a). Cells never completely ceased duplicating and a rapidly proliferating population arose earlier than in Stat3^{WT/WT} MEFs (P20 versus P24). Immortalized cell lines were completely established after 26 passages for both genotypes (two independent experiments). Similar to what we observed in the primary cells, 3T3-immortalized Stat3^{C/C} MEFs displayed increased expression of the STAT3 canonical target gene *Socs3*, confirming higher STAT3 transcriptional activity, and proliferated much faster than their wild type counterparts (Figure 1b, c). However, in contrast to primary Stat3^{C/C} MEFs, both of the immortal Stat3^{C/C} cell lines (C1 and C2) acquired the ability to grow in multi-layers, a feature typical of transformed cells (Figure 2a). Further, both C1 and C2 were able to give rise to foci in a classical focus forming assay (FFA), while immortal Stat3^{WT/WT} cells were never able to do so (Figure 2b). Immortal Stat3^{C/C} cells were also capable of anchorage-independent growth, giving rise to colonies in soft agar (Figure 2c). The ultimate test for in vitro tumor transformation is considered the ability to grow in vivo in immune-compromised mice. 10⁶ immortal Stat3^{C/C} or Stat3^{WT/WT} MEFs were inoculated subcutaneously (s.c.) into nude mice. After 10-12 days the Stat3^{C/C} cells gave rise to palpable tumors, which reached 1 cm diameter by six weeks, time of sacrifice (Figure 2e). As expected, Stat3^{WT/WT} cells were never able to give rise to tumors.

Thus, immortal Stat3^{C/C} cells display the hallmark of fully malignant tumor cells, suggesting that constitutively active STAT3 can indeed act as a first hit in tumorigenesis. Interestingly, the transformed phenotype remained completely dependent on STAT3 activity, as shown by the observation that Stat3 silencing completely abolished in vivo growth (Figure 2d, e).

Resistance to apoptosis of the immortal Stat3^{C/C} MEFs.

STAT3 has been linked to apoptosis resistance in many tumors and experimental systems, including cells over-expressing STAT3^C¹⁰. In order to assess the ability of the transformed Stat3^{C/C} cells to survive apoptotic stimuli, immortal Stat3^{C/C} and Stat3^{WT/WT} cells were exposed to UV-B light (10 J/m²), followed by apoptotic index assessment using a cytofluorimeter and Annexin V staining. Indeed, and similar to the primary cells of the same genotype, immortal Stat3^{C/C} cells were significantly protected from apoptotic cell death compared to their wild type counterparts (Figure 3a). In order to evaluate activation of the pro-apoptotic caspase cascade, the levels of active caspase-3 were measured by either immunofluorescence or western blotting after UV irradiation (Figure 3b and Supplementary Figure 1a). Caspase-3 activation was lower in Stat3^{C/C} cells, confirming a general down-regulation of the apoptotic cascade. Immortal Stat3^{C/C} cells were also protected from starvation-induced apoptosis (Supplementary Figure 1b), in agreement with the idea that STAT3 acts as an anti-apoptotic factor in response to different pro-apoptotic stimuli.

Since STAT3 was proposed to directly regulate a number of anti-apoptotic genes, we assessed a panel of pro- and anti-apoptotic mRNAs by qRT-PCR (not shown). Immortal Stat3^{C/C} cells expressed higher levels of the anti-apoptotic genes bcl-2, bcl2-l1 and bcl-3, all known STAT3 targets, which could well mediate the observed resistance to apoptosis (Figure 3c). Moreover, the levels of the inhibitor of apoptosis (IAP) gene family member birc-5, also known as survivin, were induced by 4-fold upon UV irradiation in the Stat3^{C/C} cells but not in the wild-type cells (Figure 3d). Birc-5/survivin was also previously shown to be a direct STAT3 transcriptional target, and its increased level may help in maintaining the caspase cascade activation at a low level.

Immortal Stat3^{C/C} MEFs display STAT3-dependent aerobic glycolysis.

We recently showed that constitutively active STAT3 promotes aerobic glycolysis in both primary Stat3^{C/C} MEFs and in STAT3-dependent human tumor cell lines via the transcriptional up-regulation of Hif-1 α ¹⁶. To assess glucose metabolism in the immortalized cell lines, a number of glycolysis parameters were evaluated. Similar to the primary cells, immortal Stat3^{C/C} MEFs

displayed increased levels of Hif-1 α and of several of its target genes known to be involved in glycolysis such as pyruvate dehydrogenase kinase (Pdk-1), lactate dehydrogenase (Ldh-a) and phospho-fructokinase-1 (Pfk-1) (Figure 4a). Accordingly, immortal Stat3^{C/C} MEFs produced higher lactate levels, thus confirming enhanced aerobic glycolysis (Figure 4b). Both lactate production and the Hif-1 α level were dependent on STAT3 activity, as they were normalized by STAT3 silencing. (Figure 4c, d).

Hif-1 α silencing impairs aerobic glycolysis but only partially reverts the transformed phenotype of immortalized Stat3^{C/C} MEFs.

Hif-1 α is often up-regulated in cancer, particularly in late-stage tumors, where it helps protect cells from hypoxia by activating glycolysis and stimulating angiogenesis. Hif-1 α silencing down-regulated aerobic glycolysis in primary Stat3^{C/C} MEFs, relieving their glucose dependence. Interfering with Hif-1 α expression dramatically reduced the transcription of glycolysis-related genes and the production of lactate, confirming that indeed STAT3-dependent aerobic glycolysis is mediated by Hif-1 α also in the immortal Stat3^{C/C} MEFs (Figure 5a, b). In contrast to what observed in the primary cells, however, Hif-1 α silencing also restored mitochondrial activity, as measured by mitochondrial-Ca²⁺ uptake, to levels similar to those of the wild type cells (Figure 5c). Additionally, silencing of Hif-1 α reduced the proliferation rates of immortal Stat3^{C/C} cells (not shown), but only partially reversed other transformed phenotypes. Thus, the ability of immortal Hif-1 α -silenced Stat3^{C/C} MEFs to form foci and soft agar colonies, as well as their in vivo growth rates, were significantly reduced but not abolished (Figures 4d,e). These data suggest that the oncogenic properties of a constitutively active STAT3 partly rely on Hif-1 α up-regulation and the consequent metabolic switch towards aerobic glycolysis.

DISCUSSION

Constitutive, oncogenic STAT3 activity is believed to stem from the aberrant activation of many oncogenic pathways, which ultimately induce its tyrosine phosphorylation¹. The pro-oncogenic role of STAT3 was shown for the first time by the finding that overexpression of the constitutively active mutant form, STAT3C, can transform cultured cells^{10,11,28}. Further, *in vivo*, overexpression of STAT3C was shown to enhance malignant progression of skin tumors²⁹ and trigger the onset of lung adenocarcinomas³⁰. However, STAT3 activation in tumors most often does not entail increased protein levels, in keeping with the idea that oncogenic STAT3 activation is the leading mechanism³¹ and suggesting that overexpression is probably not the best experimental system to assess the mechanisms of STAT3-mediated tumorigenesis.

We recently showed that expression of STAT3C at physiological levels can cooperate with the Neu oncogene to drive invasive breast cancer¹⁵, and that STAT3C knock-in MEFs display features of transformed cells such as increased proliferation, resistance to apoptosis and senescence, and aerobic glycolysis¹⁶. Here, we show for the first time that pre-existing physiological levels of constitutively active STAT3 are sufficient to transform primary cells to full malignancy following immortalization in a 3T3 protocol, suggesting that low and chronic activity of STAT3 may suffice as a first hit in multistep carcinogenesis. This finding is of particular relevance for the emerging key role of STAT3 in inflammation-driven cancer. It has been shown that chronic inflammation, entailing a positive loop between the transcription factor NF- κ B, the pro-inflammatory cytokine IL-6 and the IL-6-activated STAT3, is a highly predisposing condition for cancer, particularly in the colon, the liver and the breast³². In addition, to generate a favorable niche for the growth of tumor cells via the production of pro-oncogenic signals, our data show now that cells exposed to chronic IL-6 signaling, displaying continuous STAT3 activation, can behave like cells that have undergone a first oncogenic mutation. These cells will therefore be exquisitely sensitive to further mutagenic events, which are favored by the inflammatory microenvironment that contains cytokines, growth factors and reactive oxygen species.

The mechanism through which constitutively active STAT3 sensitizes cells to tumorigenic transformation is probably many-fold and observed in either our Stat3^{C/C} primary¹⁶ or immortalized MEFs: first, protection from apoptosis; second, delayed senescence; third, increased proliferation; fourth, increased glycolytic metabolism. Protection from apoptosis, induced by different stress stimuli, correlates with the increased expression of anti-apoptotic genes. Delayed senescence and increased proliferation likely correlate with a metabolic switch towards aerobic glycolysis. Aerobic glycolysis was recently proposed to give an advantage to rapidly proliferating cells, allowing them to accumulate NADPH and carbon skeletons needed for anabolic reactions as a side-product of glucose consumption and ATP production³³. Another important player in oncogenesis is c-myc, which contributes to both increased proliferation and aerobic glycolysis, and indeed the levels of c-myc¹⁵, a well known STAT3 target, are elevated in the Stat3^{C/C} MEFs³⁴.

Hif-1 α is often over-expressed in cancer cells, playing key roles in critical aspects of cancer biology, including angiogenesis³⁵, cell survival, genetic instability³⁶, invasion and metastasis³⁷, proliferation and metabolism³⁸. Indeed, Hif-1 α is a well known inducer of aerobic glycolysis, and it was not therefore surprising to find that STAT3-induced glycolysis was Hif-1 α -dependent. Interestingly, the two-fold transcriptional induction of Hif-1 α triggered by constitutively active STAT3 was enough to elevate protein levels without inducing protein stabilization, thought to be essential for enhanced Hif-1 α activity³⁹. Similar to their primary counterparts, immortal Stat3^{C/C} cells show Hif-1 α -dependent aerobic glycolysis, and Hif-1 α silencing reduced, but did not abolish, several transformed phenotypes. Thus, STAT3-induced Hif-1 α overexpression and aerobic glycolysis are important contributors to STAT3-mediated tumorigenesis.

MEF cells immortalization usually entails loss of function of either the p53 or the ARF oncosuppressors⁴⁰. Interestingly, RAS overexpression induced senescence in Stat3^{C/C} primary MEFs, similar to its effect in wild type cells⁴¹ (data not shown), possibly because RAS and STAT3 act in the same signaling cascade. Indeed, STAT3 is phosphorylated on Serine 727 following RAS signaling, and it was shown to support RAS-mediated transformation via mitochondrial localization

and function¹⁷. Via induced Ser phosphorylation, RAS might therefore increase STAT3C localization to mitochondria, thus reducing its nuclear concentration and activity. We have previously shown that nuclear constitutively active STAT3 restrained mitochondrial metabolism in both primary MEFs and STAT3-dependent tumor cells by a Hif-1 α -independent mechanism¹⁶. Also 3T3 Stat3^{C/C} cells showed decreased mitochondrial activity, which could be rescued by Hif-1 α silencing. This may reflect the intrinsic heterogeneity in the transformation process, where tumor cells may come to rely more heavily on different pathways upon transformation.

In conclusion, our data suggest that aberrantly constitutively active STAT3 can act as a first hit during tumorigenic transformation supporting tumor survival, growth and metabolism, strengthening its definition as an oncogene. Thus, drugs inhibiting STAT3 activity might be beneficial not only in the therapy of cancer but also in prophylactic treatments aimed at preventing the onset of inflammation-driven tumorigenesis.

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TITLES AND LEGENDS TO FIGURES

Figure 1. Immortalization and characterization of the Stat3^{C/C} MEFs. A) MEFs were derived from E14 embryos of the indicated genotypes and re-seeded at a density of 2×10^5 cells/10 cm² every 3 days as described in the Methods section. Population doubling (PD) was calculated at each passage and is shown from passage 10 to 25. B) SOCS3 mRNA levels were measured by Taqman RT-PCR in immortalized Stat3^{C/C} (black bars) or ^{WT/WT} (white bars) MEFs. C) Immortalized Stat3^{C/C} MEFs display increased proliferation rates. 10^4 cells were plated and cells were counted every day for 5 days.

Figure 2. Transformed features of immortalized Stat3^{C/C} MEFs. A) Phase contrast pictures showing multi-layer growth of Stat3^{C/C} MEFs. Magnification 10X. B) Focus forming assay. 30×10^4 cells were seeded and the number of colonies scored after 15 days using methyl-violet. WT, Stat3^{WT/WT}; C1= Stat3^{C/C} line No I, C2= Stat3^{C/C} line No II. C) Soft-agar growth. 2×10^4 cells (as in B) were seeded in soft agar and colony number evaluated after 30 days. D) STAT3 silencing. STAT3 levels were measured by Western Blot in cells of the indicated genotypes, plus or minus lentiviral-mediated STAT3 silencing (sh-stat3). ACTIN was used as an internal control, and GFP to assess lentiviral infection. E) In vivo tumor growth. 10^6 cells (Stat3^{C/C}, Stat3^{WT/WT} or Stat3^{C/C} silenced for STAT3 - sh-stat3) were inoculated s.c. in the flank in nude mice (n=6), and tumor growth was monitored each week for 6 weeks.

Figure 3. Immortal Stat3^{C/C} MEFs are protected from apoptotic stimuli. A) Stat3^{C/C} (black bars) or ^{WT/WT} (white bars) MEFs were treated with UV-B (10 J/m²) and stained with Annexin V after 24 hours, followed by quantification of positive cells by flow cytometry. B) Same as in A, followed by staining for active CASPASE-3 (red). Nuclei were stained in blue by Hoescht. C, D) The indicated mRNAs were measured by Taqman RT-PCR in Stat3^{C/C} (black bars) or ^{WT/WT} (white bars) MEFs. D) Cells as in A were UV-irradiated followed by RNA extraction and RT-PCR analysis after 12 hours. *, $p \leq 0,05$.

Figure 4. Aerobic glycolysis in Stat3^{C/C} MEFs. Stat3^{C/C} (black bars) or ^{WT/WT} (white bars) MEFs were assessed for mRNA levels of the indicated genes by Taqman RT-PCR (A, D) or for their accumulation of lactate in the medium 12 hours after seeding (B, C). Lactate production (C) and Hif-1 α levels (D) were also measured upon Stat3 silencing (sh-stat3). *, $p \leq 0,01$

Figure 5. Hif-1 α silencing blunts the oncogenic properties of Stat3^{C/C} MEFs. Stat3^{C/C} MEFs either silenced for Hif-1 α (striped bars) or infected with a control virus (black bars) were assessed for mRNA levels of the indicated genes by Taqman RT-PCR (A) or for their accumulation of lactate in the medium 12 hours after seeding (B). C) MEFs of the indicated genotypes were transduced with a mitochondria-targeted aequorin (AEQ), and aequorin activity was measured upon challenging with 100 μ M ATP. D,E) Stat3^{C/C} MEFs either silenced or not for Hif-1 α were tested for their ability to form foci and colonies in soft agar (D), or tested for *in vivo* growth (E) as described in the legend to Fig. 1. *, $p \leq 0,01$

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MATERIALS AND METHODS

Mice, MEFs preparation, cell lines derivation and culture. *Stat3C/C* mice¹⁵ were maintained in the transgenic unit of the Molecular Biotechnology Center (University of Turin). Procedures were conducted in conformity with national and international laws and policies as approved by the Faculty Ethical Committee. Embryos were dissected 13.5 days post coitum for MEF derivation. 3T3 cell lines were prepared as previously described²⁶. 3T3 MEFs were grown in DMEM with GLUTAMAX (Dulbecco's modified Eagle medium; Gibco-BRL, Carlsbad CA, USA), supplemented with 10% (v/v) heat-inactivated FCS (fetal calf serum; Gibco-BRL), 100 U/ml penicillin, 100 µg/ml streptomycin.

Cell proliferation, Focus Forming Assay and soft agar .

For cell proliferation rate, 1.5×10^5 cells were seeded in 6-well plates and counted at the indicated times using the Countess Automated Cell Culture (Invitrogen, Carlsbad CA, USA). Population doublings were calculated using the formula $PD = t \log 2 / \log N_t - \log N_o$.

For the focus forming assay, 30×10^4 cells were seeded in a 10 cm plate and the media refreshed every 2-3 days. After 15 days, cells were fixed in cold methanol and stained with methyl violet (Sigma Aldrich, St. Louis MO, USA). Plates were scanned and the number of colonies was calculated using the software ImageJ (<http://rsbweb.nih.gov/ij/>).

For soft agar assay, 2×10^4 cells were plated in 0.35% agar in complete growth medium in 3.5 cm wells. Colonies were counted 30 days after seeding.

Lentiviral infection.

pLKO vectors carrying either scrambled or shRNA-HIF-1 α sequences (Open Biosystems, Huntsville AL, USA) and pLVTH-GFP carrying either scrambled or shRNA-STAT3 sequences⁴² were packaged by transfecting 293T cells and used to infect cells for 24 hours, followed by puromycin selection for 48 hours.

Sub-cutaneous tumors.

Female CD-1 mice 5 to 7 weeks of age CD-1 mice (Charles River Laboratories, Inc.) were s.c. implanted with 3T3 cells. Cells were harvested using trypsin and washed with PBS. 10⁶ cells were resuspended in 200 μ L PBS and injected s.c. into the left flank of mice. All mice were monitored two times weekly for body weight. S.c. tumors were measured with a caliper weekly, and mice were sacrificed whenever the tumor exceeded 10 mm of diameter.

Real Time-PCR.

Total RNA was prepared with the PureLink Micro-to-Midi total RNA Purification System (Invitrogen). qRT-PCR reactions were performed using the Universal Probe Library system (Roche Italia, Monza, Italy). The 18S rRNA pre-developed TaqMan assay (Applied Biosystems) was used as an internal control. For primers and probes see Supplementary Information.

Immunofluorescence.

Cells plated on glass coverslips were washed in PBS, fixed in 4% paraformaldehyde, quenched with 50 mmol/L ammonium chloride, permeabilized with 0.3% Triton X-100 in PBS, saturated with 3% bovine serum albumin, and incubated with primary antibodies at room temperature for 1 h, followed by rhodamine-labeled secondary antibodies (Sigma) and then by Hoechst-dye. An Axiovert 200M Zeiss microscope for optical sectioning was used.

Western blots.

Total and nuclear protein extracts were obtained as previously described¹⁶. Samples were fractionated on SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica MA, USA).

Lactate measurements

10⁵ cells were seeded and the media were refreshed 12 hours later. Lactate was measured in cell supernatants 3 hours after changing medium using a Lactate Colorimetric Assay Kit (Abcam). Data were normalized to final cell counts.

Mitochondrial calcium measurements

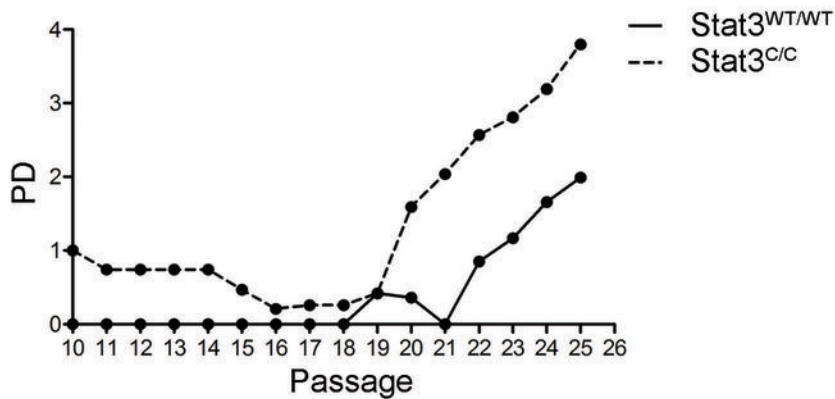
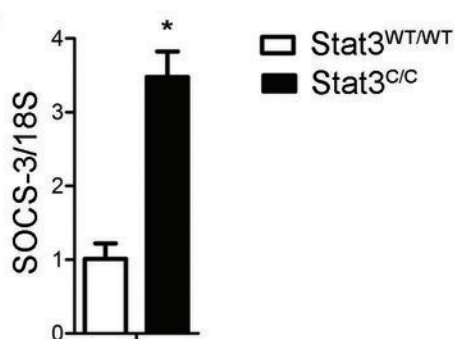
Cells were grown on glass coverslips at 50% confluence and infected with the adenovirus expressing the appropriate aequorin chimera as previously described⁴³. Measurements were carried out in KRB (125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM Na₂HPO₄, 5.5 mM glucose, 20 mM NaHCO₃, 2 mM l-glutamine and 20 mM HEPES pH 7.4, supplemented with 1 mM CaCl₂). ATP was added to the same medium. Cells were lysed with 100 μM digitonin in a hypotonic Ca²⁺-rich solution (10 mM CaCl₂ in H₂O), thus discharging the remaining aequorin pool. The light signal was collected and calibrated into [Ca²⁺] values.

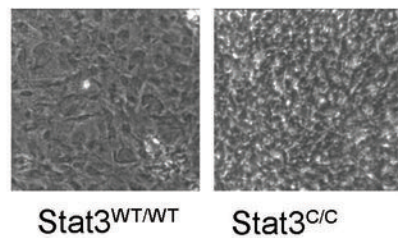
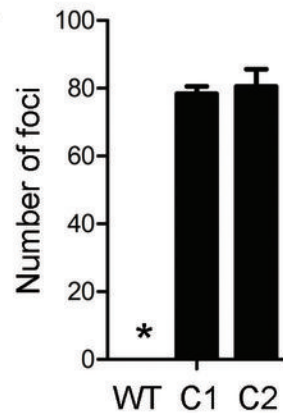
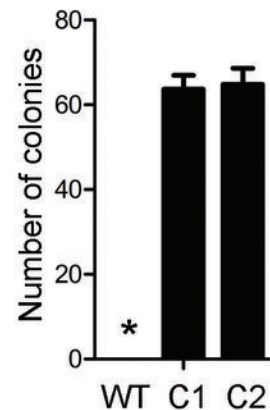
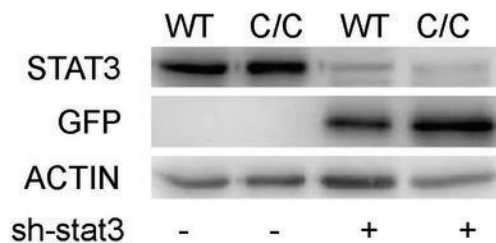
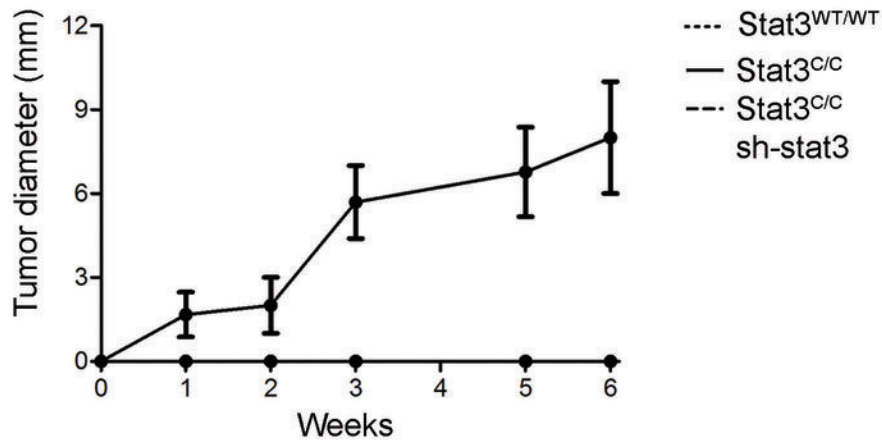
In vitro cell death.

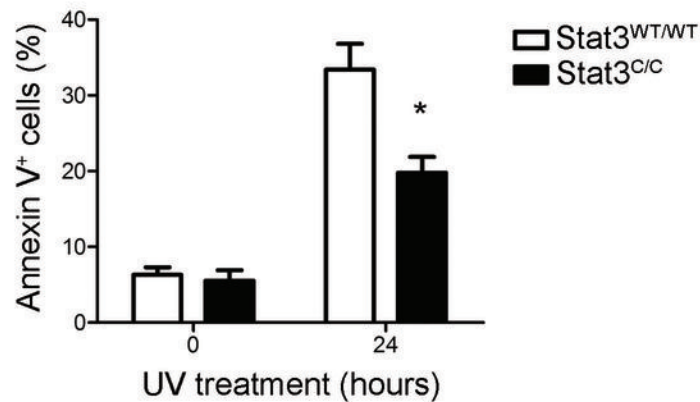
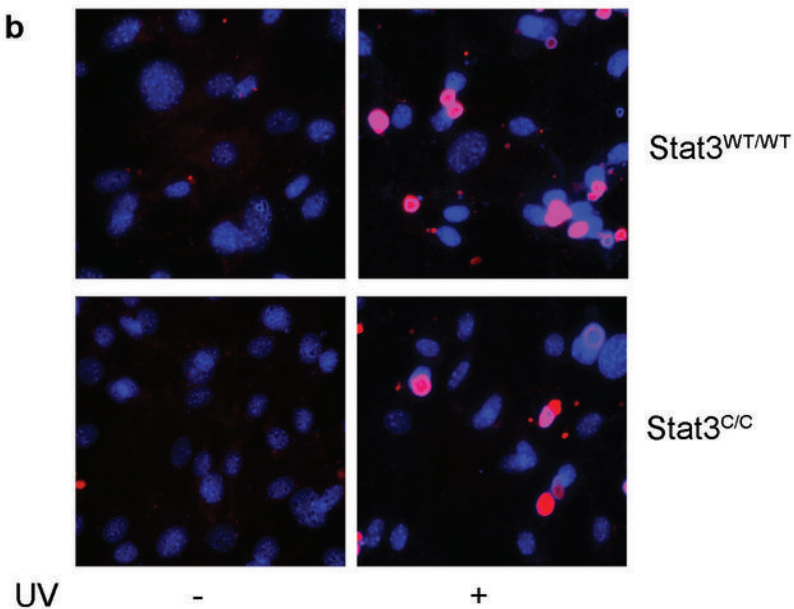
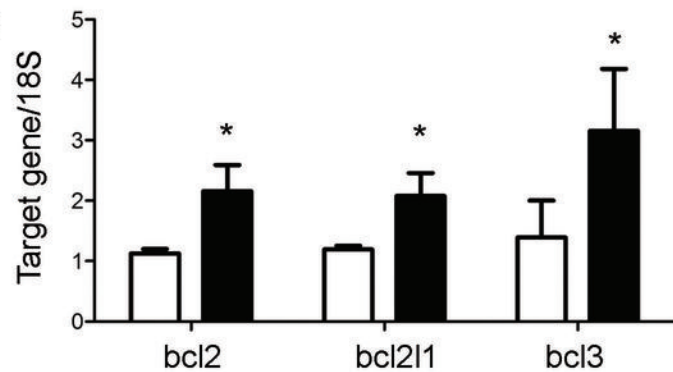
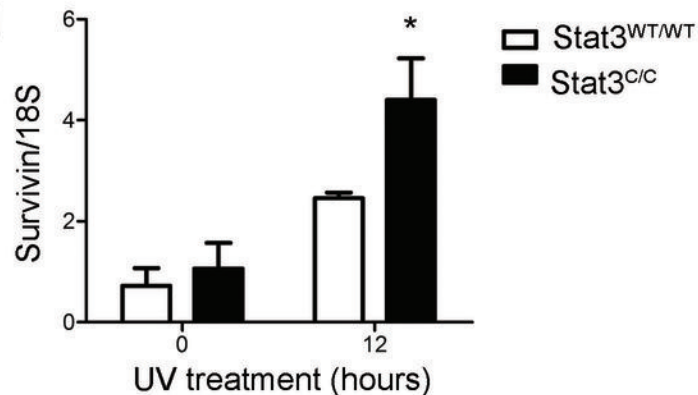
Cells were irradiated with 10 J/m² UV-C or serum-starved for 48-72 hours, followed by staining with either Annexin-V or anti-activated Caspase-3. Annexin-V emission were detected in the green channel (525 nm) and propidium iodide in the red channel (575 nm) following excitation by a 488 nm laser on a FACS Calibur cytometer (Beckton, Dickinson and Company, Franklin Lakes NJ, USA).

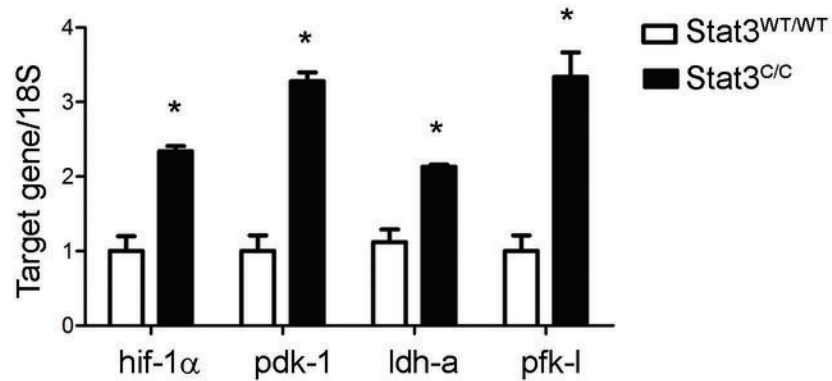
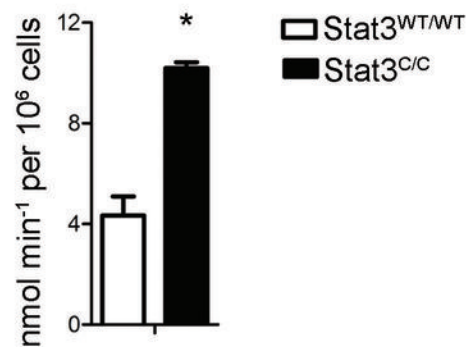
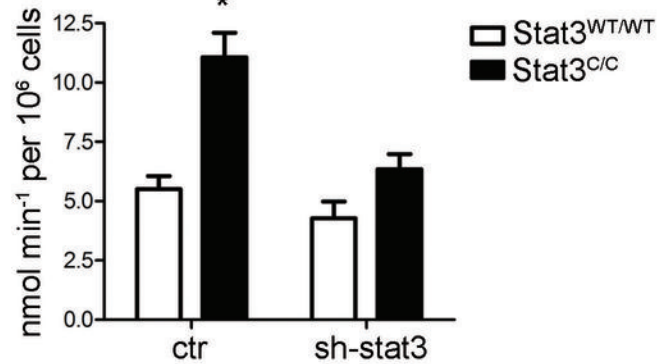
Statistical analysis.

An unpaired *t* test was used to calculate a P value for two groups, while a P value on a response affected by two factors was calculated with a two-way ANOVA.

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