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STRAP Regulates c-Jun Ubiquitin-mediated Proteolysis and Cellular Proliferation

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Abstract

STRAP is a ubiquitious WD40 protein that has been implicated in tumorigenesis. Previous studies suggest that STRAP imparts oncogenic characteristics to cells by promoting ERK and pRb phosphorylation. While these findings suggest that STRAP can activate mitogenic signaling pathways, the effects of STRAP on other MAPK pathways have not been investigated. Herein, we report that STRAP regulates the expression of the c-Jun proto-oncogene in mouse embryonic fibroblasts. Loss of STRAP expression results in reduced phospho-c-Jun and total c-Jun but does not significantly reduce the level of two other early response genes, c-Myc and c-Fos. STRAP knockout also decreases expression of the AP-1 target gene, cyclin D1, which is accompanied by a reduction in cell growth. No significant differences in JNK activity or basal c-Jun mRNA levels were observed between wild type and STRAP null fibroblasts. However, proteosomal inhibition markedly increases c-Jun expression in STRAP knockout MEFs and STRAP over-expression decreases the ubiquitylation of c-Jun in 293T cells. Loss of STRAP accelerates c-Jun turnover in fibroblasts and ectopic over-expression of STRAP in STRAP null fibroblasts increases c-Jun expression. Collectively, our findings indicate that STRAP regulates c-Jun stability by decreasing the ubiquitylation and degradation of c-Jun.

Keywords

STRAP; c-Jun; ubiquitylation; cancer

Introduction

The serine threonine kinase receptor-associated protein (STRAP) is a conserved WD40 domain protein that has been shown to regulate a wide array of signaling pathways. STRAP was initially shown to block TGF-β SMAD signaling by sterically blocking TGF-β receptor I-mediated phosphorylation of the Smad-2/3 complex [1,2]. However, TGF-β independent functions for STRAP have also been identified. For example, STRAP has been reported to bind PDK1 and stabilize p53 through direct association with NM23 [3,4,5]. While some of these associations modulate STRAP's role in Smadsignaling, STRAP can reciprocally affect

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the signal transduction pathways mediated by its binding partner. In other instances, the identification of these novel binding partners has led to undiscovered biological functions for STRAP.

c-Jun, a component of the AP-1 transcription factor complex, is recognized as an immediate early gene due to its rapid induction in response to various growth factors. Studies on AP-1 indicate that its activity is important for normal biological processes including proliferation and survival [6,7,8]. However, aberrant c-Jun activity has also been implicated in oncogenesis. As such, c-Jun activity and expression are subject to stringent regulation through control of transcription, protein synthesis and posttranslational modification. At the post-transcriptional level, phosphorylation of specific c-Jun residues appears to be critical to the regulation of both c-Jun activity and stability. JNK-mediated phosphorylation of c-Jun at S63 and S73 is required for the assembly of transcriptionally active AP-1 heterodimers [9,10]. Interestingly, JNK1 phosphorylation of c-Jun has also been reported to stabilize c-Jun [11,12]. Conversely, phosphorylation of other residues in c-Jun has been reported to limit c-Jun activity by promoting ubiquitin-mediated proteolysis. It has been shown that GSK3β-dependent phosphorylation of c-Jun at T242 is essential for F-box and WD repeat domain-containing 7 (Fbw-7) dependent degradation of c-Jun [13] whereas phosphorylation of Y170 has been reported to regulate Itch mediated ubiquitylation of c-Jun [14]. In addition to the Fbw7 and Itch, both MKK1 and constitutively photomorphogenic 1 (COP1) have also been reported to exhibit E3 ligase activity towards c-Jun [15,16]. Although each ligase recognizes a unique sequence in c-Jun, the apparent redundancy in target selection underscores the importance of regulating c-Jun expression.

There is growing evidence to suggest that STRAP over-expression exerts a largely tumorigenic influence on cells, independent of its role in TGF-β signaling. STRAP over-expression has been detected in human lung, colon, and breast cancer [17,18]. Furthermore, STRAP expression promotes anchorage independent growth of various cell lines *in vitro* as well as tumor formation in nude mice [17]. At the cellular level, STRAP over-expression may support oncogenesis through ERK activation and pRb phosphorylation [17,18]. In some experimental contexts, activation of ERK has been shown to promote S phase entry through induction of cyclin D1 [19,20], which indirectly promotes pRb phosphorylation by Cdk4/6 and dissociation from the E2F transcription factor. However, ERK activation and pRb phosphorylation may occur independently of each other as previous studies have shown that activation of the p38 and JNK MAPK pathways can also induce cyclin D1 expression [21,22]. As such, we hypothesized that STRAP regulates signaling by affecting components of the MAPK signaling pathways. Here, we report for the first time that STRAP expression promotes c-Jun activation and expression in fibroblasts by inhibiting c-Jun ubiquitylation and degradation. The increased stability of c-Jun is correlated with increased expression of the AP-1 target gene, cyclin D1, and increased proliferation. These findings may suggest a novel mechanism by which STRAP can promote tumorigenesis by stimulating cell autonomous growth.

Materials and Methods

Cell lines and plasmids

Immortalized STRAP knockout and wild type fibroblasts were generously donated by Dr. Philippe Soriano (Mount Sinai Medical Center, New York, NY). MEFs and 293T cultures were maintained in DMEM supplemented with 7% FBS. pcDNA3-c-Jun was kindly provided by Dr. Mike Engel (Vanderbilt University, Nashville, TN) and the His6-Ubiquitin expression construct was a gift from Dr. Christoph Eglert (Leibniz Institute for Age Research, Jena, Germany). Construction of the pcDNA3-STRAP-HA and pBABE puro-STRAP-HA expression vectors have been described elsewhere [1,2].

Reagents and Antibodies

MG132, cycloheximide, and sodium butyrate were purchased from Sigma (St. Louis, MO). Lithium chloride was obtained from Calbiochem (La Jolla, CA). The STRAP monoclonal antibody was purchased from BD Transduction Labs (San Jose, CA). PARP, c-Jun, cyclin D1, p53, and JNK antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-c-Jun, phospho-JNK/SAPK, c-Fos, and FosB antibodies were from Cell Signaling Technology (Beverly, MA). The cleaved caspase-3 antibody was purchased from Trevigen, Inc (Gaithersburg, MD). The c-Myc custom antibody was provided by Dr. Steve Hann (Vanderbilt University, Nashville, TN).

Generation of stable cell lines

STRAP-HA and control retrovirus were generated as previously described [23]. Early passage STRAP knockout fibroblasts were infected with retrovirus and subjected to puromycin selection to obtain polyclonal cultures.

Immunoblot analysis

For terminal experiments requiring treatments, 10 mM sodium butyrate (24 hours), 10 mM LiCl (16 hours) and 50 μM MG132 (4 hours) was added directly to the growth medium. Preparation of cell lysates, SDS-PAGE, and protein transfer to PVDF membranes was performed as previously described [23]. The membranes were immunoblotted with the indicated antibodies. Protein densities were determined using NIH Image J software.

Cell Growth Assays

For the cell counting assays, 3×10^3 cells were seeded in triplicate wells of 12-well plates. Forty eight hours after seeding, cells were trypsinized daily and counted in a hemocytometer. The total number of cells was calculated for each well. Results are expressed as the log of the total cell number ± SEM for six independent experiments. The thymidine incorporation assay has been previously described elsewhere [23]. Results are expressed as the log of the counts per minute (CPM) ± SEM for six independent experiments. Statistical analysis of cell counting and thymidine incorporation assays were performed using mixed-model ANOVA.

Reverse Transcription-PCR

Total RNA was extracted from sub-confluent MEF cultures using TRIzol reagent. Two μgs of total RNA were reverse transcribed at 37°C for one hour using MMLV reverse transcriptase (Promega, Madison, WI). The resulting cDNA was PCR amplified for 25 cycles using primers specific for c-Jun and GAPDH. The PCR reaction was carried out using the following conditions: denaturation at 95°C for one minute, annealing at 54°C for one minute, and elongation at 72°C for one minute. The specific primer sequences are as follows: c-Jun 5′-GACTGCAAAGATGGAAACGA and 5′-GGGTTGAAGTTGCTGAGGTT; GAPDH 5′-ACCACAGTCCATGCCATCAC, and 5′-TCCACCACCCTGTTGCTGTA.

Cycloheximide assays

Sub-confluent wild type and STRAP knockout fibroblasts were treated with 100 μg/ml of cycloheximide. The cultures were harvested at the indicated time points and c-Jun expression was determined by immunoblot analysis.

Ubiquitylation Assay

293T cells were transiently transfected with c-Jun, His6-Ubiquitin, and STRAP-HA expression plasmids using lipofectamine 2000 (Invitrogen, Calsbad, CA). The cell suspension was lysed in a 6 M guanidine hydrochloride buffer and ubiquitylated proteins were pulled down from the lysates with Ni-NTA resin. The bound proteins were eluted by boiling in 3X Laemmli buffer containing 350 mM imidazole. Ubiquitylated c-Jun was detected by immunoblot analysis.

Results

STRAP expression is correlated with c-Jun activation and expression

In order to examine the role of STRAP in the regulation of MAPK signaling, the kinetics of p38, ERK, and c-Jun activation was assessed in serum re-stimulated wild type and STRAP knockout cultures. STRAP expression was not associated with increased ERK or p38 activation after the addition of serum (Supplementary Fig. 1). However, serum induced c-Jun phosphorylation in wild type MEFs (Fig. 1A). Activated c-Jun has been reported to positively auto-regulate its expression through binding to AP-1 sites on its own promoter [24]. Given that c-Jun activation levels peak more rapidly than c-Jun total protein for both cultures, it is possible that the phosphorylated protein is promoting c-Jun transcription. The activation and expression of c-Jun was also examined in asynchronous cultures without re-stimulation. STRAP knockout significantly diminished c-Jun phosphorylation and expression, supporting our finding that STRAP regulates c-Jun activation and expression (Supplementary Figure 2).

Activated c-Jun promotes cell cycle progression by transcriptionally regulating genes that are necessary for the G1 to S transition, including many growth factors and cyclin D1. In accordance with the reduced phospho-c-Jun levels, STRAP deletion decreased cyclin D1 expresion by western blot (Fig. 1B) as well as cyclin D1 mRNA levels in a microarray study (unpublished data) . To determine whether STRAP generally upregulates expression of other transcription factors, MEF lysates were immunoblotted for p53, c-Myc, c-Fos, and FosB (Supplementary Fig. 3). Interestingly, c-Myc was significantly up-regulated in the STRAP knockout cultures whereas expression of p53 was significantly elevated in wild-type fibroblasts ($p<0.05$). These findings suggest that STRAP exhibits specificity towards c-Jun rather than indiscriminately up-regulating the expression of all transcription factors.

Expression of c-Jun can be regulated by transcription, post-transcriptional modification, and degradation. In order to determine whether STRAP transcriptionally regulates c-Jun expression, we examined c-Jun mRNA levels by RT-PCR (Fig. 1C). No apparent differences in steady state c-Jun mRNA were detected, suggesting that STRAP does not affect transactivation or degradation of c-Jun mRNA. To determine whether STRAP can promote c-Jun activation and expression through regulation of JNK activity, the kinetics of JNK activation in response to serum stimulation were examined in wild type and STRAP null MEFs. Western blot analysis revealed marginal differences in the relative amounts of phosphorylated JNK and total JNK between wild type and STRAP MEFs (Fig. 1D left). However, normalized p-JNK levels were not statistically significant by ANOVA, suggesting that STRAP does not promote c-Jun expression through activation of JNK (Fig. 1D right).

STRAP regulates c-Jun stability by decreasing ubiquitylation

In order to determine whether c-Jun expression levels in MEFs are regulated through proteosomal degradation, wild type and STRAP null MEFs were treated with the proteosome inhibitor, MG132, and the GSK3β inhibitor, LiCl. As shown in Figure 2A (left), 50 μM MG132 treatment markedly increased Jun expression in both wild type and STRAP

null MEFs, suggesting that proteosomal degradation modulates c-Jun protein levels in these cells. The mean relative density ± SEM from three independent experiment was plotted to show the considerable differences between the untreated and MG132 treated cultures (Fig. 2A, right). Interestingly, c-Jun protein levels in wild type and STRAP knockout MEFs were unaffected by treatment with 10 mM LiCl (Fig 2A) or another GSK3β inhibitor, SB415286 (unpublished data). These findings suggest that GSK3β/Fbw7 mediated degradation of c-Jun may not be necessary for c-Jun turnover in MEFs. Next, we tested the effect of STRAP on c-Jun ubiquitylation in 293T cells (Fig. 2B, left). Co-expression of c-Jun and His-Ubiquitin produced a ladder comprised of mono- and poly-ubiquitylated Jun protein. Over-expression of STRAP markedly decreased the ubiquitylation of c-Jun. Importantly, western blot analysis of the corresponding lysates showed approximately equal amounts of poly-ubiquitylated proteins (unpublished data), indicating that STRAP expression does not result in a global decrease in ubiquitylation. Mann Whitney analysis of fourteen independent experiments indicated that the effect of STRAP on c-Jun ubiquitylation is statistically significant, $p<0.05$ (Fig. 2B, right).

STRAP prolongs the half-life of c-Jun

In order to determine whether STRAP expression affects the half-life of c-Jun, wild type and STRAP null MEFs were treated with cycloheximide and the kinetics of c-Jun degradation were examined by western blot analysis (Fig. 3A, left). In STRAP knockout MEFs, c-Jun protein levels experienced a sustained and rapid decline over the four hour timecourse. However, c-Jun levels in wild type appeared to gradually decline and stabilize at four hours, suggesting that STRAP inhibits c-Jun degradation. The relative densities of c-Jun at various time points after cycloheximide addition were normalized to the initial density of c-Jun and plotted to show the approximate rate of c-Jun decay (Fig. 3A, right). Based on nonlinear regression analysis, the half-life of c-Jun in STRAP null fibroblasts can be estimated at one hour. While the half-life of c-Jun in wild type MEFs can't be interpolated from the normalized c-Jun values, the smaller slope indicates that the rate of c-Jun decay is markedly slower in these cells.

Stable reintroduction of STRAP rescues the c-Jun instability defect in STRAP knockout fibroblasts

To confirm that STRAP regulates c-Jun expression, STRAP null MEFs were transduced with retrovirus harboring empty vector or STRAP-HA and the effect of STRAP overexpression on c-Jun protein levels was examined by western blot (Fig. 3B, left). Relative to the wild type, STRAP knockout and vector control fibroblasts exhibited a statistically significant reduction in c-Jun protein levels (Fig. 3B, right). Ectopic expression of STRAP-HA increased c-Jun protein levels although endogenous STRAP expression levels were markedly higher in wild type. These data suggest that STRAP can rescue the c-Jun instability defect in the knockout cell line.

STRAP promotes proliferation of MEFs

A role for STRAP as an oncogene has been proposed due to its ability to antagonize TGF-β mediated growth inhibition [17]. However, STRAP over-expression has been correlated with tumorigenicity in the absence of exogenous TGF-β [17]. In cell counting assays, STRAP knockout MEFs exhibited a marked defect in proliferation relative to the wild type cultures (Fig. 4A). The STRAP knockout MEFs also exhibited a significant decrease in thymidine incorporation, suggesting that STRAP promotes cellular proliferation (Fig. 4B). Because decreased cell numbers can be attributed to increased cell death, markers for apoptosis were examined by western blot analysis (Fig. 4C). As a positive control, wild-type MEFs were treated with 10 mM sodium butyrate for 24 hours to induce apoptosis. Minimal cleaved caspase-3 and PARP were detected in the untreated cultures, whereas both markers

were present in the positive control. Furthermore, visual examination of the cultures under normal growth conditions did not reveal any morphological changes associated with cell death or reduced viability (unpublished results). Collectively, our observations suggest that STRAP promotes cellular proliferation in the absence of exogenous TGF-β.

Discussion

Here, we show that STRAP regulates c-Jun expression by inhibiting the ubiquitylation and degradation of c-Jun. It has been previously reported that STRAP decreases ubiquitylation of the p53 tumor suppressor through formation of a ternary complex with NM23 and p53 [3]. As expected, wild type fibroblasts expressed significantly more p53 than STRAP null fibroblasts. However, unlike p53, c-Jun does not co-immunoprecipitate with STRAP (unpublished results). This suggests that STRAP-mediated c-Jun stabilization occurs through a novel mechanism. Previous studies have shown that c-Jun is a substrate for the ubiquitin E3 ligase, Fbw-7 [13]. Fbw-7 mediated degradation depends on GSK3β phosphorylation of T242 in the C-terminus of c-Jun. Because treatment with the GSK3β inhibitor, LiCl, did not alter the basal level of c-Jun protein in MEFS, we do not expect c-Jun stability to be regulated by Fbw-7 in these cells. In addition to Fbw-7, the ubiquitin E3 ligases Itch and DET/COP1 have been shown to promote c-Jun degradation. Further work will be required to determine whether STRAP can influence c-Jun association with these E3 ligases.

While very little is presently known about the cellular functions of STRAP, it appears that STRAP can regulate a wide array of signaling pathways by associating with a diverse group of cellular proteins. Although the specific biological function of STRAP varies according to its binding partner, STRAP binding to these proteins generally promotes signaling pathways and processes that are frequently associated with tumorigenesis [25]. Previous studies on c-Jun indicate that its expression is important for cellular proliferation as c-Jun knockout MEFs exhibit a significant delay in cell cycle progression [6,8]. Similarly, loss of STRAP significantly impairs the proliferation of MEFs suggesting that the defect in cellular growth may be in part due to increased turnover of c-Jun. In light of the evidence that c-Jun signaling promotes tumor formation [26,27], STRAP over-expression could support tumor growth through deregulation of c-Jun expression. Alternatively, it is possible that STRAP mediated deregulation of c-Jun in stromal cells could activate oncogenic signaling pathways in nearby epithelia through AP-1 dependent secretion of growth factors and cytokines. For example, wild-type fibroblasts have been shown to exhibit a greater capacity for stimulation of benign prostatic hyperplasia (BPH) proliferation through IGF-1 paracrine signaling compared to c-Jun null fibroblasts [28]. Based upon our findings and the literature published thus far, STRAP may promote growth and tumorigenicity by regulating the TGF-β, ERK, and c-Jun signaling pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

STRAP	serine-threonine kinase receptor-associated protein
COP1	constitutively photomorphogenic 1

Research Highlights

- STRAP is specifically correlated with c-Jun expression and activation in fibroblasts
- STRAP inhibits c-Jun ubiquitylation *in vivo* and prolongs the half-life of c-Jun
- STRAP expression increases expression of the AP-1 target gene, cyclin D1, and promotes cell autonomous growth

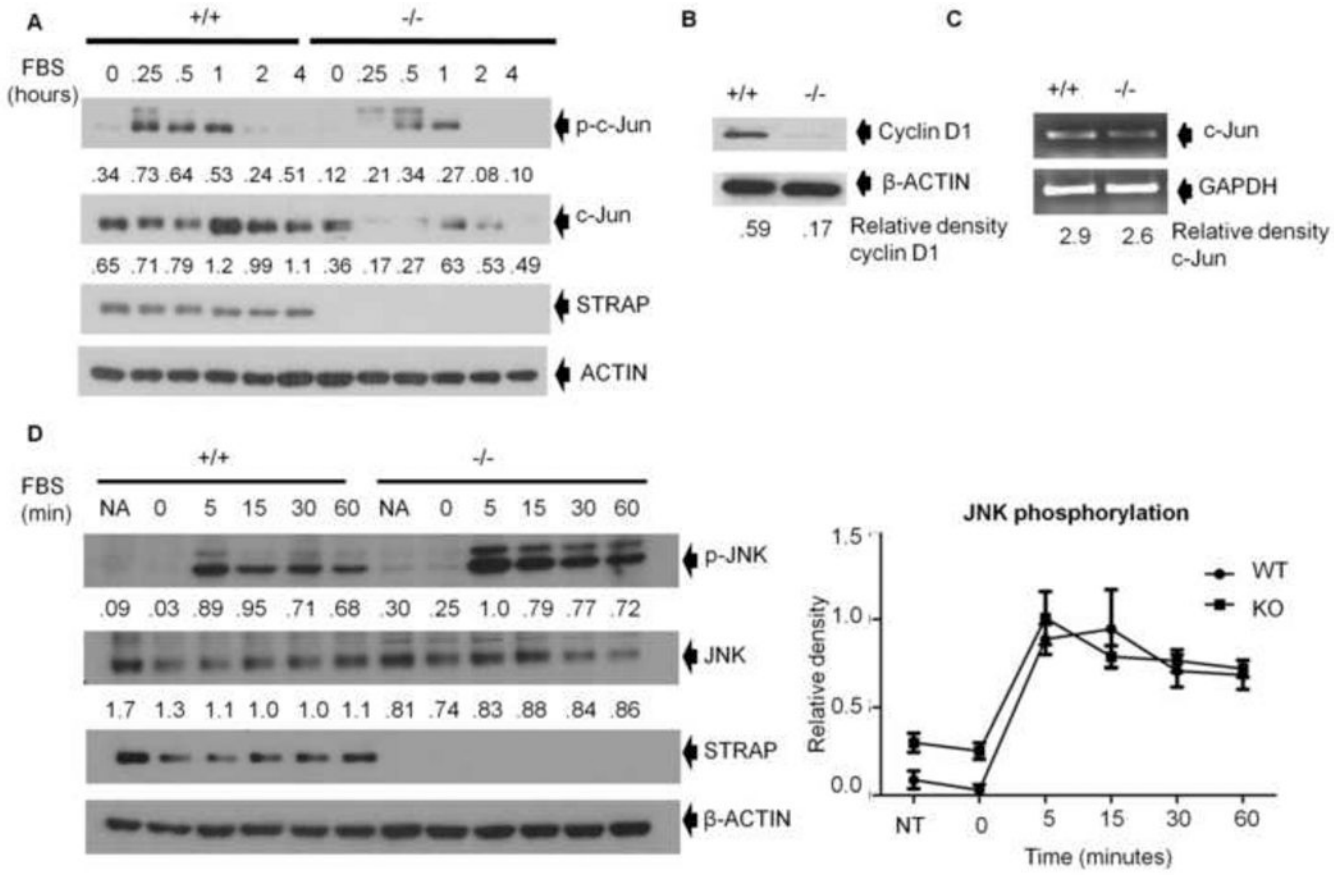

Figure 1. STRAP regulates c-Jun protein expression and activity

A. Wild type and STRAP null MEFs were serum starved overnight and re-stimulated with 10% serum for the indicated times. Lysates were immunoblotted for phospho-c-Jun and total c-Jun expression. Phosphorylated and total c-Jun protein was normalized against actin and the mean relative density from three independent experiments is shown. B. Expression of the AP-1 target gene, cyclin D1, was determined by immunoblot analysis. Cyclin D1 levels were normalized to actin and the average relative densities from three independent experiments are shown. C. Basal mRNA levels in MEFs were examined by RT-PCR. GAPDH was amplified as an internal control. c-Jun mRNA was normalized to GAPDH and the mean relative densities from three independent experiments are shown. D. Wild type MEFs were serum-starved and re-stimulated with 10% serum for the indicated time points. "NA" samples represent cultures in normal log growth phase. Lysates were immunoblotted for phospho-JNK and total JNK (left). p-JNK and JNK were normalized against actin and the mean densities from four independent experiments are shown. The mean relative density ± SEM of p-JNK is shown to the right.

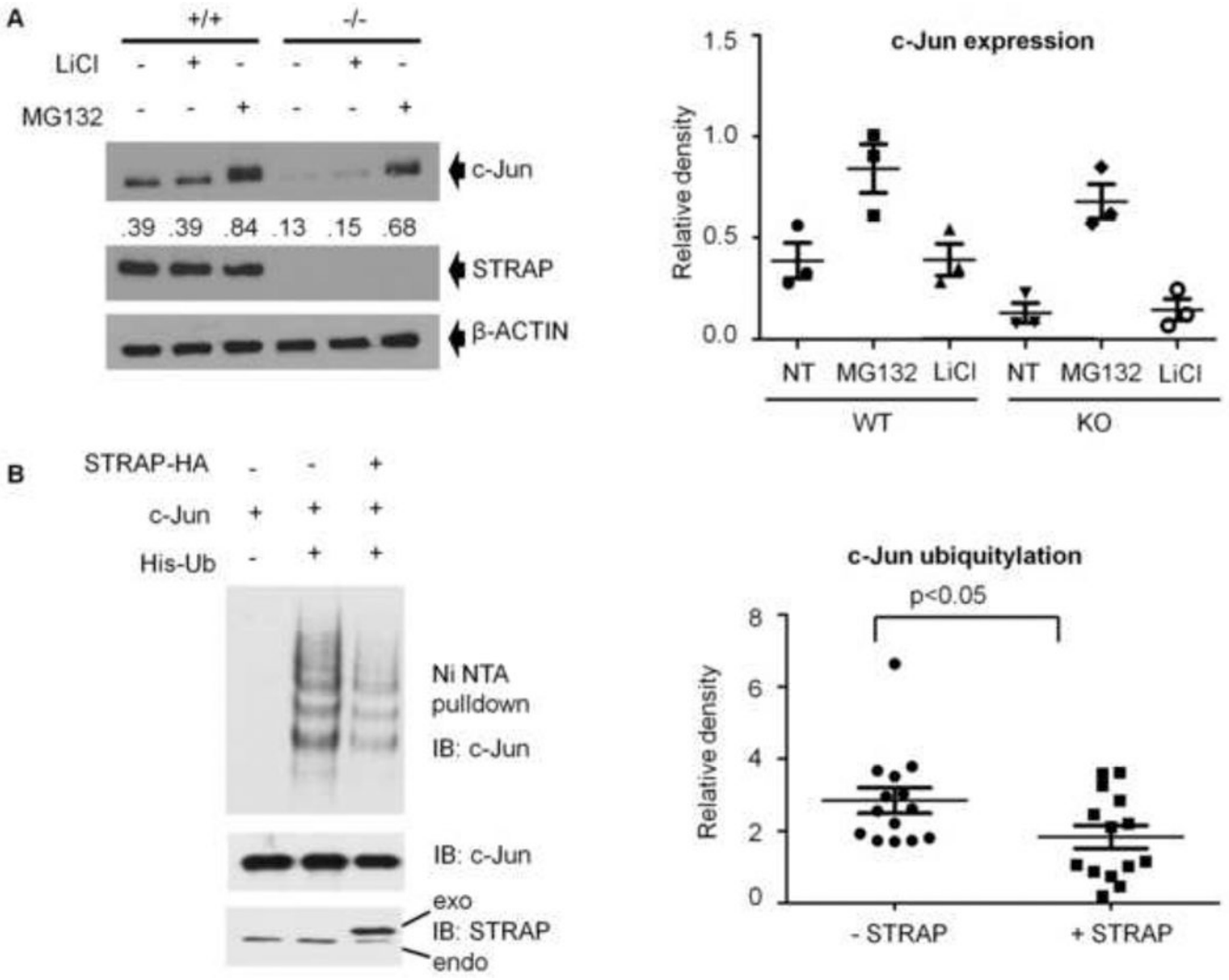

Figure 2. STRAP stabilizes c-Jun by inhibiting ubiquitin-mediated proteolysis of c-Jun

A. Wild type and STRAP null fibroblasts were treated with MG132 and LiCl. The effect of treatment on total c-Jun protein levels was analyzed by immunoblot analysis. Total c-Jun levels were normalized to actin and the mean relative densities from three independent experiments are shown (top). The mean relative densities ± SEM for each treatment were plotted (bottom). B. The ubiquitylation of c-Jun in the presence and absence of STRAP expression was assessed by transiently transfecting 293T cells with the indicated expression constructs. Ubiquitylated proteins were pulled down from cell lysates using Ni^{2+}-NTA resin and analyzed for c-Jun by immunoblot analysis. The relative expression of c-Jun and STRAP was determined by immunoblot analysis of whole cell extracts (top). Ubiquitylated c-Jun was normalized to total Jun levels in the whole cell extracts. The mean relative density ± SEM of ubiquitylated c-Jun from 14 experiments is shown. Statistical significance was determined using the Mann Whitney test (bottom).

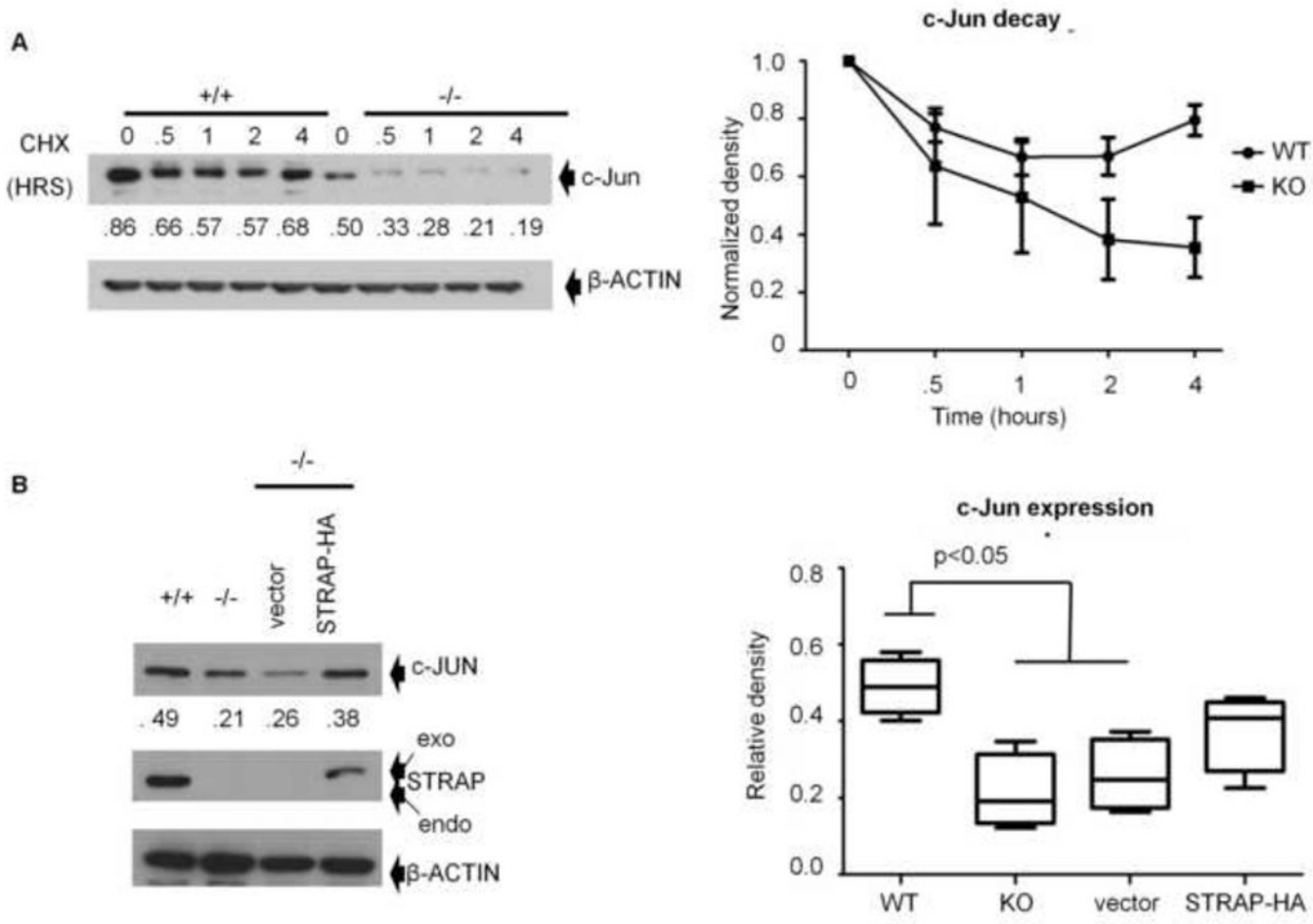

Figure 3. STRAP prolongs the half-life of c-Jun and increases c-Jun expression in STRAP knockout fibroblasts

A. Wild type and STRAP null fibroblasts were treated with cycloheximide (CHX) and lysed after 0, 0.5, 1, 2, and 4 hours of treatment. c-Jun protein levels were analyzed by immunoblot analysis. The c-Jun density was normalized against actin and the mean relative density of three independent experiments is shown (left). To show c-Jun decay over time, the relative densities (c-Jun density/Actin density) were normalized to the baseline c-Jun values for each culture. The adjusted c-Jun values are plotted ± SEM for three experiments (right). B. STRAP null fibroblasts were transduced with retrovirus to obtain polyclonal cultures expressing either an empty vector or a STRAP-HA construct. The effects of enforced STRAP expression on c-Jun protein levels were determined by immunoblot analysis (left). The c-Jun density was normalized against actin and the mean relative density from four independent experiments is shown. Statistical significance was determined using the Mann Whitney test (right).

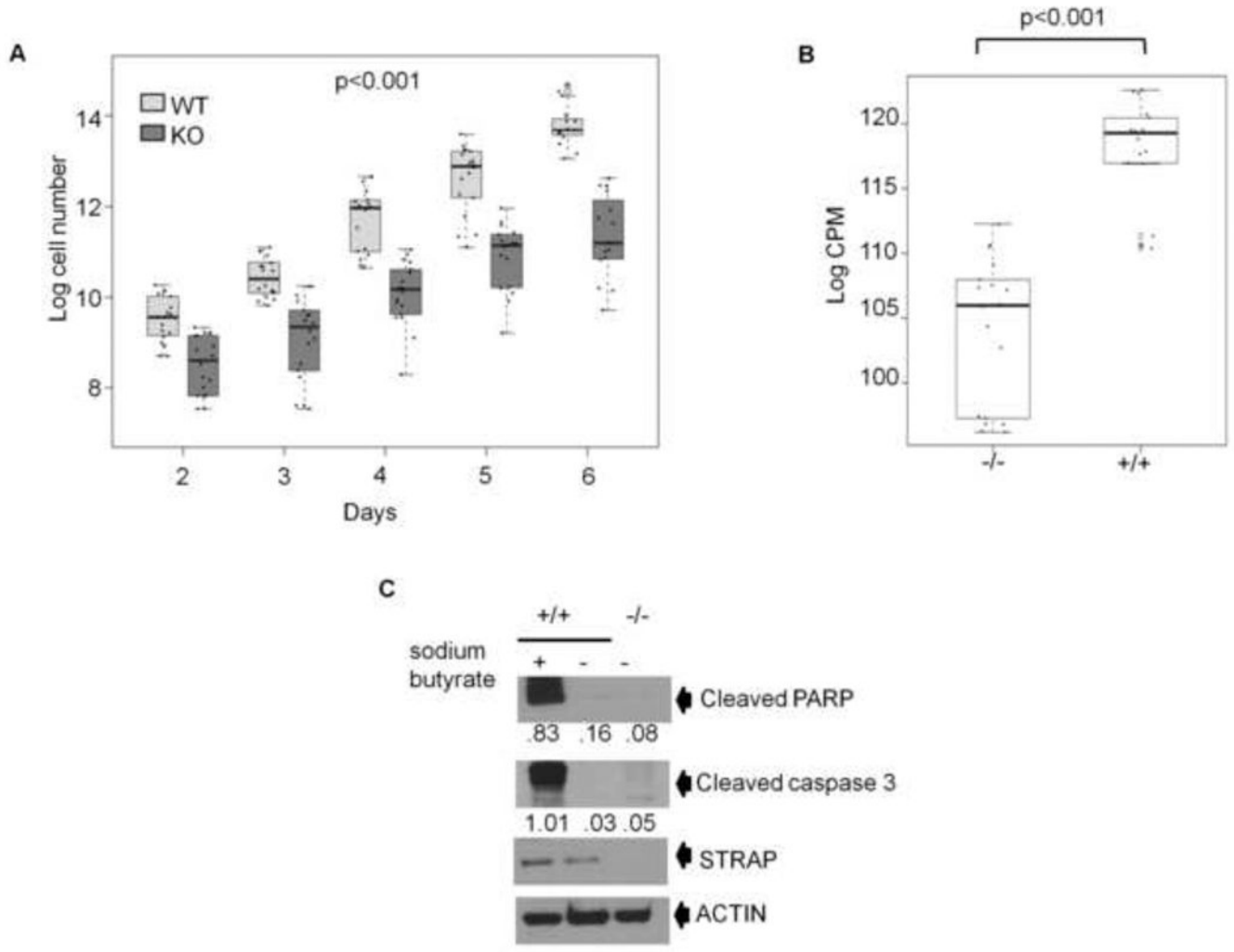

Figure 4. STRAP expression promotes cellular proliferation

A. Proliferation of wild type and STRAP knockout MEF cell lines was examined by counting cells over a five-day consecutive timecourse. The data are presented as the log of the average cell number from six independent experiments. The statistical significance between was determined using a mixed model ANOVA ($p<0.001$). B. Growth of wild type and STRAP knockout MEFs was assessed by measuring ^{3}H-thymidine incorporation 46 hours after cell culture seeding. The data is presented as the log of the counts per minute (CPM) for six independent experiments. Statistical analysis was performed using a mixed-model ANOVA ($p<0.001$). C. MEF lysates were analyzed for markers of apoptosis by western blotting with antibodies directed against cleaved caspase-3 and PARP. Wild-type MEFs were pre-treated with sodium butyrate for 24 hours as a positive control for apoptosis. The relative density of caspase-3 and PARP was determined by normalizing against the corresponding actin band. The mean relative density from three independent experiments is shown.