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DNA damage-induced alterations in chromatin contribute to genomic integrity and age-related changes in gene expression

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Abstract

Genomic instability and alterations in gene expression are hallmarks of eukaryotic aging. The yeast histone deacetylase Sir2 silences transcription and stabilizes repetitive DNA, but during aging or in response to a DNA break, the Sir complex relocates to sites of genomic instability, resulting in the desilencing of genes that cause sterility, a characteristic of yeast aging. Using embryonic stem cells, we show that mammalian Sir2, SIRT1, represses repetitive DNA and a functionally diverse set of genes across the mouse genome. In response to DNA damage, SIRT1 dissociates from these loci and relocates to DNA breaks to promote repair, resulting in transcriptional changes that parallel those in the aging mouse brain. Increased SIRT1 expression promotes survival in a mouse model of genomic instability and suppresses age-dependent transcriptional changes. Thus, DNA damage-induced redistribution of SIRT1 and other chromatin modifying proteins may be a conserved mechanism of aging in eukaryotes.

Keywords

epigenetics; sirtuin; chromatin; histone; DNA repair

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Introduction

Chromosomes are arguably the most difficult structure for an organism to maintain over a lifetime. Chromosomes break, mutations accumulate, and youthful gene expression patterns are progressively lost. Some changes in gene expression have been interpreted as beneficial responses to cellular damage (Narita et al., 2006; Niedernhofer et al., 2006). However, there are numerous stochastic changes in gene expression that have no apparent long-term benefit to the organism and may in fact be detrimental (Bahar et al., 2006). The "Heterochromatin Island Hypothesis" and related hypotheses propose that alterations in chromatin and the resulting gene expression changes can drive the aging process, but evidence is lacking (Cutler, 1995; Imai and Kitano, 1998; Vijg, 2004; Villeponteau, 1997).

Potential clues about the relationship between epigenetic changes and aging come from studies in *Saccharomyces cerevisiae*, where epigenetic changes are a primary cause of the aged phenotype. The *SIR2* gene, encoding a nutrient-responsive NAD⁺-dependent histone deacetylase, has emerged as a key regulator of health and lifespan in yeast and other organisms (Haigis and Guarente, 2006). Two major functions of Sir2 are to repress gene expression at the silent mating type loci *HML* and *HMR* (Klar et al., 1979; Rine et al., 1979) and to suppress recombination at the ribosomal DNA (rDNA) locus, which gives rise to toxic rDNA circles (ERCs) (Sinclair and Guarente, 1997). As yeast cells age, the Sir protein complex dissociates from *HM* loci and moves to the nucleolus in response to ERC accumulation causing sterility, a hallmark of yeast aging (Kennedy et al., 1997; Sinclair and Guarente, 1997; Smeal et al., 1996). Thus, a redistribution of chromatin modifying factors and the resulting changes in transcription are the cause of primary yeast aging phenotypes.

Aging is not the only stimulus that causes yeast Sir proteins to relocalize. DNA breakage causes Sir proteins to dissociate from *HM* loci and relocate to DNA breaks, in a DNA damage checkpoint dependent manner (Martin et al., 1999; McAinsh et al., 1999; Mills et al., 1999). The effect of relocalization appears to be two fold: (i) expression of *HM* genes promotes DNA repair and (ii) Sir proteins directly modify chromatin surrounding the break site, possibly to facilitate DNA repair (Lee et al., 1999; Tamburini and Tyler, 2005).

There is some evidence that related processes occur in mammals. First, cells damaged by oxidative stress *in vitro* undergo stochastic transcriptional changes that parallel those in aged heart tissue (Bahar et al., 2006). Second, a deficiency in the DNA repair factor ERCC1 accelerates aging phenotypes and generates gene expression profiles reminiscent of aged animals (Niedernhofer et al., 2006). Third, cells that senesce due to replicative aging *in vitro* or in aged tissues *in vivo* exhibit alterations in heterochromatin (Herbig et al., 2006; Narita et al., 2006) and secrete growth factors that can drive tumorigenesis (Campisi, 2005). Finally, oxidative DNA damage at promoters correlates with gene repression in the aging human brain (Lu et al., 2004) and has been linked to both transcriptional and epigenetic changes that may contribute to Alzheimer disease (Wu et al., 2008).

To date, no study has tested whether Sir2-mediated alterations in chromatin contribute to aging in mammals. Several observations, however, are consistent with this possibility. The mammalian ortholog of Sir2, SIRT1, regulates both the expression of individual genes (Picard et al., 2004; Pruitt et al., 2006; Vaquero et al., 2004) and the formation of facultative heterochromatin (Vaquero et al., 2007). SIRT1 has also been linked to the DNA damage response via regulation of p53 (Luo et al., 2001; Vaziri et al., 2001) and its interaction with Nbs1, a component of the DNA damage sensor complex MRN (Mre11-Rad50-Nbs1) (Yuan et al., 2007). Furthermore, SIRT1 has recently been implicated in the regulation of DNA methylation patterns at damaged CpG-rich DNA (O'Hagan et al., 2008). Deletion of another Sir2 homolog, SIRT6, reduces base excision DNA repair and causes an accelerated aging

phenotype in mice (Mostoslavsky et al., 2006). In this study, we map the interaction between SIRT1 and the mouse genome and identify an evolutionarily conserved DNA damage response that may drive changes in gene expression during aging.

Results

Loss of Sir2 and SIRT1-dependent silencing in response to oxidative stress

Our previous studies on the relocalization of yeast Sir2 utilized highly artificial means to induce DNA damage, such as *EcoRI* and the yeast *HO* endonuclease (Mills et al., 1999). To test whether a stress more relevant to aging results in desilencing of mating-type loci, a yeast strain carrying a GFP reporter at the *HMR* locus (*HMR::GFP*) was exposed to oxidative stress (i.e. H₂O₂). There was a tight correlation between H₂O₂ levels and *HMR* derepression (Figure 1A and Figure S1A).

An additional copy of *SIR2* (*2xSIR2*) extends replicative lifespan (Kaeberlein et al., 1999), but its effect on oxidative stress resistance and loss of silencing is unknown. When treated with H₂O₂, strains with increased Sir2 levels maintained *HMR* silencing and had more stable rDNA compared to wild type cells (Figures 1A and B). At non-lethal concentrations of H₂O₂, the *2xSIR2* strain had a significantly greater replicative lifespan (Figure 1C and Figure S1B). Thus, additional *SIR2* suppresses the toxicity, genomic instability, and desilencing caused by genotoxic stress.

We next sought to examine whether these findings are relevant to mammals. We first tested if SIRT1 associates with highly repetitive DNA such as pericentromeric major satellite repeats in mouse ES cells, a cell type that has been previously used to study satellite repeat silencing (Kanellopoulou et al., 2005). Histone H1 acetylation on lysine 26 (H1AcK26) served as a read-out for SIRT1 deacetylase activity (Vaquero et al., 2004). Using chromatin immunoprecipitation (ChIP) and quantitative PCR (q-PCR), we detected an association between SIRT1 and major satellite repeats that was disrupted by the pansirtuin inhibitor nicotinamide (NAM) (Figure 1D and 1E). This coincided with an increase in repeat transcripts (Figure 1F) and H1AcK26 (Figure 1E). Consistent with known redundancy among histone deacetylases (Zhu et al., 2004), trichostatin A (TSA), NAM and sirtinol, pan-class I/II and pan-class III (sirtuin) inhibitors, respectively, caused desilencing of major satellite repeats (Figure 1F and Figures S1D and E, (Kanellopoulou et al., 2005)), whereas no significant increase in repeat transcripts was seen after stable knock-down of SIRT1 (Figure S1C and data not shown). Ongoing work is aimed at identifying the role of other HDACs that may contribute to satellite repeat silencing.

In accordance with our observations in yeast, treatment of cells with non-cytotoxic levels of H₂O₂ greatly decreased the amount of SIRT1 bound to repeats, coinciding with an increase in H1AcK26 (Figure 1E, Figure S2 and S3). Similar to the effect of NAM and TSA, oxidative stress increased the transcription of these loci (Figure 1F), an effect that was counteracted by overexpression of SIRT1 (Figure 1G). Together, these data indicate that SIRT1 binds and can contribute to the silencing of major satellite repeats.

Global changes in promoter-associated SIRT1 in response to oxidative stress

Given the role of yeast Sir2 in silencing *HM* loci, we hypothesized that mammalian SIRT1 might regulate a number of protein-coding genes. To test this, we used ChIP in combination with a genome-wide promoter tiling array (ChIP on chip) to identify putative SIRT1 target genes. Immunoprecipitated DNA from either untreated or H₂O₂-treated cells was hybridized to a NimbleGen MM5 array to detect SIRT1- and H1AcK26-associated promoter segments (Figure 2A). Based on gene ontology (GO), overrepresented groups included genes for chromatin assembly and transcriptional repression (e.g. methyl-CpG binding proteins 2, -3

and -4, SIRT7, and several histone genes), ubiquitin-regulated protein degradation, and cell cycle regulation (Figure 2B, S4A and Table S1).

Next, we determined if SIRT1-binding and H1 acetylation patterns change in response to oxidative stress. Paralleling the yeast response, oxidative stress caused a redistribution of SIRT1 at the chromatin level (Figure 2A), such that less than 10% of SIRT1-associated promoters overlapped between untreated and H₂O₂-treated cells (Figure S4B), and the resulting binding pattern did not cluster into functional GO groups, indicating a shift to random SIRT1 distribution (Figure 2B). There was a significant negative correlation between the loss of SIRT1 binding and H1K26 acetylation ($\chi^2=12.12$, $P<0.001$, Figure 2C), supporting the notion that SIRT1 regulates these genes, at least in part, through H1 deacetylation. Together, these observations indicate that SIRT1 associates with considerably more genes than currently known and that oxidative stress causes a major change in SIRT1 distribution across the genome.

Oxidative stress causes SIRT1 dependent transcriptional deregulation

To determine how the redistribution of promoter-associated SIRT1 affects transcription, we performed a combination of microarray-based transcriptional profiling (Table S2), and q-RT-PCR analysis of SIRT1-associated and non-associated genes, comparing standard growth conditions with H₂O₂ treatment, with and without SIRT1 overexpression. We identified a diverse set of SIRT1-associated genes that significantly increased in expression upon H₂O₂ treatment, coincident with SIRT1 release, including regulators of metabolism (*pisd*, *prkag3*, *gstz1*), ion transport (*slc9a9*), cell motility (*tcte3*) and G-protein signaling (*farp2*). Of these genes, 75% were repressed by modest overexpression of SIRT1 (Figure 3) and H1K26 acetylation inversely correlated with SIRT1 binding (Figure S5A). Demonstrating specificity, SIRT1 overexpression did not repress the induction of genes that were not associated with SIRT1 (Figure 3B). The reduction in SIRT1 binding was confirmed for a random selection of promoters by q-PCR using two distinct types of genotoxic stress: H₂O₂ and methyl-methane-sulfonate (MMS) (Figure S5B). Inactivation of SIRT1 resulted in transcriptional deregulation of six of the eight SIRT1-associated loci, further corroborating a regulatory role for SIRT1 at these sites (Figure S5C). SIRT1 was recently shown to negatively regulate HDAC1 and can, thus, function both as transcriptional activator and repressor (Binda et al., 2008), which may explain why knockdown of SIRT1 does not always alter gene expression in the same direction as oxidative stress. Together, our results indicate that DNA damage induces a change in SIRT1 distribution that affects the expression of individual genes, and that this effect can be suppressed by increasing SIRT1 levels.

SIRT1 is recruited to DNA double strand breaks

Given that the yeast Sir complex redistributes from silent loci to sites of DNA repair (Martin et al., 1999; Mills et al., 1999), we wondered if SIRT1 also behaves in this way. When isolating chromatin-bound and non-chromatin-bound protein fractions from untreated or H₂O₂-treated cells, we observed a substantial, transient, and dose-dependent increase in chromatin-associated SIRT1. Treatment with MMS produced a similar effect, supporting the idea that SIRT1 is recruited to damaged DNA from promoters and from the soluble nuclear pool (Figure 4A, Figures S6A and S6B).

In yeast, the recruitment of Sir proteins to a DNA double-strand break (DSB) requires DNA damage signaling through *MEC1*, an ortholog of the mammalian PI3-kinases ATR/ATM (Martin et al., 1999; McAinsh et al., 1999; Mills et al., 1999). To test whether DNA damage signaling is required for SIRT1 redistribution, cells were treated with the PI3 kinase inhibitor wortmannin or the ATM inhibitor KU55933 prior to H₂O₂ exposure. The increase

in chromatin-associated SIRT1 was strongly reduced by both compounds (Figure 4B). We then investigated whether SIRT1 recruitment is dependent on histone H2AX, one of the immediate targets of ATM (Burma et al., 2001). In H2AX-deficient ES cells there was a 2–3 fold reduction in the amount of chromatin-bound SIRT1 in response to H₂O₂ or MMS compared to cells reconstituted with wild-type H2AX (Figure 4C and Figure S6C, (Xie et al., 2004)). Thus, efficient recruitment of SIRT1 to damaged DNA requires DNA damage signaling through ATM and H2AX.

Rad51, a critical component of the homologous DSB repair (HR) process, was recruited to chromatin concomitant with SIRT1 in response to both H₂O₂ and exposure to DSB-inducing ionizing irradiation (IR). Importantly, Rad51 recruitment was impaired in the absence of SIRT1 (Figure 4D–E). These data indicated that SIRT1 might physically associate with DSBs and perform a key step in the DNA repair process.

To test this, we employed a cell-based system in which a DSB is induced by transient transfection with a vector encoding the endonuclease *I-SceI* (Weinstock et al., 2006). This system has been used previously to identify the DSB-binding patterns of a number of DNA repair enzymes (Rodrigue et al., 2006). Nbs1, a component of the MRN complex served a positive control for binding (Berkovich et al., 2007). Concomitant with recruitment of Nbs1, SIRT1 binding was detected 24 h after transfection with *I-SceI* at the DSB site. Similar kinetics were recently shown for the interaction between SIRT1 and DSBs in CpG-rich DNA (O'Hagan et al., 2008).

Interestingly, the association of Nbs1 with the break site was delayed and strongly reduced in the absence of SIRT1 (Figures 5A and B). A similar effect was observed for recruitment of Rad51 (Figure 5C). These data show that SIRT1 physically associates with sites of DNA damage and agree with recent studies indicating that chromatin-modifying enzymes are recruited to the DSB to prepare the site for incoming DNA repair factors (Botuyan et al., 2006; Tamburini and Tyler, 2005).

SIRT1 is required for efficient DSB repair and genomic stability

DSB repair occurs through two major pathways: HR and non-homologous end-joining (NHEJ). Our Rad51 data suggested a role for SIRT1 in HR, which can be measured by determining the repair frequency with which a defective GFP gene is restored to wild-type upon *I-SceI* transfection (see Figure S7A). Inhibition of SIRT1 activity with NAM, the specific SIRT1 inhibitor S91211 (Solomon et al., 2006), or stable knockdown of SIRT1 resulted in a 25% to 50% reduction in GFP⁺ cells, indicating that SIRT1 is necessary for efficient HR-mediated repair (Figure 5D, Figure S7B). Repair by NHEJ, the prominent DSB repair pathway in G1 and post-mitotic cells, was also reduced in this assay system, but to a lesser extent (Figure S7C).

To test whether SIRT1 is necessary for the maintenance of genomic stability, SIRT1 knock-down ES cell lines were transiently exposed to H₂O₂ and analyzed for metaphase aberrations. In the absence of genotoxic stress, there was no significant difference in chromosomal stability between SIRT1-deficient and control cells, consistent with previous findings (Chua et al., 2005). Strikingly, H₂O₂ treatment caused a significant increase in chromosomal aberrations specifically in SIRT1 deficient cells (Figure 5E). The frequency of chromatid breaks was comparable between knock-down and control ES cells, but the number of chromosomal fusions, in particular dicentric chromosomes and Robertsonian translocations, was significantly higher in the absence of SIRT1 (Table S3). Chromosome fusions are generally a result of aberrantly repaired DNA breaks, further supporting a role for SIRT1 in DSB repair. Although a general DSB defect should also increase chromatid

breaks, we predominantly detected stably inherited aberrations such as fusions as metaphases were analyzed 48 h (approx. two divisions) after exposure to H₂O₂.

Increased SIRT1 levels protect from irradiation-induced cancer in mice

To test if SIRT1 promotes genomic stability *in vivo*, we used p53^{+/-} mice (Jacks et al., 1994), which, when exposed to IR, show a high incidence of malignant thymic lymphoma arising from a loss of heterozygosity (LOH) at the p53 locus (Kemp et al., 1994). To explore the role of SIRT1 in damage-induced LOH, p53^{+/-} mice were fed the SIRT1 activator resveratrol (Baur and Sinclair, 2006) three weeks prior to irradiation. Resveratrol-treated animals showed a 24% increase in survival ($\chi^2=5.0$, $P = 0.025$, Wilcoxon rank sum test) and a 45% reduction in the frequency of fatal thymic lymphomas (Figures 6A and B), resulting in a tumor spectrum highly reminiscent of non-irradiated p53^{+/-} mice (Donehower et al., 1992; Jacks et al., 1994),

Given that the effects of resveratrol may not be limited to SIRT1 activation, we generated a SIRT1 transgenic mouse strain (SIRT1^{STOP}), that allows for Cre-mediated temporal and tissue-specific overexpression of SIRT1 via the deletion of a transcriptional STOP cassette (Firestein et al., 2008). The SIRT1^{STOP} strain was crossed to p53^{+/-} mice carrying the interferon (IFN) type I inducible *Mx-cre* transgene (Kuhn et al., 1995). Mice with *Mx-cre*-dependent, IFN-inducible SIRT1 overexpression are referred to as "MISTO mice". Injection with the IFN inducer poly(I)poly(C), MISTO mice increased expression of SIRT1 in bone marrow lymphocyte progenitors (3–4 fold) as well as mature B and T cells (Figures 6C and 6D). Thymocytes had intrinsically high levels of SIRT1. Two weeks after SIRT1 induction, p53^{+/-} MISTO mice p53^{+/-} controls were exposed to 4 Gy of γ -irradiation, and monitored for tumor-related deaths. Deletion of the STOP cassette in tumor tissues was examined by Southern blot or q-PCR (Figure S8A and B).

The mean survival of MISTO mice was ~46% greater than in control animals (Figure 6E, $\chi^2=5.68$, $P=0.017$, Log-Rank test; $\chi^2=4.9$, $P=0.027$, Wilcoxon rank sum test). Furthermore, the frequency of fatal thymic lymphoma was reduced by 45% in MISTO mice, consistent with our finding in resveratrol-treated animals (Figure 6F). Tumor cells exhibited LOH at the p53 locus both in control and MISTO mice (Figure S8C and D). Together, our data indicate that increasing SIRT1 activity or quantity can increase genomic stability *in vivo* and suppress tumorigenesis.

SIRT1-associated genes are deregulated in the aged brain

Our observations indicated that SIRT1, like its yeast counterpart, is recruited to DSBs in response to genotoxic stress, resulting in a loss of silencing at repetitive DNA elements and SIRT1-regulated genes. To test if these transcriptional changes were relevant to aging, we compared the transcriptional changes caused by oxidative stress with those associated with aging using q-RT-PCR and microarrays. We chose to examine neocortex because age-related gene expression changes have been well characterized in this tissue (Lee et al., 2000; Lu et al., 2004). Interestingly, two thirds of SIRT1-bound genes that were derepressed by oxidative stress *in vitro* (Figure 3) were also derepressed during aging (Figures 7A and D). Moreover, SIRT1-target genes identified by ChIP on chip *in vitro* (see Figure 2) were significantly overrepresented amongst age-upregulated genes ($\chi^2=7.3$, $P=0.0055$; Figure 7B and Table S4). The abundance of major satellite repeat transcripts also increased significantly with age (Figure S9A).

To gain mechanistic insights, we tested whether overexpression of SIRT1 in the brain could delay these transcriptional changes, paralleling the ability of *SIR2* to suppress the expression of yeast mating type genes. The SIRT1^{STOP} transgenic mouse was crossed to a brain-

specific Cre-driver (*Nestin-cre*) to generate *Nestin-cre*; SIRT1^{STOP} mice, referred to as "NeSTO mice." Transcript levels of SIRT1-bound genes as well as age-upregulated non-SIRT1 bound (control) genes were examined in NeSTO mice with comparable SIRT1 overexpression (~10 fold, Figure 7C, Figure S9B and C) at 8–10 or 18–19 months. Strikingly, transcriptional derepression was almost exclusively observed for SIRT1-associated genes and was completely suppressed in aged NeSTO mice (Figure 7D). The only non-SIRT1 associated genes to change with ~19 months of age were inflammatory markers associated with gliosis, a characteristic of brain aging (Nichols et al., 1995). This upregulation was also repressed in NeSTO mice, indicating that there are secondary, beneficial effects of SIRT1 overexpression. The effect of SIRT1 overexpression in mice older than 19 months of age is not yet known. Together, our results confirm that SIRT1-bound genes are derepressed in the aging brain and that SIRT1 overexpression can suppress these age-related changes.

Discussion

The discovery that the yeast Sir complex relocalizes during aging and in response to DNA damage (Kennedy et al., 1995; Kennedy et al., 1997; Martin et al., 1999; McAinsh et al., 1999; Mills et al., 1999) led us to propose a model whereby aging is caused, in part, by the DNA-damage induced reorganization of chromatin, a phenomenon we have termed the "RCM response," for redistribution of chromatin modifiers (Imai and Kitano, 1998; Oberdoerffer and Sinclair, 2007; Villeponteau, 1997). Here, we present evidence that the RCM response exists in mammals, and that it may contribute to age-related changes in gene expression.

SIRT1 is recruited to DSBs and is required for efficient DNA repair

A role for chromatin modifiers in DNA repair has been convincingly shown in yeast (Tamburini and Tyler, 2005) and mammals (Bassing et al., 2002; Bhaskara et al., 2008; Botuyan et al., 2006; Celeste et al., 2002; Xie et al., 2004). Here, we show that SIRT1 binds to hundreds of promoters in the mouse genome, and that this binding pattern is altered in response to genotoxic stress, coincident with the relocalization of SIRT1 to damaged DNA.

The recruitment of SIRT1 to DSBs is reminiscent of yeast Sir2 and other histone modifying enzymes that bind to a DSB, resulting in epigenetic changes surrounding the break site (Chen et al., 2008; Tamburini and Tyler, 2005). As shown for the methylation of H4 at lysine 20, chromatin alterations surrounding a DSB can promote the recruitment of DNA repair factors such as 53BP1 (Botuyan et al., 2006). DSB associated SIRT1 may serve to deacetylate histones or DNA repair factors. Consistent with the latter, SIRT1 was shown to directly interact with Nbs1 (Yuan et al., 2007) and we show here that both proteins coexist at the break site (Figure 5B). How SIRT1 recruitment to DSBs is initiated at the molecular level will require further study, but our data indicate that ATM-mediated signaling through H2AX phosphorylation is important (Figure 4B and 4C). We further demonstrate SIRT1 is required for efficient DSB repair and genome maintenance in response to oxidative stress. The direct association with DSBs as well as the finding that SIRT1-deficient cells are checkpoint proficient suggest a DNA repair defect rather than a checkpoint defect (Cheng et al., 2003). Consistent with a role for SIRT1 in DNA repair, high doses of IR have been reported to induce a cell cycle delay in SIRT1-deficient fibroblasts (Yuan et al., 2007), reminiscent of SIRT6-deficient cells, which show a DNA repair defect and prolonged S phase but no checkpoint defect (Mostoslavsky et al., 2006).

Based on studies showing that SIRT1 can deacetylate and inactivate p53, some researchers predicted that SIRT1 will promote tumorigenesis *in vivo* (Lim, 2006). In contrast, p53^{+/-} mice with increased SIRT1 activity are less susceptible to irradiation-induced thymic

lymphoma with a 20–46% greater mean lifespan (Figure 6). It is appealing to speculate that increased SIRT1 levels protect from irradiation-induced LOH by increasing DSB repair efficiency. Our finding that MISTO transgenic mice overexpressed SIRT1 in early lymphocyte precursors but not at later stages during thymic T cell development (Figures 6C and 6D) is consistent with this hypothesis and argues against a protective role for SIRT1 at later stages of tumor progression. We cannot rule out, however, that additional protective mechanisms contributed to the protection from tumorigenesis.

Derepression of SIRT1-associated loci in response to oxidative stress and aging

SIRT1 has previously been reported to contribute to the formation of facultative heterochromatin (Vaquero et al., 2007). Our data show that SIRT1-mediated repression can also occur at constitutive heterochromatic regions such as pericentromeric DNA, as well as a number of specific genes, most prominently regulators of chromatin assembly and transcription. Oxidative stress reduces the association of SIRT1 with both repetitive loci (Figure 1E) and individual genes (Figure 2). Not surprisingly, this change in SIRT1 localization is associated with functional consequences on silencing of heterochromatic repetitive DNA (Figure 1F) as well as the expression of individual genes (Figure 3). In cells and in the aging brain, the majority of these changes were counteracted by overexpressing SIRT1 (Figure 3 and Figure 7D). Loss of SIRT1 binding, however, did not always lead to transcriptional derepression, indicating that chromatin alterations may be necessary but not always sufficient to cause transcriptional deregulation. Further work will be required to identify other chromatin modifiers or transcription factors that are involved in the RCM response. HDAC1 and HDAC2 are candidates for the RCM response given their increased binding to chromatin after DNA damage (see Figure S10).

Although a wealth of data on changes in gene expression with age has been catalogued in recent years, there is still much debate about their physiological relevance (Oberdoerffer and Sinclair, 2007; Vijg, 2004). Transcriptional changes in the elderly may be a beneficial defense response to cellular damage (Niedernhofer et al., 2006). Conversely, age-related changes in gene expression may be deleterious, yet exist because of the weak forces of natural selection at older ages. Thus, while a transient RCM response in young individuals is likely beneficial, constitutive triggering of RCM may actually contribute to aging. This duality is clearly evidenced in yeast, where the transient derepression of silent *HM* loci increases HR and resistance to DNA damaging agents (Lee et al., 1999) yet the constitutive derepression of *HM* loci in old cells is a cause of sterility (Smeal et al., 1996). In mammals, defective DNA repair is often associated with premature aging (Lombard et al., 2005). Conversely, the lack of a DNA damage response can be beneficial in situations of chronic DNA damage due to telomere dysfunction (Choudhury et al., 2007; Schaetzlein et al., 2007). Furthermore, exposure to genotoxic stress early in life seems to accelerate changes in gene expression that have been associated with age-related diseases such as amyloidogenesis (Wu et al., 2008). Interestingly, we found that constitutive overexpression of a set of age-deregulated SIRT1 target genes promotes apoptosis in primary neurons (Figure S11), however more work is needed to determine the physiological relevance of this observation.

Perspective

We have identified SIRT1 as participant in a stress response that may provide a direct link between DNA damage and gene expression changes that occur during aging. While DNA damage has been previously suggested to directly inhibit gene repression (Lu et al., 2004), our data explains how ostensibly undamaged genes may become deregulated over time. We speculate that the RCM response may also cause permanent changes to the chromatin structure at sites of repair, leading to stable transcriptional changes that accumulate over a lifetime (Oberdoerffer and Sinclair, 2007). Indeed, a recent report showed that SIRT1

recruitment to a DNA break in CpG islands can result in DNA methylation changes and heritable gene silencing (O'Hagan et al., 2008).

Because age-related transcriptional changes are not limited to SIRT1-regulated loci (Botuyan et al., 2006; Tamburini and Tyler, 2005), multiple mechanisms involving other chromatin modifiers are likely to be involved. What sets SIRT1 apart is its link to calorie restriction (CR), a dietary regimen that slows aging in mammals (Sinclair, 2005). Given that increased *SIR2/SIRT1* expression can suppress genomic instability and gene expression alterations, perhaps CR promotes genomic stability and delays aging in mammals via a similar mechanism.

Experimental Procedures

Yeast experiments

All experiments were on log phase yeast growing in YPD (2% glucose). *HMR::GFP* cells or *HMR::GFP 2xSIR2* cells (Park et al., 1999) were exposed to H₂O₂ for 30 min, followed by a 4 h recovery period, then analyzed FACS. Replicative lifespans and rDNA recombination analyses were performed as described (Lamming et al., 2005). For rDNA recombination, WT or *Sir2^{o/e} W303AR* cells were treated for 30 min with H₂O₂ (1.5 mM) prior to plating. For lifespan analysis, WT or *2xSIR2* cells were plated on regular YPD agar or agar supplemented with H₂O₂ (1 mM).

Cell culture and treatments

Mouse ES cells were cultured on gelatinized tissue culture dishes as described (Kanellopoulou et al., 2005). Stable SIRT1 overexpressing V6.5–C10 ES cells were obtained from SIRT1^{STOP} ES cells (Firestein et al., 2008) by Cre-mediated deletion of a *loxP*-flanked STOP cassette. SIRT1 knock-down ES cells were generated by lentiviral infection of V6.5–C10 ES cells (Beard et al., 2006). Cells were infected with either a luciferase-specific or a SIRT1-specific shRNA lentiviral vector (Araki et al., 2004). Generation of SIRT1 knock-out ES cells and respective wild-type cells is described (Chua et al., 2005). Cells were γ -irradiated (4 Gy, ¹³⁷Cs irradiator, Shepherd and Associates) or treated with H₂O₂ or MMS for 1 h at 37°C. Treatment with 50 μ M wortmannin (Sigma) or 25 μ M KU55933 (AstraZeneca) was started 2–3 h prior to other treatments. NAM (Sigma, 25 mM), TSA (Sigma, 0.1 μ M) or sirtinol (100 μ M) were added for the indicated times.

DRGFP-transgenic U2OS cells and I-*SceI*- or Ds-red-encoding plasmids pCBASceI and pCAGGS-Dsred are described (Weinstock et al., 2006). Stable SIRT1 knock-down and control lines were generated by lentiviral gene transfer using shRNA vectors from Open Biosystems. Transfection with pCBASce or pCAGGS-Dsred was performed using Fugene 6 transfection reagent (Roche). When indicated, cells were treated with NAM (10 mM) or S91211/EX-527 (50 μ M, (Solomon et al., 2006)) starting 2 h prior to transfection. After 48 h, cells were analyzed by FACS.

Chromatin immunoprecipitation (ChIP)

Approximately 10⁷ cells were cross-linked with 1% formaldehyde for 15–20 minutes at 37°C and quenched with glycine. Cell lysates were sonicated (Branson sonifier) and incubated overnight with rabbit α -Sir2 α (Upstate), α -Nbs1 (Novus), α -Rad51 (Calbiochem) or α -H1AcK26 (Vaquero et al., 2004). Immunoprecipitation was performed as described (Upstate) and eluates were purified using QIAgen PCR purification, followed by (q-) PCR analysis.

RNA isolation, reverse transcription, PCR analysis

Total RNA was isolated using Trizol reagent (Invitrogen), followed by DNase treatment (Turbo Dnase free, Ambion). RNA was reverse transcribed with Invitrogen's ThermoScript RT PCR system using a combination of random hexamers and oligo dT primers. PCR was performed using Taq Platinum (Roche). q-PCR was performed using SYBR green-based detection on a Roche Light Cycler or Roche LC480. See Table S5 for primers and PCR conditions.

Microarray analysis and statistics

For Nimblegen promoter tiling array analysis (Nimblegen-Roche), ChIP DNA was amplified by ligation-mediated PCR. IP and input DNA samples were labeled using 9mer Cy3 and Cy5-labeled primers. IP and total DNAs were co-hybridized to the NimbleGen MM5 minimal promoter tiling array and analyzed using NimbleScan software (NimbleGen). Peak data files were generated by searching for 4 or more probes with significant enrichment using a 500 bp sliding window. Each peak was assigned a false discovery rate (FDR) score based on randomization. Gene ontology cluster analysis of SIRT1-associated promoters (FDR \leq 0.1) was performed using the BiNGO plug-in in Cytoscape v2.5.

For Affymetrix expression analysis, total RNA was hybridized to the mouse genome 430 2.0 array. CEL files were analyzed for significance and fold changes between experimental groups using DChip software. For the comparison of Nimblegen and Affymetrix array data, Gene IDs of both arrays were matched using DAVID. Analysis was limited to genes with highly significant SIRT1 promoter enrichment (FDR $<$ 0.005) and transcriptional increase (\geq 10%, $P \leq$ 0.005). χ^2 -based P values were calculated using Pearson's Chi-squared test with Yates' continuity correction. Microarray data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE13121 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13121>).

Cellular fractionation and Western blotting

Chromatin-bound protein was purified as described (Cha et al., 2005). Primary antibodies were rabbit α -Sir2a, α -histone H3, α -Histone H4 (Upstate), rabbit α -YY1 (Santa Cruz), mouse α -GAPDH (Chemicon) and rabbit α -Rad51 (Dr. R. Scully); α -rabbit and α -mouse HRP-coupled secondary antibodies were from GE Healthcare.

Metaphase analysis

Metaphase spreads were performed as previously described (Mostoslavsky et al., 2006). At least 80 metaphases of each genotype were scored per experiment.

Mouse breeding and treatments

All mice were housed pathogen-free. SIRT1^{STOP} (Firestein et al., 2008), *Mx-cre* (gift from Dr. K. Rajewsky), *Nestin-cre* and *p53*^{-/-} mice (Jackson Laboratory) were crossed as to obtain the indicated genotypes. Experimental animals were on C57BL/6 \times 129/Sv mixed genetic background. For *Mx-cre* induction, mice were injected with 400 μ g poly(I)poly(C) (Amersham) at 6–10 weeks of age (Kuhn et al., 1995). For tumor studies, mice were γ -irradiated 10–14 days thereafter with a single dose of 4 Gy. Animals were sacrificed when moribund. When not obvious, mice were submitted to necropsy to identify the cause of death. Deaths not related to tumors and mice too decomposed for analysis were censored. Kaplan-Meier survival curves were generated from two separate cohorts of irradiated animals using JMP7 software. Resveratrol was fed at 2.4 mg/kg food as previously described (Baur et al., 2006). Two cohorts were pooled for survival analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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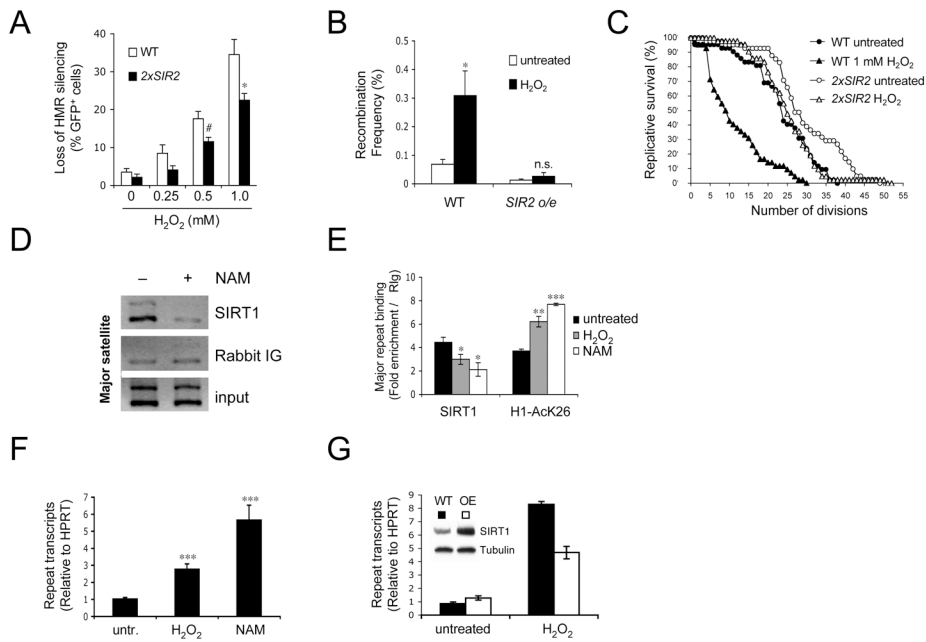
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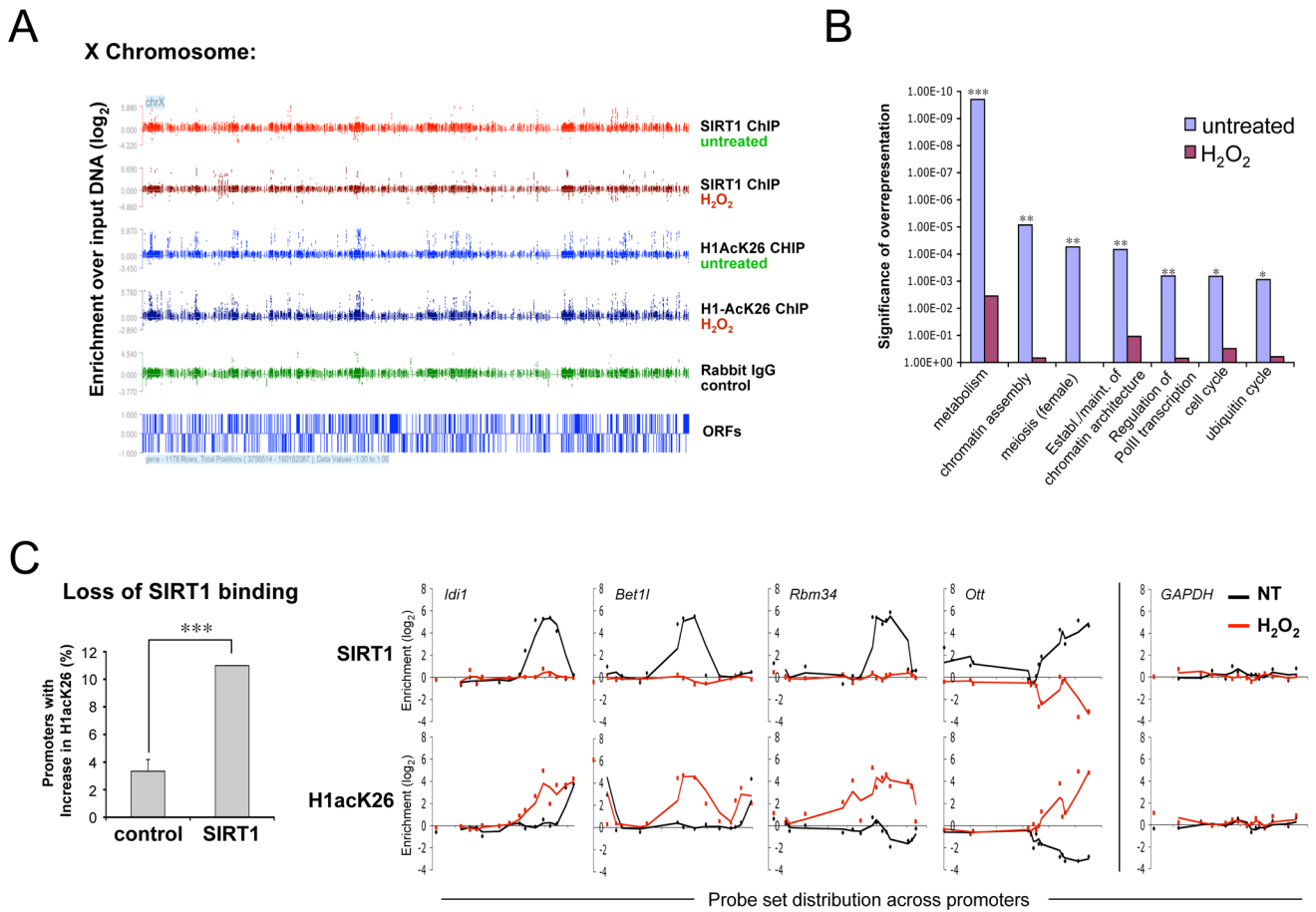
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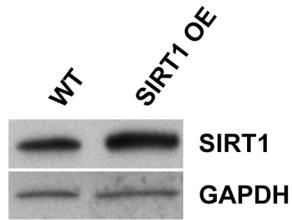
**Figure 1.**

Oxidative stress reduces Sir2/SIRT1-mediated repetitive DNA silencing in yeast and mammalian cells. (A–C). Effects of H₂O₂ on yeast aging. (A) H₂O₂ causes loss of silencing at the mating type loci. Shown is the fraction of GFP⁺ cells in *2xSIR2* and WT cells. Unless otherwise noted, P values are based on Student's two-tailed t test with # $P \leq 0.1$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. (B) Frequency of H₂O₂-induced rDNA recombination in the absence presence of extra Sir2 (WT and *Sir2 o/e*). (C) Replicative lifespan of WT and *2xSIR2* cells grown in the absence or presence of 1 mM H₂O₂. (D) Mouse SIRT1 binds to repetitive genomic DNA. ChIP for SIRT1 or control immunoglobulin (Ig) at major satellite repeats in the absence or presence of NAM (25 mM) (E) q-PCR analysis of ChIP experiments using antibodies specific for SIRT1 or H1AcK26. ES cells were left untreated or treated with H₂O₂ (2 mM) or NAM for 1 h. (F) Oxidative stress increases transcription of satellite repeat DNA. ES cells were treated with NAM for 24 h or with H₂O₂ for 1 h, followed by 23 h recovery. (G) Cells with a targeted extra copy of SIRT1 and control cells were treated with H₂O₂ and analyzed as in (F). The inset shows a Western blot of SIRT1 in WT (black) and SIRT1 overexpressing ES cells (white). Data are represented as mean +/- SEM.

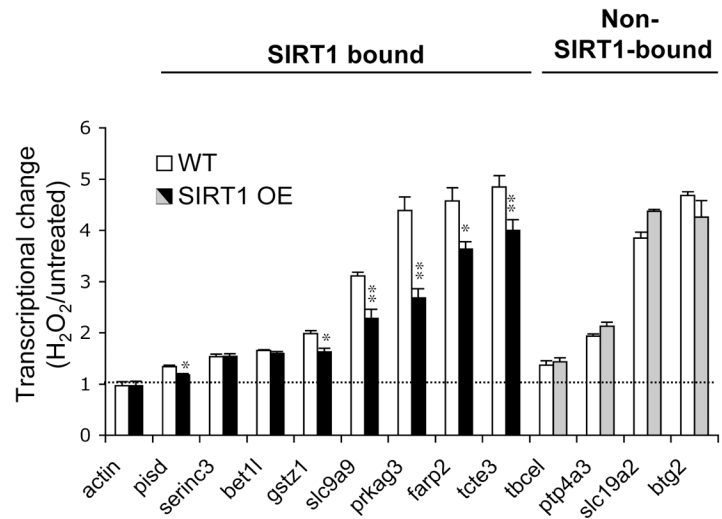
**Figure 2.**

Oxidative stress causes a major redistribution of SIRT1. (A) Distribution of SIRT1 and H1AcK26 along a representative chromosome. ES cells were either untreated or treated with H_2O_2 for 1 h, followed by ChIP for SIRT1, H1AcK26 or control Ig. The x-axis depicts probe sets spanning the promoters of annotated ORFs, signals across the y-axis reflect the \log_2 change of IP DNA over input DNA. (B) Significance of overrepresentation of selected gene ontology (GO) groups amongst SIRT1-associated genes before and after H_2O_2 treatment. Asterisks indicate significance after Benjamini-Hochberg false discovery rate correction. (C) Loss of SIRT1 binding correlates with increased H1K26 acetylation. Promoters with $\geq 2\log_2$ enrichment of SIRT1 prior to H_2O_2 treatment, but no signal thereafter were compared to non-SIRT1 associated promoters. The y-axis shows the fraction of promoters with a ≥ 2 -fold increase in H1AcK26 enrichment after exposure to H_2O_2 . SIRT1 and H1AcK26 probe set binding is shown for four of these promoters. GAPDH served as a negative control.

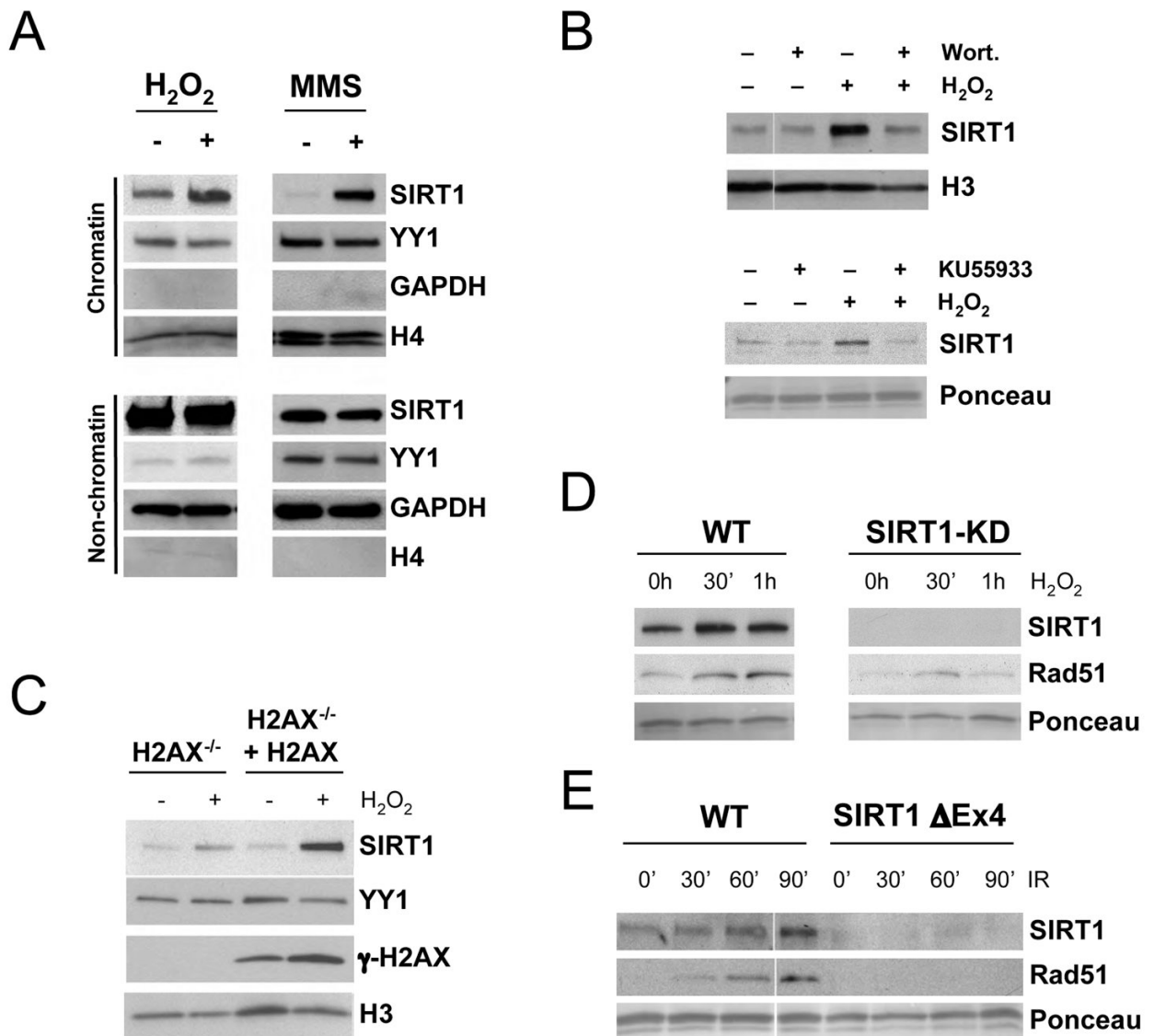
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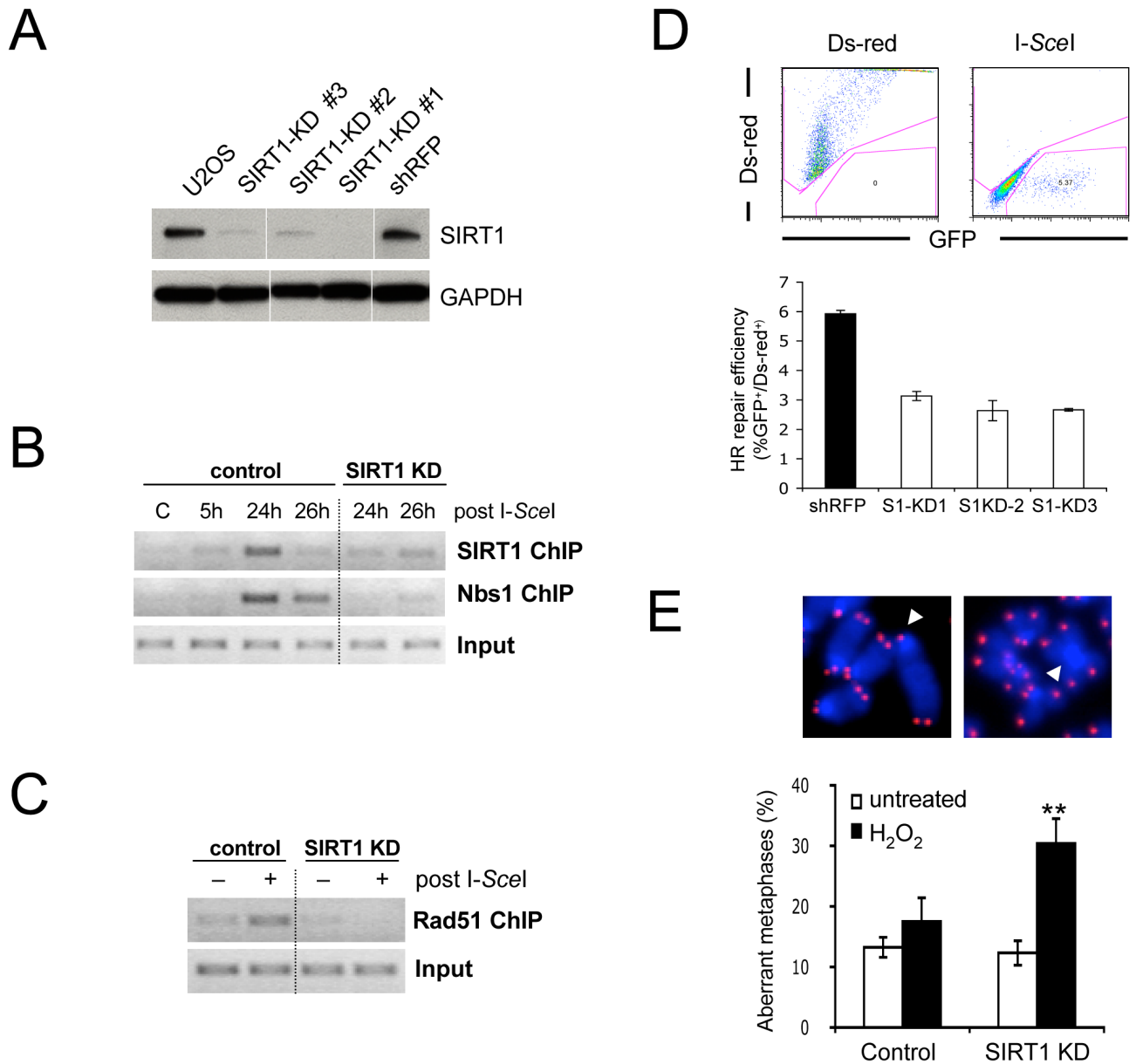
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**Figure 3.**

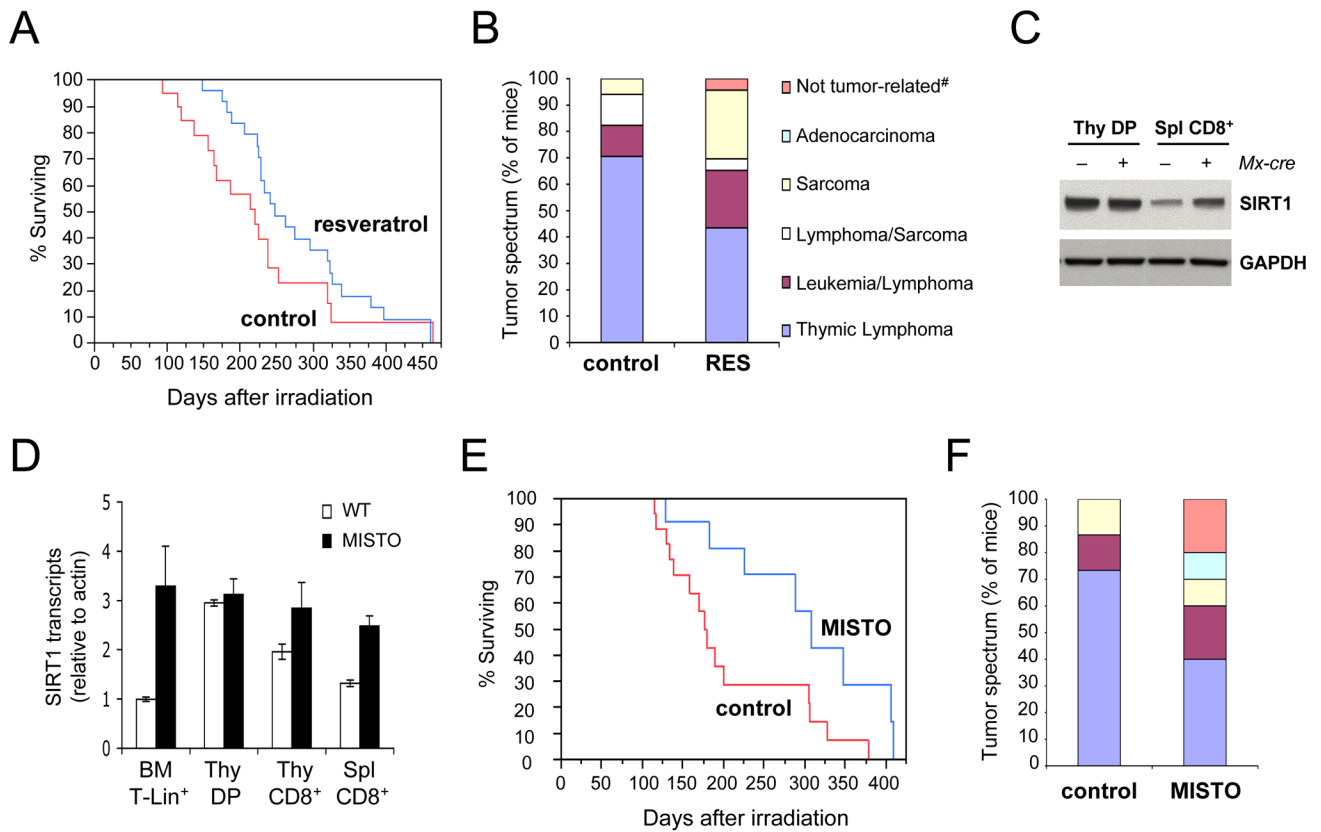
Transcriptional deregulation of SIRT1-associated genes in response to oxidative stress is repressed by increasing SIRT1 levels. (A) SIRT1 expression in wild-type (WT) and SIRT1 overexpressing (OE) ES cells. (B) q-RT-PCR analysis of putative SIRT1 target genes (SIRT1-bound), non-SIRT1-bound control genes and β -actin in wild-type (open bars) and SIRT1 overexpressing ES cells (closed bars). Shown is the fold change of expression compared to untreated samples. Data are represented as mean \pm SEM.

**Figure 4.**

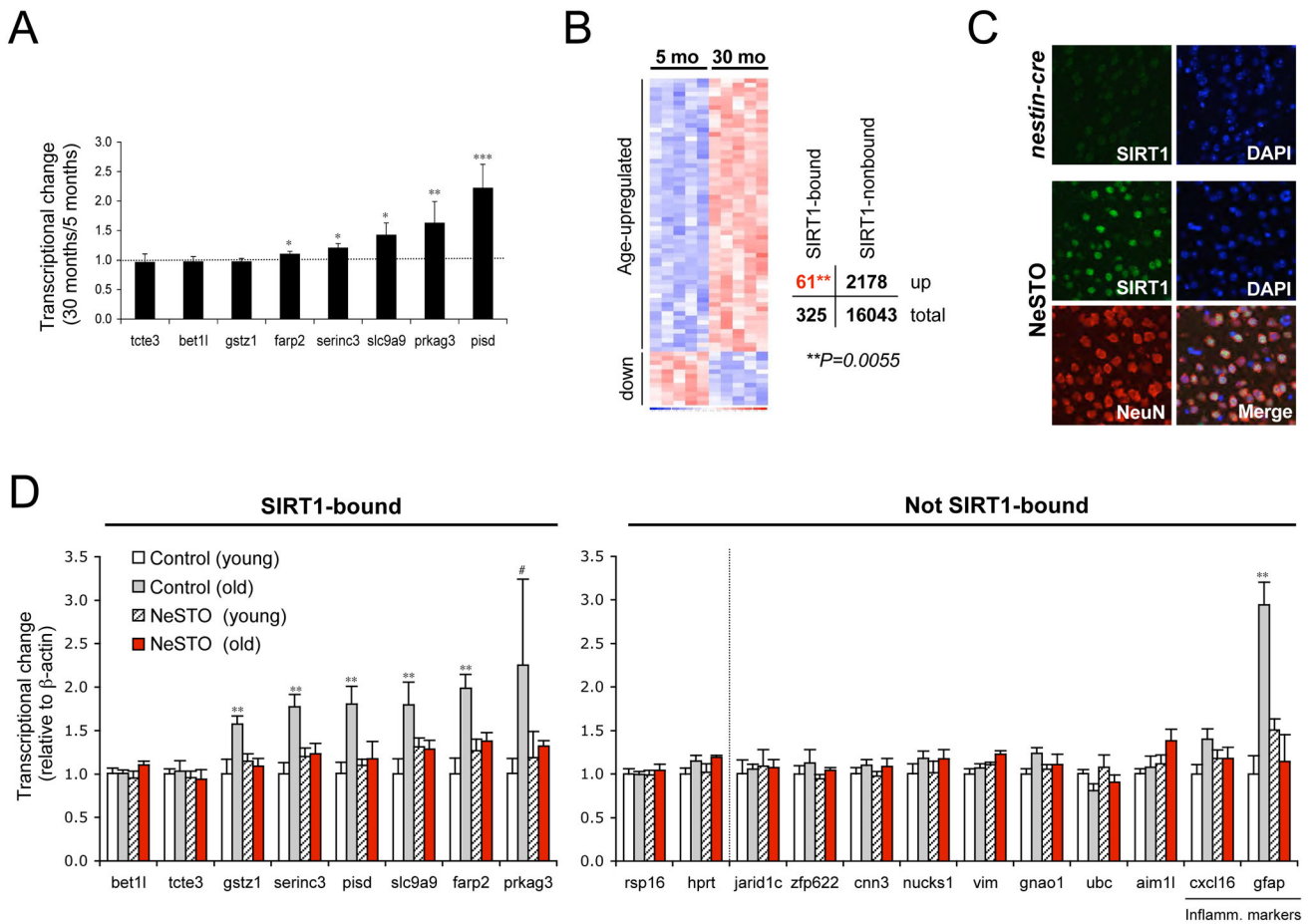
SIRT1 is recruited to chromatin upon DNA damage in an ATM-dependent manner. (A) Western blot analysis of SIRT1 and indicated control proteins in both chromatin-bound and non-chromatin-bound protein fractions from ES cells that were either left untreated or treated with H_2O_2 or MMS for 1 h. (B and C) Western blot analysis of chromatin-bound SIRT1 in response to H_2O_2 with or without pretreatment with wortmannin or KU55933 (B), and in the presence or absence of H2AX (C). (D and E) Recruitment of SIRT1 and Rad51 to chromatin in WT or SIRT1-deficient (SIRT1- Δ ex4) cells in response to H_2O_2 or IR.

**Figure 5.**

SIRT1 is recruited to DNA breaks and is required for efficient DSB repair. (A) SIRT1 expression in parental U2OS-DRGFP cells, three independent shSIRT1 expressing cell lines (SIRT1-KD) and shRFP expressing controls. (B) ChIP analysis of SIRT1 and Nbs1 binding to a DSB in shRFP (control) and SIRT1 KD cells from (A). (C) DSB ChIP analysis of Rad51 recruitment using Ds-red-transfected (-) or I-SceI transfected cells (+) from (B) 24h after transfection. (D) SIRT1 knock-down results in reduced DSB repair as measured by GFP expression (see Figure S7A). Control and SIRT1-KD cell lines from (A) were transfected as in (C) and analyzed by FACS. (E) Loss of SIRT1 causes increased genomic instability upon oxidative stress. Untreated or H₂O₂ treated shSIRT1- or control shRNA-expressing ES cells were subjected to Q-FISH analysis. Telomeres are shown in red. Arrows indicate a chromatid break (left) and a fused centromere (right). The fraction of aberrant metaphases is shown. Data are represented as mean \pm SEM.

**Figure 6.**

Increased SIRT1 activity alters tumor spectrum and increases survival of irradiated $p53^{+/-}$ mice. (A) Survival of irradiated $p53^{+/-}$ mice fed normal or resveratrol-supplemented chow (n=19 and 25, respectively). Tumor-related deaths were recorded in days after irradiation. (B) Tumor spectrum from mice in (A), legend lists dominant tumor at time of death; #no tumors detected in necropsy. (C) Western blot analysis of MACS-purified thymic CD4/CD8 double-positive (DP) T cells and splenic CD8⁺ T cells from MISTO mice (+ *Mx-cre*) and littermate controls (- *Mx-cre*). Mice were analyzed 14 days after *Mx-cre* induction; Thy: Thymus, Spl: Spleen. (D) SIRT1 mRNA expression in lymphocyte subsets from MISTO mice (closed bars) and littermate controls (open bars). BM: Bone marrow, Lin⁺: Lineage-positive. (E) Survival of MISTO and control $p53^{+/-}$ mice (n=12 and 16, respectively) in response to a single dose of 4 Gy γ -irradiation. Tumor-related deaths were recorded in days after irradiation. (F) Tumor spectrum in mice from (E), legend as in (B).

**Figure 7.**

Transcriptional deregulation of SIRT1 target genes occurs during normal aging. (A) Expression of SIRT1-associated gene from Figure 3 in the neocortex from young (5 months) and old (30 months) B6C3F₁ mice ($n \geq 6$ per group), analyzed by q-PCR. Shown is the fold change in expression in old relative to young mice. P values are based on student's one-tailed t-test. (B) Microarray expression analysis of SIRT1 target genes in the neocortex of young and old B6C3F₁ mice ($n = 5$ per group). SIRT1 target genes are significantly overrepresented amongst age-upregulated genes ($\chi^2 = 7.28$, $P = 0.0055$). (C) Immunofluorescence analysis of SIRT1 and NeuN expression in the neocortex of Nesto mice and *Nestin-cre* littermate controls. (D) q-RT-PCR analysis of SIRT1-bound genes from (A) and non-SIRT1-bound genes including housekeeping genes (*rps16*, *hprt*) and genes upregulated in 30 months old mice by microarray. Nesto mice and *Nestin-cre* littermate controls (Control) were analyzed at 8–10 months (young, $n = 3$ –5 and 6, respectively) or 18–19 months of age (old, $n = 4$ and 5, respectively). P values are based on student's one-tailed t-test. Data are represented as mean \pm SEM.