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Progressive loss of SIRT1 with cell cycle withdrawal

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Summary

Sir2 is an NAD^+-dependent deacetylase that regulates lifespan in yeast, worms and flies. The mammalian orthologs of Sir2 include SIRT1 in humans and mice. In this study, we analyzed the level of SIRT1 in human lung fibroblasts (IMR90) and mouse embryonic fibroblasts (MEFs) from mice with normal, accelerated, and delayed aging. SIRT1 protein, but not mRNA, decreased significantly with serial cell passage in both human and murine cells. Mouse SIRT1 decreased rapidly in prematurely senescent (p44 Tg) MEFs, remained high in MEFs with delayed senescence (Igf-1r–/–), and was inversely correlated with senescence-activated β-galactosidase (SA-βGal) activity. Reacquisition of mitotic capability following spontaneous immortalization of serially passaged wild-type MEFs restored the level of SIRT1 to that of early passage, highly proliferative MEFs. In mouse and human fibroblasts, we found a significant positive correlation between the levels of SIRT1 and proliferating cell nuclear antigen (PCNA), a DNA processing factor expressed during S-phase. In the animal, we found that SIRT1 decreased with age in tissues in which mitotic activity also declines, such as the thymus and testis, but not in tissues such as the brain in which there is little change in mitotic activity throughout life. Again, the decreases in SIRT1 were highly correlated with decreases in PCNA. Finally, loss of SIRT1 with age was accelerated in mice with accelerated aging but was not observed in long-lived growth hormone-receptor knockout mice. Thus, as mitotic activity ceases in mouse and human cells in the normal environment of the animal or in the culture dish, there is a concomitant decline in the level of SIRT1.

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Introduction

Cellular senescence, which refers to the process by which primary cells in culture lose their ability to divide, has been a useful model for the study of mammalian aging for decades (Hayflick & Moorhead, 1961); yet not all or even most of the molecular details by which this process occurs are known. Cellular senescence appears to parallel organismal aging, and cells from mice or humans with various premature aging syndromes undergo senescence in culture more rapidly than do cells from normal mice or humans (Lombard *et al*., 2005). How, or even if, cellular senescence contributes to organismal aging, however, is not known. Senescent cells accumulate *in vivo* as the organism ages, as well as at sites of pathology (Itahana *et al*., 2004). One theory holds that senescence of stem or progenitor cells impairs tissue renewal, limiting the ability of the organism to replace cells that die or wear out with time and leading to the deterioration of the body and frailty we associate with old age.

One molecule that is known to affect senescence is the NAD^+-dependent deacetylase Sir2 (Imai *et al*., 2000; Landry *et al*., 2000; Smith *et al*., 2000). Although first identified in yeast as a gene involved in mating type switching (Rine & Herskowitz, 1987), the *SIR2* gene is highly conserved in organisms ranging from archea to humans (Brachmann *et al*., 1995), where its role is much broader. Overexpression of Sir2 extends replicative lifespan in yeast (Kaeberlein *et al*., 1999), and Sir2 orthologs extend organismal lifespan in both worms (Tissenbaum & Guarente, 2001) and flies (Rogina & Helfand, 2004).

Of the seven Sir2 family homologues (sirtuins) in humans (Frye, 1999, 2000), SIRT1 is the most closely related to Sir2 of *Saccharomyces cerevisiae* (Frye, 2000). In contrast to yeast, SIRT1 was found not to extend replicative lifespan when overexpressed in human fibroblasts or epithelial cells under standard culture conditions (Michishita *et al*., 2005), and deletion of the normal allele of Sirt1 from heterozygous *Sirt1* +/– mouse embryonic fibroblasts (MEFs) in culture dramatically increased replicative lifespan (Chua *et al*., 2005). Thus, how SIRT1 is involved in mammalian replicative senescence remains unclear.

Observations from a number of organisms showing that changes in Sir2 expression accompany changes in the ability of cells to divide, led us to explore this link in greater detail. We examined the levels of the mammalian Sir2 ortholog, SIRT1, under conditions that alter mitotic activity both *in vitro* and *in vivo*. The results of this study imply that mammalian SIRT1 might interact with the cell cycle on multiple levels.

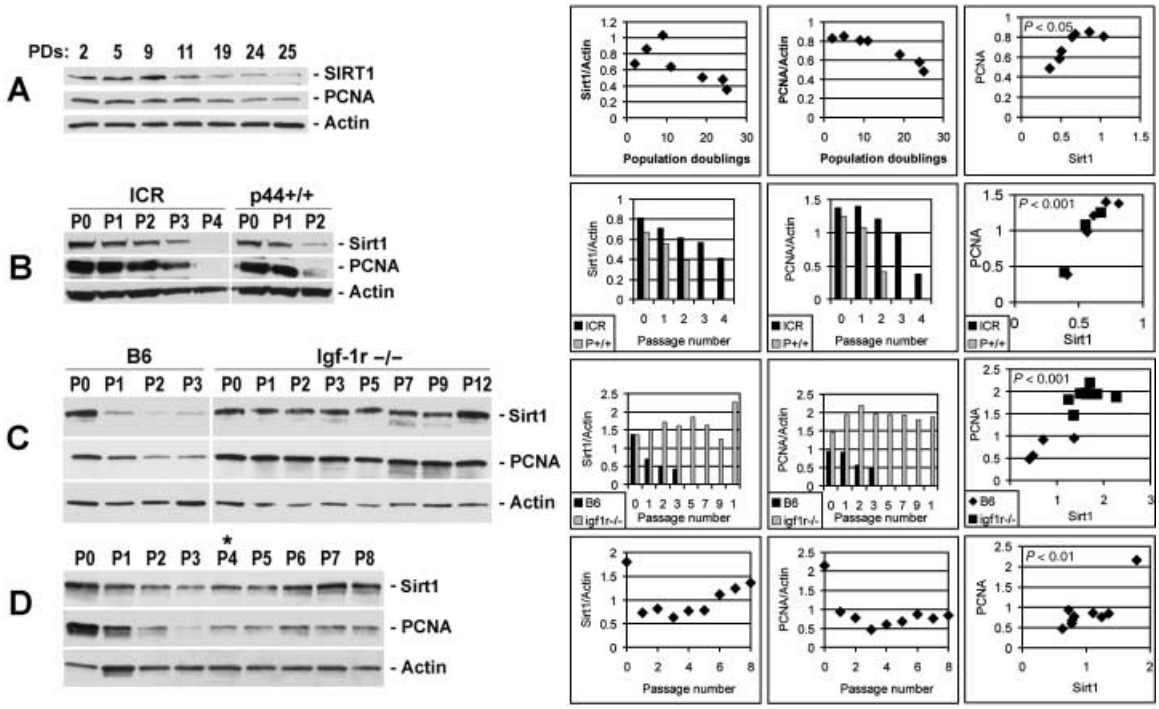

Fig. 1 SIRT1 protein level decreases with serial cell passage and is coupled to PCNA level. (A) Western blot of extracts from IMR90 human lung fibroblasts. Note the coordinated decline in SIRT1 and PCNA with serial cell passage. (B) Western blot of extracts from ICR (left) and p44+/+ (right) MEFs. Note that both SIRT1 and PCNA decrease faster in p44+/+ MEFs, which senesce prematurely, compared to NT MEFs. (C) Western blots of extracts from B6 (left) and Igf1r–/– (right) MEFs. Note that both SIRT1 and PCNA levels remain high in Igf1r–/– MEFs, which did not senesce in culture, compared to normal MEFs, which did. (D) Western blot of extracts from NT MEFs from ICR strain that underwent spontaneous immortalization at passage 4 (P4*). Note that SIRT1 and PCNA protein levels are coupled to each other and to cellular mitotic activity. Results of densitometric analysis of each blot were plotted and are shown in graphs on the right side of each figure. In each experiment, levels of SIRT1 and PCNA showed significant linear correlation (*F*-test, *P*-values indicated in graphs). NT, nontransgenic; P, passage; PDs, population doublings; B6, C57Bl6.

Results

The level of SIRT1 decreases with serial cell passage as cells stop dividing

Replicative lifespan in yeast cells is sensitive to the dosage of SIR2 (Kaeberlein *et al.*, 1999). In order to determine if such a relationship exists in mammalian cells, we analyzed the level of SIRT1 in human and murine cells during serial cell passage. Human lung fibroblasts (IMR90) were serially passaged at a ratio of 1 : 8 in 10-cm plates, and protein levels determined by Western blot analysis. As shown in Fig. 1A, as the number of population doublings of IMR90 cells increased, there was progressively less SIRT1 in the extracts. Similarly, in normal MEFs passaged at a ratio of 1 : 4 in 10-cm plates, the level of SIRT1 decreased with increasing passage number in MEFs prepared from two different strains of mice (Fig. 1B, ICR, and Fig. 1C, B6).

We next determined if the level of SIRT1 in murine cells in which senescence is accelerated or delayed relative to normal declined at a faster or slower rate, respectively. In MEFs from p44 transgenic mice, which express a short isoform of p53 (p44) and senesce more rapidly than normal MEFs (Maier *et al.*, 2004), the rate at which SIRT1 levels declined was more rapid than in nontransgenic MEFs of the same strain (ICR) (Fig. 1B, compare ICR to p44+/+). These observations were confirmed in two independent serial cell passage experiments. Conversely, MEFs that senesce more slowly in culture showed little decline in SIRT1 levels with passage number. Igf-1r–/– MEFs, for example, were serially passaged at a ratio of 1 : 4 until passage 4, then at a ratio of 1 : 8. These cells did not undergo any of the changes associated with senescence, such as cell flattening and enlargement, even after approximately 34 population doublings (passage 12; data not shown). As shown in Fig. 1C, they also showed little if any decline in SIRT1 compared to normal MEFs of the same strain (C57Bl6).

To get a rough estimate of how mitotically active the cells were at each successive passage, we determined the level of a DNA polymerase processing factor, proliferating cell nuclear antigen (PCNA), in the same extracts used for SIRT1 determination. We found a significant linear correlation between levels of SIRT1 and PCNA [Fig. 1A–D, *F*-test, (A), $P < 0.05$; (B), $P < 0.001$; (C), $P < 0.001$; (D), $P < 0.01$]. We were able to confirm this observation in MEFs that underwent spontaneous immortalization in culture. Following a decline in both SIRT1 and PCNA levels between passage 0 and passage 3, the amounts of both proteins began to increase, reaching presenescence levels again at about passage 6 (Fig. 1D).

SIRT1 is regulated at the protein, not the RNA, level

The observed decline in the level of SIRT1 could have been caused by changes at the transcriptional level or the post-transcriptional level. To distinguish between these possibilities, we analyzed the levels of *SIRT1* mRNA in serially passaged cells. The results are shown in Fig. 2 for IMR90 cells (A), normal ICR MEFs (B), and p44 transgenic MEFs (C). In contrast to protein levels, the level of *SIRT1* mRNA did not decrease significantly with passage [IMR90 cells (A): *F*-test, $P > 0.1$ for correlation between population doubling and *SIRT1* mRNA level; ICR MEFs (B): *t*-test, $P > 0.1$ between passage 0 and 3; and p44+/+ transgenic MEFs (C): *t*-test, $P > 0.05$ between passage 0 and 2]. Furthermore, when we compared protein levels in IMR90 cells (Fig. 1A) to mRNA levels (Fig. 2A), we did not find a significant linear correlation between the two (Fig. 2D: *F*-test, $P > 0.1$). Thus, the changes in the level of SIRT1 protein occur post-transcriptionally.

The level of SIRT1 increases when cells are stimulated to divide

Based on our findings with cells subjected to serial cell passage, we hypothesized that the level of SIRT1 protein in a cell might be linked to its mitotic activity. In order to test this hypothesis, we cultured IMR90 human lung fibroblasts (Fig. 3A) and normal C57Bl6 MEFs (Fig. 3B) under different serum concentrations. Both SIRT1 and PCNA increased when cells were stimulated with fetal bovine serum (FBS) and compared to nonstimulated cells. However, SIRT1 appeared to be more sensitive to serum concentration, reaching maximal induction at a lower concentration than that needed for maximal PCNA induction. When cells were treated with aphidicolin, a DNA polymerase inhibitor that should block cells in S-phase, the level of SIRT1 did not go up with serum stimulation (Fig. 3C). At the same time, the increase in PCNA level indicated that cells had entered S-phase prior to the block.

SIRT1 decreases specifically in cells that stop dividing

Next, we used histochemical techniques to correlate loss of SIRT1 with the onset of senescence in populations of normal cells undergoing successive cell divisions in culture. Cells with prominent senescence-activated β-galactosidase (SA-βGal) activity (Fig. 4, arrowheads, left panel) did not react with an antibody against SIRT1 (middle panel), while cells that showed only slight SA-βGal activity (Fig. 4, arrows) retained some SIRT1 reactivity, and cells that showed no SA-βGal activity showed high SIRT1 reactivity. Thus, the decrease in SIRT1 with senescence can be attributed to a decrease in the population of cells that continue to proliferate rather than an overall decrease in SIRT1 expression in all cells.

In summary, these data indicate that the level of SIRT1 in mammalian cells both correlates positively with mitotic activity and decreases with replicative senescence.

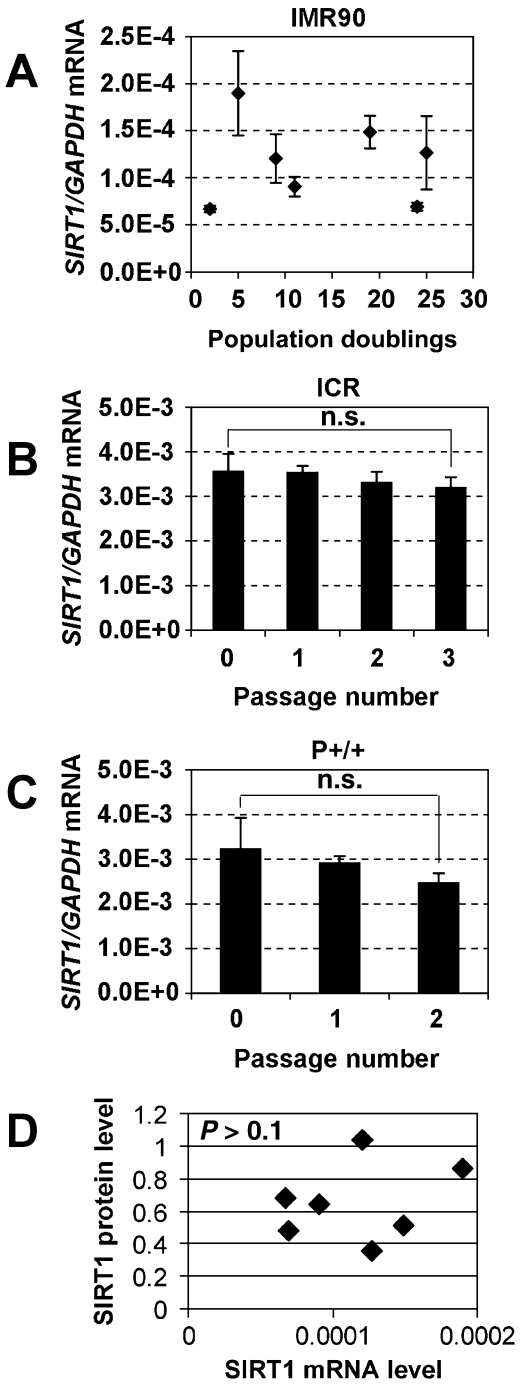

Fig. 2 SIRT1 mRNA level does not correlate with number of population doublings *in vitro*. *SIRT1* mRNA level was standardized to *GAPDH* mRNA level as determined by qRT–PCR. *SIRT1* mRNA level does not correlate with (A) number of population doublings in IMR90 human lung fibroblasts (*F*-test, $P > 0.1$), or passage number in (B) ICR MEFs (*t*-test, $P > 0.1$ between passage 0 and 3) or (C) p44+/+ transgenic MEFs (*t*-test, $P > 0.05$ between passage 0 and 2). Error bars represent the standard deviations of three measurements of each sample. There was no significant linear correlation between levels of SIRT1 mRNA and protein in IMR90 cells (D: *F*-test, $P > 0.1$).

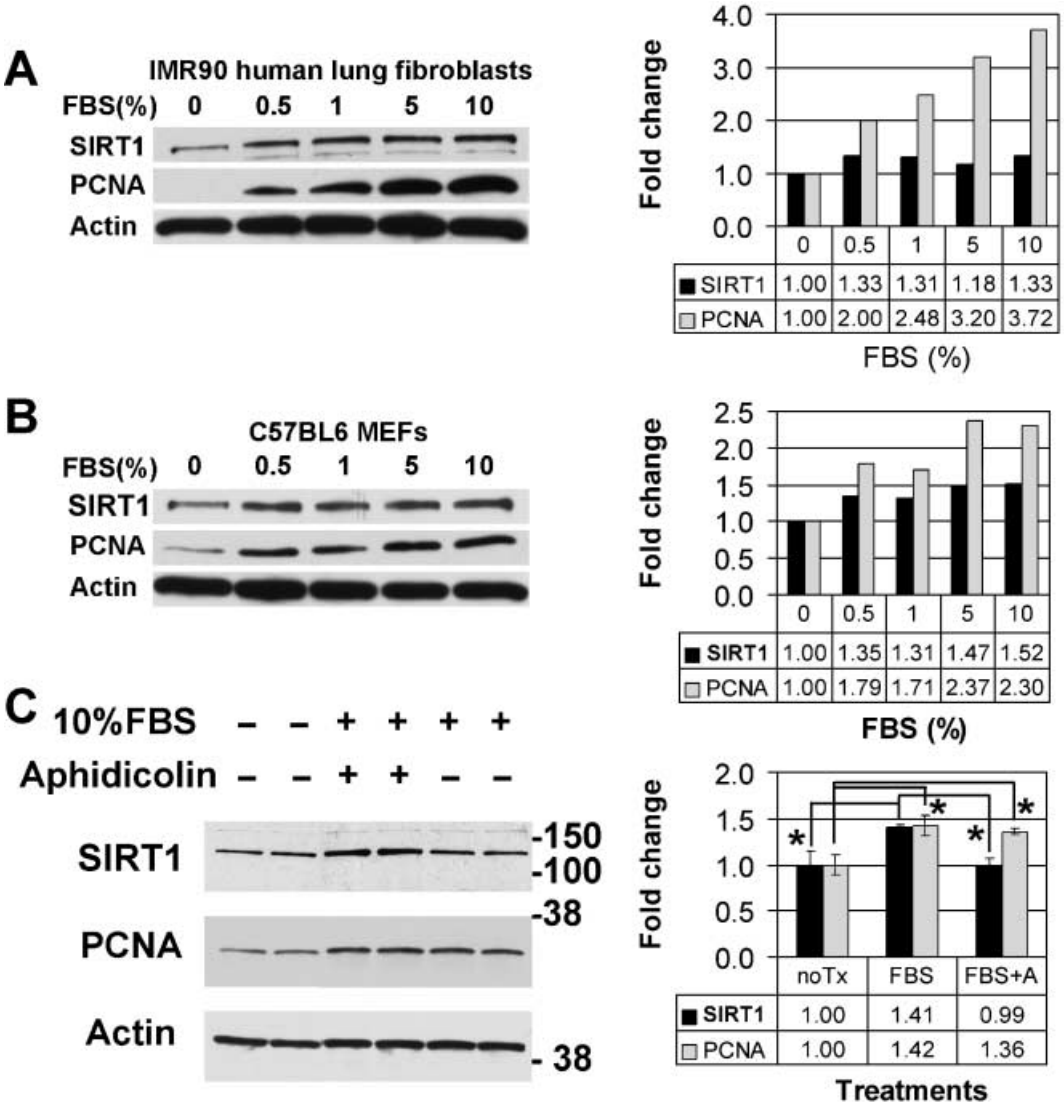

Fig. 3 SIRT1 and PCNA protein levels are coupled *in vitro*. Western blots of extracts from (A) IMR90 human lung fibroblasts and (B) C57BL6 MEFs stimulated with 0.5–10% FBS. Both SIRT1 and PCNA increased upon serum stimulation in both cell types. (C) Western blot of extracts from nontransgenic ICR MEFs treated for 24 h with 0.5% serum (no Tx), 10% serum (FBS), or 10% serum with aphidicolin (FBS+A) after 24 h of serum starvation by 0.5% serum. Asterisks (*) indicates significant difference between two groups (*t*-test, $P < 0.05$). In (C), an anti-SIRT1 monoclonal antibody was used.

SIRT1 and mitotic activity are coupled in tissues and decline in parallel during aging

In humans and mice, mitotic activity is higher in tissues that have a greater demand for regeneration and cell replacement than in tissues that undergo relatively little cell replacement. The rates of mitotic activity also change as the organism ages. We measured the levels of SIRT1 in an organ that undergoes little cell replacement (brain), and in two organs in which the demand for cell replacement is high (thymus and testis). We compared the level of SIRT1 in tissues from young adult mice (4 months of age) to those of older mice (12, 18, and 24 months of age) using Western blot analysis with antibodies against N- and C-terminal regions of SIRT1. In the brain, which consists mainly of postmitotic neurons and glial cells that remain capable of proliferation, there was no consistent change in SIRT1 level with age (Fig. 5A). The slight increase in PCNA signal may in fact reflect this glial cell mitotic activity. In all, there was no significant linear correlation between the levels of SIRT1 and PCNA in brain (*F*-test, $P > 0.1$). In the testis, where the seminiferous epithelium remains highly mitotically active throughout most of adulthood, the level of SIRT1 was maintained at a relatively constant level, with some decline observed in the oldest animals (Fig. 5B). This relatively persistent mitotic activity is reflected in the levels of PCNA, which were significantly correlated to the levels of SIRT1 at all ages (Fig. 5B: *F*-test, $P < 0.001$). In contrast, both SIRT1 and PCNA levels decreased significantly with age in the thymus (Fig. 5C). The decline in PCNA reflects the decreased

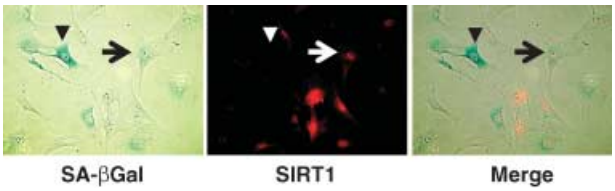

Fig. 4 SIRT1 is lost specifically in cells with decreased mitotic activity. SIRT1 immunofluorescence (middle) combined with senescence-activated β-galactosidase (SA-βGal) stain (left). Note that cells with prominent SA-βGal activity (arrowheads) did not react with an antibody against SIRT1, while cells that showed only slight SA-βGal activity retained some SIRT1 reactivity (arrows), and cells that showed no SA-βGal activity showed high SIRT1 reactivity.

mitotic activity of thymocytes during regression of the thymus, which occurs in adult animals (Taub & Longo, 2005). There was also significant linear correlation between SIRT1 and PCNA levels in thymus (*F*-test, $P < 0.001$).

To determine if these changes in SIRT1 protein level in tissues were due to changes in *SIRT1* transcription, we measured the level of *SIRT1* mRNA in the same brain, testis and thymus samples using quantitative real-time reverse transcriptase–polymerase chain reaction (RT–PCR). We found no significant correlation between the amount of *SIRT1* transcript and the age of the animal for any of the tissues tested (*F*-test, $P > 0.1$). We also did not find any significant linear correlation between the levels of *SIRT1* mRNA and protein among tissues examined (*F*-test, $P > 0.1$). These results, which are presented in Fig. 6, are consistent with similar findings in cultured cells shown in Fig. 2.

Accelerated or delayed aging alters the rate of SIRT1 decline

As a final test of the link between the level of SIRT1 and mitotic activity, we utilized two mouse models of aging, the p44 transgenic mouse, which exhibits accelerated aging, and the growth hormone-receptor knockout (GHRKO) mouse, in which aging is delayed. In the accelerated aging model, we found that SIRT1 in the testis of p44 transgenic mice decreased to a greater extent and in younger animals than in nontransgenic mice of the same strain (ICR) (Fig. 7A). This is consistent with the premature reproductive senescence and decreased spermatogenesis seen in these mice (Maier *et al*., 2004). SIRT1 levels in the thymus of p44 transgenic mice also decreased at younger ages than in nontransgenic mice (Fig. 7C).

In the GHRKO mice, on the other hand, and in their normal littermates, we found that SIRT1 and PCNA levels were maintained in the testes with age (Fig. 7B). In the thymus, however, SIRT1 levels declined with age in both GHRKO mice and normal littermates, and there was no difference in the rate of SIRT1 decline between two groups (Fig. 7D). Significant linear correlations between the levels of SIRT1 and PCNA were observed in the thymus (*F*-test, $P < 0.05$ in Fig. 7C, $P < 0.005$ in Fig. 7D). There was no significant linear correlation between the levels of SIRT1 and PCNA in testis.

These data from tissues from normally aging mice and from mutant mice in which the rate of aging is disturbed reinforce the observation that the loss of SIRT1 is directly proportional to the loss of mitotic activity, and are the *in vivo* equivalent of data obtained using cells undergoing replicative senescence in culture.

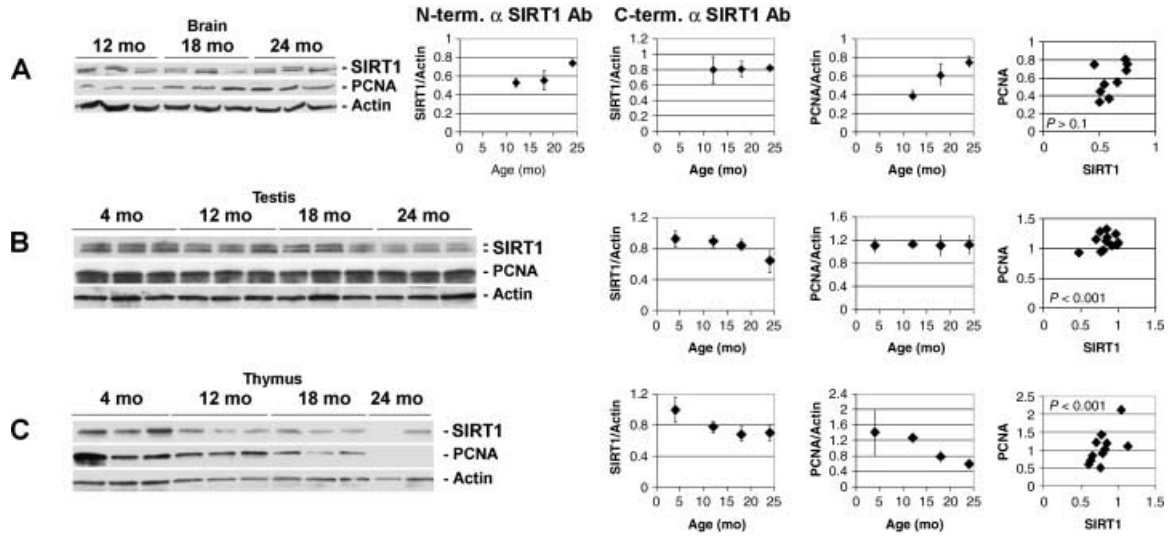

Fig. 5 SIRT1 protein level decreases with age in mitotic tissues, but not in postmitotic tissues. Western blots performed on lysates from B6 male brain (A), testis (B), and thymus (C). Densitometry results for each blot are shown on the right. There was a significant decrease in testis (*F*-test, $P < 0.02$) and thymus (*F*-test, $P < 0.005$). The brain blot showed an age-dependent increase in SIRT1 with the anti-N-terminal SIRT1 antibody, but not with the anti-C-terminal SIRT1 antibody. Age-dependent changes in SIRT1 in testis and thymus were seen with both anti-N- and C-terminal SIRT1 antibodies. Each data point represents an average of the samples from the same age, with error bars representing the standard deviations among samples. There were significant linear correlations between levels of SIRT1 and PCNA in testis and thymus (*F*-test, $P < 0.001$), but not in brain (*F*-test, $P > 0.1$). mo, month old.

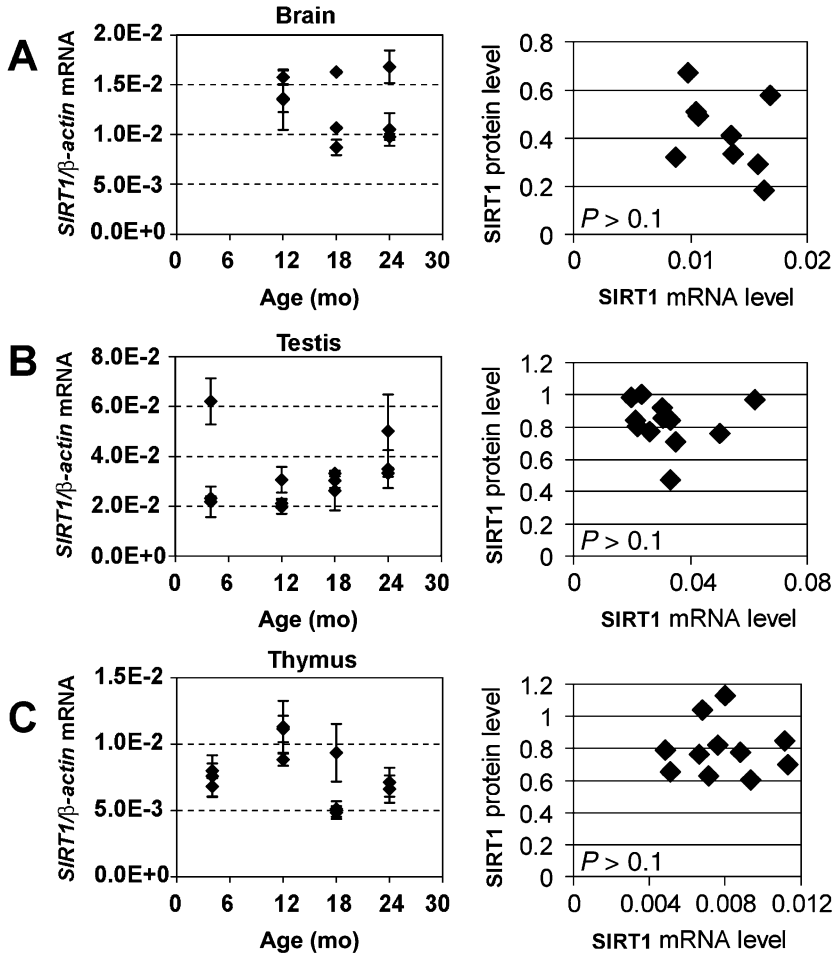

Fig. 6 SIRT1 mRNA level does not correlate with age *in vivo*. qRT–PCR determination of *SIRT1* mRNA levels in brain (A), testis (B), and thymus (C). No significant correlation with *SIRT1* mRNA level and age ($P > 0.1$ by *F*-test for brain, testis and thymus). *SIRT1* mRNA level was standardized to β-*actin* mRNA levels for each sample. The same tissues were used for these qRT-PCR data as were used for Western blots in Fig. 5A–C. Error bars represent the standard deviations of three measurements of each sample. There was no significant linear correlation between levels of SIRT1 mRNA and proteins in tissues examined (*F*-test, $P > 0.1$).

Discussion

In this study, we have explored the relationship between changes in the level of the mammalian homologues of Sir2, SIRT1 and Sirt1, and changes in mitotic activities of cells undergoing senescence in culture or as a consequence of the aging process in the animal. When we began this study, we thought that the most likely effect of SIRT1 would be to facilitate mitosis via a pathway upstream of cell cycle entry. This assumption was based largely on the effects of calorie restriction or nutrient withdrawal on the level of SIRT1 expression (Cohen *et al*., 2004; Nemoto *et al*., 2004; Rodgers *et al*., 2005), and on the effect of SIRT1 on cell survival under conditions that evoke a p53 response (Luo *et al*., 2001; Vaziri *et al*., 2001; Langley *et al*., 2002). Results of our study imply, however, that the effects of SIRT1 on lifespan might lie downstream of the cell cycle and be coupled to the cellular machinery of mitosis itself. The key distinction lies in the fact that nutrient withdrawal can affect the level of SIRT1 transcription (Nemoto *et al*., 2004), whereas mitosis appears to affect the level of SIRT1 protein post-transcriptionally. We found no evidence for fluctuations in the amount of *SIRT1* mRNA under conditions that altered mitotic activity both *in vitro* and *in vivo* in any of the cells or tissues we analyzed. What we did find was that replicative cellular senescence in human and mouse fibroblasts was accompanied by a decrease in the level of SIRT1 protein. Other studies with fibroblasts (Michishita *et al*., 2005), human lung and breast cancer cells (Ota *et al*., 2005), and primary neuroblasts (Horio *et al*., 2003) have reported similar links between low levels of SIRT1 and reduced cellular proliferation. In addition, cells from mice deficient in SIRT1 show reduced proliferative capability, a finding that is consistent with the reduced tissue volume seen in tissues such as the pituitary in these mice (Lemieux *et al*., 2005). We found, furthermore, that the level of SIRT1 varies directly with the level of PCNA, which is expressed during DNA replication (Paunesku *et al*., 2001). In cells that senesce prematurely (p44+/+ MEFs), coordinate decreases in SIRT1 and PCNA occurred at earlier cell passages than in normally senescing cells. Igf-1r-null cells grow more slowly and undergo crisis *in vitro* later than their wild-type counterparts (Sell *et al*., 1994). Loss of Igf-1r in mice confers resistance to oxidative stress (Holzenberger *et al*., 2003), which is known to play a major role in murine cell senescence *in vitro* (Parrinello *et al*., 2003). In Igf-1r–/– MEFs, these decreases in SIRT1 and PCNA were not observed while cells remained mitotically active and lacked the morphological changes typical of senescent cells. When wild-type MEFs underwent spontaneous immortalization

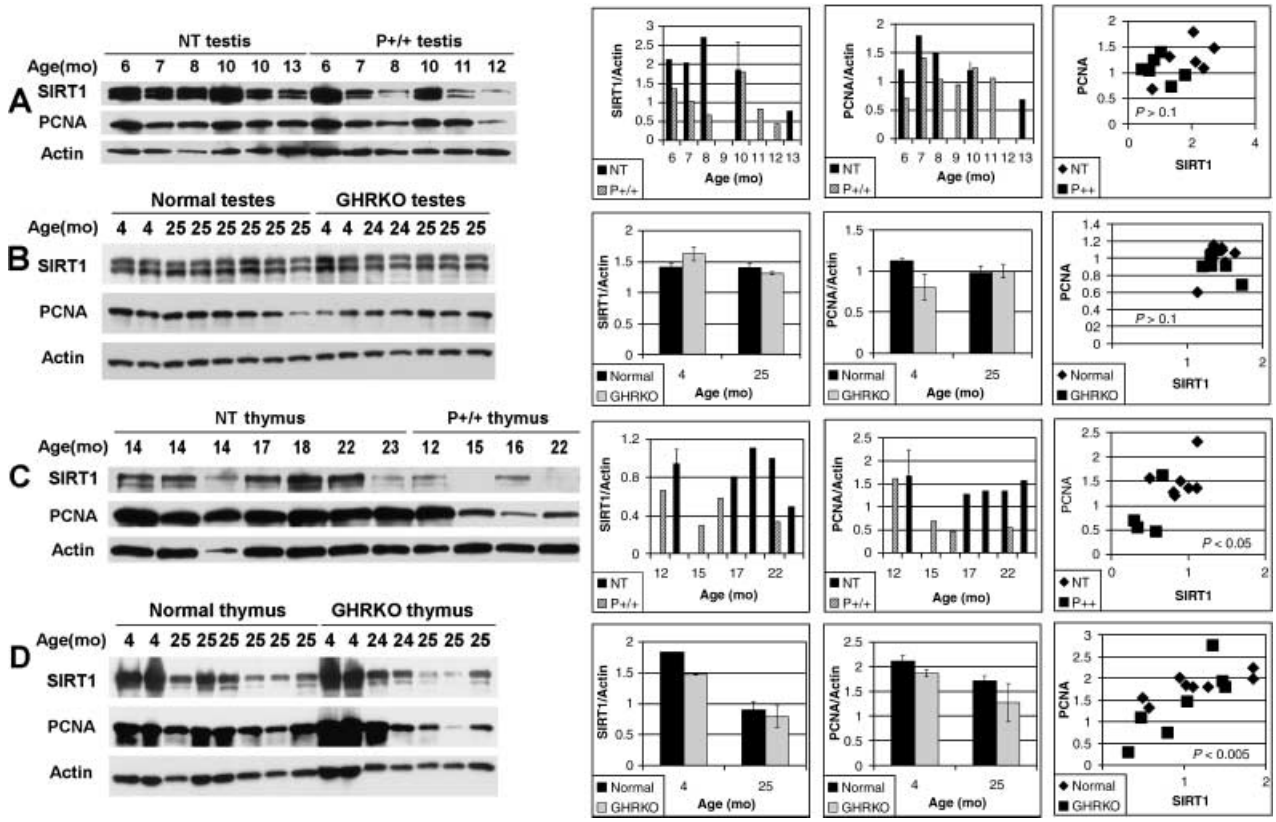

Fig. 7 The rate at which SIRT1 protein level declines *in vivo* is affected by the rate of aging. (A) Western blot of extracts from NT and p44+/+ testis. SIRT1 protein level decreases faster in prematurely aging p44+/+ testis, than in normal (NT) testis. (B) Western blot of GHRKO and normal male testis. There was no major decline in SIRT1 and PCNA levels in either genotype. (C) Western blots of SIRT1 level in NT and p44+/+ thymus. (D) Western blot of extracts from Normal and GHRKO thymus. SIRT1 level declined both in GHRKO thymus and normal counterparts, but there was no difference in the rate of decline. Numbers above the lanes of each Western blot indicate the age (month) of animal analyzed. Results of densitometric analysis of each blot are shown on the right. Significant linear correlations between levels of SIRT1 and PCNA were observed in (C) and (D), but not in (A) and (B) (*F*-test). 25 month group for GHRKO tissues include both 24 and 25 month samples. GHRKO, growth hormone receptor-knockout; mo, month old; NT, nontransgenic; P+/+, p44+/+ transgenic mice.

and re-entered the cell cycle, SIRT1 and PCNA levels went up again concomitant with increased mitotic activity. Finally, SIRT1 immunofluorescence inversely correlated with SA-βGal activity, a marker for senescence. Thus, in both human and mouse fibroblasts, the level of SIRT1 is linked to the activity of the cell cycle.

We confirmed these results by experimentally manipulating cell cycle activity with serum and a cell cycle inhibitor, aphidicolin. We found that SIRT1 level was higher in fibroblasts that were stimulated with serum, and that PCNA level changed accordingly. We found that the level of SIRT1 was very sensitive to serum stimulation, reaching maximal levels at low concentration (Fig. 3A,B), and that the increase could be blocked by aphidicolin (Fig. 3C). These data indicate that SIRT1 increases when cells are allowed to go through mitosis and suggest that cell cycle activity might stabilize the protein after S-phase. In fact, we detect two SIRT1 bands at about 110 kDa in murine tissues and cells (see Figs 1C,D, 3C, 5A,B and 7) as well as in human cells (see Fig. 3A), which could be indicative of two different post-translationally modified forms of the protein. These two bands are detected by both anti-N-terminal and anti-C-terminal SIRT1 polyclonal antibodies and an anti-SIRT1 monoclonal antibody. Post-translational modifications such as phosphorylation and ubiquitination are known to affect protein migration in gels.

The organization of DNA into chromatin and chromosomal structures plays a central role in many aspects of cell biology in eukaryotes. DNA, along with chromatin and other modifications, must all be replicated during mitosis (Ehrenhofer-Murray, 2004). Because SIRT1 deacetylates histones (Imai *et al.*, 2000), the demand for SIRT1 may go up in proliferating cells. Then, as cells stop dividing, the demand for SIRT1 would decrease and the level of the protein decline accordingly.

Replicative senescence of human and mouse cells in culture, although similar in phenotype, is thought to occur primarily by different mechanisms. In human cells senescence is linked to telomere attrition, while in mouse cells, it is linked to sublethal oxidative damage suffered under standard culture conditions (Parrinello *et al.*, 2003; Busuttil *et al.*, 2004; Shay & Wright, 2005). Normal cells senesce following any one of a number of stressors, such as DNA damage, damage to chromatin structure, and oxidation (Serrano & Blasco, 2001; Lloyd, 2002). All of these ultimately affect the ability of a cell to undergo mitosis. In this

study, we found that conditions that alter mitotic activity in fibroblasts control the level of SIRT1. We also found that the levels of SIRT1 and mitosis were linked in tissues from mice undergoing normal and accelerated aging. SIRT1 decreased with age only in tissues in which mitotic activity also slowed. There were two exceptions to this. In the thymus, SIRT1 levels declined with age in both GHRKO mice and normal littermates, and there was no difference in the rate of SIRT1 decline between two groups (Fig. 7D). We conclude that this aspect of aging may not be delayed in GHRKO mice. GHRKO mice also have low serum IGF-1, decreased length and diameter of seminiferous tubules, decreased testosterone secretion upon luteinizing hormone (LH) treatment, and decreased fertility (Chandrashekar *et al.*, 2004). These endocrinological changes can account for the loss of linear correlation between SIRT1 and PCNA in GHRKO testis (Fig. 7B). Finally, the testes of p44 transgenic mice undergo degeneration accompanied by abnormal Leydig cell proliferation (Fig. 7A) (Maier *et al.* manuscript submitted). This could account for the loss of linear correlation between Sirt1 and PCNA.

In summary, as mitotic activity ceases in mouse and human cells in the normal environment of the animal or in the culture dish, there is a concomitant decline in the level of SIRT1. These results strongly imply that the level of SIRT1 and replicative senescence may be influenced by a common mechanism, such as the machinery that drives mitosis.

Experimental procedures

Cell culture

Mouse embryonic fibroblasts were separated from subcutaneous (s.c.) tissue of embryos at 13.5 postcoital days and grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and high glucose, unless otherwise noted. IMR90 human lung fibroblasts were obtained from ATCC. Cells were cultured in 37 °C with 5% CO_2, with DMEM with high glucose (Gibco#11885) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) and penicillin/streptomycin (Invitrogen). For the serum comparison experiment, C57BL6 MEFs and IMR90 fibroblasts were plated subconfluently in 6-well plates in DMEM with 10% FBS, then starved in 0.5% FBS overnight, followed by incubation with 0%, 0.5%, 1%, 5%, or 10% FBS for 48 h. For the growth factor treatment experiment, nontransgenic MEFs from the ICR strain were starved with 0.5% FBS for 24 h, followed by 0.5% FBS, 10% FBS, or 10% FBS with aphidicolin (4 μg mL^{-1}, EMD Biosciences, San Diego, CA, USA) for 24 h.

Serial cell passage

IMR90 human lung fibroblasts were serially passaged at a ratio of 1 : 8 in 10-cm plates. MEFs from wild-type ICR strain and p44+/+ transgenic mice were serially passaged at a ratio of 1 : 4 in 10-cm plates. MEFs from wild-type C57BL6 strain were serially passaged at a ratio of 1 : 4, whereas MEFs from Igf-1r–/– mice were serially passaged at a ratio of 1 : 4 till passage 4, then at a ratio of 1 : 8 from passage 5. ICR MEFs that underwent spontaneous immortalization were serially passaged at a ratio of 1 : 8. The number of cells in each plate was measured and used to calculate estimated population doublings at each passage in IMR90 cells.

Protein isolation

Tissue samples were first pulverized in liquid nitrogen. Cells in culture were first washed with PBS. Cell lysis buffer (1/10 RIPA buffer, supplemented with 50 mM Tris, 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 0.2% sarkosyl, 1 mM DTT and 10% glycerol) in the presence of phosphatase inhibitors (Sigma, St. Louis, MO, USA), protease inhibitor cocktail (Sigma), Trichostatin A, and nicotinamide unless otherwise mentioned, were applied to pulverized tissue or cells *in vitro*, then incubated on ice for 30 min. DNA was sheared using a 25G needle syringe. Samples were centrifuged at maximum speed for 10 min in 4 °C to remove any insoluble material.

Western blot analysis

Western blots were performed using a BioRad Western blot kit (BioRad Laboratories, Inc., Hercules, CA, USA). Proteins from each sample (20–30 μg) were loaded on 4–20% gel or 10% gel, and proteins separated by molecular weight. After transfer to a Protran nitrocellulose membrane (PerkinElmer, Wellesley, MA, USA), the membranes were blocked in 5% milk/0.05% Tween-20 in PBS for 1 h, then hybridized with primary antibody overnight in 4 °C. After washing with PBS-T/milk for 5 min three times, secondary antibodies were applied and incubated for 1 h. After another three washes with PBS-T/milk for 10 min each, either Pico or Femto chemilluminescence substrates (Pierce, Rockford, IL, USA) were used to develop Western blots and X-ray films exposed accordingly. The following antibodies were used: peroxidase-conjugated AffiniPure Goat-anti-Mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA, #115-035-100, 1 : 10 000), peroxidase-conjugated AffiniPure goat antirabbit IgG (Jackson ImmunoResearch #110-035-144, 1 : 10 000), antiactin monoclonal antibody (MP Biomedicals, Solon, OH, USA, #69100, 1 : 1 000 000), anti-PCNA polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, #sc-7907, 1 : 1000), anti-SIRT1 N-terminal polyclonal antibody (Upstate Biotech, Charlottesville, VA, USA, #07-131, 1 : 1000), anti-SIRT1 C-terminal polyclonal antibody AS-16 (Sigma #S5313, 1 : 1000), and anti-SIRT1 monoclonal antibody 2G1/F7 (Upstate Biotech #05-707, 1 : 1000). For SIRT1 Western blots, anti-SIRT1 N-terminal polyclonal antibody was used unless otherwise mentioned.

Western blot densitometry

Films were scanned with a Molecular Dynamics Densitometer (GE) and densitometry was performed using ImageQuant5.0 (GE). SIRT1, PCNA, and Actin signals of each sample were measured and SIRT1 and PCNA levels of each sample were standardized to the levels of actin.

RNA and cDNA preparation

Total RNA from tissues was harvested with 1 mL of TriReagent (Molecular Research Center, Cincinnati, OH, USA) after tissues were pulverized in liquid nitrogen. After isolation of RNA, total RNA was treated with RNase-free DNase I (Roche, Basel, Switzerland) for 1 h, and RNeasy (QIAGEN, Valencia, CA, USA) was used to purify RNA. Purity and amount of RNA were determined spectrophotometrically (Eppendorf). Total RNA from cells was harvested in a similar fashion except 0.5 mL of TriReagent was used. Reverse transcription reactions to generate cDNAs were performed with SuperScript III (Invitrogen) using 1 μg of total RNA as a template and random hexamer as primers. Reactions were performed at 50 °C for 1 h for tissues and for 3 h for cells *in vitro*.

Quantitative real-time RT–PCR

Reverse transcriptase–polymerase chain reaction was performed in two steps. First cDNA was prepared as described above, then 1 μL of cDNA was used to perform real-time PCR. DNA FastStart SybrGreenI kit (Roche) and LightCycler (Roche) was used for real-time PCR. The specificity of the reaction was assessed by the melting curve analysis. Each cDNA sample was measured three times. The following primers were used. Murine *SIRT1* primers: forward (1918-33): GTAAGCGGCTTGAGGG; reverse (2132-2147): TTCGGGCCTCTCCGTA. Human *SIRT1* primers: forward (428-453): GAGATAACCTTCTGTTCGGTGATGAA; reverse (595-621): CGGCAATAAATCTTTAAGAATTGTTCG. β-*actin* primers: forward (412-431): CCCTAAGGCCAACCGTGAAA; reverse (500-520): ACGACCAGAGGCATACAGGGA. *GAPDH* primers: forward (529-548): TGCACCACCAACTGCTTAGC; reverse (595-615): GGCATGGACTGTGGTCATGAG.

Statistical analysis

Following densitometry, signal intensities were normalized to loading controls (actin) and *F*-tests were performed to check for significant correlation between age of tissues and SIRT1 signals, levels of SIRT1 and PCNA, and levels of SIRT1 mRNA and protein using Microsoft Excel. For qRT–PCR of tissues, *F*-tests were applied after SIRT1 transcript levels were adjusted to those of *β-actin*. For qRT-PCR of cells *in vitro*, after SIRT1 transcript levels were adjusted to GAPDH transcript levels, *F*-test was applied for IMR90 data. For ICR and p44+/+ data and data from cell cycle manipulation with aphidicolin, *t*-tests were performed using Microsoft Excel.

Senescence-activated β-galactosidase staining followed by SIRTt1 immunofluorescence

Wild-type MEFs from ICR strain which were passaged three times at a ratio of 1 : 4 were washed in PBS once and fixed with 0.5% glutaraldehyde in PBS for 5 min, then washed twice in PBS (pH 7.2) with 1 mM $MgCl_2$. Cells were stained in X-Gal solution consisting of PBS at pH 6.0 containing 1 mg of X-gal (5-bromo-4-chloro-3-indolyl–D-galactopyranoside), 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 150 mM NaCl, 1 mM $MgCl_2$ overnight at 37 °C. The following day, cells were rinsed in PBS once, demasked with ice-cold 0.1 N HCl for 15 min followed by incubation in ddH_2O for 5 min twice, in PBS containing 5 mM $MgCl_2$ and 0.1% Triton-X-100 for 5 min, in ddH_2O for 5 min twice and in PBS for 2 min. Sections were blocked with PBS containing 10% normal goat serum, 3% bovine serum albumin and 0.1% Triton-X-100 for 30 min, followed by incubation with anti-Sir2 antibody (Upstate #07-131, 1 : 200) overnight. Cells were incubated for 2 h with Alexa Fluor 568 goat antirabbit IgG (Molecular Probe #A11036, 1 : 300) as a secondary antibody. After washing cells with PBS, cells were mounted with Fluormount G (Southern Biotech #0100-01).

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