A rare human centenarian variant of SIRT6 enhances genome stability and interaction with Lamin A

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Abstract

Sirtuin 6 (SIRT6) is a deacylase and mono-ADP ribosyl transferase (mADPr) enzyme involved in multiple cellular pathways implicated in aging and metabolism regulation. Targeted sequencing of SIRT6 locus in a population of 450 Ashkenazi Jewish (AJ) centenarians and 550 AJ individuals without a family history of exceptional longevity identified enrichment of a SIRT6 allele containing two linked substitutions (Nlocus in a population of centenarians without a family history of exceptional longevity. Characterization of this SIRT6 allele (centSIRT6) demonstrated it to be a stronger suppressor of LINE1 retrotransposons, confer enhanced stimulation of DNA double-strand break repair, and more robustly kill cancer cells compared with wild-type SIRT6. Surprisingly, centSIRT6 displayed weaker deacetylase activity, but stronger mADPr activity, over a range of NAD+ concentrations and substrates. Additionally, centSIRT6 displayed a stronger interaction with Lamin A/C (LMNA), which was correlated with enhanced ribosylation of LMNA. Our results suggest that enhanced SIRT6 function contributes to human longevity by improving genome maintenance via increased mADPr activity and enhanced interaction with LMNA.

Keywords centenarians; lamin; longevity; SIRT6
Subject Categories Chromatin, Transcription & Genomics; DNA Replication, Recombination & Repair; Genetics, Gene Therapy & Genetic Disease

Introduction

SIRT6 is a protein deacetylase and mono-ADP-ribosylase (mADPr) enzyme that has diverse cellular functions, many of which are related to aging and longevity. SIRT6 knockout mice show premature aging and genomic instability (Mostoslavsky et al., 2006) while mice overexpressing SIRT6 display an extended lifespan (Kanfi et al., 2012; Roichman et al., 2021). Across mammalian species, SIRT6 is conserved and its activity has a strong positive correlation with maximum lifespan (Tian et al., 2019). At the molecular level, SIRT6 is involved in DNA repair, telomere maintenance, silencing of the repetitive elements including LINE1 retrotransposons, regulation of glucose homeostasis, inflammation, and pluripotency (Mostoslavsky et al., 2006; Michishita et al., 2008; Kawahara et al., 2009; Mao et al., 2011; Van Meter et al., 2014, 2016; Etchegaray & Mostoslavsky, 2015). In addition, SIRT6 is a noted tumor suppressor (Van Meter et al., 2011; Min et al., 2012; Sebastian et al., 2012). Cells lacking SIRT6 are more susceptible to malignant transformation, while SIRT6 overexpression induces apoptosis in cancer cells but not in normal cells (Van Meter et al., 2011).
SIRT6 is localized in the nucleus where it interacts with nuclear scaffold protein Lamin A/C (LMNA) (Ghosh et al., 2015), which is also implicated in human longevity. Mutations in LMNA cause a multitude of human genetic syndromes, many of them associated with premature aging, including Hutchinson Gilford progeria syndrome (HGPS) (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). Interaction with LMNA activates SIRT6 enzymatic activities (Ghosh et al., 2015). Early onset premature aging observed in SIRT6 deficient mice resembles HGPS, which led to the suggestion that abnormal SIRT6 localization and function in HGPS drives the disease pathogenesis (Ghosh et al., 2015). A coding change in SIRT6, SIRT6 D63H, leads to the loss of SIRT6 enzymatic activity and embryonic lethality (Ferrer et al., 2018).

The strong correlation between SIRT6 activity and longevity across species (Tian et al., 2019) and in genetically modified mice (Kanfi et al., 2012; Roichman et al., 2021) raises the question of whether higher SIRT6 activity may be associated with a longer lifespan in humans. Noncoding genetic polymorphisms in the SIRT6 gene region were associated with human longevity in candidate SNP analyses (TenNapel et al., 2014; Li et al., 2016; Hirvonen et al., 2017). To date, no beneficial mutations in SIRT6 associated with longevity have been functionally characterized.

In this study, we identified two novel variants of SIRT6 enriched in a population of human centenarians. One of these variants, centSIRT6 possesses altered biological activities compared with the common allele. centSIRT6 allele demonstrates enhanced mono-ADP ribosylase activity but a reduced deacetylase activity in vitro. This tradeoff in activity produces an allele that confers enhancement in DNA repair and enhanced suppression of transposable elements, as well as resistance to oxidative stress. Additionally, the centenarian allele is more efficient at killing cancer cells. Functionally, we found that the centenarian allele shows enhanced interaction with LMNA, which correlates with an increased ribosylation state of LMNA. Together, we identified a novel centenarian allele of SIRT6 that results in enhanced genome maintenance by shifting the balance between the deacetylation and mADPrib activities of SIRT6.

Results

To examine whether variants of SIRT6 are associated with human longevity we performed targeted sequencing of SIRT6 locus in a population of 450 Ashkenazi Jewish (AJ) centenarians and 550 AJ controls (individuals without a family history of exceptional longevity) (Fig 1A). Table 1 shows all the SNPs identified. We observed an association of rs350845 with living beyond 100 years (P = 0.009, Table 1). This SNP lies within a SIRT6 intron and is an eQTL for SIRT6 upregulation across 18 tissue types (Fig EV1A–C). Interestingly, this SNP is in high linkage disequilibrium LD (r² > 0.98) with two other eQTLs, rs350843 and rs350846, which also upregulate SIRT6.

In addition, two rare missense variants in perfect linkage were observed, rs183444295 (A313S) and rs201141490 (N308K), aka centSIRT6, which had nearly double the allele frequency among centenarians (1.0%) compared with both study controls (0.55%) and the AJ cohort within GnomAD (0.60%), although this difference was not statistically significant (P = 0.3, P = 0.27, respectively) due to limited population size.

Analysis of the entire GnomAD database (141,456 individuals of diverse ethnic backgrounds), uncovered an apparent enrichment of centSIRT6 allele pair among the 75+ age group compared with other age groups. Compared with other alleles at similar MAF (0.1–1%), the centSIRT6 allele was in the top 5th percentile of 75+ enriched SNPs and in the 9th percentile of all missense mutations (Fig 1B and C). We sought to characterize this missense mutation to discover its molecular impacts on SIRT6 function in the context of extreme longevity (Fig 1D).

centSIRT6 demonstrates reduced deacetylase activity in vitro

SILAC analysis of HEK293 cells expressing different SIRT6 alleles showed no difference in the protein turnover rates between the WT and centSIRT6 (Fig EV2A). To assess the biochemical properties of the different SIRT6 alleles, we generated and purified recombinant protein for each allele (Fig EV2B). Consistent with similar half-lives of wild type and centSIRT6 in vivo, we found no notable differences in the thermal stability of the SIRT6 alleles in vitro, suggesting that centSIRT6 does not grossly alter the folding or the ambient stability (Fig EV2C).

We then tested SIRT6 deacetylase activity (Jiang et al., 2013) on a myristoylated peptide to determine Kₘ for NAD⁺ for wild type and centSIRT6. While the centenarian allele displayed a slight reduction in activity (Vₘₐₓ) compared with the wild type (P = 0.02) (Fig 1E), Kₘ was not significantly different between wild type and centSIRT6 for both substrates. Quenching of SIRT6 intrinsic Trp fluorescence by NAD⁺ binding also showed that centSIRT6 has a similar affinity for NAD⁺ to the wild-type allele (Fig EV2D). Overall, these data indicate that centSIRT6 confers a slight reduction in deacetylase catalytic efficiency (as defined by Vₘₐₓ /Kₘ) (Fig 1E).

The centSIRT6 displayed significantly lower deacetylase activity and slower kinetics on recombinant nucleosomes with saturated H3K9ac and H3K18ac modifications (Fig 2A and B). Similar to the synthetic histones, the centSIRT6 allele deacetylated both H3K9ac and H3K18ac residues on nucleosomes purified from HeLa cells, at a significantly reduced activity compared with the wild-type SIRT6 (Fig EV3A). Taken together, the combined data from deacetylase and histone deacetylase experiments indicate that the centSIRT6 variant has reduced deacetylase/deacetylase activity.

To test the effect of the centSIRT6 allele on histone deacetylation activity in vivo, we generated telomerase-immortalized human HCA2 fibroblast cell lines in a SIRT6 KO background containing a cumate switch promoter driving expression of SIRT6 WT, N308K, A313S, and the centSIRT6 alleles (cstrate SIRT6 fibroblasts). Each cell line was induced with cumate to drive equal and robust levels of SIRT6 expression (Fig EV3B and C). To rule out contributions from cumate to cell viability and toxicity, cells were assessed for apoptosis and growth rate and no differences were noted (Fig EV3D and E). We quantified histone post-translational modifications from purified histones (Fig EV3F) from these cells by mass spectrometry using peptide standards as described previously (Sidoli et al., 2016). Cells expressing the centSIRT6, N308K, and A313S SIRT6 alleles showed similar global histone post-translational modification (PTM) levels at all sites known to be SIRT6 substrates including H3K9ac, H3K18ac, and H3K56ac (Fig 2C; Dataset EV1). Similarly, global levels of H3K9ac and H3K18ac measured by Western blot did not show significant changes, even when challenged with parquat-
induced oxidative damage (Fig 2D). We then sought to evaluate more direct targets of SIRT6’s deacetylase activity by performing ChIP using antibodies to H3K9ac and H3K18ac. Previous reports have identified LMNA, MsSOD, NFxB2, and NFxBia as H3K9ac targets of SIRT6, as well as SatII and SatIII as H3K18ac targets (Kawahara et al., 2009, 2011; Tasselli et al., 2016). However, similar to
what was observed for global histone acetylation, no major differences were observed between the WT and centSIRT6 alleles, though both alleles were capable of reduced acetylation at these target sites compared with the uninduced cells (Fig 2E–J). These results suggest that while centSIRT6 enzyme has reduced catalytic efficiency of deacetylation, its activity is sufficient to maintain normal steady-state levels of PTMs in vivo.

**mADPr activity is enhanced in centSIRT6**

In addition to its deacetylase activity, SIRT6 has mono-ADP ribosylation (mADPr) activity, able to mono-ADP ribosylate itself and other proteins. mADPr activity is critical to SIRT6 role in DNA damage response and LINE1 repression (Kaidi et al., 2010; Mao et al., 2011; Van Meter et al., 2014). To assess the mADPr activity, two known SIRT6 substrates were used: PARP1 and SIRT6, itself. The centSIRT6 demonstrated an enhanced auto-ribosylation rate with biotin-labeled NAD⁺ than the wild-type SIRT6 protein (Fig 3A). The SIRT6 proteins with single SNPs did not display a notable difference in self-mADPr compared with the wild type, suggesting the increase observed in the centenarian allele results from a synthetic interaction that is not cumulative between the two SNPs (Fig 3A). Following this, we utilized an antibody (Bonfiglio et al., 2020) with specificity to mADPr residues to examine the self-ribosylation efficiency using a titration of NAD⁺. As with the biotin-labeled NAD⁺, centSIRT6 displayed almost 2-fold higher maximal mADPr activity and the trend of enhanced activity was also observed upon titration of NAD⁺ (Fig 3B). After subtraction of the basal signal where zero NAD⁺ was added, Michaelis–Menten best-fit analysis of the horse-radish peroxidase signals revealed that CentSIRT6 displayed an apparent NAD⁺ affinity (Kapp NAD⁺) of 223 μM and maximal relative auto-ribosylation signal mADPrmax of 5.2e⁶, whereas wild-type SIRT6 displayed Kapp NAD⁺ =54.2 μM and mADPrmax of 1.85e⁶ (Fig 3B). In trans, mADPr activity was assessed by incubation with human PARP1, which was previously shown to be ribosylated by SIRT6 (Mao et al., 2011). Similar to self-riboseylation, the centSIRT6 protein displayed higher PARP1 ribosylation activity (Fig 3C). While both single mutants had elevated activity, the A313S protein was...
more similar to the centSIRT6 than N308K (Fig 3C). Collectively, these data show that centSIRT6 displays enhanced mADPr activity.

centSIRT6 is more efficient at suppressing LINE1s and cancer cell killing

SIRT6 suppresses the expression of LINE1 retrotransposons via its mADPr activity (Van Meter et al., 2014). The loss of silencing of these elements contributes to age-related sterile inflammation and drives progeroid phenotypes in SIRT6 KO mice (Simon et al., 2019). To assess the capacity of the centSIRT6 allele to silence LINE1 transposons, we used the cumate-inducible fibroblasts expressing alleles of SIRT6. qRT–PCR analysis of both 5’- and 3’-biased regions of an evolutionarily active family of human LINE1s (L1HS) both showed that the centSIRT6 allele enhances LINE1 suppression compared with the wild-type SIRT6, as did the N308K allele (Fig 4A).
A313S allele showed a slight trend toward stronger suppression but did not show a significant improvement over the wild-type SIRT6 when assessed for the 3’ bias. We then examined whether the centSIRT6 allele could directly inhibit LINE1 retrotransposition (RT). HCA2 cells expressing centSIRT6 demonstrated a significant reduction in successful RT events compared with wild-type SIRT6 (Fig 4B). Previous reports have demonstrated that SIRT6 executes LINE1 silencing via ribosylation of the heterochromatin scaffold.
centSIRT6 was more efficient than the wild-type SIRT6 at ribosylating KAP1 in vitro (Fig 4C). These results show that centSIRT6 is more efficient at silencing LINE1 elements, possibly through enhanced ribosylation of KAP1, which are functionally implicated in longevity.

SIRT6 is a major regulator of DNA double-strand break (DSB) repair (Mao et al., 2011). To quantify the differences between the SIRT6 alleles in promoting DSB repair, we employed in vivo GFP-based assays that measure nonhomologous end joining (NHEJ) and homologous recombination (HR) repair of a chromosomal DSB induced by I-SceI enzyme (Seluanov et al., 2010). Different alleles of SIRT6 were transiently expressed in the NHEJ and HR reporter telomerase-immortalized human foreskin fibroblast cell lines (Mao et al., 2008). We found that equivalent amounts of the centSIRT6 stimulated NHEJ 2.5-fold and HR 2-fold greater than the wild-type SIRT6 (Figs 4D and E, and EV4A). Similarly, we observed in the

Figure 4.
Cumate SIRT6 fibroblasts expressing centSIRT6 that basal levels of γH2AX foci were reduced by 50% relative to those observed in either single alleles or the wild-type SIRT6 (Figs 4F and EV4B). Taken together, these data indicate that the centSIRT6 allele elicits enhanced DNA DSB repair activity. We next compared whether different SIRT6 alleles have different effects on stress. Using the cumate SIRT6 fibroblasts, we exposed the cells to γ-irradiation and assessed the resolution of DSBs over time via γH2AX immunostaining. We observed that while both wild-type and centSIRT6-expressing cell lines had similar levels of γH2AX foci immediately after exposure, the centSIRT6-expressing cells showed an improved recovery rate (Figs 4G and EV4C). In the absence of cumulative induction, all cell lines showed no significant differences in survival after oxidative stress (Fig 4H). By contrast, we found that the centSIRT6-expressing cells more resistant to oxidative stress-induced apoptosis (Figs 4I and EV4D). Taken together, these results indicate that the centSIRT6 improves DSB repair and resistance to DNA damage.

To further confirm that centSIRT6 enhances genome maintenance we generated knock-in human embryonic stem cells carrying centSIRT6 allele. The two centenarian mutations were introduced using CRISPR/Cas9 and confirmed by sequencing. We generated two independent knock-in clones and differentiated them into mesenchymal stem cells (MSCs). The MSCs were then transfected with linearized NHEJ and HR GFP reporters (Mao et al., 2008). Cells harboring centSIRT6 allele showed higher efficiency of repair (Fig EV5A and B). These cells were also showed increased survival and a lower number of 53BP1 foci upon treatment with methyl methanesulfonate (MMS), a potent DNA-damaging agent (Fig EV5C and D).

Previous studies have demonstrated that MSCs deficient for SIRT6 have repression of the NRF2-antioxidant mediated stress response (Pan et al., 2016; Rezazadeh et al., 2019). Both RNAseq analysis and qRT–PCR of these target genes, as well as NRF2, showed no significant change between the two alleles (Fig EV5E and F). Interestingly, the centSIRT6 knock-in cells showed higher protein levels of SIRT6 (Fig EV5G), suggesting that centenarian mutations increase protein abundance, in addition to modulating SIRT6 enzymatic activities. As it was impossible to compare equal levels of SIRT6 in CRISPR knock-in cells, we relied on cumate cells for the majority of our in vivo assays.

SIRT6 overexpression induces apoptosis in cancer cells but not in normal cells (Van Meter et al., 2011). In order to assess the tumor cell killing capacity of the centSIRT6 allele, we transfected SIRT6 alleles into two common tumor cell lines, HT1080 and HeLa cells. Expression of the centSIRT6 resulted in ~2-fold fewer adherent surviving cells in both cancer cell lines compared with wild-type SIRT6 (Fig 5A). Additionally, the centSIRT6 allele triggered at least 2-fold higher levels of apoptosis than the wild-type SIRT6 allele in these cancer cell lines (Fig 5B and C). These data indicate that centSIRT6 confers a more robust anti-tumor activity than the wild-type SIRT6.

centSIRT6 displays a higher affinity for LMNA

Since the centSIRT6 mutations are located in the flexible C-terminus of SIRT6 and may influence protein–protein interactions, we compared the interactomes of the centSIRT6 and the wild-type alleles.
Figure 5.
We used antibodies against SIRT6 to immuno-precipitate SIRT6-interacting proteins from cumate SIRT6 fibroblasts and analyzed them by mass spectrometry with tandem mass tags (TMT) to permit accurate quantitation. Several interacting proteins were enriched by the centSIRT6 allele (Fig 6A; Dataset EV2). Most notable proteins showing stronger interaction with centSIRT6 than the wild-type SIRT6 were LMNA and vimentin (VIME), which showed 38 and 40 peptides, respectively. Both of these proteins are known to interact with SIRT6 (Patil & Nakamura, 2005; Ghosh et al., 2015; Huttlin et al., 2015; Gioutakis et al., 2017; Oughtred et al., 2019), and LMNA was shown to stimulate SIRT6 activity (Ghosh et al., 2015). No loss or gain of interactions was apparent in the centSIRT6 set, indicating

| Name   | Description                  | Peptides | p value   |
|--------|------------------------------|----------|-----------|
| RBP56  | TATA-binding protein-associated factor 2N | 5        | 0.0002    |
| LMNA   | Prelamin-A/C                  | 38       | 0.0025    |
| RL35   | 60S ribosomal protein L35     | 4        | 0.0056    |
| CEBPB  | CCAAT/enhancer-binding protein beta | 1        | 0.0119    |
| VIME   | Vimentin                      | 40       | 0.0188    |
| NUMA1  | Nuclear mitotic apparatus protein 1 | 2        | 0.0227    |
| NOL6   | Nucleolar protein 6           | 2        | 0.0250    |
| FRG1   | Protein FRG1                  | 2        | 0.0336    |
| A0A0B4J2E5 | Uncharacterized protein | 3      | 0.0354    |
| PHRF1  | PHD and RING finger domain-containing protein 1 | 3        | 0.0445    |
| H15    | Histone H1.5                  | 11       | 0.0516    |
| H31    | Histone H3.1                  | 3        | 0.0534    |
| NOL10  | Nucleolar protein 10          | 1        | 0.0581    |

Figure 6.

**Table:**

| Name | Description                  | Peptides | p value |
|------|------------------------------|----------|---------|
| RBP56 | TATA-binding protein-associated factor 2N | 5        | 0.0002  |
| LMNA | Prelamin-A/C                  | 38       | 0.0025  |
| RL35 | 60S ribosomal protein L35     | 4        | 0.0056  |
| CEBPB | CCAAT/enhancer-binding protein beta | 1        | 0.0119  |
| VIME | Vimentin                      | 40       | 0.0188  |
| NUMA1 | Nuclear mitotic apparatus protein 1 | 2        | 0.0227  |
| NOL6 | Nucleolar protein 6           | 2        | 0.0250  |
| FRG1 | Protein FRG1                  | 2        | 0.0336  |
| A0A0B4J2E5 | Uncharacterized protein | 3        | 0.0354  |
| PHRF1 | PHD and RING finger domain-containing protein 1 | 3        | 0.0445  |
| H15 | Histone H1.5                  | 11       | 0.0516  |
| H31 | Histone H3.1                  | 3        | 0.0534  |
| NOL10 | Nucleolar protein 10          | 1        | 0.0581  |

Figure 6.
that the effect of the novel allele could be in enhancing existing SIRT6 interactions.

LMNA is a nuclear scaffold protein playing a central role in nuclear organization and is also implicated in aging. To confirm the mass spec data on SIRT6 and LMNA interactions, we performed co-IP with SIRT6 antibodies followed by Western blot with LMNA antibodies using cumate SIRT6 fibroblasts. We found that centSIRT6 indeed, associates more strongly with LMNA than the wild-type SIRT6 allele (Fig 6B and C), and the effect was reciprocal as assessed by co-IP with LMNA antibodies followed by Western blot with SIRT6 antibodies (Fig 6B and D). Taken together these results demonstrate that centSIRT6 interacts more strongly with LMNA than the wild-type SIRT6. Given the enhanced mADPr activity of the centSIRT6 allele, we next assessed the ribosylation status of LMNA using the mADPr antibodies. SIRT6 IPs performed on the cumate SIRT6 fibroblasts, using the mADPr-specific antibody (Bonfiglio et al., 2020), showed that centSIRT6 was more ribosylated in vivo (Fig 6B and E); as demonstrated in vitro with the purified SIRT6 proteins (Fig 3A). We also conducted IPs against vimentin and TAF15 (RBP56), as both also demonstrated enhanced association with centSIRT6. Additionally, TAF15 has also been shown to be ribosylated in response to genotoxic stress (Jungmichel et al., 2013). The IPs revealed enhanced ribosylation status of vimentin and TAF15 in centSIRT6-expressing cells (Appendix Fig S2A–C). IP with a mADPr-specific antibody (Bonfiglio et al., 2020) and subsequent Western blot with an anti-LMNA antibody revealed increased ribosylation of LMNA in the presence of the centSIRT6 allele (Fig 6B and F). LMNA has been reported to be ADP-ribosylated (Jungmichel et al., 2013; Martello et al., 2016; Bilan et al., 2017; Vivelo et al., 2017; Leslie Pedrioli et al., 2018; Hendriks et al., 2019) (shown as hexagons in Fig 6G), suggesting that ADP ribosylation by SIRT6 may play a role in mediating these interactions. Taken together, our data show that centSIRT6 is more efficient at promoting DNA repair and suppressing LINE1 elements, which is likely mediated by its enhanced mADPr activity and stronger interaction with LMNA.

Discussion

We demonstrated that a novel rare variant of SIRT6 discovered in a cohort of centenarians confers beneficial effects on SIRT6 protein function. centSIRT6 performed better at stimulating DNA repair, mitigating DNA damage, suppressing LINE1 transposons, and killing cancer cells. Rare genetic variants are more likely to be deleterious than beneficial (Nelson et al., 2012), and are often associated with human disease. The only other coding SIRT6 human mutation, which had been functionally characterized, leads to embryonic lethality (Ferrer et al., 2018). SIRT6 activity is highly correlated with aging and it has been demonstrated that overexpression of SIRT6 results in increased lifespan in mice (Kanfi et al., 2012; Roichman et al., 2021). It has also been reported that SIRT6 from long-lived species can confer increased longevity in Drosophila (Tian et al., 2019). Our findings indicate that increased SIRT6 activity has a beneficial impact on cellular functions linked to longevity and may contribute to increased lifespan in centenarians.

Genetic association tests on human extreme longevity are reliable statistical power to detect small effects. To date, only two variants near APOE and FOXO3a have been associated with...
longevity in genome-wide scans (Deelen et al, 2011; Broer et al, 2015). For FOXO3a, this required a large-scale meta-analysis involving 6,036 longevity cases and 3,757 controls (Broer et al, 2015). Since there simply are insufficiently large numbers of centenarians in human populations, the genetic association has limited utility in finding loci that promote healthy aging and longevity. In this study, we instead took a candidate SNP approach for SIRT6 within a genetically isolated population of Ashkenazi Jews who exhibited extreme phenotype (>95 years old). The observed association for rs350845, which is linked to higher SIRT6 levels is nominally significant ($P = 0.049$). This association between higher SIRT6 expression and longevity is consistent with the data from Drosophila (Tian et al, 2019) and mouse (Kanfi et al, 2012) where SIRT6 over-expression resulted in lifespan extension. The missense variant centSIRT6 had twice the allele frequency in the AJ centenarians (1%) compared with controls (0.5%); however, we lacked the power to detect statistically significant differences ($P = 0.3$). To further validate this finding, a follow-up analysis of the GnomAD cohort of cohorts, which also contains AJ individuals, showed evidence of centSIRT6 enrichment among 75+ year-olds compared with all age groups. In addition to enrolling larger centenarian cohorts, future work may investigate epistasis and the co-occurrence of other SNPs in the genome with centSIRT6 and extreme longevity.

While increased SIRT6 activity correlates with a longer lifespan in model organisms (Kanfi et al, 2012; Tian et al, 2019), it has not been clear which of SIRT6’s enzymatic activities are central to longevity. The seemingly contradictory nature of the centenarian SIRT6 allele possessing decreased deacetylase activity came as an unexpected result. SIRT6 KO mice show several progeroid phenotypes and do not typically survive past 30 days (Mostoslavsky et al, 2006). In these mice, both the deacetylase activity and ribosylation activity are abrogated. In this study, we have demonstrated that a centSIRT6 possesses reduced deacetylase activity in vitro and showed nondiscernable differences in vivo. The latter differences likely result from additional protein factors or PTMs present in vivo that help maintain SIRT6 deacetylation activity at close to the wild-type levels, despite the weakened core enzyme. Interestingly, loss of SIRT6 deacetylation activity in humans or SIRT6 knockout in monkeys lead to severe developmental phenotypes rather than premature aging, suggesting that, at least in primates, deacetylation activity of SIRT6 is required for development but not necessarily for adult longevity (Ferrer et al, 2018; Zhang et al, 2018).

The extent to which ADP-ribosylation functions of SIRT6 contribute to longevity remains largely unknown. Herein we report that the centSIRT6 allele displays significantly enhanced ribosylation activity against itself, PARP1 and KAP1 in vitro and also appeared enhanced in vivo. Further, we show that centSIRT6 confers improved aspects of SIRT6 function to living cells, including DNA DSB repair, LINE1 element suppression, and tumor cell suppression, all of which showed dependence on SIRT6 ADP-ribosylation functions previously. These results suggest that enhancement of ADP-ribosylation functions of SIRT6 contributes to increased longevity. While we cannot rule out that other aspects of SIRT6 activity, such as glucose metabolism and telomere maintenance, have been impacted by these mutations and may also play a role in the longevity functions of this allele, those effects have been largely attributed to SIRT6 deacetylase activity, which appears similar or reduced compared with the WT allele. It remains possible that SIRT6 ADP ribosylation may play unanticipated roles in these processes as supported by recent reports of SIRT6 ADP ribosylation of SMARCA2 and KDM2A (Rezazadeh et al, 2019, 2020). Consistent with this idea, SIRT6 mono-ADP ribosylation (and subsequent activation) of PARP1 plays a critical role in DNA DSB repair by facilitating the recruitment of DNA repair proteins, such as MRN complex proteins (Haince et al, 2008; Van Meter et al, 2016). Improvements in DSB repair efficiency, specifically those driven by SIRT6 activity, have been shown to correlate with longevity and healthspan (Roichman et al, 2017; Tian et al, 2019). Additionally, the ribosylation of KAP1 by SIRT6 and subsequent recruitment to transposable elements plays a critical role in silencing these opportunistic elements, which have been linked to age-related sterile inflammation and DNA damage (Van Meter et al, 2014; De Cecco et al, 2019; Simon et al, 2019). These same elements have been linked to inflammation driven by senescent cells, which are prevalent in aged systems (De Cecco et al, 2019). Further investigation of the separation of function alleles of SIRT6 will be required to disentangle the contribution of its deacetylase/deacylase and ADP-ribosylation activities. Beyond its enhanced mADPR activity, centSIRT6 displays enhanced interaction with the nuclear scaffold protein LMNA. This result is especially significant for several reasons. LMNA functions as a key organizer of the nucleus, especially in maintaining heterochromatin at the nuclear periphery and LINE1 elements reside within lamina-associated domains (LADs) (Peric-Hupkes et al, 2010; Zullo et al, 2012). Aberrant processing of LMNA results in human premature aging syndrome, Hutchison Gilford Progeria, while LMNA SNPs have been identified in human centenarians (De Sandre-Giovannoli et al, 2003; Eriksson et al, 2003; Conneely et al, 2014). Fibroblasts isolated from centenarians were also shown to have increased levels of pre-LMNA, suggesting that modulated functions of LMNA are associated with both premature aging and exceptionally long-lived centenarians (Lattanzi et al, 2014; Ghosh et al, 2015) found that the catalytic domain of SIRT6 interacts with LMNA. In addition, they found that many key aspects of SIRT6 activity, such as PARP1 ribosylation and activation, as well as chromatin localization in response to DNA damage were lost in LMNA−/− MEFs. Our data suggest that a gain of function of SIRT6 may have the opposite effect of progerin on longevity. Remarkably, SNPs in LMNA were found in centenarians (Conneely et al, 2012). Fibroblasts isolated from centenarians were also shown to have increased levels of pre-LMNA, suggesting that modulated functions of LMNA are associated with both premature aging and exceptional longevity (Lattanzi et al, 2014).

Questions remain as to how centenarian SIRT6 allele together with LMNA enhances genome stability and contributes to longevity. As LMNA enhances SIRT6 enzymatic activities, the stronger interaction may result in higher SIRT6 activity (Ghosh et al, 2015). Additionally, centSIRT6 may enhance LMNA function through stronger binding or as a result of a differential interactome of ribosylated LMNA. Previous attempts to induce ribosylation of purified LMNA by SIRT6 in vitro have been unsuccessful, possibly due to the lack of PTMs and the 3D nuclear organization that may be necessary to shape the interaction between SIRT6 and LMNA. As ribosylations are notoriously difficult to detect by mass spectrometry, more work will need to be done to identify the specific residues on LMNA modified by SIRT6.
In summary, we demonstrated that centSIRT6 displays reduced deacetylation activity in vitro, and has enhanced mADPr activity both in vitro and in vivo. This change in the balance of the two enzymatic activities leads to enhanced function of SIRT6 in DNA repair, LINE1 suppression, and cancer cell killing, which are the activities that require mADPr activity of SIRT6. centSIRT6 also shows enhanced binding to LMNA, which may further promote its function in DNA repair and chromatin organization via direct stimulation of SIRT6 enzymatic activity, and by coordinating interactions of LMNA with other components of LMNA complex (Fig 7). It would be interesting to model centSIRT6 in mice, to directly test whether this allele extends lifespan. However, the C-terminus of SIRT6 that harbors the centenarian mutations is not conserved in the mouse. SIRT6 and LMNA pathways are highly linked with the aging process, indicating that the SIRT6 ribosylation activity may be the more critical of the two enzymatic functions in regards to healthy aging and may aid LMNA to organize/control nuclear protein–protein and protein-RNA interactions. Given these results, there may be a benefit to enhancing SIRT6 activity, specifically the ribosylase activity. Molecules that impact SIRT6 activity, as well as its major co-enzyme NAD⁺, have been identified and hold potential as future methods of anti-aging interventions (Li et al., 2017; Rahnasto-Rilla et al., 2018; Rajman et al., 2018). Further refining the search for interventions that specifically target the mADPr activity of SIRT6 may yield more specific therapeutics to improve lifespan and healthspan.

Materials and Methods

Human subjects

Ashkenazi Jewish population derived from an undetermined small number (estimated to be in the several thousands) of founders. External factors, including ecclesiastical edicts prohibiting all social contact with Jews, the Crusades, the establishment of the Pale of Settlement, numerous Pogroms, and ethnic bigotry, resulted in the social isolation and inbreeding of the Ashkenazi Jews. This history resulted in both cultural and genetic homogeneity and has made this population useful for the identification of genetic traits. In our study, a centenarian is defined as a healthy individual living independently at 95 years of age or older and control is defined as an individual without a family history of unusual longevity; parents of controls survived to the age of 85 years or less. The participants’ ages were defined by birth certificates or dates of birth as stated on passports. This study group consisted of 450 Ashkenazi Jewish centenarians and 550 Ashkenazi Jewish controls that were previously collected as part of a longevity study at the Albert Einstein College of Medicine (Barzilai et al., 2003). The ages of centenarians were 100.4 ± 3.3 years (mean ± SD) and the ages of controls were 78.3 ± 8.1 years (mean ± SD) at the time of sequencing. 72% of centenarians and 52.55% of controls were female. The BMIs of centenarians were 26.9 ± 4.8 (mean ± SD) and the BMIs of controls...
were $22.8 \pm 3.6$ (mean $\pm$ SD). Informed written consent was obtained in accordance with the policy of the committee on clinical investigations of the Albert Einstein College of Medicine, New York, NY. An additional 5,185 AJ controls were derived from GnomAD database.

**Cell lines**

HEK293 SIRT6 overexpression lines were generated by transfecting HEK293 cells with linearized CMV-SIRT6 plasmids via jetPRIME transfection reagent and selecting stably integrated clones. To generate normal human fibroblasts expressing WT and centenarian alleles of SIRT6 under the control of cumate-inducible promoter (cumate SIRT6 fibroblasts), constructs containing SIRT6 alleles under the control of cumate promoter were integrated into the genome of SIRT6 fibroblasts via the PiggyBac Transposon Vector System. Endogenous SIRT6 was knocked out in these cells using CRISPR/Cas-9 (Tian et al., 2019). NHEJ and HR reporter assays were conducted using telomerase-immortalized HCA2 human fibroblast cell lines containing integrated reporter constructs (I9A and H15C) (Mao et al., 2008; Seluanov et al., 2010). HT1080 and HeLa cell lines were used to assess anti-tumor activity. Human H7 ESCs (WiCell Research) and their genetically modified derivatives were maintained on Matrigel in mTeSR Plus medium (Stem Cell Technology) and used to generate MSCs.

**Cell culture**

All cell lines were maintained in humidified incubators at 5% CO$_2$, 5% O$_2$, at 37°C. Cells were grown in Eagle’s minimum essential medium with 15% fetal bovine serum and 1x penicillin/streptomycin, with the exception of the HEK293, HT1080, and HeLa cells, which were cultured in DMEM with D-Glucose and L-Glutamine. Human MSCs were cultured on Gelatin-coated plate in hMSC culture medium ($\alpha$MEM (Gibco), 10% fetal bovine serum (GeminiBio), 1% penicillin/streptomycin (Gibco) and 1 ng/mL bFGF (ThermoFisher)). The cell lines are routinely tested for mycoplasma contamination.

**Sequencing of SIRT6 gene in Ashkenazi Jewish Centenarians**

The sequencing of SIRT6 gene was a part of the pooled target capture sequencing (capture-seq) project aiming to discover centenarian-enriched rare variants in genes involved in conserved longevity assurance pathways (Ryu et al., 2018). SIRT6 was selected as a candidate longevity-associated gene. All SIRT6 variants discovered in the capture-seq of 1,000 AJ samples (450 centenarians vs. 550 controls) are provided in Table 1. Plots of SIRT6 expression and eQTL status for rs350845 were downloaded from the GTEx website.

**GnomAD centSIRT6 enrichment**

GnomAD analysis was performed on the 2.1.1 release of combined exome and whole-genome sequences representing 125,748 exomes and 15,708 whole genomes. Chromosome 19 variants were downloaded from the GnomAD database, and since the centSIRT6 allele is rare (0.5% - 1%) we filtered for SNPs between 0.1% and 1% frequency. Our goal was to build a distribution of allele occupancy in the 75+ age group normalized against all age groups. While this is not a traditional association study per se, it does allow us to witness allele frequency bias in older vs. younger age brackets.

$$\text{Normalized over75 allele counts} = \frac{\text{allele counts in over75}}{\text{allele counts all ages}}$$

A normalized value of 0 represents a complete absence of the allele in 75+, and higher values represent greater occupancy of that allele in the 75+ age bracket compared with all ages. Alleles with a value of 1 were filtered from consideration, as these may have represented alleles only observed in cohorts comprised of older individuals. In total, 66,441 SNPs were measured, of which 3,307 were missense.

**Gene editing at SIRT6 locus**

CRISPR/Cas9-mediated knock-in was performed using Alt-R CRISPR-Cas9 System (IDT) and HDR Donor Oligos. Cas9 nuclease, sgRNA (GAATCTCCACCCGGATCAA) targeting double variant site and single-strand DNA oligo donors (ssODNs) containing SIRT6 double variant were ordered from IDT. To generate SIRT6 double variant knock-in hESCs, $2 \times 10^5$ individualized hESCs were resuspended in 10 μL Resuspension Buffer R (Invitrogen) containing CRISPR ribonucleoproteins (Cas9 protein + sgRNA) and ssODNs and were then electroporated using NEON Transfection System (Invitrogen). After electroporation, cells were seeded on Matrigel-coated plates in mTeSR Plus with 1x RevitaCell Supplement (Gibco). After 48 h expansion, cells were dissociated by Accutase and 10,000 cells were seeded on CytoSort™ Array (10,000 microwells, CELL Microsystem). Once cells were attached, microwells containing a single colony were automatically picked and transferred to a 96-well plate by CellRaft AIR System (CELL Microsystem). The expanded clones on a 96-well plate were further genotyped by TaqMan genotyping assay (rs201141490, rs183444295, ThermoFisher) and Sanger sequencing.

**MSC differentiation**

hESCs-derived embryoid bodies were first produced using AggreWell (Stem Cell technology) and were then plated on Matrigel-coated plates in hMSC differentiation medium ($\alpha$MEM (Gibco), 10% fetal bovine serum (GeminiBio), 1% penicillin/streptomycin (Gibco), 10 ng/mL bFGF (ThermoFisher) and 5 ng/mL TGFβ (Thermofisher)) for around 10 days till fibroblast-like cells were confluent. These fibroblast-like cells were maintained in an hMSC culture medium on Gelatin-coated 10 cm dishes for two passages and were further sorted by FACS machine (BD FACS Aria II) to purify CD73/CD90/CD105 tri-positive hMSCs.

**Western blotting**

Cells and reactions were collected using a 2x Laemmli solution and incubated on ice for 15 min, during which time the samples were passed through a large gauge needle several times and vortexed every 5 min. Samples were then spun at 14,000 RPM to remove
debris, and the supernatant was transferred to a new tube. Samples were heated in boiling water for 20 min before being centrifugated at 14,000 RPM for 1 min and loaded into a BioRad Criterion 4-20 gel. After transfer to PDVF membrane and blocked (5% dehydrated milk) for 2 h at RT, membranes were incubated overnight antibodies in 2.5% blocking buffer at 4°C. Membranes were washed 3x with TBST for 10 min each before secondary antibody in 1x TBST was added for 2 h incubation at RT. Membranes were washed 3x with TBST for 10 min and then imaged.

The following antibodies were used: H3 (Abcam ab500)-1:5,000, H3K9ac (Abcam ab4441)-1:1,000, H3K18ac (Abcam ab1191)-1:1,000, β-tubulin (Abcam ab6046)-1:10,000, SIRT6 (Cell Signaling #12486)-1:1,000, Lamin A/C-1:1,000 (Abcam ab108595, Millipore 05–714), γH2AX (Millipore 05–636), PARP1(Abcam ab227244)-1:1,000, Vimentin-1:1,000 (Abcam ab92547); TAF15-Abcam ab134916; mADPr 1:500 (AbD33204 and AbD33205)(Bonfiglio et al, 2020).

**Immunoprecipitation**

Cells were plated and grown with equivalent dosages of cumate for 48 h prior to IP. In brief, cells were collected via trypsin and centrifugation and then lysed on ice using IP Buffer (20 mM HEPES pH = 8, 0.2 mM EDTA, 5% glycerol, 150 mM NaCl, 1% NP40) + COMplete protease inhibitor for 10 min. The lysate was sonicated at 25% output for 10 pulses, and then, debris was pelleted by centrifugation at 4°C at 13,000 RPM for 10 min. Supernatant was transferred to a new tube and precleared with A/G Sepharose beads at 4°C for 1 h on a rotator. Beads were removed by centrifugation and transfer of cleared sample to new tubes. 50 μl of the sample was reserved as input control. Samples were incubated overnight at 4°C with 5 μg of antibody (SIRT6-Cell Signaling #12486; PARP1-Abcam ab227244; Lamin-A/C-Millipore 05–714; Vimentin-Abcam ab92547; TAF15-Abcam ab134916; mADPr 1:500 (AbD33204 and AbD33205)(Bonfiglio et al, 2020), then with 30 μl of 25% Sepharose for 2 h at 4°C. Samples were spun down, and beads were washed 5x with IP buffer. Final resuspension with 100 μl IP buffer.

**DNA repair assays**

Both NHEJ and HR efficiency were assessed as previously described (Seluanov et al, 2010). In brief, I-Sce1, SIRT6 or HPRT control, and DsRed plasmid were transfected into telomerase-immortalized human foreskin fibroblasts containing chromosomally integrated NHEJ or HR reporter cassettes (I9A or H15C) cells (Mao et al, 2011). Anti-γH2AX (05–636) and anti-53BP1 (MAB3804) antibodies were purchased from Millipore. Apoptosis in fibroblasts was measured using the Annexin V Staining Kit (Roche).

**Quantitative RT–PCR**

Total RNA was isolated from cells at 80% confluence using Trizol Reagent and then treated with DNase. cDNA was synthesized using Superscript III (Life Technologies) cDNA kit with the oligo-dT primer. qRT–PCR was performed on the BioRad CFX Connect Real-Time machine with SYBR Green Master Mix (BioRad) using 30 ng of cDNA per reaction with 3x reactions/sample. All primer sets were tested for specificity and efficiency targeting the human LIP1 evolutionary active family of LINE1 elements. Actin was assayed using QuantumRNA Actin Universal primers (Thermo AM1720). For NRF2 pathway gene expression, total RNA was isolated from cells using RNeasy Plus Mini Kit (QIAGEN). cDNA was synthesized using PrimeScript RT Master Mix (Takara), qRT–PCR was performed with PowerUp SYBR Green Master Mix (Thermofisher) in QuantStudio 6 Pro Real-Time PCR System (Thermofisher). All primer sets were tested for specificity and efficiency. hLINE1 ORF1 Fwd-GTCACAAAGAACACAA, Rev-GTCTGAAATGTCCTCGTGAC; hLINE1 ORF2 Fwd-GCGACAGTTTGCAATCTGACTG, Rev-CTGGTCTGCTCTGTATTGGGT; 18S Fwd-GTAAACCCGTTCACCCCCATT, Rev-CAATTGTCGTAATGCG; NRF2 Fwd-TCAGCGAGGAGAAAGAATGATGA, Rev-CCATGGTTTCTGATCTGAGTGT; NQ01 Fwd-CCAGAGAGTTTGTGATAT; Rev-AAAAGCCCTACACACAGTTG, GCLC Fwd-GAATTTGGAAAATGGGCAATTG, Rev-CTAGATGATACCGAGGTTTGGAA; GCLM Fwd-TGACATGAGATGACCATGCT, Rev-TCACGAATACGGCTGAA.

**Immunofluorescence and apoptosis**

γH2AX and 53BP1 immunostaining was carried out as previously described(Mao et al, 2011). Anti-γH2AX (05–636) and anti-53BP1 (MAB3804) antibodies were purchased from Millipore. Apoptosis in fibroblasts was measured using the Annexin V Staining Kit (Roche).

**MMS treatment**

3x10⁴ hMSCs were seeded on a gelatin-coated 96-well plate and hMSCs were treated with DNA-damaging agents for 48 h upon reaching 90% confluence. CellTiter 96 AQueous One Solution Cell Proliferation Kit (MTS assay, Promega) was used to measure cell viability according to the manufacturer’s protocol. DNA-damaging agents employed in this assay were as follows: Methyl methanesulfonate (0.125/0.25/0.5 mM, MMS).

**Gamma irradiation**

Cells were grown to 75% confluence prior to transfected slides. A Cs-137 irradiator was used to deliver 2Gy of radiation to cells. Cells were transported in a 37°C container and media was replaced postexposure.

**Paraquat treatment**

Cells were maintained to 75% confluence, at which point fresh media lacking sodium pyruvate and containing paraquat was added. Cells were maintained for 24 h in treated media, followed by...
replacement with fresh media lacking sodium pyruvate. 48 h post-treatment, the cells were stained and evaluated for apoptosis.

**SIRT6 protein purification**

His-tagged SIRT6 cDNA was cloned into pET11a vectors and transformed into Rosetta-Gami E. coli cells. Cells were grown in presence of antibiotics and then protein production was induced with 0.5 mM IPTG 2 h before harvest. Cells were spun down and lysed on ice for 1 h using a solution of 50 mM Tris–HCl (pH = 7.5), 300 mM NaCl, 10% glycerol, and 10 mM imidazole with EDTA-free protease inhibitor (Sigma #P8849) and 1 mg/ml egg white lysozyme followed by sonication with a Branson instrument. After the removal of cell debris by centrifugation, the lysate was incubated with Ni2+ and 10% glycerol, and 10 mM imidazole with EDTA-free protease inhibitor (Sigma #P8849) and 1 mg/ml egg white lysozyme followed by sonication with a Branson instrument. After the removal of cell debris by centrifugation, the lysate was incubated with Ni2+ -NTA agarose overnight. Solution with beads was placed in gravity column and washed with lysis solution, followed by 2 volumes of wash buffer (lysis buffer + 30 mM imidazole). Finally, the protein was eluted with elution buffer (lysis buffer + 500 mM imidazole) and fractions were collected. Protein concentration was assessed by BCA assay and run on an SDS-PAGE gel. The 3–4 highest concentration fractions were pooled and dialyzed against storage buffer (50 mM Tris–HCl pH = 7.5, 150 mM NaCl, 1 mM DTT, 5% glycerol).

**Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC)**

Turnover experiments were performed using HEK293 cell lines stably expressing SIRT6 WT or centSIRT6. Cells were cultured for one week until confluent in MEM for SILAC (Thermo) supplemented with 10% dialyzed FBS (Gibco), L-glutamine, L-arginine 15C, 15N (Cambridge Isotopes), and L-lysine 15C, 15N2 (Cambridge Isotopes). The media was changed to a normal culture medium, and cell pellets were harvested at 0, 2, 4, 6, 8, 12, and 24 h postmedia change. Cell pellets were lysed in buffer containing 8 M urea, 75 mM NaCl, 50 mM Tris, pH 8.5, with a protease inhibitor cocktail (Roche). Pellets were vortexed for 30s and sonicated 5x 10 s with 1 min rest on ice in between sonication steps. The lysate was centrifuged at 15,000 xg for 10 min, and then, the supernatant was collected.

**SIRT6 deacylation activity**

The substrate for this reaction is a synthetic TNF-derived peptide first developed by Schuster _et al_ (2016) was synthesized by Gen-Script. Reactions were performed with 150 mM NaCl, 20 mM Tris, 5% glycerol, 1 mM β-mercaptoethanol, 2 μM SIRT6, and substrate concentration ranges of 7–1,000 μM NAD+ and 2–88 μM peptide. Reactions were performed at 37°C and fluorescence was measured using 310/405 nm excitation/emission spectra on a Tecan Spark 20 M plate reader. Readings were taken every 30s and initial rates were calculated from the relative fluorescence increase over a minimum of 6 min.

**Histone analysis**

Histones were purified from cumate SIRT6 fibroblasts (SIRT6 KO primary human foreskin fibroblasts constitutively expressing the catalytic subunit of telomerase and various alleles of SIRT6 via a cumate-inducible promoter) using an established cumate extraction method followed by propionylation of lysines prior to trypsin digestion to enhance coverage (Shechter _et al_, 2007; Sidoli _et al_, 2016). A data-independent analysis (DIA) mass spectrometry (MS) method was employed for quantification of modified peptides after samples were separated by nano-LC using EpiProfile (Sidoli _et al_, 2015) or, alternatively, after direct infusion followed by a one-minute acquisition and analysis with EpiProfileLite (Sidoli _et al_, 2019).

**Analysis of SIRT6 and LMNA protein–protein interactions by mass spec**

Cumate SIRT6 fibroblasts were used to compare interactomes for centSIRT6 and the wild-type SIRT6. Nuclear extracts were prepared using a hypotonic lysis buffer. Nuclei isolated from approximately 2.5x107 cells were resuspended in one ml of extraction buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM DTT, 0.25%NP-40, 1x Roche Complete protease inhibitors, as well as kinase/phosphatase inhibitors—1 mM NaVO4, 10 mM beta-glycerophosphate, 1 mM sodium pyrophosphate, 1 mM NaF), and nuclei were disrupted by passage through a 27 gauge needle followed by sonication (3 pulses at constant 20% power) with a Branson Sonifier on ice. Next, samples were filtered through a 0.2 μm MWCO filter to remove any insoluble material (and reduce non-specific binding). Proteins were quantified using the BCA assay, and equal amounts of wild-type SIRT6 and centSIRT6-derived extract were divided into 5 replicates each. Three replicates of extract received 4 μg of anti-SIRT6 antibody (Cell Signaling #1248) or anti-Lamin A (Lamin A/C-Millipore 05–714) and 75 μl of Millenyi protein G μMACS magnetic particles. Two of the replicates were mixed with normal rabbit IgG (Cell Signaling #2729) as controls for non-specific binding to the particles. Samples were rotated at 4°C for 6 h followed by separation by the magnet. Samples were washed with 3 additional ml of extraction buffer and eluted with 100 μl of boiling elution buffer (5% SDS with 50 mM Tris–HCl pH 7.5). After removal of SDS with S-columns (ProtiFi; Huntington, NY) and trypsin digestion, peptides were resuspended in MS-grade water and labeled with tandem mass tags (TMT 10-plex; Thermo Fisher). Samples were resolved by nano-electrospray ionization on an Orbitrap Fusion Lumos MS instrument (Thermo) in positive ion mode using a 30 cm homemade column packed with 1.8 μm C-18 beads to resolve the peptides. Solvent A was 0.1% formic acid and solvent B was 100% acetonitrile (CAN) with 0.1% formic acid. The length of the run was 2 h with a 90 min gradient. CID (35% collision energy) was used for MS2 fragmentation. HCD (60% collision energy) was used for MS3 detection of TMT groups. Other details of the run parameters may be found in the embedded run report of the RAW data file uploaded to the ProteomeXchange database. Peptide assignments were made using Proteome Discoverer and Sequest and MS3 ions were used for quantitation. False discovery rates (FDR) were estimated using a Decoy Database Search with Target FDR (strict) set to 0.01 and Target FDR (relaxed) set to 0.05. Validation was based on q-value. In the consensus step, ions with a co-isolation threshold above 30% were excluded. Normalization between replicates was achieved using the total protein approach (Wisniewski, 2017) where peptide counts for a single protein were divided by the sum of all proteins in the lane. Proteins appearing in specific antibodies (either anti-SIRT6 or anti-Lamin A) compared with normal IgG serum with
were prepared on ice and moved to 37°C. All reagents were obtained from Epicypher. Common reagents were prepared as anti-H3K18ac antibodies. 15 min before being run in Western analysis using anti-H3K9ac and incubation. The reaction was quenched with direct application of 1 μl. Five μl of SIRT6 protein was combined with 100 fm PARP1, 20 mM Tris–HCl pH = 8, 10 μM ZnCl2, 10 μM MgCl2, 10% glycerol, 300 μM NAD+, 1 mM DTT, 0.1 μg/ml salmon sperm DNA and ddH2O up to 50 μl. All reagents were prepared on ice and moved to 37°C for the duration of the incubation. The reaction was quenched with direct application of 1 volume of 2x Laemmli buffer with BME. Samples were boiled for 15 min before being run in Western analysis using anti-H3K9ac and anti-H3K18ac antibodies.

**Deacetylase Assay**

Three μg of SIRT6 protein was combined with 0.5 μg histones, 1 or 5 mM NAD+, 30 mM Tris–HCl pH = 8, 4 mM MgCl2, 1 mM DTT and ddH2O up to 50 μl. Designer nucleosomes with relevant PTMs were obtained from Epicypher. Common reagents were prepared as a master mix prior to the addition of SIRT6 protein. All reagents were prepared on ice and moved to 37°C for 30 min upon addition of SIRT6. The reaction was quenched with direct application of 1 volume of 2x Laemmli buffer with BME. Samples were boiled for 15 min before being run in Western analysis using anti-H3K9ac and anti-H3K18ac antibodies.

**PARP1 Ribosylation Assay**

Five μg of SIRT6 protein was combined with 100 fm PARP1, 20 mM Tris–HCl pH = 8, 10 μM ZnCl2, 10 μM MgCl2, 10% glycerol, 300 μM NAD+, 1 mM DTT, 0.1 μg/ml salmon sperm DNA and ddH2O up to 50 μl. All reagents were prepared on ice as a master mix and moved to 30°C for 30 min upon addition of SIRT6. The reaction was quenched with direct application of 1 volume of 2x Laemmli buffer with BME. Samples were boiled for 15 min before being run in Western analysis using anti-PADPR antibody (Abcam Ab14459).

**KAP1 Ribosylation Assay**

KAP1 ribosylation was conducted using the protocol in Van Meter et al., 2014, with Biotin-NAD+ (Tocris 6,573) in leu of [³²P] NAD+. Five μg of KAP1 protein was used in each reaction. Reactions were prepared as a master mix prior to the addition of SIRT6. Samples were boiled and then run on SDS–PAGE gel and then transferred to PDPF membrane. Membranes were probed using HRP streptavidin (Sigma RABHRP3).

**SIRT6 Ribosylation Assay**

Three μg of SIRT6 was combined with 50 mM Tris–HCl pH = 7.5, 1 mM DTT, 10 μM ZnCl2, 150 mM NaCl, 25μM Biotin-NAD+ (Tocris 6,573), and ddH2O up to 20 μl. All reagents were combined on ice in a master mix except for SIRT6. Master mix was aliquoted into reaction tubes and SIRT6 was added and incubated at 37°C for 3 h. The reaction was quenched with 1 volume of 2x Laemmli buffer with BME. Samples were boiled and then run on SDS–PAGE gel and then transferred to PDPF membrane. Membranes were probed using HRP-streptavidin (Sigma RABHRP3).

**Chromatin Immunoprecipitation (ChIP)**

Cells were harvested at 80% confluency after 48 h with or without cumate. Trypsin was used to remove cells from the plate and then neutralized with PBS containing FBS and spun down to collect the cell pellet. The cell pellet was washed with cold PBS 1x prior to using Abcam Ab500 ChIP Kit. H3K9ac (Abcam ab4441), H3K18ac (Abcam ab1191), and SIRT6 (Cell Signaling #12486) antibodies were used. The manufacturer’s protocol was used with the following considerations: Fixation time = 10 min at 37°C, Sonication settings (Qsonica Instrument) = 12.5 min, 15 s on/off, 30% output, 4”. qPCR primers from (Kawahara et al., 2009; Tasselli et al., 2016) were used.

**Thermostability**

Four μM SIRT6 protein was combined with 1x SYPRO Orange dye in storage buffer (50 mM Tris–HCl pH = 7.5, 150 mM NaCl, 1 mM DTT, 5% glycerol) to 50 μl. Samples were placed in a qRT–PCR plate and run on a BioRad CFX Connect Real-Time machine using a Melt Curve protocol (30°C-75°C, 0.5°C steps in 10 s intervals).

**Statistics**

Unless otherwise noted, the Student’s t-test was used to determine statistical significance between groups. All tests were two-tailed and P-values were considered significant below a 0.05 threshold. For two-way ANOVA tests, a Bonferroni correction was applied. Cell culture experiments utilized at least two separately derived cell lines for each genotype and were performed in triplicate unless noted otherwise. In vitro assays were done in triplicate using two or more separate protein isolation preps. Blinding was not conducted for assays.

**Data availability**

All sequencing data have been deposited in the Sequence Read Archive (SRA) under the bioproject number

- Sequencing data, DNA damage genes PRJNA669033 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA669033/)
- Capture-seq data, Sequence Read Archive PRJNA669034 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA669034)
- RNAseq data, Sequence Read Archive PRJNA682192 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA682192)
- The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD035689 and 10.6019/PXD035689

**Expanded View** for this article is available online.

**Acknowledgements**

We would like to extend a special thanks to Dr. Ivan Matic for providing the mADPr antibodies. We are thankful to Kevin Welle and Jennifer Hryhorenko of Mass Spectrometry Resource Lab for their help and advice. We would like to
thank Meenal kishisundaram Balasubramaniam and Robert Shmookler-Reis for their help in exploring SIRT6 structural changes. Funding was provided by the US National Institute of Health grants AG056278 (VC), AG027237 (VG), AG064706 (VC), AG064704 (VC), AG046320 (AS), AG047200 (VG and AS), AG051449 (VG and AS), AG056278 (YS), AG076040 (YS), AG057433 (YS), AG061521 (YS), AG055501 (YS), AG057341 (YS), AG057706 (YS), AG057909 (YS), and AG17242 (YS), a grant GCRLE-1320 (YS) from the Global Consortium for Reproductive Longevity and Equality at the Buck Institute, made possible by the Bai-Echo Foundation, a grant from the Michael Antonon Foundation, and a grant from the Simons Foundation (YS).

Author contributions
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Disclosure and competing interests statement
VG serves on the SAB of Genflow and DoNotAge. Other authors declare no competing interests or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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Expanded View Figures

**Figure EV1.** rs350845 (chr19:4174953:A>G) is a cis-eQTL for SIRT6 upregulation across multiple tissues.

A. GTEx tissue types are shown sorted by m-value, which is the posterior probability that an eQTL exists for SIRT6 in a specific tissue from a cross-tissue meta-analysis.

B. Single-tissue eQTL P-value vs. m-values.

C. Violin plots showing SIRT6 expression differences across three example tissues with m-value = 1. Reference allele is G and alternate allele is A, and each panel shows the normalized expression of SIRT6 in a different tissue. Values beneath genotypes represent a number of individuals.
Figure EV1.
Figure EV2. Turnover rate and biochemical properties of purified SIRT6 proteins.

A Protein turnover rate. SILAC analysis on HEK293 cells expressing SIRT6 variants.
B Coomassie staining of SIRT6 purified from Rosetta-Gami E. coli cells. Proteins were purified at the University of Rochester (Roc) and the Ichor Therapeutics facility in Ithaca. All in vitro experiments were performed with both protein preparations and the data was consistent between the two preps.
C Thermostability of purified SIRT6 proteins. Data represent two replicates with two technical replicates each using SIRT6 from the Roc and Ichor preps. n = 4 TR; error bars = SD. Student’s t-test, two-tailed.
D Tryptophan fluorescence curves for SIRT6 variants with titrated concentrations of NAD+.

Figure EV3. HeLa histone deacetylation by recombinant SIRT6, and SIRT6 expression in cumate-inducible cell lines.

A In vitro deacetylation rates of purified SIRT6 proteins with histones purified from HeLa cells. H3K9ac, H3K18ac, and H3 are immunoblots. SIRT6 loading Coomassie stain. Control lanes, from the same gels, were repositioned for consistency. Uncut gels are shown in Appendix Fig S1. Asterisks indicate P < 0.05 compared with WT. n = 3 TR; error bars = SD. Student’s t-test, two-tailed.
B SIRT6 expression in cumate-inducible telomerase-immortalized HCA2 human fibroblast cell lines. SIRT6 alleles were integrated into the genome of SIRT6 knockout HCA2 cells using PiggyBac Transposon Vector System. Different dosages of cumate and resulting SIRT6 abundance were used to determine the dose needed to achieve equivalent SIRT6 expression for each cell line. Cells were normalized by count and total protein. Subsequent experiments utilizing cumate-inducible SIRT6 fibroblasts were controlled for SIRT6 abundance using these data. For each cell line, the red box indicates the corresponding cumate dosage for equivalent expression (WT = 60 μg/ml, N308K = 30 μg/ml, A313S = 30 μg/ml, and Cent = 75 μg/ml). These concentrations were used in experiments with these cells.
C qRT–PCR expression analysis of SIRT6 using standardized dosages of cumate (colored bars are cells treated with cumate, and empty bars are uninduced controls). Cumate dosage corresponds to red boxes in Appendix Fig S2B. Actin was used for standardization and samples were normalized to uninduced WT. n = 3 BR; error bars = SD. Student’s t-test, two-tailed.
D Apoptosis assay of HCA2 cells treated with varying doses of cumate. n = 3 TR; error bars = SD. Student’s t-test, two-tailed.
E Population doubling rate of HCA2 cells treated with different doses of cumate to assess the impact on cell division.
F Coomassie-stained SDS–PAGE of histone preparations for quantitative mass spectrometry.

Source data are available online for this figure.
Figure EV3.

Figure EV4. Quantification of SIRT6 expression and FACS gating for DNA repair assays.

A. Representative images of FACS analysis for DNA DSB repair assays (Fig 4D and E).
B. Representative images of basal γH2AX immunostaining (Fig 4F). Dashed outline denotes nucleus borders. Scale bar 10 μm.
C. Representative images of γH2AX immunostaining after γ-irradiation (Fig 4G). Scale bar 10 μm.
D. Representative images of FACS traces for paraquat sensitivity assay (Fig 4H and I).
Figure EV4.
Figure EV5. Analysis of CRISPR/Cas9-edited human MSC cell lines.

A, B Double strand repair efficiency in wild-type and centSIRT6 hMSCs. DSB repair reporter constructs were integrated into hMSCs. After 72 h recovery, reactivation of the GFP reporter was measured by flow cytometry. Stimulation of NHEJ or HR was calculated as the ratio of GFP+/DsRed+ positive cells. Asterisks indicate \( P < 0.05 \). \( n = 3 \) BR; error bars = SD. Student’s t-test, two-tailed.

C Cell viability in MMS-treated wild-type and centSIRT6 hMSCs. hMSCs were treated with MMS for 48 h and cell viability was evaluated by MTS assay. Data were normalized to the control group (0 mM). Asterisks indicate \( P < 0.05 \). \( n = 6 \) BR; error bars = SD. Student’s t-test, two-tailed.

D Immunofluorescence staining of 53BP1 in wild-type and centSIRT6 hMSCs under MMS treatment. Numbers of 53BP1 foci in the nuclei of wild-type and centSIRT6 hMSCs with or without MMS (0.25 mM) treatment were quantified. >600 nuclei from 10 TR images were scored. Asterisks indicate \( P < 0.05 \). Error bars = SD. Student’s t-test, two-tailed.

E qRT–PCR of NRF2 stress response pathway genes in wild-type and centSIRT6 hMSCs. \( n = 3 \) BR; error bars = SEM. Student’s t-test, two-tailed.

F RNAseq expression of NRF2 stress response pathway genes in wild-type and centSIRT6 hMSCs. \( n = 4 \) BR; error bars = SEM. Student’s t-test, two-tailed.

G Western blot analysis of SIRT6 in wild-type and centSIRT6 hMSCs. \( \beta \)-Actin was used as a loading control and used to normalize quantification. Asterisks indicate \( P < 0.05 \). \( n = 2 \) BR; error bars = SD. Student’s t-test, two-tailed.

Source data are available online for this figure.
Figure EV5.
Appendix Table of Contents
Appendix Figure S1 ........................................................................................................1
Appendix Figure S2 ........................................................................................................2
A, B Uncut blots and gels shown in EV3A. SIRT6 was Coomassie stained on separate gels. Equal volumes of the reaction were loaded on three separate gels (4-20% BioRad stain-free SDS-PAGE) to evaluate H3K9ac, total H3, and SIRT6.
Appendix Figure S2: Immunoprecipitation of other MS-identified centSIRT6 interacting proteins

A IP experiments on lysates from cumate-induced fibroblasts expressing wild type or centSIRT6 alleles with antibodies to TAF15 and Vimentin. SIRT6 expression was induced 48 hours prior to IP. The IP experiments were repeated three times. One representative set of IPs is shown.

B TAF15 IP from cumate-inducible SIRT6 fibroblasts followed by Western blot with antibody to mADPr residues (Bonfiglio et al., 2020). Asterisks indicate p<0.05. n=3; error bars=SD. Students t-test, two tailed. Abundance normalized to input.

C Vimentin IP from cumate-inducible SIRT6 fibroblasts followed by Western blot with antibody to mADPr residues (Bonfiglio et al., 2020). Asterisks indicate p<0.05. n=3; error bars=SD. Students t-test, two tailed. Abundance normalized to input.