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SIRT1 Acts as a Nutrient-sensitive Growth Suppressor and Its Loss Is Associated with Increased AMPK and Telomerase Activity

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SIRT1, the mammalian homolog of SIR2 in *Saccharomyces cerevisiae*, is an NAD-dependent deacetylase implicated in regulation of lifespan. By designing effective short hairpin RNAs and a silent shRNA-resistant mutant SIRT1 in a genetically defined system, we show that efficient inhibition of SIRT1 in telomerase-immortalized human cells enhanced cell growth under normal and nutrient limiting conditions. Hematopoietic stem cells obtained from SIRT1-deficient mice also showed increased growth capacity and decreased dependency on growth factors. Consistent with this, SIRT1 inhibition was associated with increased telomerase activity in human cells. We also observed a significant increase in AMPK levels up on SIRT1 inhibition under glucose limiting conditions. Although SIRT1 suppression cooperated with hTERT to promote cell growth, either overexpression or suppression of SIRT1 alone had no effect on life span of human diploid fibroblasts. Our findings challenge certain models and connect nutrient sensing enzymes to the immortalization process. Furthermore, they show that in certain cell lineages, SIRT1 can act as a growth suppressor gene.

INTRODUCTION

Loss of silencing of mating type loci in *Saccharomyces cerevisiae* (Hopper and Hall, 1975; Haber and George, 1979) led to discovery of a gene named MAR1 (mating-type regulator 1; Klar *et al.*, 1979) also known as SIR2 (silent information regulator 2; Rine *et al.*, 1979). Increased dosage of SIR2 extends replicative lifespan in certain strains of *S. cerevisiae* (Kaeberlein *et al.*, 1999) and increases longevity of *Caenorhabditis elegans* (Tissenbaum and Guarente, 2001). Studies also have shown that CR (calorie restriction) may mediate lifespan extension through SIR2 (Lin *et al.*, 2000) or HST2 (Lamming *et al.*, 2005). However, newer studies have challenged these notions and shown that CR-dependent replicative lifespan extension occurs in a SIR2/HST2-independent manner (Kaeberlein *et al.*, 2004; 2006).

In the case of chronological lifespan in yeast, SIR2 mediates the opposite effect (limiting lifespan). In one study, deletion of SIR2 promoted chronological lifespan extension under CR (Fabrizio *et al.*, 2005). Early studies on SIR2 of *S. cerevisiae* suggested that SIR2 has an ADP-ribosylating activity in vitro (Moazed, 2001). This subsequently led to uncovering an activity capable of deacetylating synthetic acetylated histone substrates in vitro (Imai *et al.*, 2000), generating *O*-acetyl ADP-ribose (Tanner *et al.*, 2000; Tanny and Moazed, 2001). The in vivo deacetylation targets of mammalian SIR2 homolog (SIRT1) are nuclear factors such as p53 (Luo *et al.*, 2001; Vaziri *et al.*, 2001, Langley *et al.*, 2002, Michishita *et al.*, 2005), FOXO (Brunet *et al.*, 2004; Nemoto *et al.*, 2004), Ku (Cohen *et al.*, 2004a), acetylated histones (Vaquero *et al.*, 2004), and nuclear factor (NF)-κB (Yeung *et al.*, 2004).

More recently novel activators of SIRT1 such as HIC1 and AROS have also been identified that activate SIRT1 and promote deacetylation of its targets such as p53 (Chen *et al.*, 2005; Kim *et al.*, 2007). SIRT1 is also suggested to act as a nutrient sensor in response to caloric restriction (Cohen *et al.*, 2004b; Nemoto *et al.*, 2004). In *S. cerevisiae*, Sir proteins have been shown to have critical roles in response to DNA damage and are mobilized from telomeres to sites of DNA strand breaks (McAinsh *et al.*, 1999; Mills *et al.*, 1999) and are involved in maintenance of telomeric silencing (Moretti *et al.*, 1994). Synthesis of de novo telomere repeats is achieved by telomerase an enzyme originally detected as an RNP (ribosenucleotide protein) complex in *Tetrahymena* (Greider and Blackburn, 1985) and subsequently in human cells (Morin, 1989). The mammalian telomerase is composed of a reverse transcriptase catalytic subunit (hTERT; Harrington *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997) and an RNA template (hTR; Feng *et al.*, 1995). Inactivation of telomerase in *Mus musculus* has revealed roles in cell survival and maintenance of genomic integrity via telomere maintenance (Blasco *et al.*, 1997; Lee *et al.*, 1998). Telomere maintenance and regulation in mammals is achieved by collaborative effects of telomerase and telomere-binding proteins (van Steensel and de Lange, 1997). Protective effects of telomeres on chromosome ends may be achieved via function of specialized protein complexes including TRF1/

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TRF2/TIN2 (Smogorzewska and de Lange, 2004) and other single-strand G-rich telomere-binding proteins such as Pot1 that regulate accessibility of telomeres to telomerase (Baumann and Cech, 2001; Colgin *et al.*, 2003). Human diploid fibroblasts have a finite lifespan and undergo senescence upon completion of a fixed number of cell doublings (Hayflick and Moorhead, 1961). At least a part of this molecular clock is thought to operate through telomere erosion or dysfunction with each division in normal cells that ultimately triggers initiation of cellular senescence (Harley *et al.*, 1990). Consistent with this model telomerase is reactivated in immortal human cells (Counter *et al.*, 1992; Kim *et al.*, 1994).

Further direct findings indicate that reconstitution of telomerase activity in vivo in primary mortal human fibroblasts causes bypass of senescence and leads to cell immortality (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998). Consistent with this model, human germ cells maintain their telomeres (Allsopp *et al.*, 1992), and human embryonic and adult hematopoietic stem cells express telomerase (Chiu *et al.*, 1996). This telomerase activity in hematopoietic stem cells is not sufficient to prevent telomere shortening and may confer a finite self-renewing capacity (Vaziri *et al.*, 1994). Telomerase has since been widely used as a marker for identification of human pluripotent stem cells (Shamblott *et al.*, 2001).

Here we investigate the role of SIRT1 in regulation of replicative life span and cell growth in primary, telomerase-immortalized human cells and murine hematopoietic stem cells under normal and nutrient-limiting conditions. We designed effective short hairpin RNA (shRNA) constructs that are able to reduce SIRT1 protein expression significantly. By suppressing endogenous SIRT1 in human cells we show that SIRT1 can negatively regulate cell growth, and this is associated with an increase in telomerase activity levels. Extension of these findings to an animal model indicates that hematopoietic stem cells from mice lacking SIRT1 show a greater proliferative capacity under conditions of stress. We propose that SIRT1 is a nutrient-sensitive growth suppressor in certain cell types. Therefore our findings have implications for growth of normal and immortal cells.

MATERIALS AND METHODS

Cell Culture and Cell Lines

All cell strains were grown either in DMEM + 10% fetal bovine serum (FBS) in 60–100-mm Petri dishes (Greiner, Frickenhausen, Germany). A rapid plasmid-based system (Vaziri *et al.*, 2001) was used to generate all retroviruses (Imgenex, San Diego, CA). In brief, pSRP (pSUPER-Retro-Puro), pSRP-shSIRT1(HS6), pSRP-shSIRT1(HS11) and pSRP-shControl, pBabe-Ires-Neo, pBabe-Ires-Neo-SIRT1-R, and pBabe-puro-wtSIRT1 vectors and packaging plasmid (Imgenex) were transfected into 293T cells using Fugene 6, and supernatants were used to infect the target cells carrying mCAT1. Cells were typically infected with pSRP-based viruses at multiplicity of infection (MOI) of ≈20, two times sequentially and subsequently selected in 1 μg/ml puromycin or 200–400 μg of G418. Wild-type or SIRT1-R viruses were put in at ≈2–5 MOI.

Cell Culture in Absence of Nutrients

Initially, cells were grown in growth medium (H21 medium, Invitrogen, Carlsbad, CA; cat no 12800) with 10% FBS (Invitrogen) under low density in 60-mm dishes. Twenty-four hours later, the exponentially dividing cells were washed once with phosphate-buffered saline (PBS; −Ca and −Mg). Media on the cells was subsequently changed with 4 ml of α-MEM without glucose and serum (89-5118EF, Invitrogen, with base media to which aspargine, arginine, methionine, isoleucine, L-valine, and ascorbic acid with antibiotics were added; OCI, Toronto, Ontario, Canada, Media Department). Duplicate dishes were used to estimate the total number of cells in the plates. For each cell line the cells were trypsinized, neutralized by addition of α-MEM without glucose with 2% FBS, and counted on a hemocytometer using trypan blue exclusion. The average live cell count was then calculated. Cell counts were performed every 24 h after the addition of the α-MEM without glucose. Cells were collected at different time points (0, 4, 8, 12, and 15 h) after addition of α-MEM without glucose and subjected to lysis as described below under immunoblotting.

Telomerase Assays

TRAP (telomere repeat amplification protocol) assays were performed as previously described (Kim and Wu, 1997). Typically, within 2–10 population doublings (PDs) after selection, CHAPS lysates were prepared from cells, and aliquots were frozen. For rescue experiments cells from ≈PD 93 were used to prepare lysates. On thawing, the lysates were subjected to protein quantification using the quick-start Bradford assay system (Bio-Rad, Hercules, CA). Twenty-six–cycle PCR-TRAPs were performed in linear range of the assay using 50–300 ng of total protein lysate per reaction. TRAP products were resolved on 15% polyacrylamide large gels and exposed to phosphorimager screens.

Design of SIRT1 shRNA Expression Vectors, shRNA-resistant Silent Mutant

More than 12 shRNAs were designed to find the most effective set. The most effective we developed was HS6 (Qiagen, Chatsworth, CA). The second sequence (HS11) was based on a published sequence (Ota *et al.*, 2006).

The SIRT1 shRNA sequences (bold) used as insert in pSRP (pSuper-Retro-Puro, OligoEngine, Seattle, WA) vector were as follows: HS6: GATCCCC**AGCGATGTTTGATATTGAA**TTCAAGAGA**TTCAATATCAAACATCGCT**TTTTTA. HS11: GATCCCC**GATGAAGTTGACCTCCTCA**TTCAAGAG**ATGAGGAGGTCAACTTCATC**TTTTTA. The control shRNA sequence was as follows: GATCCCC**TTCTCCGAACGTGTCACGT**TTCAAGAGA**ACGTGACACGTTCGGAGAA**TTTTTA.

A PCR-based strategy was used to introduce six silent mutations in the SIRT1 region targeted by the HS6 shRNA (for sequence, see Supplementary Figure 1A). The resulting mutant named SIRT1-R was subcloned in the PBabe-INeo vector. This vector PBIN-SIRT1-R and the backbone (PBIN) were subsequently used to infect puromycin-resistant target cells expressing pSRPshControl and pSRPshSIRT1(HS6) for a genetic rescue experiment.

Immunoblotting

Cells were harvested by trypsinization (0.05%) and neutralized with either DMEM + 10% FBS (for nutrient experiments, MEM without glucose + 2% FBS). Cells were spun and washed in $PBS^{-/-}$ twice, and the pellets were lysed in 0.5% NP40, 150 mM NaCl, and 50 mM Tris in presence of 1× complete miniprotease inhibitor mix (Roche, Indianapolis, IN; 10× stock, 1 tablet in 10 ml water), for 30 min with occasional vortexing. Cell lysates were centrifuged at 12,000 rpm for 20 min at 4°C. Protein content of lysates was measured by Bio-Rad Quick Start protein assay (500–0201). Protein, 10–50 μg, was resolved on NuPAGE (Novex, Encinitas, CA) 4–12% Bis-Tris gradient gels, transferred to PVDF membranes (Bio-Rad) and blocked in 5% skim milk. The membrane was incubated in: 1:5000 dilution for anti-SIRT1 (Vaziri *et al.*, 2001), 1:500 for 2 h for hTERT (Santa Cruz Biotechnology, Santa Cruz, CA; H-231: SC-7212), 1:20,000 for β-actin (Abcam, Cambridge, MA) 10–20 min, 1:2000 dilution of Phospho-AMPK-α (Thr172)(40H9) and total AMPK-α (23A3) (Cell Signalling, Beverly, MA; kit 9957) for 2 h. For AMPK experiments membranes were first immunoblotted with total anti-AMPK-α antibody, and the levels were measured. To prevent residual carry over, the membrane was subsequently stripped and after testing for clearance was subjected to the phospho-AMPK-α antibody for detection of active form.

The membrane was washed twice in 0.05% TBST buffer for 20 min. Peroxidase conjugated AffinPure goat anti-rabbit horseradish peroxidase IgG (H+L) secondary antibody or anti-mouse (Jackson ImmunoResearch, West Grove, PA) were used at a concentration of 1:30,000 for 45 min in 1% milk was used. After washing, the membrane was then incubated with Super signal west, dura, or femto maximum substrate (Pierce, Rockford, IL) for 2 min and exposed to film for up to 30 min.

Chromatin Immunoprecipitation

Cells (10^7) were cross-linked in plates by addition of 1% formaldehyde for 10 min, followed by the addition of glycine to a final concentration of 0.125 M to stop the cross-linking reaction. Hela-pSRP-controlshRNA and Hela-pSRPshSIRT1 cells ($n = 10^7$) were used per immunoprecipitation reaction mixture. Cells were washed twice in PBS and lysed in 1 ml of cell lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% NP-40, 1× protease inhibitors) on ice for 10 min. The nuclei were pelleted at 5000 rpm and lysed in nuclei lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, and 1% SDS, including protease inhibitors on ice for 10 min. The chromatin was sonicated eight times, 15 s each on ice. The samples were precleared by incubating with 20 μl of blocked protein G agarose beads (Roche) containing 1.5 μg of sea urchin sonicated sperm DNA for 15 min. The protein-chromatin complexes were incubated with no antibody, 3 μl of antiacetylated histone H4 antibody (06-866; Upstate Biotechnology, Lake Placid, NY), anti-SIR2 antibody (2 μl), or rabbit serum (2 μl) at 4°C overnight. Each reaction mixture was then incu-

bated with 20 μl of protein G beads for 30 min at room temperature. The protein G agarose beads were pelleted, and the supernatant from the no-antibody sample was used as total input chromatin (input). The protein G agarose pellets were washed twice in dialysis buffer (2 mM EDTA, 50 mM Tris, pH 8.0) and four times in immunoprecipitation (IP) wash buffer (100 mM Tris, pH 9.0, 500 mM LiCl, 1% NP-40, 1% deoxycholic acid). The protein-chromatin complexes were eluted from the protein G agarose beads twice in IP elution buffer (50 mM $NaHCO_3$, 1% SDS), followed by reverse cross-linking in 0.3 M NaCl along with 1 μg of RNase-A at 67°C for 5 h. The reactions were precipitated with 2.5 volumes of ethanol at −20°C overnight. The reaction mixtures were then centrifuged at 13,200 rpm for 20 min, and the pellets were air-dried and resuspended in 100 μl of Tris-EDTA–proteinase K buffer (final reaction concentrations, 10 mM Tris, pH 7.5, 5 mM EDTA, 0.25% SDS, and proteinase K (1 U) and incubated at 45°C for 2 h. Subsequently, the samples were purified by phenol-chloroform extraction. NaCl (final concentration of 0.14 M), and 2.5 volumes of ethanol were then added, and the samples were allowed to precipitate overnight at −20°C. The samples were centrifuged at 13,200 rpm for 20 min, and the pellets were air dried and resuspended in 50 μl of water. Two microliters of the purified DNA was used for each PCR. In addition the input DNA was diluted 1:20, and the same volume was used in the PCR reaction. The PCR was performed with the following primers: Forward : 5′-acgtggcggagggactg, and Reverse: 5′-gccagggcttcccacgt.

PCR conditions were as follows: 94°C for 3 min, followed by 32 cycles at; 94°C for 0.45 min; 65°C for 0.30 min; and 72°C for 0.30 min. The ChIP (chromatin immunoprecipitation) PCR products were analyzed on a 2% agarose gel and analyzed using the Bio-Rad imaging system.

Population Doubling Assays

Primary BJ cells infected with pSRPshControl and pSRPshSIRT1(HS6) were grown in DMEM + 10% FBS and were subjected to a standard replicative lifespan assay. Late passage BJ fibroblasts strain ~7 PDs away from senescence was infected with pM-hTERT-IRES-EGFP vector (MSCV-based vector, Weinberg lab). Immediately after green fluorescent protein (GFP) was expressed these BJT cells were either infected with pSRP, pSRP-shControl, or pSRP-shSIRT1(HS6) viruses. After selection in 1 μg of puromycin for 4 d, the resistant cells were split and grown for a standard population-doubling analysis.

RT-PCR Analysis

For RT-PCR analysis, Trizol reagent was used to purify total RNA from cells. First-strand cDNA synthesis was performed as described by manufacturer (Amersham Biosciences, Piscataway, NJ). The sequence of primers used is described elsewhere (Nakamura *et al.*, 1997). The resulting cDNA were quantified on a Turner fluorometer, and equal DNA amounts were used for the PCR amplification. PCR amplification was performed using 25 cycles in presence of a ^{32}P-labeled forward hTERT primer. Products were resolved on 15% polyacrylamide gels and exposed to phosphoimager screens, and bands were quantified using Image Quant (Molecular Dynamics, Sunnyvale, CA/Amersham). hTERT signals were normalized to the GAPDH signal. Quantitative-PCR on an ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA) with SYBR Green chemistry (Qiagen). The cDNA preparation was similar to that of RT-PCR use; however, the template was used at a final concentration of 500 ng/reaction in a 20 μl total reaction volume. Each sample had been run through the Q-PCR (quantitative PCR) analysis in triplicate on freshly synthesized cDNA, using a no-template negative control for each sample set of cDNA and primers. Each 20-μl reaction contained 10 μl of SYBR Green master mix, 2 μl of template cDNA or water, 1 μl of forward and reverse primer mix at 0.6 μM each/reaction, and 7 μl of nuclease-free water.

Hematopoietic Stem Cell Analysis and Culture

The Sirt1 knockout strain (from Dr. Fred Alt, Harvard Medical School) was back-crossed five times onto a C57BL6 background before performing this study. In all experiments, young mice (3–9 wk old) were used. Mice were fed with a standard diet and maintained in a temperature- and light-controlled room (228C, 14L:10D; light starting at 0700 h), in accordance with the guidelines of the Laboratory Animal Services at the University of Hawaii and the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources National Research Council (DHEW publication 80-23, revised in 1985). The protocol for animal handling and treatment procedures was reviewed and approved by the Animal Care and Use Committee at the University of Hawaii. Hematopoietic stem cells (HSCs) were analyzed using flow cytometry as previously described (Allsopp *et al.*, 2002; Rossi *et al.*, 2005; Yilmaz *et al.*, 2006). Briefly, whole bone marrow (WBM) was flushed from the tibia and femur bones, and cells were stained with antibodies to c-Kit, Sca-1, plus a lineage cocktail, as well as either antibodies to Flk2 and CD34, or CD150 (SLAM). All analysis and cell sorting was performed on a FACS Aria (Becton-Dickinson). For HSC culture, complete media consisted of X-Vivo 15 media (BioWhittaker, Walkersville, MD) plus 5×10^{-5} M 2-mercaptoethanol, Steel factor (10 ng/ml), IL-3 (30 ng/ml), IL-6 (10 ng/ml), IL-11 (10 ng/ml), Tpo (10 ng/ml), and Flt3 ligand (10 ng/ml). Cells were cultured in standard tissue culture incubators at 5% CO_2.

RESULTS

Inhibition of SIRT1 and Telomerase Activity

Low hTERT-expressing primary human BJT diploid fibroblasts, Hela cells, and Lovo cells were infected with pSRP-shSIRT1(HS6), pSRP-ShSIRT1(HS11), and pSRP-shControl (a control shRNA). Cells were selected in puromycin and kept under selection for the remainder of experiments. SIRT1 expression was effectively reduced to varying degrees in all cell types by shSIRT1 (Figure 1, A–D). The HS6 shRNA was more effective than HS11 (Figure 1D). In all cell lines in which SIRT1 shRNA was stably expressed, we observed an increase in telomerase activity as measured by TRAP (Figure 1, E–G). As an additional control we ran the TRAP reaction in the presence of an internal control for which similar results was observed (Figure 1H). Furthermore, in order to rule out the possibility of off-target effects, we took two strategies. First, we used a second shRNA (HS11) for SIRT1 suppression (Figure 1, D and I), and most importantly we designed a shRNA-resistant SIRT1 gene, (SIRT1-R) in which we introduced six silent mutations in the region targeted by HS6. Expression of this shRNA-resistant mutant (Supplementary Figure 1B) of SIRT1 in BJT cells blocked the ability of shSIRT1(HS6) to induce telomerase activity (Figure 1J). It is noteworthy to mention that we found that higher than physiological quantities of wild-type SIRT1 or a SIRT1H363Y mutant can lead to enhancement or suppression of telomerase activity, respectively (Supplementary Figure 1C). Hence by using two independent shRNAs and a genetic rescue experiment we show that SIRT1 suppression is associated with an increase in telomerase activity and is not due to off-target effects.

SIRT1 Suppression and hTERT

To determine the mechanism through which SIRT1 suppresses hTERT activity, we performed RT-PCR and immunoblotting in shSIRT1-expressing cells. When SIRT1 was suppressed in Hela cells, an ≈3-fold increase in the level of hTERT protein was observed (Figure 2A), and this was accompanied by a 0.3-fold increase in the levels of hTERT mRNA both by in-gel RT-PCR (Figure 2B) and an insignificant but reproducible increase of 0.3–0.5-fold by real-time quantitative PCR (Figure 2, C and E). We conclude that SIRT1 controls endogenous and exogenous hTERT expression possibly at the level of RNA stability and/or through changes in chromatin structure at the hTERT promoter. To test this model, we performed ChIP experiments 240 nucleotides upstream of ATG in the hTERT promoter (Figure 2F). We used a pan-acetyl antibody against acetylated histone H4 and found that Hela cells in which SIRT1 was suppressed contained more total acetylated H4 on hTERT promoter than control cells expressing endogenous SIRT1. Furthermore, a small amount of SIRT1 was associated with hTERT promoter (Figure 2F) in control cells but not in SIRT1 knockdown cells. These results indicate that there is a transcriptional component (albeit small) to the observed effect. When we performed effective knockdown of SIRT1 in BJ-hTERT cells, we found that compared with controls, the BJ-hTERT-pSRP-SIRT1 cells showed a slower migrating band (Figure 2D). Although this suggests a posttranslational component by acetylation in stabilization of hTERT, further experimentation is required to show that the effect is directly through posttranslational modification of hTERT.

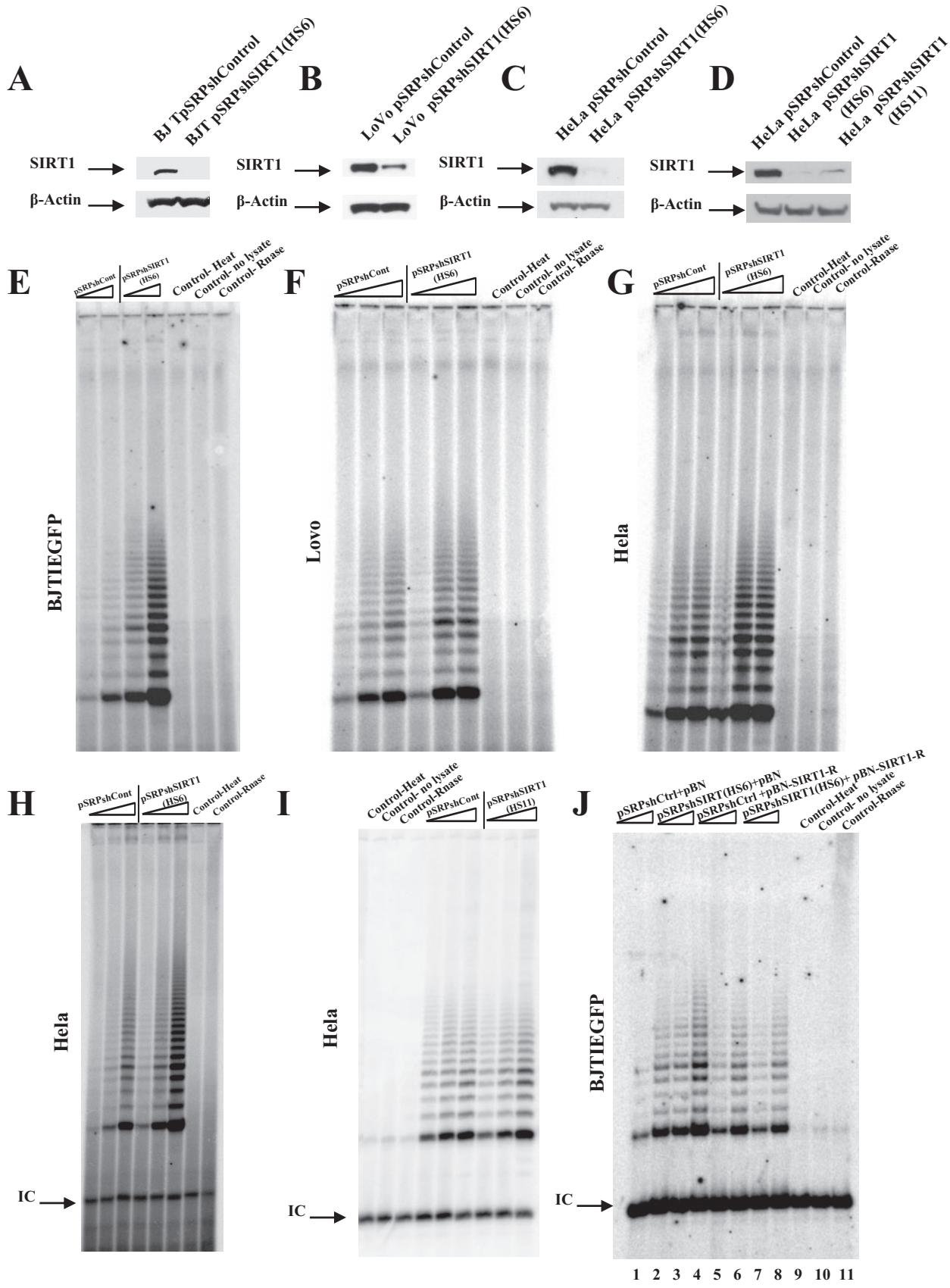

Figure 1. Effect of SIRT1 suppression on telomerase activity in human cells. (A) Suppression of SIRT1 in BJ-hTERT cells (BJT). BJT cells were infected with a control shRNA expressing retrovirus pSRP-shControl or SIRT1 knockdown virus pSRP-shSIRT1(HS6). Cell lysates were subjected to immunoblotting using an anti-SIRT1 antibody or a β-actin antibody. (B) Suppression of SIRT1 in Lovo cells. The same retroviral vectors as in A and in analysis were used. (C) Suppression of SIRT1 in Hela cells. The same retroviral constructs as in A were

SIRT1 Inhibition Cooperates with hTERT to Promote Cell Growth under Normal and Low Nutrient Conditions

Having shown that SIRT1 suppression is associated with increased telomerase activity, we wanted to determine if SIRT1 and telomerase functionally cooperate in a replicative lifespan assay in human cells. The shRNA-mediated repression of SIRT1 in primary BJ fibroblasts did not affect replicative lifespan in a long-term assay (Figure 3A). Furthermore, no significant effect on replicative lifespan was observed when SIRT1 was overexpressed (Figure 3B). Next, we asked if SIRT1 repression affected the growth of ectopic hTERT-expressing BJ cells. We first introduced hTERT in the same primary BJ cells, and then subjected these telomerase-positive cells to infection with the shSIRT1(HS6), control shRNA virus, or the backbone virus (pSRP). We observed that the population doubling time of cells expressing shSIRT1 was significantly reduced compared with cells infected with pSRP or pSRP-shControl (Figure 3, C and D) and that the endogenous SIRT1 was effectively repressed in the shSIRT1-expressing cells (Supplementary Figure 1D.). Hence the ability of SIRT1 to control the growth of BJ cells is observed only in the presence of hTERT expression. Ectopic expression of SIRT1-R reversed the enhanced growth phenotype of BJ cells expressing hTERT and shSIRT1(HS6) (Figure 3D). Hence the results of this genetic rescue experiment indicate that the effect of the SIRT1 shRNA in enhancement of cell growth is specific to SIRT1 and is independent of any off-target effects of the shRNA used.

Effect of Glucose Withdrawal on SIRT1-depleted BJT Cells

When cells are exposed to glucose withdrawal they are known to undergo cell cycle arrest followed by death. When we exposed BJT-pSRP-shControl and BJT-pSRP-shSIRT1(HS6) cells expressing telomerase to nutrient withdrawal by exposing them to media containing no glucose, we found that BJT cells expressing ectopic telomerase with no SIRT1 expression could survive and divide much longer in the initial phases of glucose withdrawal (Figure 3E). Although both control and knockdown cells died at approximately the same time (5th day; Figure 3E). In the control BJT cells, the levels of SIRT1 were gradually increased after glucose depletion and activated AMPK levels gradually increased with time. However, in SIRT1 suppressed BJT cells subjected to glucose withdrawal there was a significant increase early on in total AMPK levels and phosphorylated AMPK protein levels at 4–8 h (AMPK-α Thr 172 phosphorylation).

Increased Proliferative Capacity of Hematopoietic Stem Cells in Animals Lacking SIRT1

To extend our findings to a more physiological system, we assayed the effect of SIRT1 deficiency on the establishment of the primitive HSC compartment, by quantitating the total number of HSCs and multipotent progenitors in BM from young (3–9 wk) Sirt1 knockout (*Sirt11*$^{-/-}$) and control mice by flow cytometry using rigorous cell surface criteria for isolating HSCs and progenitor cells (Rossi *et al.*, 2005) (Supplementary Figure 1). These analyses showed that establishment of neither the HSCs nor multiprogenitor subsets were significantly impacted in the absence of Sirt1 (Figure 4A). Similar results were observed when alternative markers for isolating HSCs were used (Kiel *et al.*, 2005; not shown). These results suggest that Sirt1 does not play an important role in establishing HSC homeostasis in young adult mice housed in a stress-free environment.

To assess the capacity of young Sirt1-deficient HSCs to proliferate in response to mitogenic stimuli, we purified HSCs from *Sirt1*$^{-/-}$ and control mice by fluorescence-activated cell sorting, cultured the cells in cytokine-rich media, and then quantitated the total number of progeny cells generated after 7 d (Figure 4B). Strikingly, these experiments revealed that the *Sirt1*$^{-/-}$ HSCs exhibited a three- to fivefold ($\approx$20,000 cells) increased proliferative capacity compared with Sirt1+/− HSC controls (Figure 4B). To address the capacity of Sirt1-deficient HSCs to proliferate under conditions of nutrient deprivation, we clone sorted HSCs from *Sirt1*$^{-/-}$ or *Sirt1*$^{+/-}$ mice into individual wells of Terasaki plates containing cytokine-deprived media and monitored the number of wells in which cell proliferation could be detected (i.e., wells containing two or more cells). As shown in Figure 4, a significantly greater number of *Sirt1*$^{-/-}$ HSCs were capable of proliferating in media in the presence of single cytokines with either IL-3 (Figure 4C) or SCF (Figure 4D) compared with control HSCs, indicating that Sirt1-deficient HSCs have a greater capacity than controls to proliferate under these restrictive conditions.

To determine whether Sirt1 expression per se is affected by cell proliferation, we purified HSCs, as described above, and either immediately isolated RNA from resting HSCs (HSC-R), or cultured HSCs in complete media for 4 d before RNA isolation from actively cytokine stimulated HSCs (HSC-S). As shown in Figure 4E, real-time RT-PCR analysis of Sirt1 mRNA levels relative to Hprt reveals a small (about twofold) but significant increase in Sirt1 levels in cytokine stimulated HSCs. Although this result suggests that Sirt1 expression may be cell cycle dependent in HSCs, it is important to note that cytokine-stimulated HSCs undergo extensive differentiation in vitro. Thus it remains to be determined to what extent the regulation of Sirt1 levels has physiologically relevant affects on the proliferation of HSCs in vivo.

DISCUSSION

Here we present results showing that SIRT1, the NAD-utilizing deacetylase enzyme is a negative regulator of growth under normal and restrictive conditions in certain cell lineages. Consistent with this notion, efficient inhibition of SIRT1 deacetylase was associated with an increase in telomerase activity that is required for survival and long-term cell growth. Our data indicate that the effect of SIRT1 on telomerase activity is mediated through the catalytic

Figure 1 (cont). used. (D) Suppression of SIRT1 in Hela cells using control shRNA retrovirus and two shSIRT1 (HS6) and shSIRT1 (HS11) constructs.(E) Parental BJ cells expressing ectopic pM (MSCV)-hTERT-IEGFP were infected with pSRP-shControl RNA or pSRP-shSIRT1 (HS6) virus. Within 6 PDs after selection in puromycin, cells were lysed in CHAPS lysis buffer, and equal protein quantities (50 and 300 ng) were subjected to TRAP analysis to determine telomerase activity. Control RNase and heat treatments all contained 300 ng of protein lysate. (F) Lovo cells were infected twice with the viruses and were subjected to TRAP analysis. Protein amounts of 50, 200, and 600 ng were used in the TRAP reaction. (G) Same as F except Hela cells were used. (H) Same as in G except internal controls were included using HeLa cell extracts (10, 50, and 200 ng). (I) A second shRNA (HS11) was used to suppress SIRT1 in Hela cells and TRAP analysis was performed as in H. (J) Same cells as in A (BJ-hTERT-IEGFP with and without shRNA against SIRT1) were infected with pBabe-neo (PBN) control vector or with an shRNA-resistant silent SIRT1-R expressing construct (PBN-SIRT1-R) generating four additional lines. Two protein concentrations (50 and 200 ng) from four cell line were subjected (total of 16) to the TRAP analysis. The first six lanes are experimental controls. Lanes 7 and 8 are results of rescue experiments. The last three lanes are negative controls for the TRAP reaction.

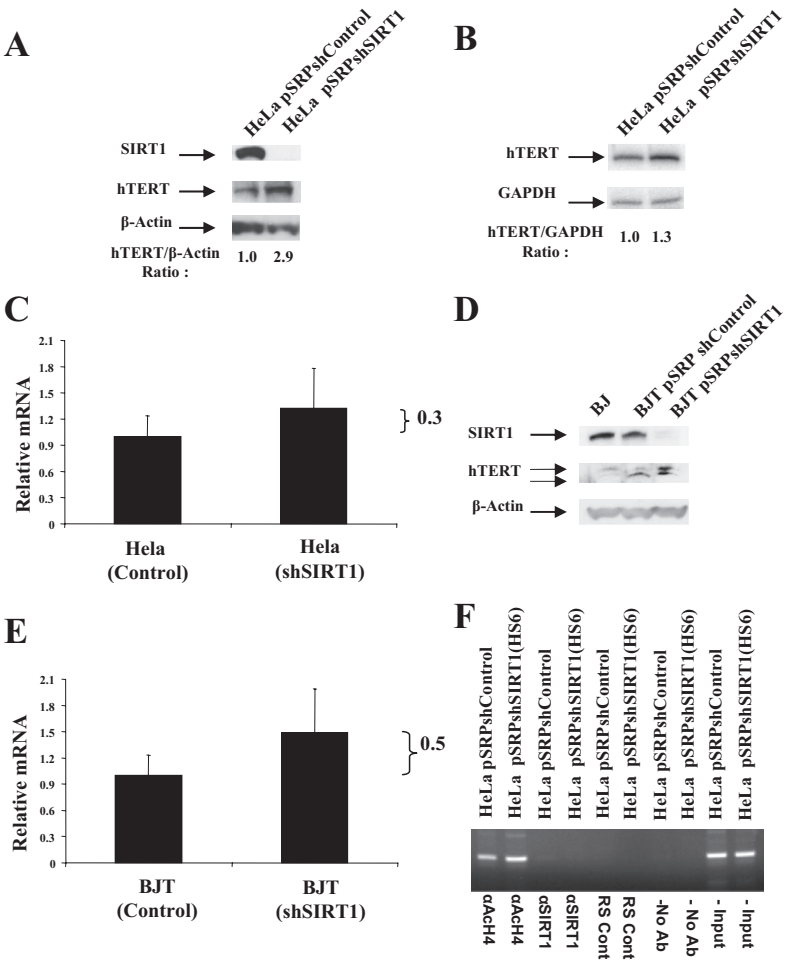

Figure 2. Regulation of hTERT. (A) Effect of SIRT1 suppression on hTERT protein in Hela cells. Western blot analysis was performed on cell lysates, and they were subjected to immunoblotting with anti-SIRT1, anti-hTERT, and anti-β-actin antibodies. (B) Effect of SIRT1 suppression on hTERT mRNA in Hela cells. Total RNA was isolated from Hela-pSRP-shControl and Hela-pSRPshSIRT1 cells and hTERT mRNA was quantified by quantitative radioactive in-gel PCR as described (Nakamura *et al.*, 1997). The ratio of hTERT/GAPDH is shown. (C) Quantification of hTERT mRNA in Hela and Hela-pSRPshSIRT1 cells by real-time Q-PCR. The values shown are normalized to an internal GAPDH control. (D) Regulation of hTERT protein in BJT cells. BJT-pSRP-shControl and BJT-pSRPshSIRT1 cell lysates were resolved on 4–12% gradient gels, and immunoblotting was performed using an anti-hTERT rabbit antibody. Primary BJ cells in the first lane were used as negative control. (E) Effect of SIRT1 suppression on hTERT mRNA in BJT cells. Same as in C except that BJT and BJT-pSRPshSIRT1 cells were used. (F) ChIP of hTERT promoter using the antibodies shown. Hela control and SIRT1(HS6) knockdown cells were used in each ChIP reaction as shown. Antibodies used were against total acetylated H4 and SIRT1. Controls were rabbit serum (RS) and no antibody reactions. For details consult *Materials and Methods*.

subunit of telomerase, hTERT. On SIRT1 inhibition there is a small increase in hTERT mRNA level and a significant increase in levels of hTERT protein. This increase in mRNA correlated with lack of SIRT1 at proximal regions of hTERT promoter and an increase in total H4 acetylation at the hTERT promoter. Cell lines expressing either endogenous hTERT under its native promoter or primary human diploid fibroblasts expressing ectopic hTERT showed increased levels of hTERT protein and activity upon SIRT1 suppression. We find that the suppression of SIRT1 and its effects on telomerase are independent of how hTERT is expressed (i.e., under native or ectopic viral promoters). Interestingly, we also observed an hTERT doublet in BJT cells in which SIRT1 was expressed suggesting a post-translational role for SIRT1 in regulation of hTERT protein stability. However, further experimentation is required to investigate if this is caused by increased hTERT acetylation.

The increased telomerase activity and cell growth phenotype observed could be rescued by a silent mutant SIRT1-R protein that is resistant to repressive effect of shRNA directed to SIRT1, showing that the effect observed was specific. Our results point toward a functional interaction between SIRT1 and hTERT; however, the basis for this genetic interaction is unknown, and it is possible that the effect of SIRT1 on hTERT is not direct and is mediated via other proteins. Furthermore, given the diverse range of SIRT1 targets the effects observed on hTERT maybe one factor that contributes to the observed cellular phenotype.

Overexpression of SIR2 extends replicative lifespan of single-cell eukaryotes such as *S. cerevisae* and chronological lifespan of multicellular protostomes such as *C. elegans*. We reasoned that if the lifespan-inducing functions of the mammalian SIR2 homolog SIRT1 is conserved, this should reflect itself in either survival or replicative lifespan of vertebrate cells with long life spans, such as somatic cells of *Homo sapiens*.

When we overexpressed wild-type SIRT1 in mortal normal human diploid BJ fibroblasts, we observed no significant effect on replicative lifespan, consistent with published data (Michishita *et al.*, 2005). We also performed the reverse experiments by suppressing SIRT1 to near detection limits in primary BJ fibroblasts, and we still did not observe any effects on replicative life span. Because it has been shown before by us and others that ectopic expression of hTERT and reconstitution of its activity causes life span extension in human cells, we reasoned that inhibition of SIRT1 may have an effect on telomerase-induced extension of lifespan. If primary BJ cells were first infected with an hTERT-expressing virus and sequentially were subjected to SIRT1 inhibition, there was an increased efficiency in cell growth reflected by a decrease in the population doubling time. This effect could be mediated through telomeres or other indirect effects on cell survival. It is however clear from our data that SIRT1 suppression promotes cell growth in the presence of ectopic telomerase activity. Our findings in human cells are consistent with that of others who have shown that murine fibroblasts deficient for Sirt1(Sir2α) have a higher frequency of immortalization (Chua *et al.*, 2005). In contrast, others have shown that in different cell types such

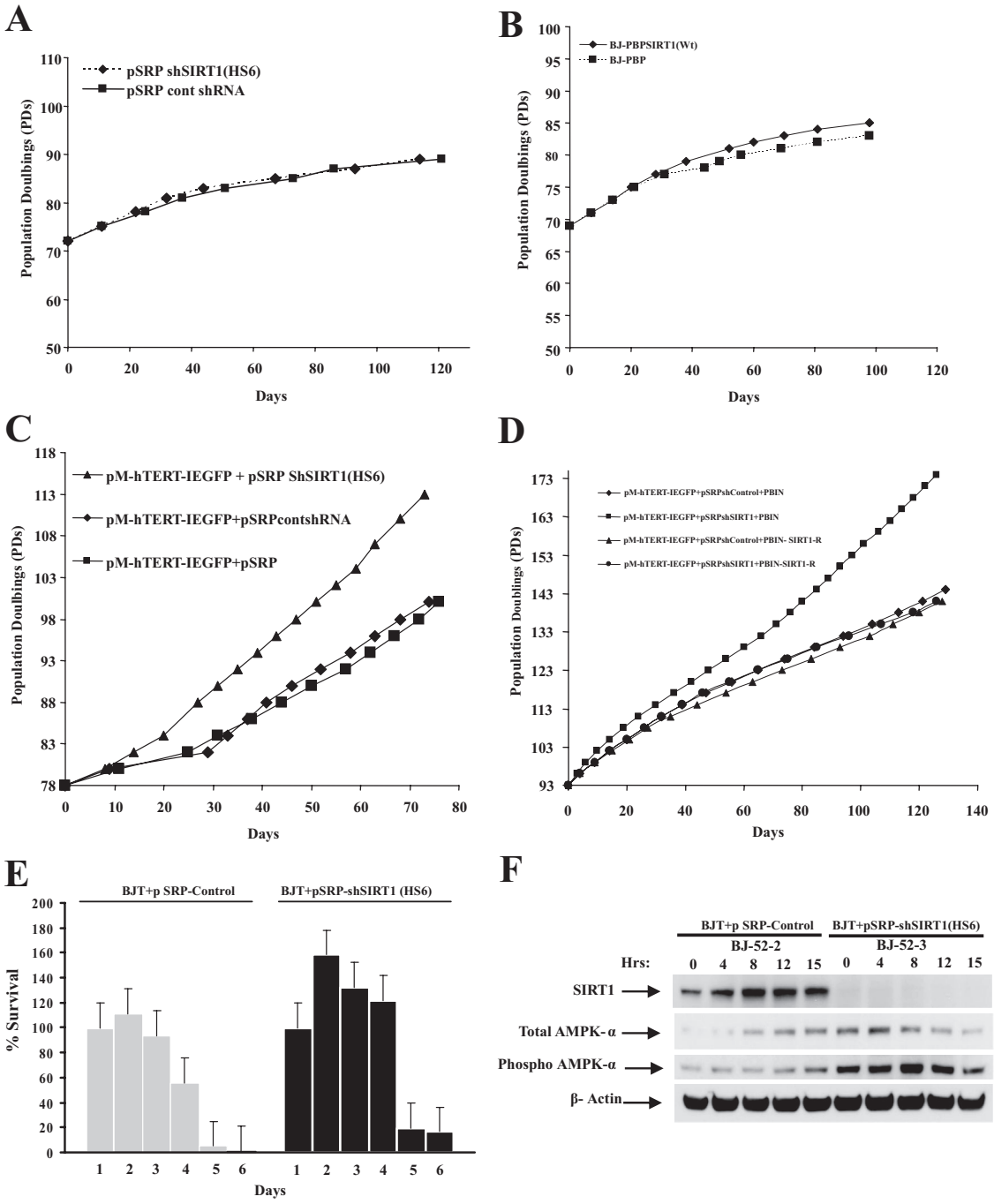

Figure 3. Cooperative effects of SIRT1 knockdown and hTERT expression on cell growth and survival. (A) SIRT1 suppression effects on lifespan of primary BJ fibroblasts. Late-passage BJ cells (≈7 PD before senescence) were infected with either pSRP-shControl or pSRPshSIRT1 (HS6) and pSRP control shRNA vector viruses and cells were subjected to selection in puromycin. (B) Overexpression of wild-type SIRT1 in primary BJ fibroblasts. Same as in A, except for the overexpression constructs (pBabe-Puro-wtSIRT1) and pBabe-Puro control vector were used. (C) Effects of SIRT1 knockdown on growth of BJT (hTERT-IRES-EGFP) fibroblasts. Late-passage BJ fibroblasts were infected with an hTERT containing virus, and the resulting BJT cells were subsequently infected by pSRP-shControl or BJ-pSRPshSIRT1 (HS6) viruses and subjected to a standard replicative lifespan assay. (D) Rescue of the biological effect of shRNA by an shRNA-resistant mutant. BJT cells were infected with pSRP-shControl or BJ-pSRPshSIRT1(HS6) viruses and subsequently infected with the rescue construct PBN-SIRT1-R or PBN control alone. Cells were kept under puromycin and neomycin selection throughout the experiments. BJT cells and their rescue counterparts generated were subjected to a long-term replicative assay as before to asses the ability of SIRT-R to rescue the phenotype of BJT cells expressing the SIRT1 shRNA. (E) BJT cells expressing control of SIRT1 shRNA were subjected to glucose withdrawal on day 0 and cell viability was measured for a week. Experiments were performed in duplicate dishes. Error bars, SEM. (F) Same strains as in E were subjected to glucose withdrawal and at the shown time point (hours after withdrawal), and cell lysates were prepared and subjected to immunoblotting with an anti-SIRT1, anti-phosphor-AMPK-α (Thr-172), total AMPK-α and β-actin antibodies.

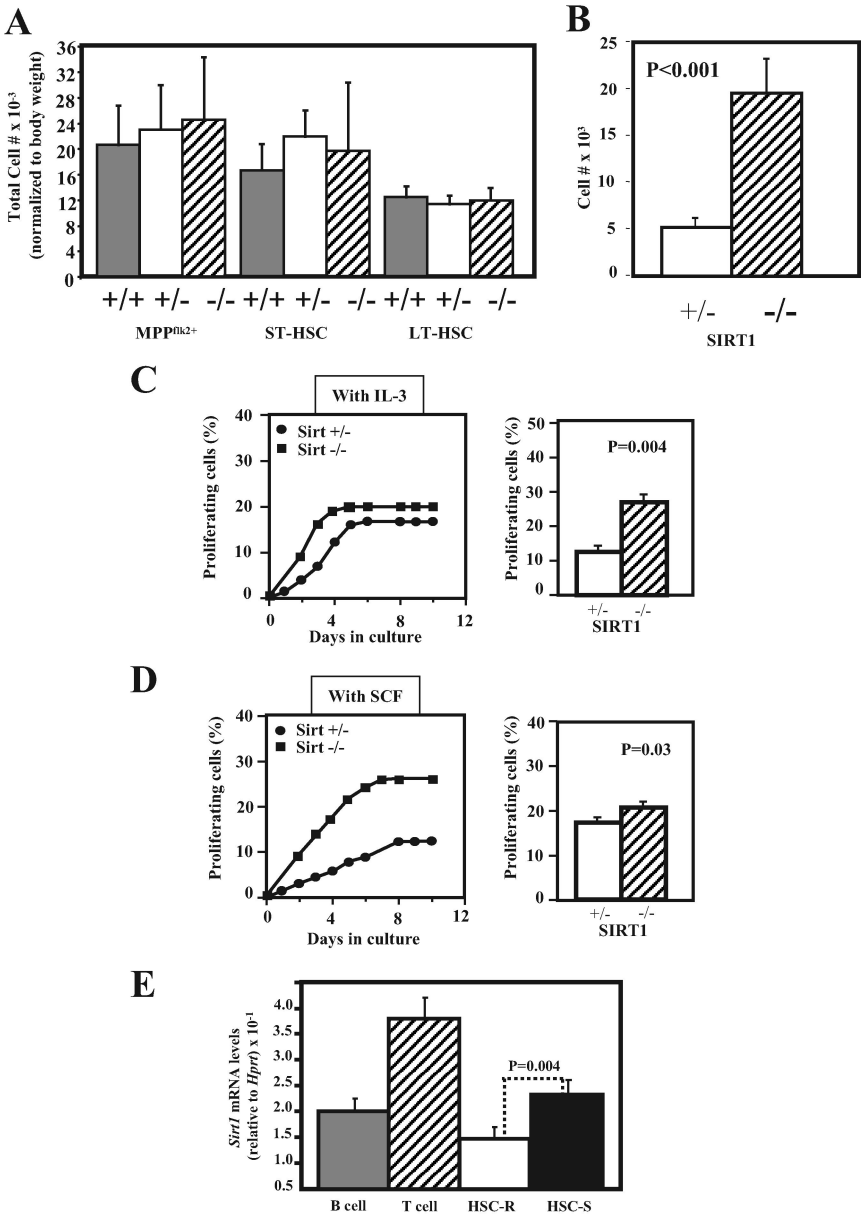

Figure 4. Analysis of the effect of Sirt1 deficiency on HSCs and progenitor frequency and proliferation. (A) Analysis of the affect of Sirt1 deficiency on HSCs and progenitor numbers. Bone marrow was harvested, red blood cells were lysed, and the remaining white cells were stained with fluorophor-conjugated antibodies to markers for HSCs and progenitors (see *Materials and Methods* for details). Multipotent progenitors and short-term and long-term HSCs are defined as the c-Kit$^+$Sca-1$^+$Lin^{neg}Flk2$^+$CD34$^+$ fraction, the c-Kit$^+$Sca-1$^+$Lin^{neg} Flk2^{neg}CD34$^+$ fraction, and the c-Kit$^+$Sca-1$^+$Lin^{neg}Flk2^{neg}CD34^{neg} fraction of WBM, respectively. The average total cell number for each type of cell, normalized to body weight, is shown (for each bar, n $\geq$ 3). Error bars, SD. (B) Quantitative analysis of cell numbers after 1 wk of growth in complete media. BM was stained as described in *Materials and Methods*, and 100 HSCs were sorted directly into individual wells of a 48-well plate containing complete media. Bars represent average counts from five wells. HSCs are defined as the c-Kit$^+$Sca-1$^+$SLAM(CD150)$^+$Lin^{neg}Flk2^{neg} fraction of WBM. (C) Left, graph represents read out over time of Terasaki plates containing single HSCs per well. The HSCs were sorted into Terasaki plates containing serum free X-Vivo media plus IL-3, and plates were monitored daily for the number of wells containing proliferating cells (i.e., more than one cell). Right, graph represents average value of frequency of proliferating HSCs from four Terasaki plates. HSCs are defined as the c-Kit$^+$Sca-1$^+$SLAM(CD150)$^+$Lin^{neg}Flk2^{neg} fraction of WBM. For all experiments, mice were 3–9 wk old. p values represent results from Student's *t* test. (D) Same experiments as in C were performed except that cells were sorted into media containing Steel factor. (E) Analysis of Sirt1 mRNA levels in resting and proliferating HSCs. HSCs (n = 1000) were purified from young adult mice (n = 3), and RNA was either extracted immediately for analysis of resting HSCs (HSC-R) or cells were stimulated to proliferate in media (X-Vivo15 serum-free media [Stem Cell] plus 20 ng/ml Steel factor, 10 ng/ml IL-6, 30 ng/μl IL-3, 2 mM L-Glu, and 50 μM mercaptoethanol) for 5 d for analysis of proliferating HSCs (HSC-S). Both T-cells (CD-3+B220negMac1neg) and B-cells (B220+CD-3negMac1neg) were purified from the bone marrow as a reference. RNA was extracted using Trizol, cDNA was synthesized using Superscript III (Invitrogen), and real-time PCR was performed using primers specific for Hprt (reference) and Sirt1 yielding single amplicons of 100 and 150 bp, respectively. Forty cycles of PCR was performed in triplicate for all samples using an iCycler real-time PCR machine (Bio-Rad). For each cell type, the average level of Sirt1 is shown, relative to Hprt.

as endothelial cells SIRT1 suppression has the opposite effect: its loss induces cell cycle arrest (Ota *et al.*, 2007). Given the range of substrates currently identified for SIRT1 and their increasing number, it is possible that the contradicting growth-promoting and growth-suppressing properties observed are cell type or species specific.

Extension of our in vitro results to hematopoiesis under adverse conditions caused by lack of growth factors is consistent with the notion that SIRT1 is a growth suppressor. Although we observed no appreciable difference in HSCs or progenitor frequencies in young *Sirt1*$^{-/-}$ mice, the in vitro proliferative capacity of Sirt1-deficient HSCs were significantly elevated in both complete media and under cytokine-deprived conditions containing a single growth factor. These results were consistent with that of immortalized human BJT cells lacking SIRT1 expression that showed higher proliferation under normal or glucose-deprived conditions. We found that consistent with the role of activated AMPK in response to low glucose (Salt *et al.*, 1998), cells lacking SIRT1 showed an earlier peak in both total levels and activated phospho-AMPK-α protein upon glucose deprivation. Activation of AMPK hence may allow survival in response to an energy shortage. Although this finding suggests that SIRT1 may regulate AMPK, others have found that induction of AMPK by the SIRT1 activator resveratrol is SIRT1 independent (Dasgupta and Milbrandt, 2007). Although a useful marker of energy status and survival, AMPK induction observed here maybe due to a complex and indirect effect of SIRT1 on cell survival under ATP-limiting conditions.

It is possible that under nutrient-restrictive conditions, SIRT1 acts as a growth suppressor to limit division in high-capacity progenitor cells. This limitation may be a physiological response to save on usage of macromolecules required for survival of pre-existing stem cells. Hence, SIRT1 can modulate the division and survival capacity of stem cells in response to nutrient availability. Our results have significant implications for survival of adult stem cells under stress and would be of interest to examine whether SIRT1 has similar effects in other types of stem cells. They also indicate that specific chemical inhibitors of SIRT1 may enhance survival or pluripotency in adult or embryonic human or murine stem cells.

Evidence suggests that calorie restriction is associated with decreased age-associated tumor incidence (Weindruch, 1992). Furthermore, the beneficial biological effects of calorie restriction in increasing lifespan have been well documented. Therefore, it is possible that in human cells, calorie restriction can increase SIRT1 activity, which in turn can suppress immortalizing genes such as telomerase. Therefore increased SIRT1 activity would then suppress tumor incidence and therefore only indirectly leads to extension of lifespan. Hence the effects of induction of molecules such as SIRT1 on longevity of complex multicellular vertebrates may be mediated indirectly via stimulating its tumor suppressor functions and hence reduce death due to cancer. We predict that overexpression of SIRT1 in mice would primarily result in suppression of certain types of tumors. Based on our results and models, SIRT1 overexpression may have no functional effect on the network of human genes promoting somatic cell chronological/replicative survival, leading directly to increased longevity. Current lack of a unifying evolutionary conservation in longevity functions of SIR2 however should not detract from its fundamental roles in cellular survival and growth from yeast to mammals.

Our findings underscore the importance of nutrient-dependent pathways and propose that SIRT1 is a nutrient-sensitive growth suppressor that may act as an important barrier to retard the growth of certain nutrient-sensitive immortal tumor cells.

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