SIRT6 deacetylates H3K18ac at pericentric chromatin to prevent mitotic errors and cellular senescence

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Pericentric heterochromatin silencing at mammalian centromeres is essential for mitotic fidelity and genomic stability. Defective pericentric silencing has been observed in senescent cells, aging tissues, and mammalian tumors, but the underlying mechanisms and functional consequences of these defects are unclear. Here, we uncover an essential role of the human SIRT6 enzyme in pericentric transcriptional silencing, and show that this function protects against mitotic defects, genomic instability, and cellular senescence. At pericentric heterochromatin, SIRT6 promotes deacetylation of a new substrate, residue K18 of histone H3 (H3K18), and inactivation of SIRT6 in cells leads to H3K18 hyperacetylation and aberrant accumulation of pericentric transcripts. Strikingly, depletion of these transcripts through RNA interference rescues the mitotic and senescence phenotypes of SIRT6-deficient cells. Together, our findings reveal a new function for SIRT6 and regulation of acetylated H3K18 at heterochromatin, and demonstrate the pathogenic role of deregulated pericentric transcription in aging- and cancer-related cellular dysfunction.

Large-scale chromatin changes occur during aging, cancer, and other disease processes1–3. The mammalian chromatin regulatory factor SIRT6, a member of the sirtuin family of nicotinamide adenine dinucleotide (NAD⁺)-dependent enzymes, has essential roles in aging, metabolism, and cancer biology6. SIRT6 deficiency in mice leads to severe metabolic defects, genomic instability, inflammatory disease, and accelerated tumorigenesis7–12, whereas overexpression of SIRT6 can extend lifespan in mice13. Notably, downregulated SIRT6 expression has been observed in human cancers10,14,15 and is associated with advanced donor age in primary human fibroblasts16. Thus, deciphering the molecular functions of SIRT6 should contribute to elucidating fundamental mechanisms of aging and disease biology. Many functions of SIRT6 are linked to its activity at chromatin, where it catalyzes NAD⁺-dependent deacetylation of histone H3 on acetylated K9 and K56 (H3K9ac and H3K56ac, respectively)17–19. SIRT6 associates with telomeric chromatin, where it prevents telomere instability, replicative cellular senescence, and impaired silencing of telomere-proximal genes17,20. At promoters of active genes, SIRT6 represses gene networks associated with aging, metabolism, and tumor progression8,10,12,21,22. SIRT6 also coordinates nuclear responses to DNA damage by stabilizing the association of DNA repair and chromatin-remodeling factors at DNA breaks23,24, and by regulating the activity of DNA-processing and DNA-repair factors25,26. Notably, in addition to deacetylating histone H3, SIRT6 can also deacetylate nonhistone proteins25,27, and catalyze mono-ADP-ribosylation26,28 and deacetylation of long-chain fatty acyl groups29. Thus, elucidating the different enzymatic activities and substrates of SIRT6 that are linked to distinct genomic contexts and cellular processes should provide important insights into chromatin and nuclear signaling mechanisms.

Constitutive heterochromatin is present at noncoding regions of the genome and functions to silence transcription or genomic rearrangement of repetitive DNA elements. A classic example of constitutive heterochromatin is pericentric heterochromatin at mammalian centromeres, which occurs at arrays of tandem satellite (Sat) DNA repeat elements (mainly Sat II and Sat III) that are enriched in GGAAT motifs30,31. Notably, deregulated transcription of pericentric satellite repeats occurs in pancreatic and other cancers3, and is associated with premature and physiologic aging of mammalian cells and tissues1,2,4. However, it is not yet known whether these pericentric silencing defects have a causal role in triggering cellular dysfunction associated with cancer and aging.

In this study, we set out to identify new mechanisms of chromatin regulation by SIRT6 and to characterize their importance in cellular function. We show that SIRT6 maintains pericentric heterochromatin silencing at human centromeres through deacetylation of a new substrate, and this activity is important for protecting against mitotic errors, genomic instability, and cellular senescence.

RESULTS

H3K18ac is a physiologic SIRT6 substrate

We have previously carried out in vitro screens for NAD⁺-dependent SIRT6 deacetylase activity on a set of acetylated histone peptides by mass spectrometry17,18. Here, we extended this analysis to new peptides and found that SIRT6 catalyzed robust deacetylation of acetylated H3K18 (H3K18ac) (Fig. 1a). SIRT6 also efficiently deacetylated

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Received 4 June 2015; accepted 11 March 2016; published online 4 April 2016; corrected online 15 April 2016 (details online) doi:10.1038/nsmb.3202
H3K18ac on nucleosome substrates, whereas a catalytically inactive SIRT6 mutant protein (H133Y) did not (Fig. 1b). In contrast, H3K18 deacetylation by SIRT6 was much less efficient on free histone H3 than on the nucleosome substrates (Supplementary Fig. 1a). This observation suggests that the physiologic role of SIRT6 in H3K18 acetylation occurs in the context of chromatin, as previously reported for its H3K9ac- and H3K56ac-deacetylase activities. SIRT6 also promoted H3K18ac deacetylation when overexpressed in cells, whereas the mutant SIRT6 H133Y protein did not (Fig. 1c). Finally, global H3K18ac levels were increased in SIRT6-deficient mouse embryonic fibroblasts (MEFs), and reconstitution with wild-type mouse SIRT6 protein or a catalytically inactive SIRT6 H133A (HA) mutant. Uncropped gel images are shown in Supplementary Data Set 1.

SIRT6 promotes H3K18 deacetylation at pericentric chromatin
To determine how SIRT6 affects the genomic landscape of H3K18ac, we performed unbiased chromatin immunoprecipitation coupled with sequencing (ChIP–seq) in SIRT6-depleted or control U2OS osteosarcoma cells. Genome-wide analysis of the H3K18ac ChIP–seq peak profiles revealed that in the SIRT6-deficient cells, H3K18ac occupancy was dramatically increased (P = 9 × 10−20) at pericentric regions (average signal at peaks within ~100 kb of centromere ‘gaps’, where sequence is missing from annotated genomes) (Fig. 2a,b). H3K18ac hyperacetylation was also clear at individual pericentric, but not subtelomeric, regions (Fig. 2c). In contrast, although pericentric H3K9ac and H3K56ac levels were subtly increased in the SIRT6-depleted cells, these changes were much less substantial than those observed for H3K18ac (Supplementary Fig. 2b). Surprisingly, although SIRT6 binds and deacetylates H3K9ac and H3K56ac at subtelomeric regions and transcription start sites, SIRT6 inactivation did not significantly alter H3K18ac levels at these sequence elements, at least at the genomewide level of this analysis (Fig. 2a and Supplementary Fig. 2a).

In an independent approach, we aligned the H3K18ac ChIP–seq reads to consensus sequences of pericentric repeats as well as other families of repetitive DNA elements. SIRT6-depleted cells showed increased H3K18ac levels at pericentric sequences but not at telomeric and other repeat sequences (Fig. 2d and Supplementary Fig. 2c). Moreover, H3K18ac levels were also not robustly altered at multiple chromosome-specific and consensus α-satellite sequences at centromeric core regions (Supplementary Fig. 2d). Thus, the effects of SIRT6 on H3K18ac levels appear to be specific to pericentric satellite sequences.

Direct ChIP–qPCR confirmed the ChIP–seq findings and revealed a robust increase in H3K18ac levels at pericentric satellite repeats in SIRT6-depleted cells (Fig. 2e) as well as deacetylation of pericentric H3K18ac in cells overexpressing recombinant wild-type SIRT6 but not catalytically inactive SIRT6 H133Y protein (Fig. 2f and Supplementary Fig. 2e). We also confirmed that the effects of SIRT6 activity at pericentric heterochromatin were specific for H3K18ac, because H3K9ac and H3K56ac ChIP signals were not reproducibly altered at pericentric or centromere core sequences after either overexpression or depletion of SIRT6 (Supplementary Fig. 2f,g). Together, these data indicate that SIRT6 promotes site-specific deacetylation of H3K18ac at centromeres but not at its other known genomic targets.

SIRT6 promotes silencing of pericentric heterochromatin
We next examined the association of SIRT6 protein with pericentric satellite DNA sequences by ChIP–qPCR. SIRT6 occupancy was specifically enriched at Sat II and Sat III sequences. Importantly, the ChIP signal was decreased in SIRT6-depleted cells, thus demonstrating the specificity of the SIRT6 ChIP (Fig. 3a and Supplementary Fig. 3a). We also observed the interaction of SIRT6 with centromeres by coimmunoprecipitation of the centromere-specific marker CENP-A with SIRT6 (Fig. 3b) and by colocalization of SIRT6 with CENP-A by confocal microscopy (Fig. 3c).

To examine the functional importance of H3K18 deacetylation by SIRT6, we compared levels of pericentric satellite transcripts in SIRT6-depleted and control U2OS cells by qRT–PCR. SIRT6-deficient cells exhibited markedly increased levels of Sat II and Sat III transcripts, whereas negative-control 5S ribosomal RNA levels were unaltered. In contrast, we observed only minimal changes at α-satellite transcripts, and these might reflect indirect effects of the pericentric chromatin alterations on adjacent centromere core sequences (Fig. 3d). We also confirmed the increase in pericentric satellite transcripts in SIRT6-depleted cells by northern blot analysis, which revealed transcripts ranging in size from <1 kb to >9 kb in nuclear, but not cytoplasmic, RNA fractions (Supplementary Fig. 3b), similarly to previous observations for human Sat III transcripts induced by heat shock. Consistently with the derepressed pericentric transcription, Sat II and Sat III sequences in the SIRT6-deficient cells also exhibited increased levels of activated RNA polymerase (Pol) II phosphorylated at S2 and triphosphorylated H3 K36 (H3K36me3), a marker of active transcription (Fig. 3e and Supplementary Fig. 3d). Together, these
Figure 2 SIRT6 selectively regulates H3K18 deacetylation at pericentric chromatin. (a) H3K18ac ChIP–seq analysis at pericentric and subtelomeric heterochromatin. Genome-wide peak profiles (left) and relative box-and-whisker plots (right) show average H3K18ac occupancy in SIRT6 knockdown (SIRT6 KD1) or control cells (Co), within 100-kb windows from centromeric gaps (pericentric) or telomere gaps (subtelomeric). For box-and-whisker plots: center line, second quartile; top and bottom box limits, upper and lower quartile, respectively; top of upper whisker and bottom of lower whisker, maximum and minimum, respectively (two-tailed Student’s t-test, n = 51 peaks for pericentric and n = 50 peaks for subtelomeric regions). (b) Western blot showing SIRT6 levels in U2OS cells after lentiviral transduction of two different SIRT6 shRNAs (SIRT6 KD1 and SIRT6 KD2). Uncropped gel images are shown in Supplementary Data Set 1. (c) UCSC Genome Browser views showing H3K18ac ChIP–seq levels at representative pericentric and subtelomeric regions of chromosome (chr) 4, in control and SIRT6 KD1 U2OS cells. The y axis is normalized read count. (d) ChIP–seq H3K18ac levels at two pericentric consensus sequences (PCT cons1 and PCT cons2) in SIRT6 KD1 and control cells. Telomeric sequences, β-actin, and histone H4 are shown as controls. Graph shows values of forward and reverse paired-end reads (r1 and r2). (e) ChIP–qPCR showing H3K18ac enrichment in U2OS SIRT6 KD1 and KD2 cells at pericentric Sat II and Sat III repeats, and at control 5S ribosomal DNA (5SR) region (mean ± s.e.m.; n = 3 independent knockdown experiments). (f) ChIP–qPCR showing H3K18ac enrichment in U2OS cells overexpressing SIRT6 WT or the SIRT6 H133Y (HY) catalytic mutant at pericentric repeats (mean ± s.e.m. n = 4 technical replicates). Similar results were observed in two independent cell cultures. *P < 0.05; **P < 0.01; ***P < 0.001; when not indicated, P > 0.05 (one-tailed Student’s t-test). Source data to panels d–f are available online.

data suggest that SIRT6 is important for maintaining transcriptional repression at pericentric heterochromatin.

Surprisingly, however, the dramatic loss of satellite-transcript silencing was not accompanied by clear decreases in H3K9me3 or HP1α, typical markers of silent heterochromatin, in both ChIP–qPCR or unbiased ChIP–seq assays (Fig. 3f and Supplementary Fig. 3e,f). This result contrasts with the almost complete loss of H3K9me3 previously shown to be associated with silencing defects at pericentric heterochromatin in cells doubly deficient for the H3K9me3 methyltransferase enzymes Suv39H1 and Suv39H2 (refs. 34,35). Thus, our data suggest that H3K18 deacetylation by SIRT6 silences transcription at pericentric heterochromatin by a mechanism that is at least partly independent of or downstream of the H3K9me3–HP1α heterochromatin-maintenance pathway.

We next considered possible mechanisms for how regulation of pericentric H3K18ac levels by SIRT6 might lead to changes in satellite-repeat transcription. Recent work has reported that SIRT6 promotes transcriptional silencing at LINE-1 retrotransposable elements by regulating the transcriptional repressor protein KAP1 (ref. 28). Although KAP1 has been detected at pericentric chromatin16,37, to our knowledge, a role for KAP1 in silencing pericentric satellite repeats has not been reported. KAP1 contains a C-terminal PHD finger–bromodomain module, which is found in many chromatin-associated transcription–regulatory proteins. Interestingly, whereas bromodomains generally bind acetylated lysines, the KAP1 bromodomain lacks several conserved residues that mediate acetyl-lysine binding, and it does not bind multiple acetyl-lysine peptides38.

We found that, consistently with these observations, the KAP1 protein (both endogenous and recombinant) bound specifically to unmodified histone H3 peptides, whereas acetylation on H3K18 disrupted this interaction (Fig. 3g,h). In a control experiment, the AP9 YEATS domain bound the H3K18ac but not the unmodified peptide, as previously shown19. This result suggested that deacetylation of H3K18ac by SIRT6 may be important for KAP1 retention at pericentric satellite repeats, and in the absence of SIRT6, H3K18 hyperacetylation triggers KAP1 release and transcriptional derepression. In further support of this model, we found that inactivation of SIRT6 in cells specifically reduced KAP1 occupancy at pericentric satellite repeats (Fig. 3i and Supplementary Fig. 3g,h). Importantly, depletion of KAP1, similarly to depletion of SIRT6, led to increased satellite-transcript expression without affecting H3K9me3 levels (Fig. 3j and Supplementary Fig. 3j). Together, these findings suggest a new mechanism in pericentric transcriptional silencing, in which SIRT6-dependent changes in acetylation state at H3K18 are ‘read’ by acetylation-sensitive binding of a known transcriptional repressor.

Pathologic pericentric transcripts trigger cellular defects

Although pericentric repeat sequences are largely transcriptionally repressed, low-level transcription of satellite DNA transcripts has crucial roles in the assembly and function of pericentric heterochromatin itself40–42. However, the observation that deregulated transcription of pericentric transcripts is detected in aging and cancer has led to the suggestion that aberrant accumulation of these transcripts might have pathogenic consequences. Here, we examined the functional...
Figure 3 SIRT6 depletion disrupts pericentric chromatin silencing and leads to aberrant accumulation of satellite transcripts. (a) ChIP–qPCR showing SIRT6 occupancy at pericentric Sat II and Sat III repeats (mean ± s.e.m.; n = 3 technical replicates). Neg, negative-control intergenic region. Fold change, percentage input of SIRT6 ChIPs normalized to values in SIRT6-knockdown cells (SIRT6 KD). (b) Anti-Flag immunoprecipitation (IP) from nucleosome preparations from cells expressing Flag–SIRT6 or empty vector (control). CENP-A western blots detect centromeric nucleosomes. (c) Confocal microscopy images (representative of 10) of U2OS cells expressing EGFP–SIRT6, stained for CENP-A after in situ detergent extraction of nucleoplasmic protein. DAPI, DAPI 4′,6-diamidino-2-phenylindole nuclear stain. Scale bars, 5 μm. (d) Pericentric satellite, centromeric α-satellite (cent, αSat), and control SS ribosomal RNA (55S) transcript levels in U2OS SIRT6 KD cells, determined by qRT–PCR (mean ± s.d.; n = 3 technical replicates). (e) ChIP–qPCR for RNA Pol II phospho-S2 (Pol II S2P) in U2OS cells (mean ± s.e.m.; n = 3 technical replicates). (f) ChIP–qPCR for H3K9me3 in U2OS cells (mean ± s.e.m.; n = 3 independent knockdown experiments). (g, h) Immunoblots of peptide pulldowns with nuclear extracts (g) or recombinant proteins (h). The AF9 YEATS domain (which binds H3K18ac) and GST protein are shown as controls. Peptides used encompass amino acids 10–27 of histone H3. Similar results were observed in two independent experiments. (i) ChIP–qPCR for KAP1 in control or SIRT6-knockout (KO) U2OS cells (mean ± s.e.m.; n = 3 technical replicates). (j) Pericentric Sat III transcript levels in KAP1-deficient (KD) U2OS cells, determined by qRT–PCR (mean ± s.e.m.; n = 3 independent knockdown experiments). In a, d, e, f, i and j, *P < 0.05; **P < 0.01; ***P < 0.001; when not indicated, P > 0.05 (one-tailed Student’s t test). In a, e and i, results are representative of three different experiments from independent cell cultures. Uncropped gel images are shown in Supplementary Data Set 1. Source data to panels a, d–f, i and j are available online.

Consequences of the accumulation of pericentric satellite transcripts in SIRT6-deficient U2OS cells. Confocal microscopy revealed dramatically increased (>25%) abnormal mitoses in the SIRT6-deficient cells with multipolar spindles and supernumerary centrosomes (Fig. 4a–c), as well as disorganized or asymmetric mitoses (Supplementary Fig. 4a). Moreover, the SIRT6-depleted cells exhibited increased chromosome mis-segregation and aneuploidy, as detected with a chromosomal instability assay for aneuploidy that quantifies centromere-containing micronuclei (cytoplasmic bodies containing DNA lost from nuclei during anaphase) (Fig. 4d and Supplementary Fig. 4b). These observations indicate that SIRT6 protects against the genomic instability associated with chromosome segregation errors during mitosis.

We next asked whether the mitotic defects in SIRT6-deficient cells are dependent on impaired pericentric heterochromatin silencing rather than other effects of SIRT6 loss. Strikingly, depletion of the Sat III transcripts in SIRT6-deficient U2OS cells by short interfering RNA (siRNA)-mediated RNA interference markedly reversed the mitotic defects in these cells (Fig. 4e and Supplementary Fig. 4c). This rescue could not have resulted from nonspecific siRNAs effects, because mitotic defects were not decreased by the Sat III siRNAs in SIRT6-proficient control cells (Fig. 4e). These findings indicate that the aberrant accumulation of satellite transcripts in SIRT6-deficient cells has a pathogenic role in triggering mitotic dysfunction.

Increased levels of pericentric satellite transcripts have been observed in senescent human fibroblasts, cells from human progeroid patients, and tissues from aged mice1,2,4, thus suggesting that deregulated satellite transcription might contribute to cellular senescence or other aging-related cellular dysfunction. In addition, cellular senescence may be triggered by mitotic defects as a tumor-suppressive response in cancer cells, thus preventing propagation of potentially oncogenic genomic changes. We have previously shown that SIRT6 loss in primary human fibroblasts leads to premature replicative cellular senescence during long-term serial passaging, owing to telomere dysfunction17. However, the effect of SIRT6 inactivation on the senescence of cancer cells such as U2OS osteosarcoma cells (which do not undergo telomere-dependent replicative senescence) has not been described. We detected significantly increased numbers of senescent U2OS cells (over ~20-fold higher than those of control cells) within a week of SIRT6 depletion by lentiviral short hairpin RNAs (shRNAs) (Fig. 4f), and we observed increased senescence as early as 72 h after transient transfection of SIRT6 siRNAs (Fig. 4h and Supplementary Fig. 4d). The acute nature of the observed cellular senescence suggested that it occurs via a mechanism different from the previously described telomere-dependent replicative senescence in primary human fibroblasts17. Indeed, we did not observe signs of telomere dysfunction in SIRT6-depleted U2OS cells in this acute time frame (data not shown), consistently with previous results20. Moreover, like the mitotic errors, the senescence phenotype of SIRT6-depleted cancer cells was rescued by depletion of the Sat III transcripts (Fig. 4h and Supplementary Fig. 4c,d). Notably, demonstrating the generality of our findings, the increased levels of Sat III transcripts and associated cellular senescence were also present after depletion of SIRT6 in other
cancer cell lines (HeLa and A549 cells) (Supplementary Fig. 4f), and depletion of Sat III transcripts in A549 cells reversed this senescence (Supplementary Fig. 4g). Finally, we found that ectopic expression of Sat III transcripts in U2OS cells led to increased levels of senescence (Supplementary Fig. 4e), and previous work has shown that overexpression of satellite transcripts leads to increased abnormal mitoses44. Thus, the data indicate that pathologic accumulation of pericentric satellite transcripts is both necessary and sufficient for inducing mitotic errors and acute cellular senescence. We conclude that H3K18 deacetylation and pericentric chromatin silencing by SIRT6 protect against mitotic errors and cellular senescence by preventing transcription of pathogenic satellite DNA elements.

DISCUSSION

Here, we identified H3K18ac as a new SIRT6 substrate and showed that SIRT6 has a highly specific and limiting role in determining H3K18ac levels at pericentric heterochromatin. We showed that depletion of SIRT6 in U2OS cancer cells leads to dramatic site-specific hyperacetylation of H3K18 and pathologic deregulated transcription at pericentric satellite repeat elements. These observations suggest that SIRT6 deacetylase activity is required for maintaining pericentric heterochromatin silencing.

H3K18ac is enriched at promoters of active genes45, and we have previously shown that SIRT7, a member of the sirtuin family closely related to SIRT6, selectively deacetylates H3K18ac at a subset of these promoters, thereby regulating expression of genes involved in maintenance of oncogenic transformation46. In contrast, our ChIP-seq experiments revealed few promoters with increased H3K18ac levels (data not shown) in SIRT6-deficient cells. These observations suggest that SIRT6-dependent deacetylation of H3K18ac may not have a large role in gene repression, at least under basal conditions. However, it remains possible that SIRT6-dependent deacetylation of H3K18ac might occur at specific genes under certain conditions, such as under stress or to compensate for physiologic or induced decreases in SIRT7 levels. In contrast to our findings for SIRT6, SIRT7-deficient cells did not exhibit increased levels of pericentric H3K18ac or pericentric satellite transcripts (Supplementary Fig. 3c and data not shown).

These observations suggest that SIRT6 and SIRT7 have distinct functions in maintaining deacetylation of H3K18ac at gene promoters versus constitutive heterochromatin. A previous study has shown that, similarly to our results for SIRT6, the sirtuin SIRT1 localizes at pericentric heterochromatin and contributes to silencing of pericentric satellite repeats in mouse embryonic stem cells47. In that study, however, no derepression of pericentric satellite transcripts was observed in SIRT1-deficient embryonic stem cells, and the authors have proposed that other deacetylase enzymes may compensate for SIRT1 in pericentric heterochromatin silencing47. It is possible that SIRT6 might provide such a compensatory function.

Previous studies of pericentric heterochromatin maintenance have linked derepression of satellite transcription to dramatic or complete loss of pericentric H3K9me3, owing to combined inactivation of the H3K9me3 methyltransferases SuV39H1 and SuV39H2 (refs. 34,35).
or the H3K9me1 methyltransferases Prdm3 and Prdm16 (ref. 48). Similarly, increased satellite-transcript accumulation in human mesenchymal stem cells, owing to depletion of the Werner syndrome factor WRN, has recently been linked to abolishment of pericentric H3K9me3 levels49. In contrast, our studies show that in SIRT6-depleted cells, dramatic increases in pericentric satellite-transcript levels are associated with only subtle or negligible alterations in pericentric H3K9me3. These observations suggest that maintenance of pericentric heterochromatin silencing by SIRT6 and H3K18ac deacetylation may constitute a new mechanism, which is independent of the classic H3K9me3 heterochromatin maintenance pathway. Consistently with this possibility, previous studies have reported observations similar to ours, in which increased satellite DNA transcription occurs without dramatic H3K9me3 changes. For example, inhibition of DNA methylation in MEFs by 5-Aza-2’-deoxycytidine treatment increases pericentric satellite transcription without altering pericentric H3K9me3 and HP1 levels50. Our data are also consistent with models in which SIRT6-dependent changes in H3K18 acetylation may contribute to pericentric transcriptional silencing. In this model, changes in acetylation state at H3K18 are read by acetylation-sensitive binding of the transcriptional co-repressor KAP1 (ref. 38). We showed that KAP1 specifically binds unmodified histone H3 sequences, whereas acetylation on H3K18 disrupts this interaction. Moreover, KAP1 associates with pericentric satellite sequences, but in SIRT6-deficient cells, H3K18 hyperacetylation triggers KAP1 release and transcriptional derepression.

Notably, our data do not exclude that other mechanisms might also contribute to the silencing function of SIRT6 at satellite repeats. For example, previous work has shown that SIRT6 promotes heterochromatic silencing at LINE1 retrotransposable elements by ADP-ribosylating KAP1, thereby stabilizing the repressive KAP1–HP1α complex at these elements28. It is possible that KAP1 ribosylation by SIRT6 might also contribute to pericentric heterochromatin silencing. However, at LINE1 elements, loss of silencing in the absence of SIRT6 is associated with drastic reductions in H3K9me3 and HP1α levels, which we did not observe at pericentric heterochromatin. Moreover, in our ChIP–sequencing experiments, SIRT6 did not significantly affect H3K18ac levels at endogenous LINE1 elements (Supplementary Fig. 2c). Thus, SIRT6 and KAP1 may use distinct mechanisms to promote heterochromatin silencing at pericentric satellites versus L1 retrotransposons. At pericentric heterochromatin, this mechanism is either independent of or downstream of H3K9me3 changes. Interestingly, KAP1 has been reported to regulate Pol II pausing, and KAP1 depletion leads to increased active transcriptional elongation at numerous genes31. Thus, it is possible that the mechanism of SIRT6 and KAP1 in pericentric transcriptional silencing may involve regulation of Pol II pausing.

Growing evidence indicates that heterochromatin breakdown and accumulation of pericentric satellite transcripts occur in senescent cells, aging tissues, and human cancers31–44, but whether the silencing defects have a causal role in triggering cellular dysfunction in these contexts has not been determined. Here, we have shown that pericentric heterochromatin defects in SIRT6-deficient cells lead to mitotic errors, genomic instability, and acute cellular senescence; remarkably, these defects are rescued by depletion of the pericentric transcripts. These findings demonstrate that the pathologic accumulation of satellite transcripts is a cause, rather than consequence, of aging- and cancer-related cellular dysfunction. This conclusion is supported by the observation that ectopic expression of satellite transcripts induces senescence (Supplementary Fig. 4g) and mitotic defects44.

Similarly to our observations with SIRT6-depleted cells, cells from patients with Hutchinson-Gilford Progeria syndrome also exhibit pericentric heterochromatin defects and satellite-transcript accumulation, and undergo premature cellular senescence4. Intriguingly, a recent study has reported that SIRT6 is downregulated in HGPS cells, and SIRT6 overexpression in these cells can rescue their senescence phenotype52. The mechanism by which this occurs is still unknown, but it might at least partly involve resiliency of pericentric transcripts by SIRT6. Our study uncovers a new mechanism and molecular substrate through which SIRT6 protects against cellular dysfunction associated with aging and cancer.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Data have been deposited in the Gene Expression Omnibus database under accession code GSE69809.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank O. Gozani and members of the laboratories of K.F.C. and O. Gozani for useful discussions, and S. Paredes, T. Hong, and L.D. Boxer for technical assistance. We thank X. Shi (University of Texas M.D. Anderson Cancer Center) for providing bacterial expression vectors for the AF9 YEATS domain and Z. Yang (Stanford University) for KAP1 expression vectors. This work was supported by grants from the US National Institutes of Health (NIH) to K.F.C. (R01 AG028867, R56 AG059997), the Department of Veterans Affairs to K.F.C. (Merit Award), the Paul F. Glenn Laboratories for the Biology of Aging (K.F.C.), and fellowship awards to L.T. (Italian Foundation for Cancer Research fellowships abroad, American Italian Cancer Foundation postdoctoral research fellowship, and Stanford Dean’s fellowship) and to Z.O. (Walter and Idun Berry postdoctoral fellowship). Work in the laboratory of W.L. was funded in part by grants from the Cancer Prevention Research Institute of Texas (RP150292) and the NIH (R01HG007538 and R01CA193466). Research of K.F.C. is partly funded by Daiichi Sankyo Co., Inc.

AUTHOR CONTRIBUTIONS

L.T. and K.F.C. conceived the project, designed the experiments and wrote the manuscript. L.T. performed in vitro and cellular deacetylation assays. ChIP and ChIP–seq experiments, RNA expression analysis, microscopy, and cell biology experiments. Y.X. and W.L. performed bioinformatic analyses for the ChIP–seq experiments and contributed to the corresponding manuscript sections. W.Z. performed ChIP experiments in KAP1-depleted cells and contributed to analysis of KAP1 in SIRT6-depleted cells. R.I.T. contributed to the deacetylation assay on H3K18ac peptides, analysis of satellite transcripts, and manuscript editing. Z.O. performed ChIP experiments in SIRT6-overexpressing cells. F.S. purified nucleosomes for deacetylation assays.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Cell culture, overexpression and RNA interference. Human 293T and U2OS cell lines (ATCC) were cultured in MEM supplemented with 10% FBS, 2 mM l-glutamine and pen/strep. HeLa and A549 cells (ATCC) were cultured in DMEM with 10% FBS, 2 mM l-glutamine and pen/strep. MEFs were generated from 13.5-day-old WT and SIRT6-KO mouse embryos with standard methods, as previously described\(^7\). MEFs were grown in Advanced DMEM supplemented with 10% FBS, 2 mM l-glutamine, 0.1 mM 2-mercaptoethanol and pen/strep. MEFs were propagated with previously described 3T3 protocols for spontaneous immortalization\(^5\). Stable SIRT6-, SIRT7- and KAP1-knockdown and SIRT6-knockout cells were generated by lentiviral transduction. For lentiviral packaging, 293T cells were cotransfected with pV-Pack- VSV-G, Δ8, and p5icoR containing shRNAs directed against SIRT6, SIRT7, KAP1 or control constructs, or LentriCRISPRv2 containing guide RNA directed toward the SIRT6 gene or a luciferase control, and viral supernatant was collected after 48 h. For transduction, cells were incubated with virus-containing supernatant in the presence of 8 µg/ml polybrene. After 48 h, infected cells were selected for 72 h with puromycin (1.5 µg/ml).

Target sequences were as follows: SIRT6 KD1, 5′-AAGAATGTGCGCAAG TGTAAGA-3′; SIRT6 KD2, 5′-AAAGTCTCCTAAGATCAATAA-3′; SIRT7 KD, 5′-CACCTTTTCCTGTGAGGAAGCAGGA-3′ as previously described\(^46\); and KAP1 KD, 5′-GTGCAACCATGTGAGGATT-3′. For CRISPR SIRT6 deletion, guide RNA was directed toward the SIRT6 target sequence CCTCCGGAGGTGAACCGGCTT CG.

U2OS cells stably expressing Sat III were generated by lentiviral transduction, as described for SIRT6 knockdown. A previously described Sat III sequence\(^54\) of 131 bp, containing repeats of (GGAAAT) in CAAC(C/A)CAGAT or variations of this module, was cloned into the pI56RRL-EE1-GEFPU3H1 lentivector used for lentiviral packaging.

U2OS cells and MEFS stably expressing Flag-tagged human or mouse SIRT6, or SIRT6 catalytic mutants (human H133Y or mouse H133A), were generated by retroviral transduction. For retroviral packaging, 293T cells were cotransfected with pVPack-VSV-G, pVPack-G and pBabe vectors containing SIRT6, SIRT6 catalytic mutant, or empty vector. Retroviral and packaging conditions were selected as performed for lentiviral transduction.

siRNA transfections were performed with Dharmafect reagent (Dharmacon), according to the manufacturer's instructions and with the following target sequences: siRNA control, 5′-TGTTTTCATCATCGACTA-3′; siRNA SIRT6 (siS6), 5′-GAATGTGGCGAAGTGAAGA-3′; and siRNA SIRT6 (siSat III), 5′-TGGAATGTGGCGAAGTGAAGA-3′, as previously described\(^13\).

Antibodies. A list of antibodies used in this study is provided in Supplementary Table 1. Validation of each primary antibody is provided on the manufacturers' websites or in ref. 55.

Histone deacetylation assays and mass spectrometry. In vitro histone deacetylation assays were performed as described previously, with minor modifications\(^17\). Briefly, 2 µg of recombinant SIRT6, 0.4 µg of acetylated histone tail peptide or full-length calf thymus histone H3 (Roche), and 0.5 µg mononucleosomes purified from HeLa cells were incubated in HDAC buffer (50 mM Tris-HCl, pH 8.0, 2 mM NAD+, and 150 mM NaCl) for 4 h at 30 °C. The reaction mixture was analyzed by western blotting or mass spectrometry.

Purification of human SIRT6 protein from baculovirus-infected insect cells was as described previously\(^25\). Histone peptides were synthesized at the Yale W. M. Keck peptide synthesis facility, and liquid chromatography–mass spectrometry was performed at the Stanford University, Protein and Nucleic Acid Facility.

To determine histone acetylation levels in cells, 293T and U2OS cells were transiently transfected with pcDNA3 or pBabe vectors containing Flag-tagged wild-type SIRT6, the SIRT6-HY catalytic mutant, or an empty vector. Whole cell lysates were collected after 48 h. Western blot analysis of histone acetylation levels was performed with the modification-specific antibodies listed in Supplementary Table 1.

Immunofluorescence. Cells were fixed with 4% paraformaldehyde, washed, permeabilized with 0.1% Triton X-100 for 10 min, blocked with 2% bovine serum albumin/PBS, and immunostained with antibodies listed in Supplementary Table 1. For analysis of SIRT6 colocalization with CENP-A, U2OS cells were transiently transfected with pEGFP-N2-Flag SIRT6, detergent-extracted to remove nucleoplasmic proteins with Triton X-100 buffer (0.5% Triton X-100, 20 mM HEPES-KOH, pH 7.9, 50 mM NaCl, 3 mM MgCl\(_2\) and 300 mM sucrose) for 5 min at 4 °C, washed in PBS, and fixed in 3% paraformaldehyde and 2% sucrose in PBS for 10 min at room temperature. Cells were then repermeabilized, blocked, and immunostained for CENP-A as described above. Coverslips were mounted with ProLong Gold Antifade reagent with DAPI (Invitrogen) and imaged with a Zeiss LSM700 confocal laser-scanning microscope.

Coimmunoprecipitation. Cells were harvested, washed in PBS, and suspended in cell lysis buffer (10 mM HEPES, pH 7.5/7.9, 0.34 M sucrose, 10% glycerol, 10 mM KCl, 1.5 mM MgCl\(_2\), and protease inhibitors) containing 0.2% NP-40 and were kept on ice for 20 min. Cell extracts were centrifuged at 800g for 10 min at 4 °C to separate the nuclear pellet from the cytoplasm. The nuclear pellet was suspended in cell lysis buffer containing 2 mM CaCl\(_2\) and was kept at room temperature for 10 min. Nucleosomes were prepared by digestion with micrococcal nuclease (10 U/µl) at 37 °C for 5 min. The reaction was terminated by addition of 2 mM EGTA and 200 mM KCl (final concentrations), incubated 10 min at 4 °C, and centrifuged at 16,000g for 30 min to separate the insoluble chromatin pellet from the soluble chromatin fraction. The soluble chromatin was incubated with anti-Flag M2 agarose beads (Sigma) for immunoprecipitation. Elution was performed by boiling the beads in Laemmli loading buffer.

Chromatin immunoprecipitation (ChIP). ChIP was performed as previously described\(^26\), with antibodies listed in Supplementary Table 1. Rabbit anti-mouse IgG antibody was used as a negative control. Immunoprecipitates were collected with Protein A/G-agarose beads (Thermo Scientific) and washed sequentially with low-salt and high-salt wash buffers (Upstate Biotechnology). DNA–protein complexes were eluted in elution buffer (1% SDS and 0.1 M NaHCO\(_3\)), heated to 65 °C overnight, and deproteinized by treatment with proteinase K at 45 °C for 1 h.

ChIP of histone marks was performed as previously described\(^24\), with antibodies listed in Supplementary Table 1. Rabbit IgG antibody was used as a negative control in all the experiments. After elution, DNA was washed and recovered with a PCR purification kit (Qiagen) and assayed by RT–PCR on a LightCycler 480 with SYBR green Master Mix. Fold enrichment was calculated as percentage input and was normalized to total H3. Sequences of primers for centromeric core regions (17a, 21a, 21b and Xa), pericentric Sat II, 3S ribosomal DNA regions, and LINE1 elements were as previously described\(^28,37\). Sequences of other primers used are listed in Supplementary Table 2. The efficiency of the primers used for RT–PCR was verified to be >90% through a standard curve. For repetitive regions, the immunoprecipitated DNA was diluted at least ten-fold, so that the qPCR data were within the linear range of amplification for each primer. The ChIP signals were then normalized to the input samples; this calculation controlled for any variability in the amount of chromatin used and any potential differences in the number of repetitive elements.

ChIP–seq and computational analysis. ChIP assays for H3K18ac, H3K9ac, H3K9me3 and H3K9me3 were performed as described above, and DNA samples were used for ChIP–seq analysis. Sequencing libraries were prepared with the Illumina TruSeq DNA Sample Preparation Kit according to the manufacturer’s protocol. DNA fragments were gel-purified, PCR-amplified, and sequenced with paired-end sequencing technology on an Illumina HiSeq 2000 platform. Two independent biological replicates of H3K18ac ChIP–seq showed similar results.

For generation of ChIP–seq mapping and peak calling, raw reads were aligned to human genome hg19 with bowtie2 v2.1.0 in paired-end mapping mode, with parameters ‘--k 10’--non-deterministic’ to allow up to ten nonunique mappings per read\(^58\). The mapping output was processed to randomly select only one alignment per read pair. Whole-genome ChIP–seq profiles were generated with DANPOS v2.2.2 (ref. 59). The ChIP–seq peaks were called with the ‘dpos’ mode in DANPOS to identify peaks at nucleosome resolution. The pericentric and telomeric peak profiles were generated by averaging all peaks within a 100-kb
neighborhood of centromeric and telomeric regions. The centromere and telomere locations were obtained from the cytoband table and gap table in the UCSC genome database. For pericentric (PCT) and centromeric (CT) mapping, PCT and CT consensus or chromosome-specific sequences, and control sequences were obtained from Eymery et al. A telomeric (TTAGGG)₆ sequence was also analyzed as control. Because these sequences were shorter than the sequencing read length (101 bp), the number of reads containing the analyzed sequences were counted, and to two mismatches were allowed. The read counts for each sample were normalized to units of reads per million total reads (RPKM = read count / total reads/1,000,000).

For RepeatMasker mapping, the annotated Repbase repeat family elements were downloaded from the UCSC database. The total number of nucleotides that overlapped with each repeat family region was counted and normalized to nucleotides per kilobase pair per million reads (NPKM = overlapped nucleotide count / total size of repeat regions / total reads/1,000,000).

RT-PCR. Total RNA was extracted from cells with TRIzol (Invitrogen), per the manufacturer’s protocol. Subsequently, the RNA was treated with RQ1 DNase I (Promega) for 30 min at 37 °C to remove any contaminating DNA. Total RNA (0.5 µg) was reverse-transcribed with a SuperScript III first-strand synthesis system for RT–PCR (Invitrogen) with random primers or with a specific primer for Sat III, as previously described. qPCR for SIRT6 expression levels were assayed with a TaqMan Gene Expression Assay (Applied Biosystems). For all other transcripts, RT–qPCR was performed with SYBR Green master mix and a LightCycler 480 II. Relative mRNA levels were normalized to a specific primer with the following modifications: 10 µl of Dynabeads MyOne Streptavidin T1 (Invitrogen) was saturated with 1 µg of specific histone H3 biotinylated peptides. For peptide pulldowns with recombinant proteins, we followed the same protocol with the following modifications: 10 µl of Dynabeads MyOne Streptavidin T1 (Invitrogen) was saturated with 1 µg of specific histone H3 biotinylated peptides. After washes, the beads were incubated with 1 µg of recombinant protein.

Northern blotting. RNA purifications from nuclear and cytoplasmic fractions and northern blotting were performed as previously described.

Senescence-associated β-galactosidase assay. Senescence-associated β-galactosidase activity was detected with a Senescence Cells Histochemical Staining Kit (Sigma-Aldrich) according to the manufacturer’s instructions. Cells were scored with a Leica DM5000B microscope.

Peptide pulldowns. For peptide pulldowns with nuclear extracts, 10 µl of streptavidin–Sepharose beads (GE Healthcare) was saturated with 10 µg of specific histone H3 biotinylated peptides (encompassing amino acids 10–27 of H3) for 45 min at 4 °C under rotation in peptide buffer (50 mM, Tris pH 7.5, 150 mM NaCl, and 0.1% NP-40), then washed three times in the same buffer. Beads were then incubated with 560 µg of HeLa cytoplasmic extract for 4 h at 4 °C under rotation in peptide buffer. Beads were then washed three times in the same buffer and resuspended in Laemmli buffer for immunoblot analysis.

For peptide pulldowns with recombinant proteins, we followed the same protocol with the following modifications: 10 µl of Dynabeads MyOne Streptavidin T1 (Invitrogen) was saturated with 1 µg of specific histone H3 biotinylated peptides. After washes, the beads were incubated with 1 µg of recombinant protein.