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Genomic Instability and Aging-like Phenotype in the Absence of Mammalian SIRT6

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SUMMARY

The Sir2 histone deacetylase functions as a chromatin silencer to regulate recombination, genomic stability, and aging in budding yeast. Seven mammalian Sir2 homologs have been identified (SIRT1–SIRT7), and it has been speculated that some may have similar functions to Sir2. Here, we demonstrate that SIRT6 is a nuclear, chromatin-associated protein that promotes resistance to DNA damage and suppresses genomic instability in mouse cells, in association with a role in base excision repair (BER). SIRT6-deficient mice are small and at 2–3 weeks of age develop abnormalities that include profound lymphopenia, loss of subcutaneous fat, lordokyphosis, and severe metabolic defects, eventually dying at about 4 weeks. We conclude that one function of SIRT6 is to promote normal DNA repair, and that SIRT6 loss leads to abnormalities in mice that overlap with aging-associated degenerative processes.

INTRODUCTION

The silent information regulator (SIR) genes are nonessential genes required in *trans* for transcriptional repression of several genomic loci in the budding yeast *S. cerevisiae* (Klar et al., 1981; Rine and Herskowitz, 1987). Sir2 is unique among these factors in belonging to a large family of closely related proteins present both in prokaryotic and eukaryotic species (reviewed in Dutnall and Pillus [2001]). Sir2 functions in a complex with other Sir proteins to repress transcription at the silent mating type loci and subtelomeric sequences. In addition, Sir2 is part of another multicomponent complex in the nucleolus, where it represses recombination and transcription of rDNA repeats (Gotta et al., 1997; Gottlieb and Esposito, 1989; Straight et al., 1999). Sir2 exerts its effects on transcriptional and recombinational silencing via modulation of chromatin structure (Fritze et al., 1997) by catalyzing NAD-dependent deacetylation of histones (Imai et al., 2000; Smith et al., 2000).

S. cerevisiae Sir2 promotes longevity in yeast mother cells (Kaeberlein et al., 1999). Cells lacking Sir2 have a reduced replicative life span, and cells with extra copies display

a longer life span. Sir2 extends yeast life span by inhibiting recombination in the rDNA repeats, since the recombinational excision and subsequent accumulation of extrachromosomal rDNA circles promotes senescence (Kaeberlein et al., 1999; Sinclair and Guarente, 1997). Increased Sir2 activity in *C. elegans* and *Drosophila* also extends life span (Tissenbaum and Guarente, 2001; Wood et al., 2004); however, the mechanism of life span extension seems to differ from that in yeast. Thus, Sir2-driven increased longevity in *C. elegans* is dependent on the Daf-16 transcription factor, a critical mediator in the insulin-like signaling pathway, which promotes formation of long-lived dauer larvae under unfavorable environmental conditions and increases stress resistance and longevity in adults (Kenyon, 2001). Sir2 family members also were suggested to mediate effects of caloric restriction (CR; Sohal and Weindruch, 1996), a dietary regimen capable of extending life span in diverse organisms from yeast to mammals (Cohen et al., 2004; Howitz et al., 2003; Lin et al., 2004; Rogina and Helfand, 2004; Wood et al., 2004). CR likely decreases production of reactive oxygen species (ROS), a potential source of persistent DNA damage proposed to contribute to aging (Balaban et al., 2005).

The evolutionary conservation of a role for Sir2 in regulating life span has fueled speculation that such a role also may be conserved in mammals (Blander and Guarente, 2004; North and Verdin, 2004). There are seven mammalian Sir2 family members, designated SIRT1–SIRT7 (Frye, 2000), with SIRT1 being the most highly related to *S. cerevisiae* Sir2. However, unlike yeast Sir2, which deacetylates histones exclusively, SIRT1 possesses a large list of substrates, including p53 and forkhead transcription factors, which modulate cellular resistance to oxidative and genotoxic stress (Blander and Guarente, 2004). In addition, SIRT1, like Sir2, has been shown to modify chromatin and silence transcription of integrated reporter genes via histone deacetylation (Vaquero et al., 2004). However, potential roles of SIRT1 in life span regulation have not yet been documented. SIRT2 and SIRT3 possess deacetylase activity but are cytoplasmic proteins with functions that diverge from those of ySir2 (Blander and Guarente, 2004). Little information is available about the functions of the other mammalian SIRTs.

Mammals employ various pathways to repair specific types of DNA damage. Mutant mice defective in individual DNA repair pathways frequently show increased genomic instability and hypersensitivity to specific DNA-damaging agents. Double-strand breaks (DSBs), such as those induced by ionizing radiation (IR), are repaired by nonhomologous end-joining (NHEJ) or homologous recombination (HR). Single-stranded DNA lesions are repaired via nucleotide excision repair (NER) or base excision repair (BER), depending on the type of lesion (Hoeijmakers, 2001). In this context, bulky photodimer single-strand lesions induced by UV irradiation are primarily repaired by NER, whereas more simple types of single-stranded lesions usually are repaired by BER. The BER pathway may repair up to one million nucleotides per cell per day (Holmquist, 1998) and repairs single-strand breaks (SSBs) that arise spontaneously, such as those from endogenous alkylation, oxidation, and deamination events, as well as small, nonhelix-distorting DNA lesions induced by chemical mutagens such as the alkylating agent methyl-methane sulphonate (MMS; (Lindahl and Barnes, 2000; Sancar et al., 2004). Defects in NHEJ, HR, and NER have been shown to cause aging-like phenotypes in mice and humans. However, there have been no mouse models to study potential effects of BER with respect to aging; since mutations of BER components either are lethal or confer no obvious phenotype (Lombard et al., 2005).

In this study, we have focused on elucidating the functions of the mammalian SIRT6 protein. We find that SIRT6 is a chromatin-associated protein that is required for normal BER maintenance of genomic integrity in cells. Moreover, SIRT6 deficiency in mice leads to the development of an acute degenerative aging-like phenotype.

RESULTS

SIRT6 Is a Chromatin-Associated Protein Expressed in Most Tissues

To gain insight into SIRT6 function, we characterized subcellular and tissue distribution patterns of SIRT6 expression. SIRT6 protein was predominantly nuclear, as assessed by immunostaining mouse embryonic stem (ES) cells with an anti-SIRT6 antibody and human HT1080 cells into which we retrovirally transduced a FLAG-tagged SIRT6 protein (Figures 1A and 1B). Analysis of SIRT6 RNA and protein revealed SIRT6 expression in most mouse tissues (Figures 1C and 1D), with particularly high protein levels in thymus, skeletal muscle, and brain. Overall, these findings are consistent with those of other recent studies (Liszt et al., 2005; Michishita et al., 2005).

To probe the possibility that SIRT6 might function in the context of chromatin, we asked whether it is associated with a chromatin-enriched biochemical fraction. Isolated nuclei were separated into nucleoplasmic and chromatin/nuclear matrix subfractions, and SIRT6 protein detected by Western analysis. Endogenous SIRT6 protein in mouse ES cells, and retrovirally expressed FLAG-SIRT6 protein in HT1080 cells cofractionated with histones, almost exclusively within the chromatin/nuclear matrix subfraction (Figures 1E and 1F). As a control, the SIRT1 protein was present in both the nucleoplasm and the chromatin/nuclear matrix subfractions in these cells, as expected (Vaquero et al., 2004; reviewed in Guarente and Picard [2005]). We conclude that SIRT6 is preferentially associated with chromatin within the nucleus.

Generation of SIRT6-Deficient Cells and Mice

To gain insight into SIRT6 function we generated SIRT6-deficient embryonic stem (ES) cells by replacing *SIRT6* with a *LacZ* gene introduced in frame into the exon 1 (see Figure S1A in the Supplemental Data available with this article online). Appropriately targeted ES cells were confirmed by Southern blotting (Figure S1A) and used to generate mice heterozygous for the SIRT6-inactivating mutation (referred to as $SIRT6^{+/-}$). $SIRT6^{+/-}$ were interbred to obtain

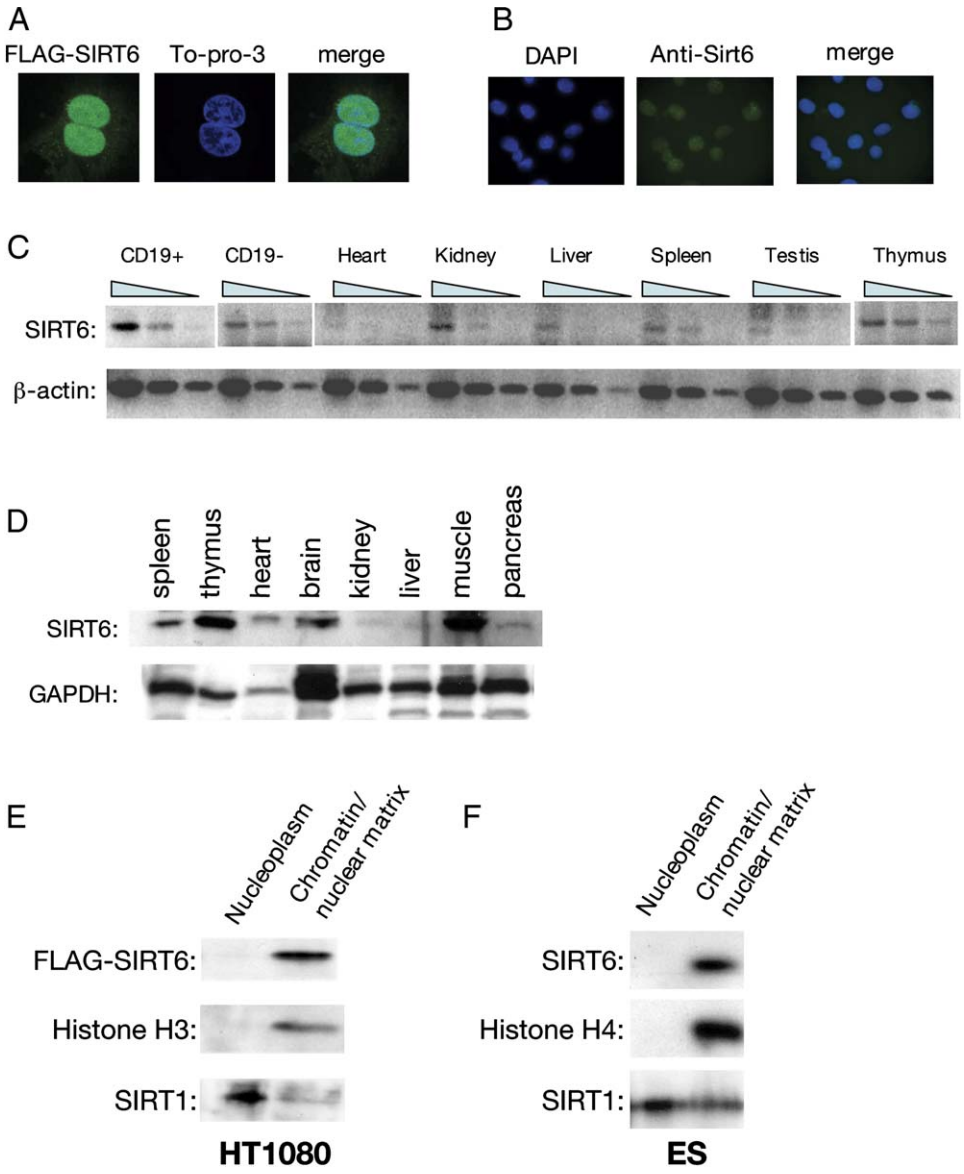


Figure 1. SIRT6 Is a Nuclear Protein Expressed in Many Different Tissues

(A) HT1080 cells were infected with a retrovirus encoding an N-terminal FLAG-SIRT6 fusion. SIRT6 protein was visualized with an antibody directed against the epitope tag, and cells were counterstained with To-pro-3 to visualize nuclei.

(B) A rabbit antiserum raised against the C terminus of SIRT6 protein was used to stain embryonic stem (ES) cells.

(C) One microgram of RNA from the indicated tissues was used in RT-PCR reactions with specific primers recognizing either SIRT6 or a control transcript (β actin).

(D) Protein lysate from the indicated tissues was probed with SIRT6 antiserum or GAPDH as control.

(E) Subcellular fractions were prepared from HT1080 cells infected with a retrovirus encoding an N-terminal FLAG-SIRT6 fusion. Western analysis of the fractions was carried out to detect FLAG-SIRT6 and histone H3, SIRT1, or SIRT5, a cytoplasmic protein (not shown), as controls.

(F) Indicated subcellular fractions were prepared from mouse ES cells and probed with anti-SIRT6, anti-H4, or anti-SIRT1 antibodies.

homozygous mutant (SIRT6$^{-/-}$) mice and SIRT6$^{-/-}$ mouse embryonic fibroblasts (MEFs). We also generated SIRT6$^{-/-}$ ES cells via the high G418-selection method (Mortensen et al., 1992 and data not shown). We confirmed absence of SIRT6 RNA and protein in SIRT6$^{-/-}$ cells by RT-PCR and Western blotting, respectively (Figure S1B). We also analyzed SIRT6$^{+/-}$ mice for βGal staining and confirmed expression of lacZ from the SIRT6 promoter in most tissues, both in adult mice and in embryos during development (Figure S1C and data not shown).

SIRT6-Deficient MEFs and ES Cells Show Impaired Proliferation and Increased Sensitivity to DNA-Damage Agents

To gain insight into the cellular effects of SIRT6 deficiency, we compared proliferation rates of SIRT6$^{-/-}$ and wild-type

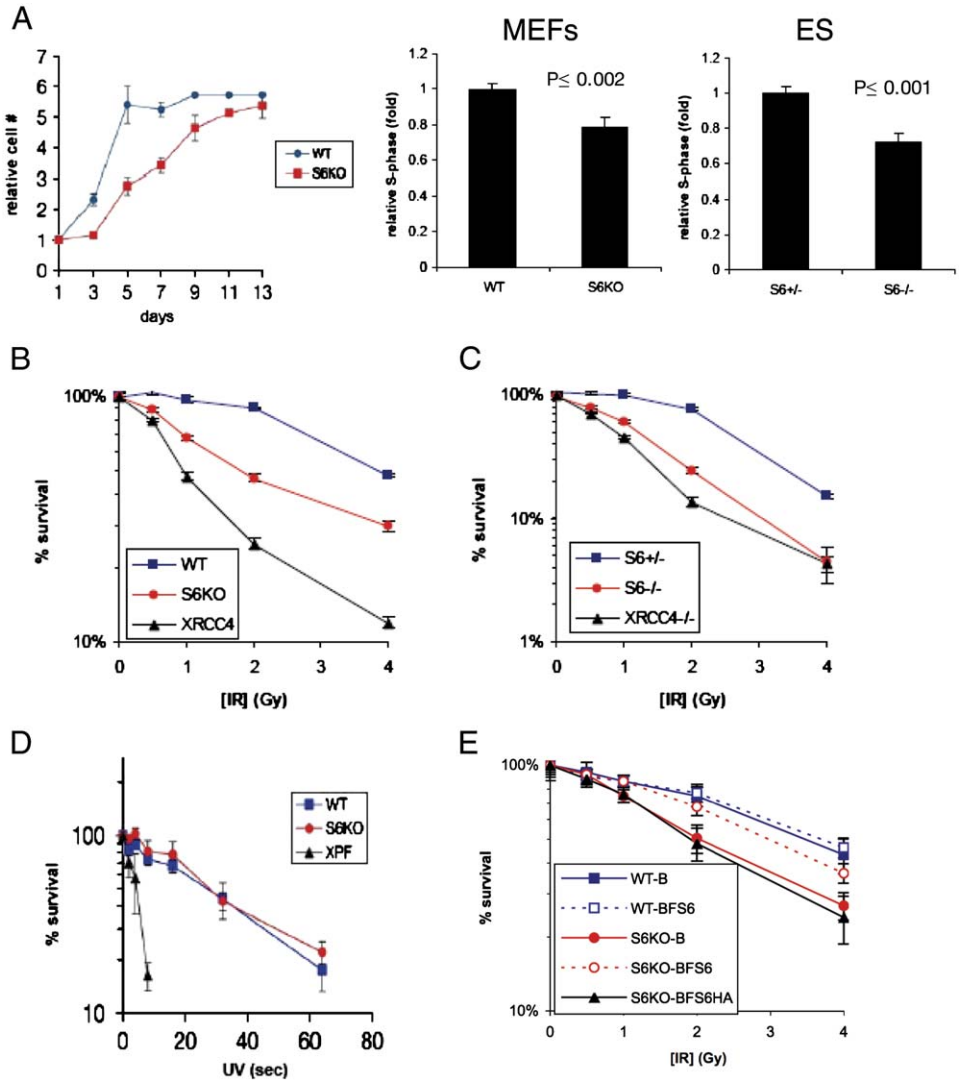


Figure 2. Impaired Proliferation and IR Sensitivity of SIRT6-Deficient MEFs and ES Cells

(A) Left: SIRT6-deficient MEFs or littermate controls were seeded at low density and the cell numbers quantified every other day as shown. Right: SIRT6-deficient ES cells or late-passage MEFs were labeled with BrdU and subjected to flow cytometry to determine the fraction of cells in S phase.

(B and C) SIRT6-deficient MEFs (B) or ES cells (C) were subjected to indicated doses of IR and the number of cells (B) or colonies (C) present 1 week later quantified. XRCC4-deficient MEFs (B) or ES cells (C) were used as positive controls.

(D) SIRT6 deficiency is not associated with UV sensitivity. SIRT6-deficient MEFs were subjected to indicated doses of UV and the cell numbers quantified 1 week later. XPF-deficient MEFs were used as a positive control.

(E) SIRT6-deficient MEFs or littermate controls were infected with an empty retrovirus (pBABE, B), a retrovirus encoding FLAG-tagged SIRT6 (BFS6), or a retrovirus encoding a catalytic mutant FLAG-tagged SIRT6 (BFS6HA). The cells were subjected to irradiation and sensitivity determined as above.

In all panels, error bars indicate the standard error of the mean.

control MEFs in culture (Figure 2A). Multiple independent SIRT6$^{-/-}$ MEF lines grew more slowly than wild-type controls. Analysis of BrdU incorporation revealed that exponentially growing SIRT6$^{-/-}$ MEF cultures had a smaller fraction of S phase cells than control wt cultures (Figure 2A). Similarly, exponentially growing SIRT6$^{-/-}$ ES cell cultures had a reduced fraction of BrdU-incorporating cells compared to controls (Figure 2A). Thus, absence of SIRT6 reduces the proliferative rate of multiple cell types.

To test for SIRT6 roles in DNA repair, we compared the sensitivity of SIRT6$^{-/-}$ MEFs and ES cells to IR and UV damage. Notably, SIRT6$^{-/-}$ MEFs and ES cells both exhibited increased sensitivity to IR (Figures 2B and 2C), albeit less than cells deficient in XRCC4, a core NHEJ factor. In contrast, SIRT6$^{-/-}$ MEFs exhibited normal UV sensitivity, indicating that SIRT6 deficiency confers hypersensitivity only to specific types of DNA damage (Figure 2D). To confirm that increased IR-sensitivity of the SIRT6$^{-/-}$ cells was a consequence of

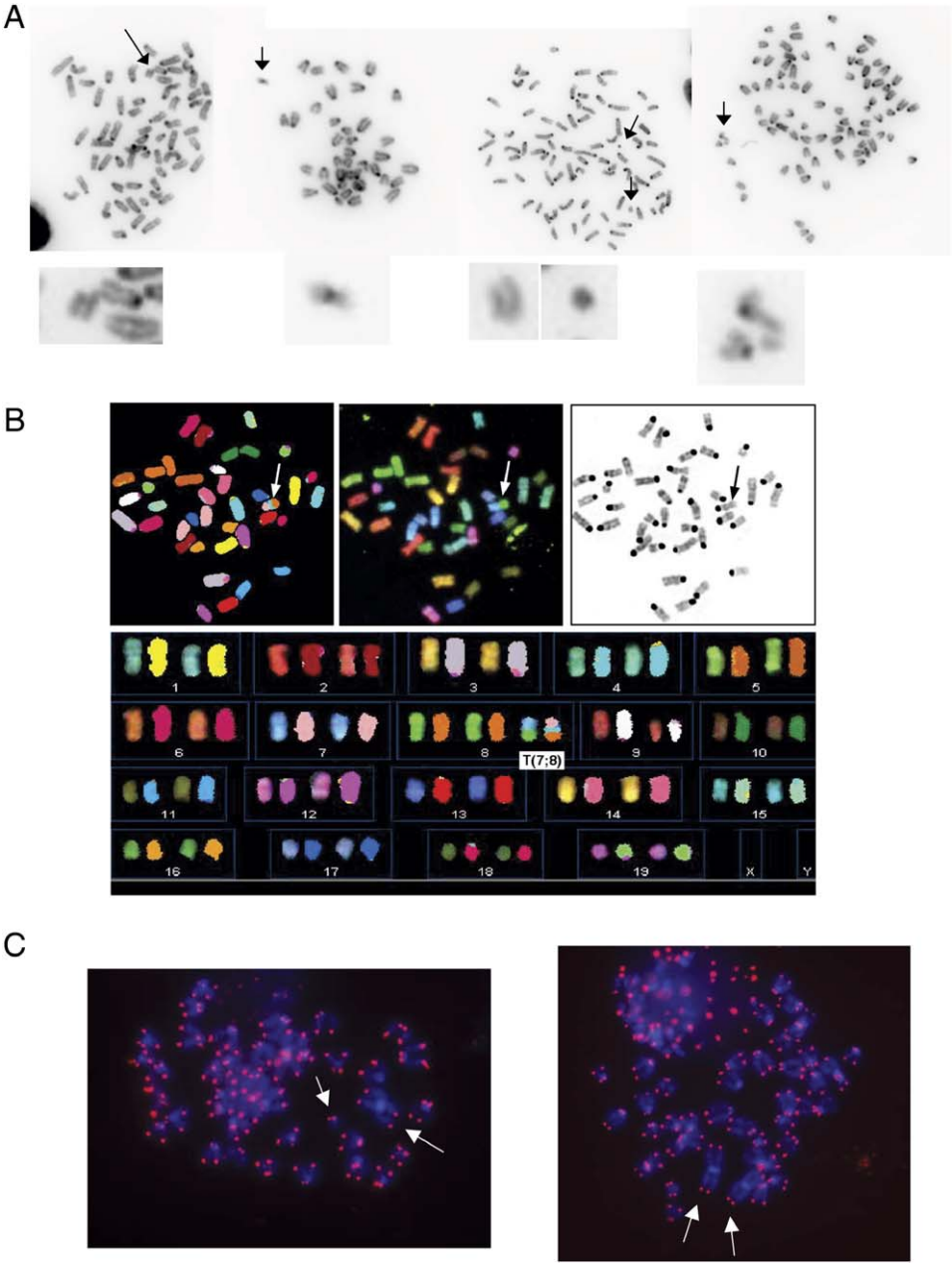


Figure 3. Genomic Instability in SIRT6-Deficient Cells

(A) DAPI staining was performed on metaphases from SIRT6-deficient MEFs.

(B) SIRT6-deficient MEF metaphases were subjected to spectral karyotype analysis to identify translocations.

(C) Q-FISH staining was performed on SIRT6$^{-/-}$ ES metaphases.

SIRT6 deficiency, SIRT6$^{-/-}$ MEFs were reconstituted with a retroviral SIRT6 expression vector, which restored SIRT6 expression (Figure S2) and largely reversed the increased IR hypersensitivity of SIRT6$^{-/-}$ MEFs (Figure 2E). Notably, expression of a catalytic mutant form of SIRT6 did not rescue the IR sensitivity (Figure 2E), indicating that the putative enzymatic activity of SIRT6 is required for its function in DNA repair.

Genomic Instability in SIRT6-Deficient Cells

To investigate whether SIRT6 suppresses genomic instability, metaphase chromosomes were prepared from SIRT6$^{-/-}$ MEFs and analyzed for abnormalities. Strikingly, chromosomal aberrations were significantly increased in SIRT6$^{-/-}$ cells (Figure 3A; Table 1), with increased frequency of several types of anomalies, including fragmented chromosomes, detached centromeres, and gaps. Increased genomic

Table 1. Increased Genomic Instability in SIRT6-Deficient Cells

(A) MEFs

	DAPI			SKY	
	wt	KO		wt	KO
Total Metaphases	96	96		24	24
Fragments/breaks	4	23	Fragments	0	2
Gaps	5	18	Translocations	0	6
Detached centromeres	2	5	Dicentric	0	1
Other aberrations	0	7			
Abnormal metaphases (%)	8 (7%)	36 (38%)		0 (0%)	9 (34%)
	$p < 0.0001$			$p < 0.01$	

(B) ES Cells—Q-FISH

	wt	XRCC4$^{-/-}$	KO1	KO2
Metaphases	50	50	50	50
Translocations	0	1	1	2
Breaks/fragments	5	10	14	13
Other aberrations	0	1	1	0
Total metaphases with abnormalities (%)	4 (8%)	11 (22%)	13 (28%)	12 (26%)

(A) MEFs were prepared from 13.5-day-old embryos, and metaphases were prepared from passage 1–2 cells. Cells were either stained with DAPI, or SKY was performed. Results include four independent experiments.
(B) ES cells of the indicated genotypes were grown, and metaphases prepared as described in Experimental Procedures. Q-FISH was performed, and aberrations scored as indicated.

instability of SIRT6$^{-/-}$ MEFs was evident in both early and late passage cultures (data not shown). Spectral karyotype analysis (SKY) revealed chromosomal translocations in SIRT6$^{-/-}$ MEFs (Figure 3B; Table 1). Karyotype analysis of the SIRT6$^{-/-}$ ES cells also revealed increased genomic instability (Figure 3C; Table 1). Together, these results indicate that SIRT6 plays a general role in maintaining genome integrity.

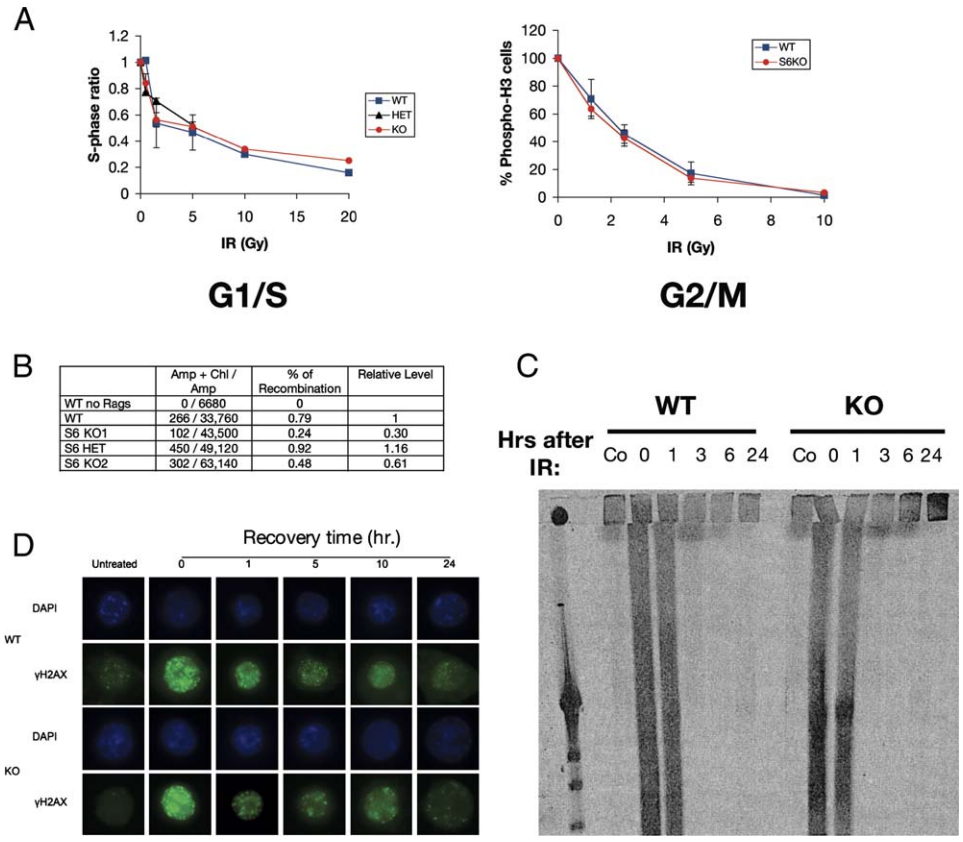
Intact Cell Cycle Checkpoints and DSB Repair in SIRT6-Deficient Cells

Genomic instability can result from either direct defects in DNA repair or faulty cell cycle checkpoints. We tested whether SIRT6 is required for normal cell cycle checkpoints in response to IR. Cells were irradiated with a range of doses and collected following BrdU labeling or staining with anti-phospho H3, to assess the G1/S and the G2/M checkpoints, respectively. In this analysis, SIRT6$^{-/-}$ and wt cultures were indistinguishable in exhibiting dose-dependent decreases in S phase and mitotic fractions, indicating that the G1/S and G2/M cell cycle checkpoints are not affected by absence of SIRT6 (Figure 4A).

We then assessed integrity of DNA DSB repair pathways in SIRT6$^{-/-}$ cells. To test NHEJ, an extrachromosomal plasmid-based recombination assay was used to quantify repair of DNA DSBs introduced by the RAG endonuclease into an immunoglobulin V(D)J recombination substrate (Hesse et al., 1987; Taccioli et al., 1993). Based on this semiquantitative NHEJ assay, SIRT6$^{-/-}$ MEFs repaired RAG-generated DSBs within the range of wt cells (Figure 4B). This conclusion was confirmed by our finding of normal lymphocyte development in young SIRT6$^{-/-}$ mice (see below). To test for chromosomal DSB repair, we performed pulse field gel electrophoresis (PFGE) following exposure of SIRT6$^{-/-}$ or control cells to high doses of IR for various lengths of time and found that SIRT6$^{-/-}$ cells showed similar DSB repair to wt cells (Figure 4C). Further, we analyzed formation and clearance of γH2AX foci, an early marker of the DSB response (Thiriet and Hayes, 2005) and again observed no difference between wt and SIRT6$^{-/-}$ cells (Figure 4D). Together, these observations suggest that the increased DNA damage sensitivity of SIRT6$^{-/-}$ cells is unlikely to result from impaired checkpoints or DSB repair pathways.

SIRT6 Deficiency Compromises the BER Pathway

We next tested whether SIRT6 deficiency affects repair of DNA lesions other than DSBs. Because NER is the main pathway responsible for repairing UV generated bulky adducts and SIRT6$^{-/-}$ MEFs showed normal sensitivity to UV irradiation (Figure 2D), we focused on BER. Monofunctional alkylating agents such as MMS and ROS generation due to oxidative agents such as H_2O_2 generate lesions that are



	Amp + Chl / Amp	% of Recombination	Relative Level
WT no Rags	0 / 6680	0	
WT	266 / 33,760	0.79	1
S6 KO1	102 / 43,500	0.24	0.30
S6 HET	450 / 49,120	0.92	1.16
S6 KO2	302 / 63,140	0.48	0.61

Figure 4. SIRT6-Deficient Cells Possess Intact Checkpoint Responses, End-Joining, and DSB Repair Pathways

(A) SIRT6-deficient MEFs and controls were subjected to various doses of IR. Left: 24 hr postirradiation, cells were pulsed with BrdU and analyzed via flow cytometry for BrdU staining and DNA content to assess the fraction of cells in S phase. Right: 1 hr following irradiation, cells were fixed and stained for the mitotic marker H3 to assess for the fraction of cells in S phase.

(B) MEFs of the indicated genotypes were transfected with V(D)J recombination substrates and RAG endonuclease expression plasmids as described (Taccioli et al., 1993). After 2 days, plasmids were recovered and transformed into bacteria. The ratio of Amp^r+Chl^r/Amp^r bacterial colonies in this semiquantitative assay roughly (with a factor of 5) reflects the level of V(D)J recombination (Hesse et al., 1987), which in turn reflects NHEJ (Taccioli et al., 1993).

(C) MEFs of the indicated genotypes were subjected to 10 Gy of IR. Following irradiation, genomic DNA was prepared at the time points indicated and analyzed via pulsed field gel electrophoresis to assess DSB repair.

(D) ES cells of the indicated genotypes were subjected to 20 Gy of ionizing radiation, followed by fixation and staining for γ-H2AX foci at the indicated time points. Cells shown are representative of the entire slide.

In all panels, error bars indicate the standard error of the mean.

primarily repaired by BER. In this regard, cells defective in different components of the BER pathway, such as Polβ and PARP-1, showed increased sensitivity to these agents (Sobol et al., 1996; Trucco et al., 1998). When SIRT6-deficient MEFs were exposed to MMS or H_2O_2, they showed markedly increased sensitivity compared to wt cells (Figure 5A). In contrast, cells deficient for XRCC4 showed increased sensitivity only to high doses of these agents, likely due to the high density of SSBs generated during repair under these conditions leading to DSBs. We also found that $SIRT6^{-/-}$ ES displayed increased sensitivity to MMS and H_2O_2 (Figure 5A). Notably, reconstitution with recombinant wild-type SIRT6, but not the catalytically inactive SIRT6 mutant, reversed the MMS and H_2O_2 hypersensitivity of SIRT6-deficient MEFs (Figure 5B). Together, these results suggest that the regulation of genomic stability by SIRT6 might be due to a role of SIRT6 in the context of BER.

SIRT6 Deficiency-Induced Hypersensitivity Is Rescued by Overexpression of the dRP Lyase Domain of Polβ

Previous studies have demonstrated that the rate-limiting step in BER is the removal of the 5′-deoxyribose phosphate (dRP) by the dRP lyase domain of Polβ, the major polymerase used for BER in mammalian cells (Sobol et al., 1996; Srivastava et al., 1998). In addition, the isolated dRP lyase domain of Polβ is sufficient to reverse the MMS hypersensitivity of Polβ-deficient cells (Sobol et al., 2000). Furthermore, overexpression of the Polβ dRP lyase domain increased resistance to DNA damage in immortalized wt MEFs (Sobol

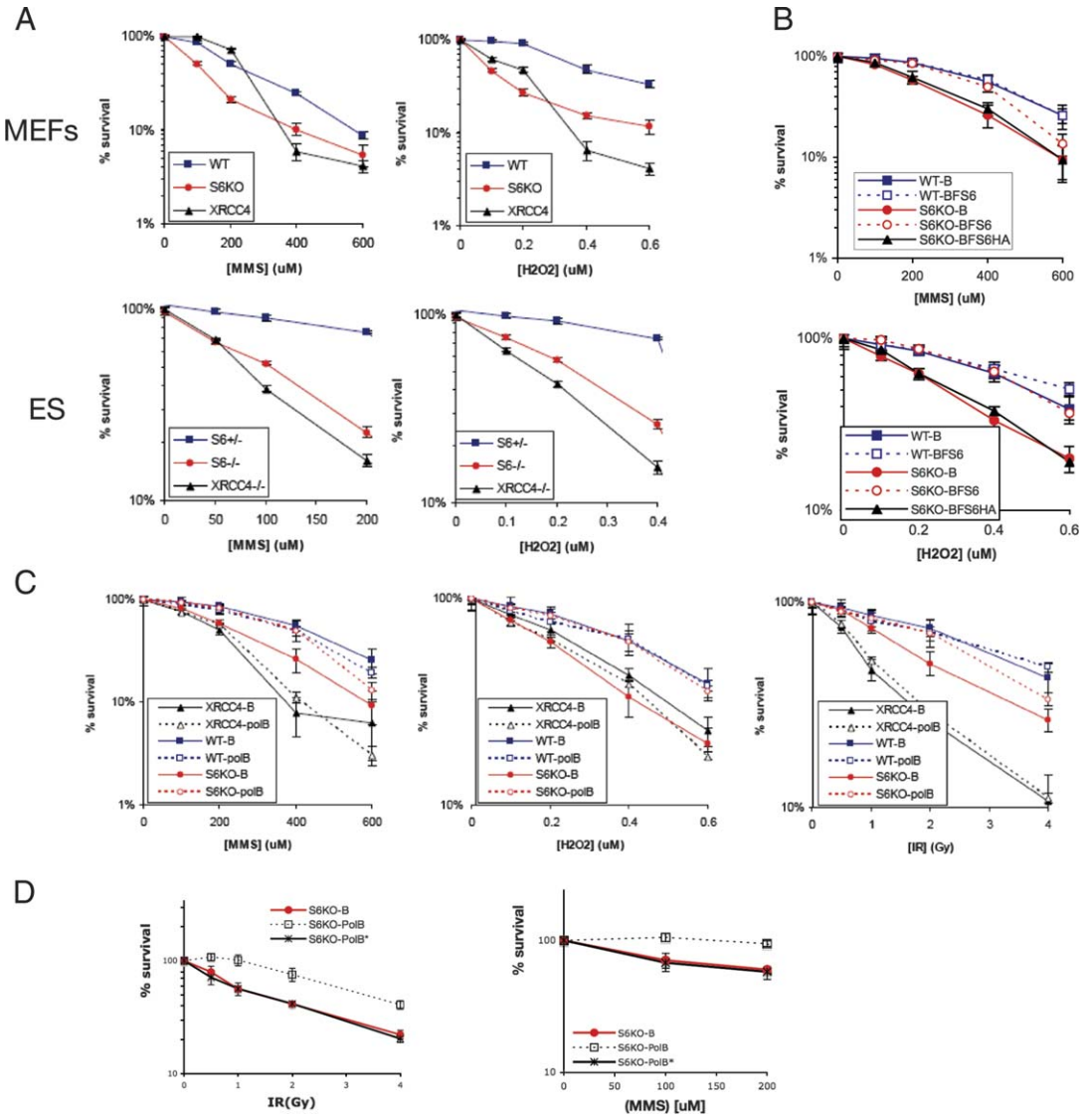


Figure 5. SIRT6 Functions in Base Excision Repair

(A) MEFs (upper) or ES cells (lower) were treated with the doses of MMS or hydrogen peroxide (H_2O_2) indicated, and cell number (MEFs) or colony number (ES cells) were quantitated 1 week following treatment. XRCC4-deficient MEFs or ES cells were included as positive controls.

(B) SIRT6-deficient MEFs or littermate controls were infected with an empty retrovirus (pBABE, B), a retrovirus encoding FLAG-tagged SIRT6 (BFS6), or a retrovirus encoding a catalytic mutant form of FLAG-tagged SIRT6 (BFS6HA) and subjected to the treatments indicated above.

(C) Wild-type, SIRT6-deficient, or XRCC4-deficient MEFs were infected with empty retrovirus (B) or a retrovirus encoding the N-terminal dRP lyase domain of polymerase β (Sobol et al., 2000) and then tested for sensitivity to MMS, hydrogen peroxide, and IR.

(D) SIRT6$^{-/-}$ MEFs were infected with an empty retrovirus (B), a retrovirus encoding the N-terminal dRP lyase domain of Polβ, or a catalytic inactive N-terminal mutant (Polβ*) and tested for sensitivity to IR or MMS.

In all panels, error bars indicate the standard error of the mean.

et al., 1996). Based on these results, we hypothesized that overexpression of the N-terminal Polβ dRP lyase domain might overcome BER defects imposed by lack of SIRT6. Strikingly, overexpression of the Polβ N-terminal domain in SIRT6$^{-/-}$ MEFs via retroviral transduction restored the MMS, IR, and H_2O_2 sensitivity to wt levels (Figures 5C and S2B). This effect was not a nonspecific consequence of overexpression of the Polβ dRP lyase, as overexpression of this domain in XRCC4-deficient MEFs did not overcome IR hypersensitivity (Figure 5C). Finally, expression of a catalytically inactive form of the N-terminal domain did not rescue sensitivity of the cells, indicating that rescue was dependent on the dRP lyase activity (Figure 5D). Because Polβ functions exclusively in BER, these results further argue that SIRT6 promotes DNA repair and genomic stability by influencing BER.

Normal Expression of BER Factors and Efficient Formation of XRCC1 and PAR Foci in SIRT6-Deficient Cells

To test whether SIRT6 affects expression of any of the core BER factors, protein levels of Polβ, XRCC1, LigaseIII, Ape1, and PARP-1 were assessed in SIRT6$^{-/-}$ and control MEFs. None of these showed differences between wt and KO cells (Figure S3). In addition, we tested whether SIRT6 colocalizes with BER factors. In this regard, XRCC1 forms foci upon MMS and H_2O_2 treatment that colocalize with sites of poly-ADP ribose (PAR) formation (El-Khamisy et al., 2003). In contrast to XRCC1, SIRT6 showed diffuse nuclear staining following exposure of cells to either MMS or H_2O_2 (Figure S4A and data not shown). In addition, both XRCC1 and PAR foci formed normally in SIRT6-deficient cells (Figure S4B). Together, these results suggest that SIRT6 might function in BER through a mechanism that does not directly involve regulation of BER factors.

SIRT6-Deficient Mice Develop a Progeroid Degenerative Syndrome

To analyze the role of SIRT6 in vivo, we generated and analyzed SIRT6$^{-/-}$ mice (Figure 6A). SIRT6$^{-/-}$ mice were born at Mendelian frequency (SIRT6$^{-/-}$ 48 [22%], n = 223) and showed no abnormalities at birth (data not shown). The mice developed normally for the first two weeks, with no apparent histologic abnormalities, though reduced body size became apparent early after birth (Figure 6A). Strikingly, at ~3 weeks of age, the mice underwent several acute degenerative processes and failed to thrive, invariably dying at around postnatal day (P) 24 (Figures 6A and 6B). Occasional SIRT6-deficient mice that died prior to day 20 also exhibited the same deterioration. Defects observed in these mice include acute loss of subcutaneous fat, lordokyphosis, a colitis consisting of erosion of the superficial colonic epithelium, and a severe lymphopenia associated with increased lymphocyte apoptosis (Figures 6C–6G). Indeed, flow cytometric analyses revealed a 50-fold reduction in the number of CD4$^+$–CD8$^+$ double-positive (DP) cells in the thymus and a 10 fold-reduction in the number of splenic lymphocytes and progenitor B cells in the bone marrow (data not shown). In addition, SIRT6$^{-/-}$ mice exhibited osteopenia, with 30% reduction in bone mineral density (Figure 6H), a finding consistent with the lordokyphosis. Since bones are still developing in mice at this age, we cannot distinguish between developmental versus degenerative defects in bone mineralization. Food intake of the SIRT6$^{-/-}$ mice was normal, as assessed by the amount of milk in the digestive track (data not shown), excluding malnutrition as a cause for the observed phenotypes.

The profound decrease in lymphocyte numbers in SIRT6$^{-/-}$ mice could be due either to a cell-intrinsic effect of SIRT6 deficiency or to systemic, non-cell-autonomous defects. To distinguish between these possibilities, donor bone marrow cells from 12-day-old SIRT6$^{-/-}$ -mice or wt littermates, were mixed with wild-type competitor cells and transplanted into lethally irradiated hosts. In these experiments, SIRT6-deficient cells contributed to repopulation of the lymphocyte compartment as efficiently as normal competitor cells and were equally represented in these mice even 5 months after transplant (Figure 7A and data not shown). These results indicate that the lymphocyte defect of SIRT6$^{-/-}$ mice is not cell intrinsic and suggest that disappearance of the lymphoid cells reflects the response of these cells to systemic alterations caused by lack of SIRT6. In this context, thymocytes from SIRT6$^{-/-}$ mice were examined for hypersensitivity to DNA-damaging agents. Notably, thymocytes from 21-day-old mice demonstrated an increased sensitivity to IR, MMS, and H_2O_2 (Figure S5), but thymocytes from 12-day-old mice exhibited normal sensitivity (data not shown). Similar to the bone marrow transplant experiments, these results indicate that the lymphocyte defects are not cell intrinsic but rather the response of these cells to systemic defects.

Severe Metabolic Defects in the Absence of SIRT6

We assayed SIRT6$^{-/-}$ mice for systemic defects that might explain the observed phenotypes. Thymocytes are extremely sensitive to glucocorticoid-induced apoptosis (Wyllie, 1980). However, SIRT6-deficient mice exhibited normal serum glucocorticoid levels (data not shown). Insulin-like growth factor IGF-1 confers upon lymphocytes resistance to apoptosis (Pifer et al., 2003), and age-associated lymphocyte decline is associated with low levels of IGF-1 (Taub and Longo, 2005). Strikingly, serum IGF-1 levels were severely reduced in SIRT6$^{-/-}$ mice (Figure 7B), with lower levels of serum IGF-1 than found in mice with a liver-specific deletion of the IGF-1 gene (Yakar et al., 2001). Notably, IGF-1 levels were low, even in 12-day-old SIRT6$^{-/-}$ mice, before any of the other phenotypes were evident. Serum glucose, although normal in 12-day-old animals, decreased sharply afterwards and by day 24, reached the limit of detection (Figure 7C). Thus, SIRT6 function is required for proper glucose homeostasis and maintenance of normal IGF-1 levels.

DISCUSSION

SIRT6 Influences DNA Repair and Suppresses Degenerative Pathologies

The yeast SIR2 protein is a chromatin regulator that silences DNA recombination and, thereby, regulates life span. We show that SIRT6 is associated with chromatin and that cells deficient for this factor display defective BER and elevated levels of spontaneous genomic instability. Moreover, SIRT6 deficiency in mice leads to aging-like degenerative processes. In mammals, regulation of genomic stability has been linked to both tumor suppression and aging (Lombard et al., 2005). While etiologies of SIRT6-deficient mouse pathologies remain to be determined, some may reflect functions of SIRT6 in BER and genome stabilization. Overall, our findings that SIRT6 regulates genomic stability on the cellular level and aging-associated pathologies at the organismal level demonstrate that the SIRT6 protein has certain functions predicted for the mammalian SIRT proteins based on analogies to yeast Sir2 (Blander and Guarente, 2004;

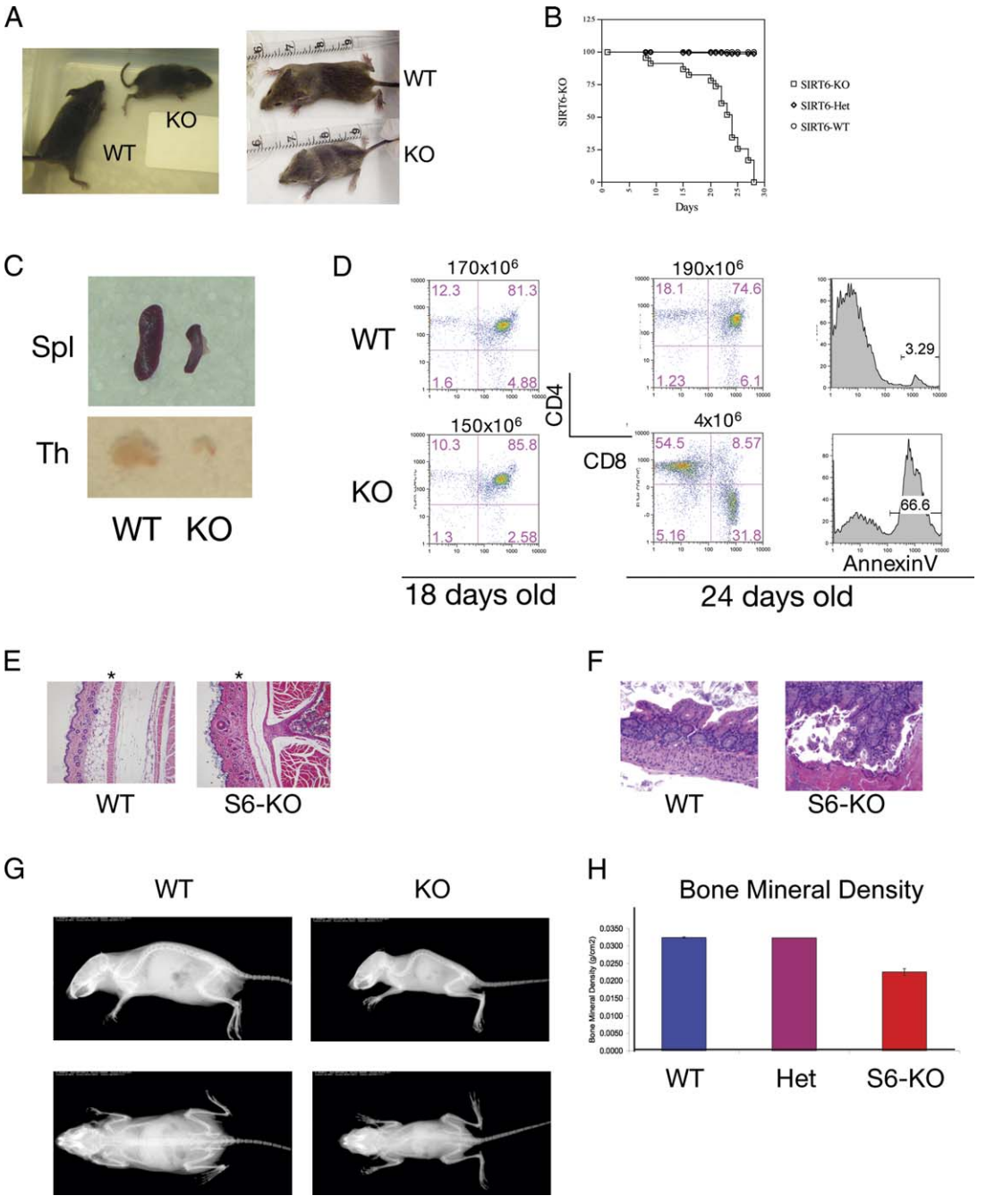


Figure 6. SIRT6-Deficient Mice Suffer from an Acute, Lethal Degenerative Phenotype

(A) wt and KO mice, 21 days old.

(B) Kaplan-Meier curve representing the percent survival of SIRT6$^{-/-}$ (n = 23), SIRT6$^{+/-}$ (n = 148), and SIRT6$^{+/+}$ (n = 86) cohort mice versus age in days.

(C) Spleen and thymus from 23-day-old wt and KO SIRT6 mice.

(D) Thymocyte numbers and profiles were measured in SIRT6-deficient mice at various weeks after birth by staining for CD4 and CD8 to reflect major developmental stages such as CD4^{+}/CD8^{+} double-positive (DP) thymocytes and annexin V to reveal apoptotic cells.

(E) Histologic analysis demonstrates an absence of subcutaneous fat in 3-week-old SIRT6-deficient animals.

(F) Histological analyses of SIRT6-deficient colons reveals colitis with prominent epithelial sloughing.

(G and H) Osteopenia and lordokyphosis in SIRT6-deficient mice. X-irradiation and bone mineral density analysis were performed on 3.5-week-old mice of the indicated genotypes. Results are average of 5 mice per genotype.

In all panels, error bars indicate the standard error of the mean.

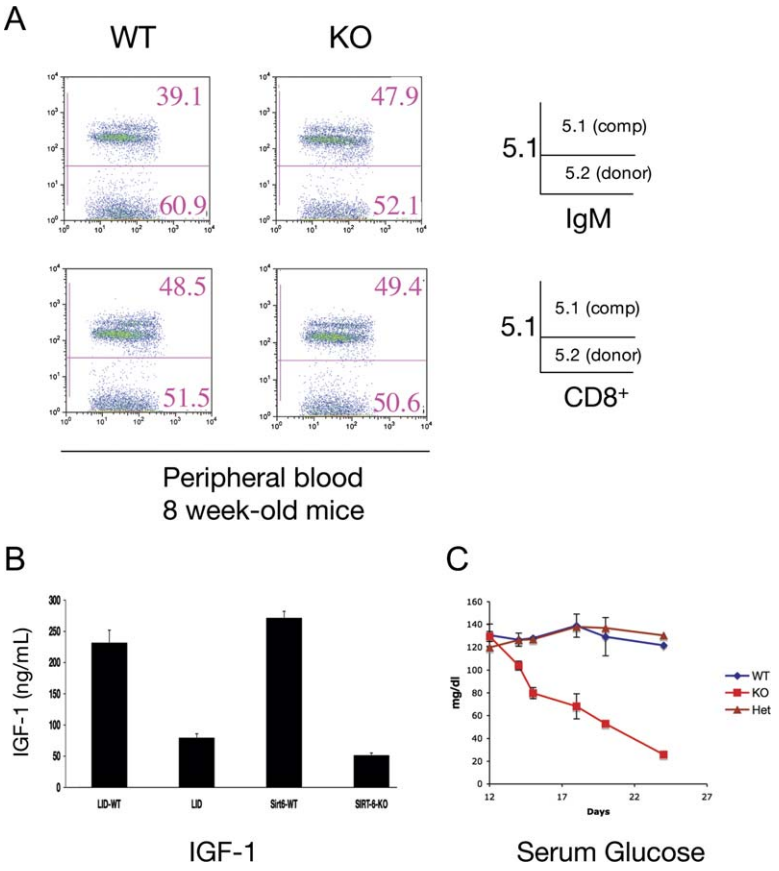


Figure 7. SIRT6$^{-/-}$ Mice Exhibited Non-Cell-Autonomous Defects in Lymphocytes and Severe Metabolic Defects

(A) Bone marrow was purified from 12-day-old SIRT6-deficient (KO) and control (wt) mice both carrying the alloantigen CD45.2 [5.2], and 1×10^6 cells were injected into lethally irradiated mice, together with equal amounts of competitor cells (alloantigen CD45.1 [5.1]). Cells were stained with either 5.1-PE and IgM-FITC (top), 5.1-PE and CD8-FITC (bottom), or 5.1-PE and CD4-FITC (data not shown) to distinguish B and T cells, respectively. FACS analysis from 8-week-old mice is shown.

(B) Blood was collected from 19-day-old mice of the indicated phenotypes, and IGF-1 levels in serum was measured. Liver-IGF-1-deficient mice (LID) were used as controls. Results are average of 10 mice per genotype.

(C) Blood was collected from mice of the indicated age, and serum glucose was measured. Results are average of 10 mice per genotype.

In all panels, error bars indicate the standard error of the mean.

North and Verdin, 2004). We note, however, that specific pathways affected by the absence of Sir2 in yeast and SIRT6 in mammalian cells, thus far, appear different.

SIRT6 and BER

Our findings indicate that SIRT6 is functionally linked to BER. BER is the main pathway for repair of spontaneously occurring single-stranded DNA lesions. Repair of single-strand lesions by BER via monofunctional glycosylases involves introduction of SSBs at lesions by the APE1 endonuclease (Barnes and Lindahl, 2004). This reaction leaves a 5′-deoxyribose phosphate (dRP) at the SSB, which must be removed following gap-filling by Polβ. Removal of dRP is rate limiting in this branch of BER and is catalyzed by the Polβ dRP lyase domain, which can rescue MMS sensitivity of Polβ-deficient cells (Srivastava et al., 1998). SIRT6 deficiency leads to spectrum of sensitivities to H_2O_2, MMS, and IR that are consistent with a BER defect. Moreover, overexpression of the Polβ dRP lyase domain rescues hypersensitivity of SIRT6-deficient cells to these agents. Thus, SIRT6 appears to function upstream of the Polβ dRP lyase reaction in this branch of BER. On the other hand, SIRT6 appears dispensable for DSB repair and for repair of UV damage which is normally handled by NER, suggesting that SIRT6 has a specific influence on BER. Notably, a trypanosome *T. brucei* Sir2 family member, TbSIR2RP1, also protects against MMS-induced DNA damage (Garcia-Salcedo et al., 2003). Thus, a function for Sir2 homologs in BER may be evolutionarily conserved.

It remains unknown how SIRT6 influences BER and whether it does so directly or indirectly. Thus far, we have not detected a physical association between endogenous SIRT6 and BER factors (Figures S6A and S6B). In addition, neither Polβ levels nor those of other core BER factors appear markedly affected by SIRT6 deficiency (Figure S3). As Polβ lyase activity can be inhibited via acetylation (Hasan et al., 2002), SIRT6 theoretically might regulate Polβ via deacetylation. While in vitro deacetylase activity had not been found for SIRT6 (Liszt et al., 2005; Michishita et al., 2005; North et al., 2003), we detected low-level deacetylation of Polβ when incubated with purified SIRT6 (Figure S7A). However, the significance of this finding is unclear; since in vivo Polβ acetylation levels, within the resolution of our assays, remained unchanged in SIRT6$^{-/-}$ cells (data not shown).

SIRT6$^{-/-}$ extracts performed BER on a naked DNA substrate at levels similar to wt extracts (Figures S7B and S7C), raising the possibility that SIRT6 may regulate BER indirectly. In this regard, Polβ activity is hampered on chromatinized templates (Beard et al., 2003; Nilsen et al., 2002). Given that dRP removal is the rate-limiting step in BER, rescue of SIRT6$^{-/-}$ cells with the Polβ dRP domain theoretically could reflect ability of this 8 kDa protein fragment to overcome chromatin restraints imposed by lack of SIRT6. Notably, the trypanosomal TbSIR2RP1 protein was proposed to

promote chromatin accessibility following MMS-induced DNA damage by catalyzing histone ADP ribosylation and deacetylation (Garcia-Salcedo et al., 2003). While SIRT6 has an ADP-ribosylation activity in vitro (Liszt et al., 2005; Figure S8), it remains unclear whether the activity occurs under physiologic conditions. Based on existing information, it is tempting to speculate that SIRT6 may promote BER by creating accessibility for BER factors via modification of histones or other chromatin-related factors.

SIRT6 Deficiency and Aging-Related Degeneration

ROS have been implicated in the pathogenesis of aging (Finkel and Holbrook, 2000). As oxidative DNA damage is repaired primarily by BER, impairment of this process might cause aging-related phenotypes. In this regard, nuclear BER and Polβ activity has been reported to decrease with age, and this decline can be prevented by CR (Cabelof et al., 2002; Cabelof et al., 2003). As targeted disruption of BER genes often leads to early lethality due to critical functions in development or to no phenotype, likely due to functional redundancy (Hasty et al., 2003), the SIRT6-deficient mouse potentially could provide a useful model of defective, presumably hypomorphic, BER. In this context, there are a number of similarities between the progeroid phenotypes of SIRT6-deficient mice and those of mice with NER deficiencies (de Boer et al., 2002; Murai et al., 2001). XPA/CS and XPA/TTD mice are normal at birth but show decreased size shortly after and die around day 22, with kyphosis, lack of subcutaneous fat, and cachexia, all degenerative phenotypes reminiscent of those of SIRT6-deficient mice. However, the XPA/CS and XPA/TTD animals differ in certain respects from SIRT6-deficient mice; most notably, they lack lymphocyte depletion. Conversely, the XPA/CS deficient mice develop severe ataxia due to depletion of cerebellar cells, a phenotype not observed in the SIRT6-deficient mice. However, in both cases, affected organs are normal at birth and show elevated apoptosis after a period of weeks. In this regard, there may be differential requirements for the BER and NER pathways in certain tissues, or some phenotypes could be unrelated to the repair defects, particularly in the case of SIRT6 deficiency.

Severe Metabolic Defects in the Absence of SIRT6: The Insulin-Signaling Connection

Our bone marrow transplantation experiments indicate that lymphocyte attrition in SIRT6-deficient mice is not a cell-autonomous defect. During normal aging, depletion of lymphocytes has been linked to a diminished response to circulating factors (Allman and Miller, 2005; Stephan et al., 1997). Furthermore, circulating growth factors, like IGF-1, can protect lymphocytes against corticosteroid-induced apoptosis (Pifer et al., 2003). In this context, thymic atrophy has been correlated with pituitary gland deficiency and low levels of circulating neuroendocrine hormones (Taub and Longo, 2005). In addition, housing conditions dramatically affect lymphocyte homeostasis (Dorshkind et al., 2003). Thus, lymphocyte homeostasis is extremely sensitive to changes in systemic factors, as well as to levels of stress. In this regard, SIRT6-deficient mice exhibited a severe reduction in circulating IGF-1 levels, a defect that could explain some of their phenotypes, and serum glucose was reduced to levels that may lead to their demise. This dramatic imbalance in glucose metabolism clearly indicates that SIRT6 plays an important role in regulating organismal homeostasis.

Perturbations in IGF-1 and insulin signaling have been linked to alterations in the rate of aging in multiple organisms (Kenyon, 2005). In *C. elegans*, Sir2 regulates life span via the Daf-16 transcription factor, a critical mediator in the insulin-like signaling pathway (Tissenbaum and Guarente, 2001). Although decreased IGF-1 signaling has been correlated with increased life span in worms, flies, and mice (Kenyon, 2005), several mouse models of premature aging in fact are associated with lower serum IGF-1 or insulin levels, such as ATM (Peretz et al., 2001) and Klotho-deficient mice (Mori et al., 2000). In addition, reduced serum IGF-1 is observed in elderly humans (reviewed in Lombardi et al. [2005]). Thus, the role of IGF-1 in life span regulation is complex. In theory, SIRT6 might play a role in insulin signaling, similar to Sir2 factors in other lower organisms. However, as in the premature aging mouse models described above, it remains unclear whether the altered serum IGF-1/insulin levels of SIRT-6-deficient mice directly contribute to aging-like phenotypes or, alternatively, reflect compensatory alterations. In this regard, it will be of interest to determine whether SIRT6 is involved in regulating the IGF-1 response to CR (Bartke, 2005). Finally, it is notable that mice in which the apoptotic function of cytochrome C was disrupted exhibited quite similar phenotypes to those of SIRT6-deficient mice and also involved defects in the insulin signaling pathway (Hao et al., 2005).

Perspective

We show that mammalian SIRT6 protein plays a key role in DNA repair and maintenance of genomic stability in cells. Moreover, we show that SIRT6 is necessary to maintain organismal health and to prevent the development of several progeroid pathologies. Further studies of SIRT6-deficient cells may elucidate molecular mechanisms that regulate the BER pathway, while SIRT6-deficient mice may provide a model for deciphering elements that influence the development of progeroid symptoms and allow evaluation of potential roles of defective BER in such degenerative processes. In these contexts, it will be of particular importance to elucidate the relationship, if any, between the metabolic and DNA repair defects of the SIRT6-deficient mice.

EXPERIMENTAL PROCEDURES

Chromatin Fractionation

Cell fractionation was performed as previously described (Mendez and Stillman, 2000).

Western and Immunostaining Analysis

Western analysis was carried out as previously described (Cheng et al., 2003). The antibodies used and their sources were as follows: anti-mouse SIRT6 (Liszt et al., 2005), anti-DNA polymerase β (NeoMarkers), anti-α-tubulin (Sigma). For immunostaining analysis, cells were grown in

coverslips and fixed in 1% paraformaldehyde as described (Bassing et al., 2002). The LacZ staining of embryos was performed as described (Whiting et al., 1991).

Construction of the Targeting Vector and Generation of SIRT6-Deficient Mice

The SIRT6 KO targeting vector was constructed by replacing exons 1 to 6 with a LacZ gene inserted in frame after the first 21 bp of exon1. The construct was generated using the VelociGene recombination method (Valenzuela et al., 2003). Chimeric mice were generated by injection of targeted ES clones into C57BL6/J blastocysts. Male chimeras were mated with 129SvJ females to generate F1 heterozygous mice, which were interbred to generate homozygous KO mice.

Generation of MEFs, Metaphase Analysis, and DNA-Damage Assays

MEFs were generated from 13.5-day-old embryos by using standard methods. Metaphases were prepared as previously described (Zhu et al., 2002). Q-FISH was performed as previously described (Chua et al., 2005), using a Cy3-labeled PNA telomeric probe (Cy3-$(TTAGGG)_3$). To assay for sensitivity to DNA damage, 5×10^4 MEFs were plated into 6-well plates and 12 hr later either γ irradiated, UV irradiated, or treated with H_2O_2 or MMS for 24 hr at the doses indicated.

DNA Repair Assay and γH2AX Analysis

The DNA repair assay was performed as described (Wong et al., 2000). For γH2AX analysis, the indicated cells were cultured on slides, irradiated with 20 Gy, followed by fixation in 4% paraformaldehyde at the indicated time points. γH2AX immunostaining was performed as described (Bassing et al., 2002).

Extrachromosomal VDJ Recombination Assay in MEF Cells

V(D)J recombination of plasmid substrates was performed as described (Hesse et al., 1987; Taccioli et al., 1993).

Cell Cycle Analysis

BrdU incorporation was assayed with anti-BrdU antibodies (BD Pharmingen) according to the manufacturer's instructions. Briefly, 5×10^5 cells were irradiated with the indicated doses of IR, and 24 hr later were pulsed with BrdU for 4 hr, harvested, stained with FITC-conjugated anti-BrdU antibodies and propidium iodide, and cell cycle profiles analyzed by flow cytometry. For G2/M analysis, cells were irradiated with the indicated doses of IR, and 1 hr later harvested and stained with anti-phospho H3 antibodies (Upstate), a specific mitotic marker (Wei et al., 1998).

Retroviral Infection

Reconstitution of MEFs was performed as previously described (Cheng et al., 2003). The different SIRT6 cDNAs (wt and mutant) and the dRP lyase 8 kDa Polβ cDNAs (wt and mutant) were amplified by PCR and cloned into the pBabe-puro vector. MEFs were infected by incubation with virus and 2 μg/ml polybrene and 48 hr later, selected in 2.5 μg/ml puromycin. Cells were allowed to recover from selection for 48 hr and then plated for the different experiments.

Histological Analysis

Mouse tissue was fixed in Bouin's fixative, embedded in paraffin, sectioned at 6 μm, and hematoxylin/eosin staining was performed by standard methods.

IGF-1 and Glucose Measurements

IGF-1 was measured with commercially available radioimmunoassay (RIA) kits from Linco Research, Inc. and Diagnostic Systems Laboratories, Inc. Serum glucose was measured from tail blood using the Elite glucometer (Elite) kit, following manufacturer's instructions.

Bone Mineral Density Analyses

Whole-body bone mineral density was measured by dual energy X-ray absorptiometry (DXA) using a GE Lunar Piximus II densitometer.

Bone Marrow Transplantations (BMT)

Competitive repopulation studies were performed as previously described (Mostoslavsky et al., 2005). Briefly, CD45.1 recipient mice were lethally irradiated with two doses of 7 Gy, 3 hr apart, 1 day before BMT. 1×10^6 whole marrow cells from CD45.1 mice were mixed with 1×10^6 whole marrow cells purified from either CD45.2 control or SIRT6$^{-/-}$ mice (CD45.2 as well), and injected retroorbitally. Peripheral blood was obtained every 4 weeks and stained as indicated. Following gating on B or T cells, respectively, cells were gated on CD45.1-PE, and the relative chimerism calculated. All antibodies were from BD Biosciences.

Supplemental Data

Supplemental Data include eight figures and can be found with this article online at http://www.cell.com/cgi/content/full/124/2/315/DC1/.

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