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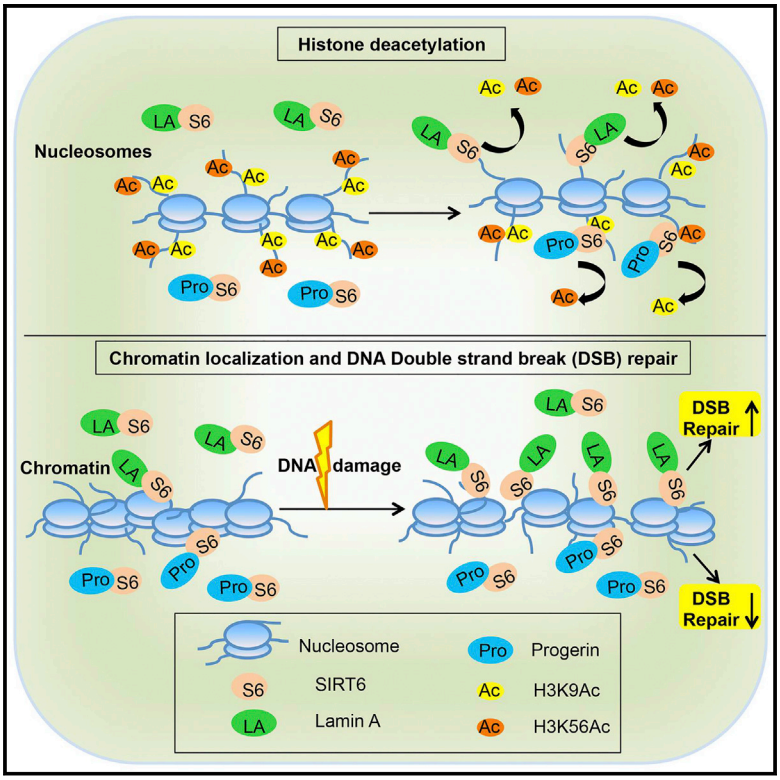
Lamin A Is an Endogenous SIRT6 Activator and Promotes SIRT6-Mediated DNA Repair

Graphical Abstract



Highlights

- Lamin A directly binds and activates SIRT6 toward histone deacetylation
- Lamin A promotes SIRT6-mediated downstream functions upon DNA damage
- Progerin exhibits impaired activating effect on SIRT6
- HGPS fibroblasts display defective SIRT6-dependent DNA repair upon irradiation

Authors

Shrestha Ghosh, Baohua Liu, Yi Wang, Quan Hao, Zhongjun Zhou

Correspondence

zhongjun@hku.hk

In Brief

SIRT6 is a mammalian sirtuin involved in numerous cellular functions. Here, Ghosh et al. identify lamin A as a SIRT6 activator. Lamin A, but not progerin, enhances SIRT6-dependent histone deacetylation and promotes SIRT6-mediated molecular events upon DNA damage. Impaired SIRT6 functioning is identified as an underlying mechanism in HGPS.

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Lamin A Is an Endogenous SIRT6 Activator and Promotes SIRT6-Mediated DNA Repair

Shrestha Ghosh,[1,2] Baohua Liu,[1,3] Yi Wang,[1] Quan Hao,[1] and Zhongjun Zhou[1,2,*]
[1]School of Biomedical Sciences, LKS Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Pok Fu Lam, Hong Kong
[2]Shenzhen Institute of Innovation and Research, The University of Hong Kong, Nanshan, Shenzhen 518000, China
[3]School of Medicine, Shenzhen University, Shenzhen 518060, China
*Correspondence: zhongjun@hku.hk
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SUMMARY

The nuclear lamins are essential for various molecular events in the nucleus, such as chromatin organization, DNA replication, and provision of mechanical support. A specific point mutation in the *LMNA* gene creates a truncated prelamin A termed progerin, causing Hutchinson-Gilford progeria syndrome (HGPS). SIRT6 deficiency leads to defective genomic maintenance and accelerated aging similar to HGPS, suggesting a potential link between lamin A and SIRT6. Here, we report that lamin A is an endogenous activator of SIRT6 and facilitates chromatin localization of SIRT6 upon DNA damage. Lamin A promotes SIRT6-dependent DNA-PKcs (DNA-PK catalytic subunit) recruitment to chromatin, CtIP deacetylation, and PARP1 mono-ADP ribosylation in response to DNA damage. The presence of progerin jeopardizes SIRT6 activation and compromises SIRT6-mediated molecular events in response to DNA damage. These data reveal a critical role for lamin A in regulating SIRT6 activities, suggesting that defects in SIRT6 functions contribute to impaired DNA repair and accelerated aging in HGPS.

INTRODUCTION

In the past few years, there has been a significant advancement in our understanding of SIRT6, one of the seven mammalian sirtuins with various catalytic activities (Giblin et al., 2014). SIRT6, predominantly a nuclear protein (Michishita et al., 2005), has been reported to catalyze both deacylation and mono-ADP ribosylation (Gertler and Cohen, 2013). Although SIRT6 was initially known to deacetylate histone H3 at lysine 9 and 56 both globally and at specific gene loci (Michishita et al., 2008, 2009; Kawahara et al., 2009), it was later identified to also deacetylate CtIP, a non-histone protein mediating DNA end resection after DNA damage (Kaidi et al., 2010). PARP1, an important protein in genomic maintenance and aging (Mangerich and Bürkle, 2012), is a known substrate of SIRT6 for mono-ADP ribosylation (Mao et al., 2011). SIRT6 is also known to mediate its own mono-ADP ribosylation (Liszt et al., 2005). Apart from PARP1, SIRT6 has been reported to mediate mono-ADP ribosylation of the nuclear corepressor Kap1, thereby aiding in the packaging of L1 retrotransposons into transcriptionally repressive heterochromatin (Van Meter et al., 2014). Recently, hydrolysis of long-chain fatty acyl moieties from lysine residues has been described as the third enzymatic activity of SIRT6 (Jiang et al., 2013). Several independent studies have revealed and elucidated the roles of SIRT6 in a spectrum of biological processes, such as DNA damage repair, cellular metabolism, inflammation, cancer, and aging (Kugel and Mostoslavsky, 2014). The in vivo biological functions of SIRT6 have been well illustrated and appreciated through studies in *Sirt6* knockout mice. Loss of Sirt6 in mice leads to severe signs of degenerative progeroid phenotypes, attributable to the defects in DNA damage repair (Mostoslavsky et al., 2006). Additionally, exogenous mammalian SIRT6 could extend lifespan in male, but not female, mice (Kanfi et al., 2012), further supporting that SIRT6 is critical in regulating aging processes and longevity. SIRT6 is known to mediate DNA double-strand break (DSB) repair via homologous recombination (HR) and non-homologous end-joining (NHEJ) repair pathways (Kaidi et al., 2010; Mao et al., 2011). Overexpression of SIRT6 in human fibroblasts stimulates HR repair and delays replicative senescence (Mao et al., 2012). SIRT6 is also known to stabilize DNA-PKcs (DNA-PK catalytic subunit) at DSB foci for the repair process (McCord et al., 2009). In addition, SIRT6 has been reported to be responsible for recruiting the chromatin remodeler SNF2H to the DNA break sites and thus preventing genomic instability (Toiber et al., 2013). These data suggest that SIRT6 contributes to aging processes and lifespan regulation largely via its functions in the maintenance of genomic integrity (Van Meter et al., 2011b). Apart from genome maintenance, SIRT6 also plays crucial roles in tumor suppression (Sebastián et al., 2012; Min et al., 2012; Van Meter et al., 2011a) and metabolic regulation (Dominy et al., 2012, Elhanati et al., 2013). Although SIRT6 has been implicated in a plethora of cellular functions, the regulation of its activity in DNA damage repair remains largely elusive, and no endogenous modulator has been reported so far (Gertler and Cohen, 2013).

The nuclear lamins, together with their interacting partners, form a complex meshwork of proteins to provide mechanical support to the nuclear envelope and maintain proper chromatin organization and transcriptional regulation (Luperchio et al., 2014). They act as a bridge between the inner nuclear membrane and chromatin (Ciska and Moreno Díaz de la Espina, 2014; Kind

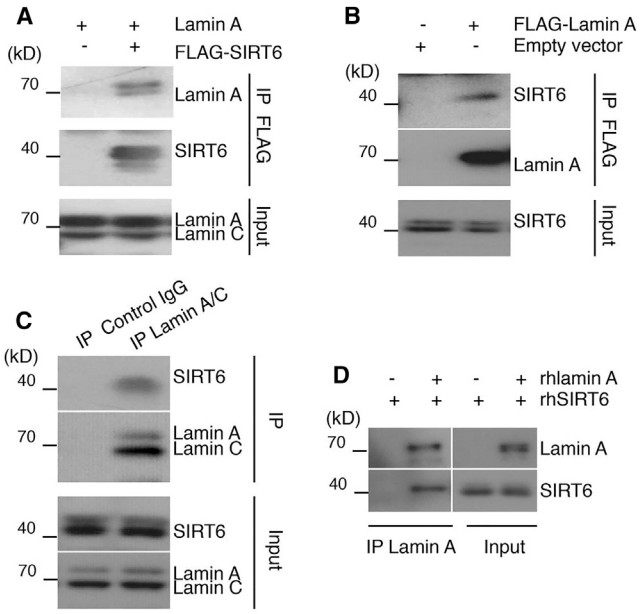

Figure 1. Lamin A Directly Interacts with SIRT6

(A) Lamin A and FLAG-SIRT6 were ectopically expressed in HEK293 cells, and co-immunoprecipitation was done using FLAG antibodies. Western blotting shows the presence of lamin A in anti-FLAG immunoprecipitates only from samples overexpressing FLAG-SIRT6. IP stands for immunoprecipitated samples.

(B) FLAG-lamin A and empty vector (EV) were ectopically expressed in HEK293 cells. By western blotting analysis, SIRT6 could be detected in anti-FLAG immunoprecipitates from the sample containing FLAG-lamin A.

(C) Western blotting showing that endogenous SIRT6 could be pulled down from whole-cell lysates of HEK293 cells by lamin A antibodies.

(D) Recombinant human SIRT6 (rhSIRT6, 2 μg) was co-immunoprecipitated with recombinant lamin A (rhlamin A, 2 μg) in test tubes using antibodies against lamin A.

See also Figures S1 and S6.

and van Steensel, 2010). The *LMNA* gene gives rise to four types of A-type lamins via alternative splicing, with lamin A and lamin C being the two most predominant species found in mammals and lamin AΔ10 and lamin C2 being the less expressed ones (Cau et al., 2014). Mutations in the *LMNA* gene lead to a variety of disorders in humans, collectively called laminopathies (Schreiber and Kennedy, 2013). A de novo single base substitution from C to T at position 1,824 in *LMNA* gene exposes a cryptic splicing site and gives rise to truncated prelamin A, termed as progerin, with a 50-amino-acid deletion at the C terminus (Eriksson et al., 2003), leading to Hutchinson-Gilford progeria syndrome (HGPS), a severe form of early-onset premature aging disorder (Liu and Zhou, 2008). Progeroid cells are characterized by morphological abnormality, misshaped nucleus, heterochromatin loss, delayed DNA damage response, and mislocalization of nuclear proteins (Prokocimer et al., 2013; Krishnan et al., 2011).

Sirt6$^{-/-}$ mice exhibit several progeroid phenotypes such as reduced body size and weight, acute degenerative phenotypes, loss of subcutaneous fat, osteopenia, and failure to thrive as well as severe metabolic defects, many of which resemble the chronic abnormalities found in HGPS patients such as extreme short stature, loss of body weight, lipodystrophy, osteolysis, and early mortality at a mean age of 13 years (Mostoslavsky et al., 2006; Hennekam, 2006). Similar to HGPS cells, *Sirt6*$^{-/-}$ cells display severe impairment in DNA damage repair and premature senescence. However, alopecia, which is characteristic of nearly all HGPS patients, is not so evident in *Sirt6*$^{-/-}$ mice (Mostoslavsky et al., 2006; Musich and Zou, 2011). We have recently reported that lamin A acts as an activator of SIRT1 (Liu et al., 2012). Given that loss of *Sirt6* results in compromised genomic maintenance and *Sirt6*$^{-/-}$ mice recapitulate many of the progeroid phenotypes found in HGPS, it is plausible that there might be a functional link between SIRT6 activities and lamin A. In the present study, we show that lamin A directly interacts with SIRT6 and promotes SIRT6-mediated histone deacetylation. Lamin A also facilitates SIRT6-mediated events in DSB repair. On the other hand, progerin exhibits a striking impairment in stimulating SIRT6 activities. The functions of SIRT6 in HGPS fibroblasts are significantly compromised in response to DNA damage, indicating impaired SIRT6 activities as a contributing underlying mechanism in HGPS.

RESULTS

Lamin A Directly Interacts with SIRT6

To identify the potential role of lamin A in the regulation of SIRT6 functions, we first examined the interaction between the two proteins. We ectopically expressed FLAG-SIRT6 and lamin A in HEK293 cells and pulled down SIRT6 by FLAG antibodies. As shown in Figure 1A, lamin A was observed in the anti-FLAG immunoprecipitates. Reciprocally, SIRT6 was pulled down together with FLAG-lamin A by anti-FLAG antibodies in HEK293 cells (Figure 1B). To further confirm this observation, we examined their endogenous interaction. As shown in Figures 1C and S6A, antibodies against lamin A could successfully pull down SIRT6 in HEK293 cells. Similar results were also observed in mouse embryonic fibroblasts (MEFs), and no Sirt6 was pulled down by lamin A antibodies in MEFs lacking lamin A (Figure S1A). This is in accordance with the proteomic studies performed using mass spectrometry analyses stating potential interaction between SIRT6 and lamin A (Simeoni et al., 2013; Miteva and Cristea, 2014). To further analyze whether lamin A is in direct physical association with SIRT6, we performed a co-immunoprecipitation assay using recombinant proteins (rhSIRT6 and rhlamin A, 2 μg of each) in vitro. As shown in Figure 1D, lamin A could pull down SIRT6 in vitro. Taken together, these data demonstrate that lamin A can directly interact with SIRT6.

The Core Domain of SIRT6 Is Crucial for Its Interaction with Lamin A

The different domains of SIRT6 have been reported to be involved in several independent as well as interdependent functions (Tennen et al., 2010). The N-terminal extension is crucial for its catalytic activity and chromatin association, while the C-terminal extension is required for its proper nuclear localization. Interestingly, a point mutation in the catalytic core domain of SIRT6 impairs not only the catalytic activity but also its association with chromatin (Tennen et al., 2010). To understand which domain of SIRT6 is responsible for binding with lamin A, we

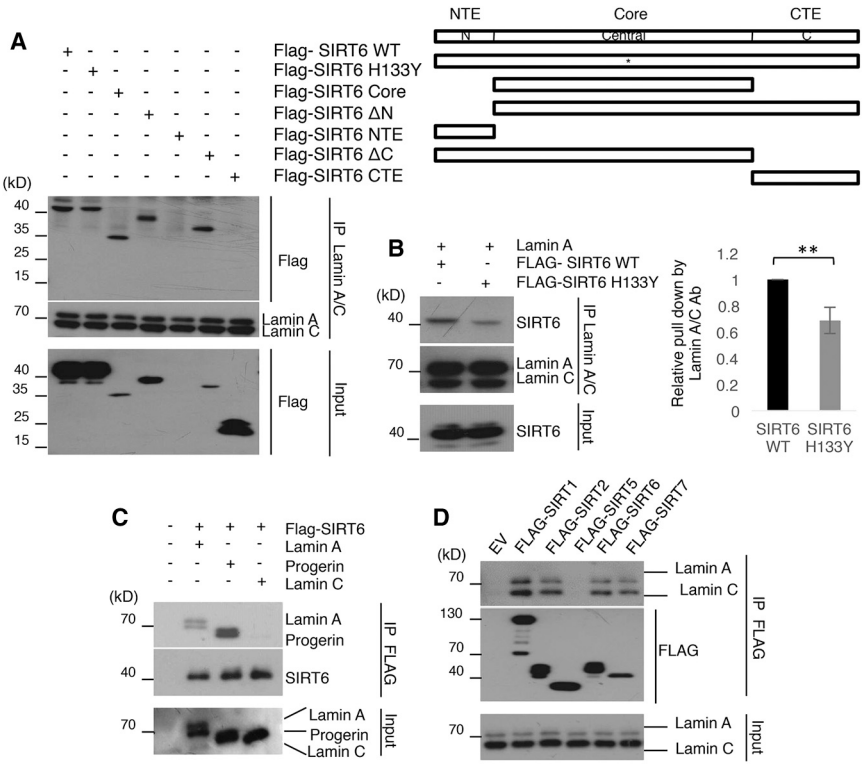

Figure 2. The Core Domain of SIRT6 Is Crucial for Its Interaction with Lamin A

(A) FLAG-tagged full-length and different-domain-deleted mutants of SIRT6 (diagrammatically represented on the right) were transiently expressed in HEK293 cells and co-immunoprecipitated by antibodies against lamin A. Western blotting was performed using FLAG antibodies. Lamin A/C western blotting served as internal control.

(B) FLAG-tagged wild-type SIRT6 and its catalytic mutant H133Y (containing a point mutation in its catalytic core domain) were ectopically expressed in HEK293 cells and co-immunoprecipitated by lamin A. Western blotting was performed using FLAG antibodies. Data are from three independent experiments; $^{**}p < 0.01$.

(C) Equal amounts of lamin A, progerin, and lamin C DNA constructs (5 μg each) were transiently expressed in HEK293 cells along with FLAG-SIRT6 (5 μg) and co-precipitated by FLAG antibodies. Western blotting was performed using lamin A antibodies that recognize lamins A and C and progerin.

(D) Equal amount of FLAG-tagged sirtuin 1, 2, 5, 6, and 7 constructs (10 μg) were ectopically expressed in HEK293 cells and immunoprecipitated by FLAG antibodies. SIRT1, 2, 6, and 7 displayed clear interaction with lamin A, while SIRT5 exhibited no detectable binding in the western blotting. EV stands for empty vector.

See also Figures S1 and S6.

examined the interaction of SIRT6 with lamin A using SIRT6 mutants with different domain deletions. As shown in Figures 2A and S6B, all SIRT6 mutants containing catalytic core domain could be pulled down by endogenous lamin A in HEK293 cells. The NTE domain (N-terminal extension) of SIRT6 was hardly detectable despite several trials whereas the CTE (C-terminal extension) domain could not be pulled down by lamin A antibodies (last lane in Figure 2A), even though an appreciable expression could be observed in the input. This suggests that lamin A interacts with SIRT6 via the catalytic core domain of SIRT6. Interestingly, the binding of lamin A with SIRT6 catalytic point mutant (SIRT6 H133Y) was significantly reduced (Figure 2B; $n = 3$, $^{**}p < 0.01$), indicating that the conformation of catalytic core domain of SIRT6 is crucial for its binding with lamin A.

Lamins A and C and progerin share an identical N-terminal region and vary from each other only in their C terminus, as diagrammatically represented in Figure S1C. To analyze the region of lamin A involved in the interaction with SIRT6, we ectopically expressed FLAG-SIRT6 along with lamin A, progerin and lamin C in HEK293 cells. As shown in Figure 2C, while lamin A and progerin could be observed in the anti-FLAG immunoprecipitates, lamin C pull-down was hardly detectable even after overexpression, suggesting that the N terminus of lamin A is dispensable for this interaction to take place and the C terminus dictates binding to SIRT6. However, upon prolonged exposure, a faint band of lamin C was detectable in the anti-FLAG immunoprecipitates (data not shown), which is likely due to indirect interaction given the existence of lamin A and lamin C in a complex (Luo et al., 2014). The existence of prelamin A in lamin A expressing cells is likely a consequence of saturated activity of prelamin A processing enzyme(s) due to large amount of lamin A precursor expression, since plasmids expressing full-length cDNA of *LMNA* gene have been used in the study (Figure 2C). To narrow down the C-terminal amino acids of lamin A binding with SIRT6, we synthesized three peptides containing fragments from the C-terminal domain of lamin A (schematically represented in Figure S1D). We biotinylated the peptides, followed by incubation with recombinant SIRT6, and then performed pull-down by using Streptavidin beads. As shown in the silver staining (Figure S1D), peptide 1 (containing the last 80 amino acids of lamin A) and peptide 2 (containing the 30 amino acids common to both lamin A and progerin), but not peptide 3, could pull down SIRT6. This suggests that the 30 amino acids common to both lamin A and progerin are crucial for interacting with SIRT6. We also tested the potential interaction between lamin A and different sirtuins by co-immunoprecipitation in HEK293 cells expressing FLAG-tagged sirtuins and observed clear interaction of lamin A with SIRT1, 2, 6, and 7 (Figure 2D). However, mitochondrial SIRT5 showed no detectable interaction. To confirm this observation, the interactions between lamin A and different sirtuins were examined by pulling down endogenous lamin A (Figures S1B and S6C). Again, lamin A was found to interact with nuclear sirtuins. No interaction was detected between lamin A and mitochondrial sirtuin SIRT5 (Figures S1B and S6C).

Lamin A, but Not Progerin, Enhances SIRT6 Deacetylase Activity

The immediate question following these observations is what the biological relevance of the interaction is. SIRT6 has been reported to be responsible for deacetylation of histone H3 at lysine

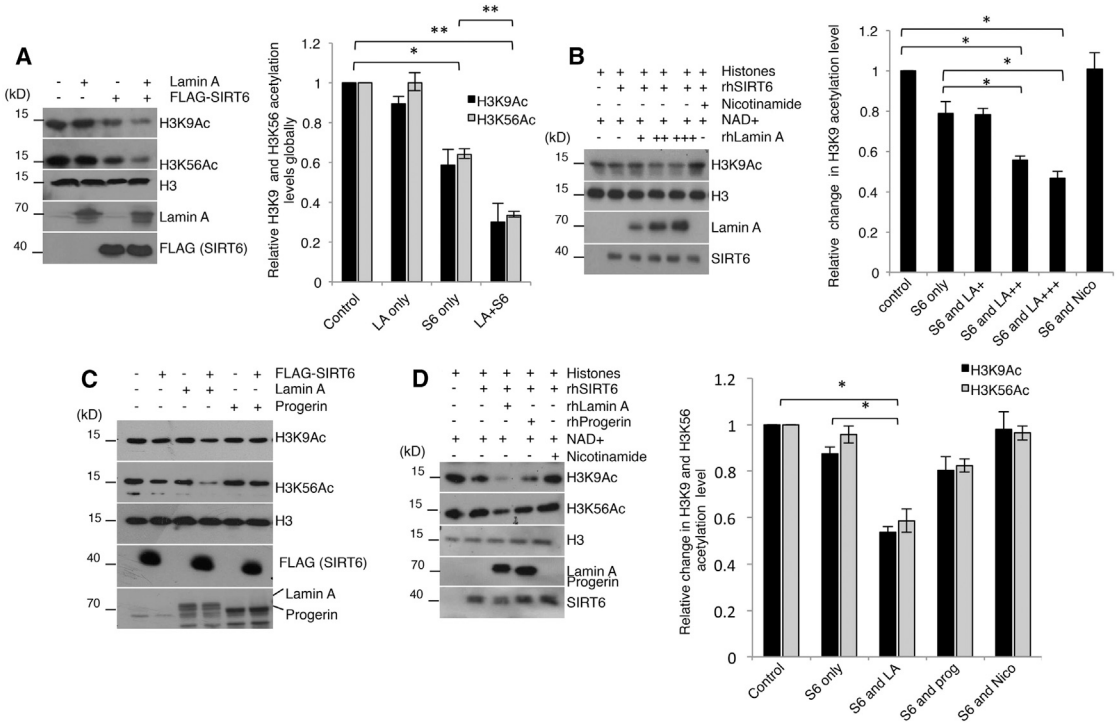

Figure 3. Lamin A, but Not Progerin, Enhances SIRT6 Deacetylase Activity

(A) SIRT6 and lamin A were ectopically expressed in HEK293 cells, either individually or in combination. Western blotting was performed in whole-cell lysates collected 48 hr posttransfection. The quantification represents mean ± SEM relative to non-transfected control (n = 3). *p < 0.05, **p < 0.01. LA and S6 stand for lamin A and SIRT6, respectively.

(B) Acid-extracted histones (2 μg) were incubated with recombinant SIRT6 (rhSIRT6, 1 μM) in the presence or absence of increasing concentrations of recombinant lamin A (rhlamin A). The concentrations of rhlamin A used were 0.2 μM, 0.3 μM, and 0.5 μM, respectively. Western blotting was performed to examine the acetylation level of H3K9. The graph shows quantification of data with respect to control (i.e., histones only) and represents mean ± SEM (n = 3). *p < 0.05. Nico stands for nicotinamide.

(C) An equal amount of either lamin A or progerin construct (5 μg each) was transiently expressed in HEK293 cells either alone or along with SIRT6 construct (5 μg). Western blotting was performed with whole-cell extract collected 48 hr after transfection to examine levels of H3K9 and H3K56 acetylation.

(D) In vitro deacetylation assay was performed using acid extracted histones (2 μg, the substrate for SIRT6) and recombinant SIRT6 (1 μM) in the presence of either recombinant lamin A (0.5 μM) or recombinant progerin (0.5 μM). Western blotting was performed to examine the acetylation of H3K9 and H3K56. The quantification, all relative to control (i.e., histones only), represents mean ± SEM (n = 3). *p < 0.05.

See also Figures S2 and S6.

9 and 56 (Michishita et al., 2008, 2009; McCord et al., 2009). Hence, we decided to test whether lamin A could regulate SIRT6 deacetylase activity by analyzing the acetylation levels of H3K9 and H3K56 in HEK293 cells with ectopic expression of lamin A and SIRT6. As shown in Figures 3A and S6D, ectopic lamin A alone did not have any significant effect, possibly due to saturation of SIRT6 activity by the endogenous lamin A. As expected, the acetylation of histone H3 at lysine 9 and 56 was significantly reduced upon ectopic expression of SIRT6. Interestingly, a further reduction in H3K9 and H3K56 acetylation was observed in cells with both ectopic lamin A and ectopic SIRT6, as compared to that with ectopic SIRT6 alone (n = 3, *p < 0.05, **p < 0.01). As a negative control, we also examined the acetylation levels of histone H3 at lysine 18, which is not a target of SIRT6. No detectable changes in H3K18 acetylation were observed in cells either with ectopic SIRT6 alone or with SIRT6 and lamin A together (Figure S2A). To test if the enhanced histone deacetylation by lamin A is SIRT6 deacetylase specific, SIRT6 H133Y mutant was employed to see if the deacetylase inactivation would abolish the increased histone H3K9 deacetylation. As shown in Figure S2B, no significant change in H3K9 acetylation level was observed in cells with ectopic SIRT6 H133Y mutant in the presence or absence of ectopic lamin A. This suggests that the deacetylation of histones is SIRT6 specific and further reduction of acetylation in the presence of lamin A and SIRT6 is also SIRT6 specific. Loss of lamin A in MEF cells ($Lmna^{-/-}$ MEFs) also resulted in increased acetylation of H3K9 and H3K56, but not H3K18, in comparison with wild-type (WT) MEFs (Figure S2C), thus further advocating the essence of lamin A in SIRT6-mediated histone deacetylation. Since lamin A could affect the function of other sirtuins, we performed in vitro deacetylation assay using acid extracted histones, recombinant SIRT6 (rhSIRT6, 1 μM), and recombinant lamin A (rhlamin A, with varying concentrations of 0.2, 0.3, and 0.5 μM) proteins, to further examine whether lamin A-mediated enhancement in histone deacetylation is SIRT6 specific. As

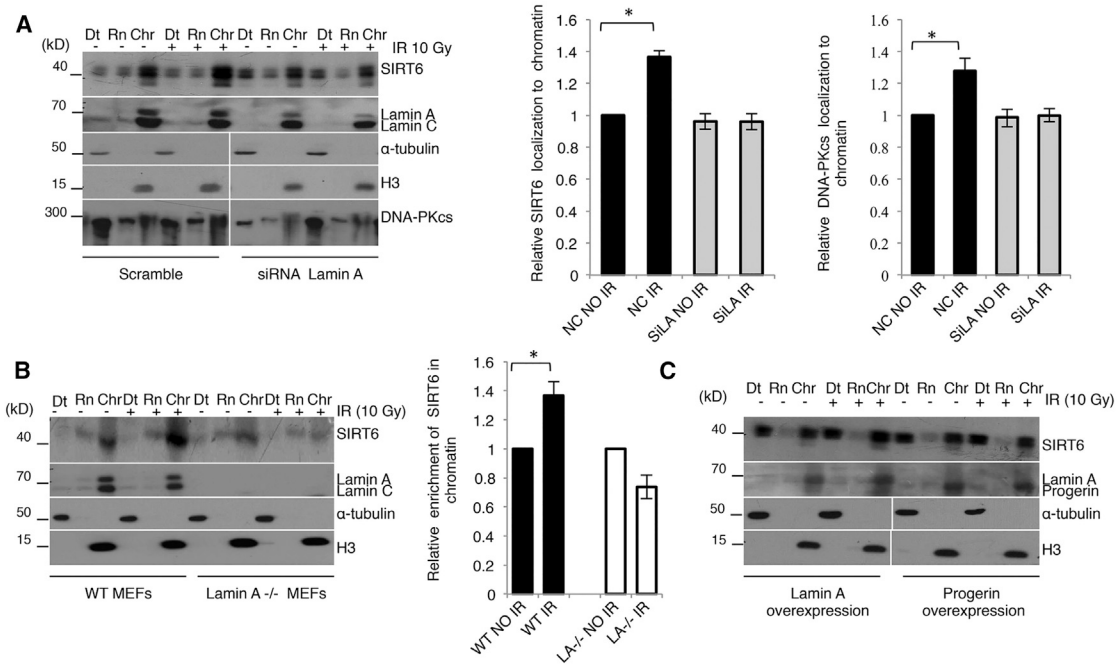

Figure 4. Lamin A Is Required for Efficient Chromatin Localization of SIRT6 upon DNA Damage

(A) HEK293 cells were transiently transfected with either siRNA against lamin A or scramble siRNA. Cell lysates were collected and fractionated as described in chromatin purification protocol (see Experimental Procedures) before and after 10 Gy gamma irradiation. SIRT6 level was determined by western blotting in different fractions. Dt, detergent-extractable portion; Rn, RNase-extractable portion; Chr, purified chromatin portion. Bar graphs show quantified data, with respect to corresponding non-irradiated controls, and represent mean ± SEM (n = 3) (SIRT6, uppermost panel; DNA-PKcs, lowermost panel). *p < 0.05. NC, NO IR, and siLA stand for negative control, no irradiation, and siRNA against lamin A, respectively.

(B) Wild-type (WT) mouse embryonic fibroblasts (MEFs) and *Lmna*$^{-/-}$ (LA−/−) MEFs were treated with 10 Gy gamma irradiation. Cell lysates were fractionated to determine Sirt6 levels. Dt, detergent-extractable portion; Rn, RNase-extractable portion; Chr, purified chromatin portion. Bar graphs show quantified data (SIRT6 for the uppermost panel), with respect to corresponding non-irradiated controls, representing mean ± SEM (n = 3). *p < 0.05.

(C) 10 μg of either lamin A or progerin was ectopically expressed in HEK293 cells with 10 Gy gamma irradiation. Cell lysates were fractionated to determine Sirt6 levels by western blotting. Dt, detergent-extractable portion; Rn, RNase-extractable portion; Chr, purified chromatin portion. Two independent experiments were performed and similar data were obtained.

See also Figure S2.

shown in Figure 3B, SIRT6-mediated H3K9 deacetylation could be enhanced by lamin A in vitro (three independent experiments, *p < 0.05), suggesting that lamin A could directly activate SIRT6 toward histone deacetylation. Although progerin exhibited stronger binding with SIRT6 (Figures 2C and S2D), it had a negligible effect on the deacetylase activity of SIRT6 on histones H3K9 and H3K56 after ectopic expression in HEK293 cells (Figure 3C). This observation was confirmed in the in vitro deacetylation assay, as evidenced by the comparable levels of both H3K9 and H3K56 acetylation in the presence and absence of progerin (Figure 3D) (data from three independent experiments; *p < 0.05). Enhanced binding but diminished activating effect of progerin further prompted us to investigate whether progerin could activate SIRT6 with increasing concentration. As shown in Figure S2E, increasing concentration of progerin (0.2 μM, 0.3 μM, and 0.5 μM) did not result in enhanced deacetylase activity of SIRT6 toward H3K9 in vitro. Taken together, these data indicate that while both lamin A and progerin interact directly with SIRT6, lamin A, but not progerin, is able to enhance SIRT6 deacetylase activity toward H3K9 and H3K56.

Lamin A Enhances Efficient Chromatin Localization of SIRT6 upon DNA Damage

It is reported that enhanced localization of SIRT6 to chromatin upon DNA damage facilitates DNA damage repair (DDR) signaling (McCord et al., 2009). However, the molecular mechanism regulating this localization remains largely unknown. To test if lamin A may play a role in the regulation of SIRT6 recruitment to chromatin upon DNA damage, we examined gamma irradiation (IR) induced-chromatin enrichment of SIRT6 in HEK293 cells where lamin A was knocked down by small interfering RNA (siRNA) (Figure S2F). We followed a stringent chromatin fractionation protocol to obtain purified chromatin-bound proteins as previously described by Drouet et al. (2005) and McCord et al. (2009) (as seen from the enrichment of histones only in the chromatin-bound fraction). The protocol allowed for extraction of detergent-extractable soluble nuclear and cytoplasmic fragment, followed by RNase-extractable fragment, and finally the chromatin-associated proteins. As shown in Figure 4A, the enrichment of SIRT6 on chromatin after DNA damage (3rd and 6th lanes) was abolished upon lamin A knockdown (9th and 12th lanes) (data from three independent experiments, *p < 0.05).

To exclude the possibility that compromised chromatin enrichment of SIRT6 upon IR in lamin A knockdown cells may be the result of an off-target effect, we examined SIRT6 recruitment to chromatin in *Lmna*$^{-/-}$ MEFs. As shown in Figure 4B, compared to WT MEFs, loss of lamin A significantly compromised the enrichment of Sirt6 to chromatin in response to DNA damage (data from three independent experiments, *$p < 0.05$). Intriguingly, SIRT6 localization to chromatin could be observed even in absence of lamin A, thus suggesting the presence of other regulatory factors that aid in the basal level of SIRT6 recruitment to chromatin. Notably, no obvious changes in the total SIRT6 levels were observed in either lamin A knockdown or lamin A-depleted cells as compared with controls (Figures S2F and S2G). In addition, no apparent induction in the total level of SIRT6 protein was observed 30 min postirradiation (Figures S2F and S2G). Similarly, neither ectopic lamin A nor ectopic progerin resulted in significant changes in the total level of SIRT6 (Figure S2H). However, HEK293 cells expressing ectopic progerin exhibited impaired SIRT6 enrichment in chromatin upon DNA damage, in comparison with cells expressing ectopic lamin A (Figures 4C and S2I). To further substantiate this observation, the recruitment of DNA-PKcs to chromatin upon DNA damage was examined, as SIRT6 on chromatin facilitates DNA-PKcs recruitment (McCord et al., 2009). As expected and consistent with defective SIRT6 enrichment, DNA-PKcs localization to chromatin upon irradiation was significantly compromised in HEK293 cells when lamin A was knocked down (Figure 4A) (data from three independent experiments, *$p < 0.05$). Unfortunately, the mobilization of DNA-PKcs to chromatin in *Lmna*$^{-/-}$ MEFs could not be detected due to the inability of the antibodies to recognize mouse DNA-PKcs. Taken together, these data suggest that efficient recruitment of SIRT6 onto chromatin upon DNA damage requires lamin A and loss of lamin A or the presence of progerin compromises the recruitment of SIRT6 to chromatin in response to DNA damage.

Lamin A Promotes SIRT6-Mediated DNA Repair

SIRT6 plays a vital role in DNA DSB repair (Etchegaray et al., 2013; Beauharnois et al., 2013). For example, SIRT6 mediates HR repair via NAD^+-dependent CtIP deacetylation (Kaidi et al., 2010). Our previous study showed that a progeroid mutation in the *LMNA* gene jeopardizes HR repair, which contributes to HGPS (Liu et al., 2005). Interestingly, loss of Sirt6 in mice results in defective DNA repair and accelerated aging similar to that in HGPS, suggesting a potential link between lamin A and SIRT6 in DNA repair and accelerated aging. Moreover, a recent study showed striking defects in HR and NHEJ repair pathways in cells lacking lamin A/C (Redwood et al., 2011). We therefore hypothesized that lamin A might play a role in promoting SIRT6-mediated DSB repair and the defects observed in cells lacking lamin A/C might result from dysregulated SIRT6 function. Thus, to investigate possible role of lamin A in SIRT6-mediated DNA repair, we examined whether lamin A is required for SIRT6-mediated deacetylation of CtIP upon DNA damage. As shown in Figure 5A, acetylated CtIP was decreased 10 min after DNA damage in WT MEFs, whereas this CtIP deacetylation immediately after DNA damage was not observed in MEFs lacking lamin A (data from three independent experiments, *$p < 0.05$). Ectopic lamin A in HEK293 cells further enhanced CtIP deacetylation upon DNA damage (Figure S3A). However, such an activating effect of lamin A overexpression was abolished after knocking down SIRT6 using small hairpin RNA (shRNA) (Figure S3A). Moreover, progerin overexpression attenuated the basal SIRT6-mediated CtIP deacetylation (Figure S3A), thus suggesting a possible dominant-negative role of progerin in regulating SIRT6 functions. Compared with WT MEFs, the increased association of CtIP with SIRT6 was significantly compromised in *Lmna*$^{-/-}$ MEFs 30 min after DNA damage (Figure 5B). This suggests that delayed SIRT6 recruitment to chromatin due to loss of lamin A leads to decreased CtIP recruitment and hence attenuated CtIP deacetylation. In line with this, reconstitution of lamin A in *Lmna*$^{-/-}$ MEFs enhanced CtIP deacetylation in *Lmna*$^{-/-}$ MEFs 30 min after IR, whereas progerin reconstitution in *Lmna*$^{-/-}$ MEFs exhibited no dramatic effect on CtIP deacetylation in response to IR (Figure 5C) (data from three independent experiments, *$p < 0.05$). Given SIRT6 is the only known deacetylase for CtIP (Kaidi et al., 2010), the observation that knocking down Sirt6 attenuated lamin A-mediated enhanced deacetylation of CtIP upon DNA damage (Figures S3A and S3B) further confirmed that lamin A-mediated enhancement of CtIP deacetylation is SIRT6 dependent. Again, ectopic expression of progerin did not result in further reduction of CtIP acetylation in *Lmna*$^{-/-}$ MEFs upon DNA damage (Figure S3B). These data therefore suggest that lamin A facilitates SIRT6-mediated CtIP deacetylation in response to DNA damage. On the other hand, progerin compromises SIRT6-mediated CtIP deacetylation upon DNA damage.

To further substantiate the finding that lamin A is an endogenous activator of SIRT6 and facilitates SIRT6-mediated DNA repair, we examined PARP1 mono-ADP ribosylation, a SIRT6-mediated event that has been rendered crucial for the NHEJ pathway of DSB repair (Mao et al., 2011). The mono-ADP ribosylation imparts PARP1 a slightly higher molecular weight detectable in SDS-PAGE (Mao et al., 2011). We exploited this property to investigate whether lamin A influences SIRT6-mediated PARP1 mono-ADP ribosylation. As shown in Figures 5D and S6E, the increase in SIRT6-mediated PARP1 mono-ADP ribosylation in WT MEFs upon DNA damage was abolished in *Lmna*$^{-/-}$ MEFs, as evidenced by the higher molecular weight of PARP1 in the SIRT6 immunoprecipitates than in the input (data from three independent experiments, *$p < 0.05$). Reconstitution with lamin A, but not progerin, into *Lmna*$^{-/-}$ MEFs restored SIRT6-mediated PARP1 mono-ADP ribosylation in response to DNA damage (Figure 5E). We also performed neutral comet assay (as previously described in Olive and Banáth, 2006) on WT and *Lmna*$^{-/-}$ (LA−/−) MEFs after SIRT6 overexpression to determine any possible effect on DNA DSB repair in response to DNA damage. As shown in Figures S3D and S3E, comet tail moments were significantly reduced in WT MEFs after SIRT6 overexpression upon IR (data from three independent experiments, *$p < 0.05$, **$p < 0.005$), thus indicating fewer DNA DSBs. However, this dramatic effect upon SIRT6 overexpression was severely compromised in *Lmna*$^{-/-}$ MEFs, thus suggesting that SIRT6-mediated DNA damage repair is regulated by lamin A (Figures S3D and S3E). Taken together, these findings signify lamin A as an endogenous activator of SIRT6, regulating both deacetylation

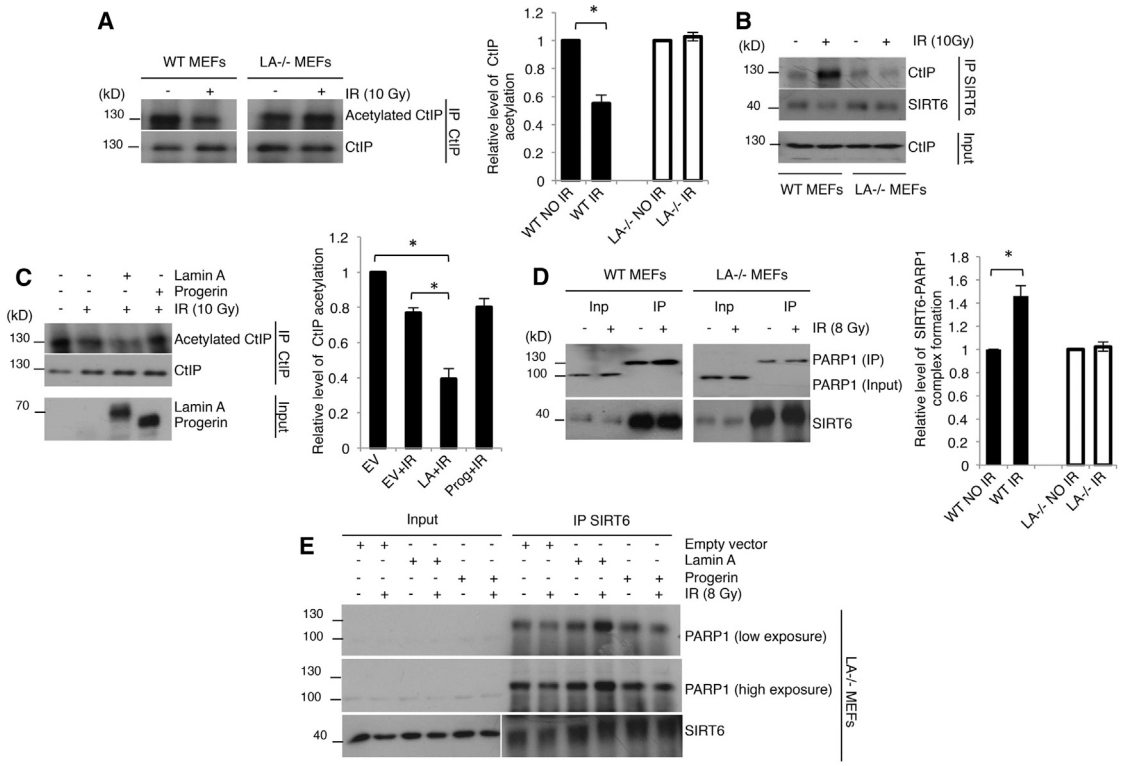

Figure 5. Lamin A Promotes SIRT6-Mediated DNA Repair

(A) Acetylation of CtIP in response to 10 Gy gamma irradiation in wild-type (WT) and *Lmna*$^{-/-}$ (LA−/−) MEFs. CtIP was immunoprecipitated before and after irradiation. Total and acetylated CtIP were determined by western blotting. Graph shows quantified data, relative to corresponding non-irradiated control, representing mean ± SEM (n = 3). *p < 0.05.

(B) Endogenous CtIP was co-immunoprecipitated using SIRT6 antibodies in wild-type and *Lmna*$^{-/-}$ MEFs 30 min post gamma irradiation (10 Gy). Western blotting shows levels of CtIP pulled down by SIRT6.

(C) *Lmna*$^{-/-}$ MEFs were reconstituted with either lamin A or progerin to determine the acetylation of endogenous CtIP in response to DNA damage (10 Gy gamma irradiation). Cell extracts were collected 30 min postirradiation. Western blotting was performed with total cell lysates and immunoprecipitated CtIP. Graph shows quantified data, relative to empty vector transfected non-irradiated control, representing mean ± SEM (n = 3). *p < 0.05.

(D) Western blotting to determine SIRT6-mediated PARP1 mono-ADP ribosylation. PARP1 was pulled down by SIRT6 antibodies to determine PARP1 mono-ADP ribosylation in response to 8 Gy gamma irradiation. Western blotting shows PARP1 protein levels in the input and immunoprecipitated samples in wild-type and *Lmna*$^{-/-}$ MEFs before and after irradiation. Note that PARP1 in the co-immunoprecipitated sample is mono-ADP ribosylated. Graph shows quantified data with respect to corresponding non-irradiated controls, and data represent mean ± SEM (n = 3). *p < 0.05.

(E) *Lmna*$^{-/-}$ MEFs were reconstituted with either lamin A or progerin to analyze SIRT6-mediated PARP1 mono-ADP ribosylation upon DNA damage (8 Gy gamma irradiation). Cell lysates were collected 2 hr after irradiation, immunoprecipitated with SIRT6 antibodies, and subjected to western blotting to determine mono-ADP-ribosylated PARP1 levels in IP samples.

See also Figures S3 and S6.

and mono-ADP ribosylation activities of SIRT6, thus facilitating SIRT6-mediated DNA DSB repair upon DNA damage.

Since SIRT6 could be regulated by lamin A, we investigated if lamin A was also a substrate of SIRT6 for deacetylation or mono-ADP ribosylation. To address this, we immunoprecipitated lamin A from *sirt6*$^{+/+}$ and *sirt6*$^{-/-}$ MEFs and performed western blotting using antibodies against acetylated lysine residues. As shown in Figure S3F, lamin A was not detected by antibodies against acetylated lysine. We also performed in vitro deacetylation assay using recombinant proteins (rhSIRT6 and rhlamin A) but could not detect any acetylation of lamin A (Figure S3G). In addition, we performed in vitro mono-ADP ribosylation assay to test if lamin A could be ADP ribosylated by SIRT6. We employed rhSIRT6 and rhlamin A in the assay and included rhPARP1 as a positive control. As shown in Figure S3H, PARP1, but not lamin A, was mono-ADP ribosylated by SIRT6 as determined by SIRT6-mediated transfer of radiolabeled ADP-ribose moieties to PARP1. These data suggest that lamin A is unlikely to be a substrate of SIRT6.

As progerin is attached to the inner nuclear membrane (INM) and is responsible for disruption of lamin-heterochromatin binding (Goldman et al., 2004; McCord et al., 2013), we hypothesized that increased interaction between progerin and SIRT6 may restrict the access and availability of SIRT6 to the chromatin, where it plays crucial roles in response to DNA damage. To test this, we fractionated HEK293 cells after ectopic expression of lamin A or progerin and analyzed the localization of SIRT6 in different subcellular fractions. As shown in Figure S3C, progerin

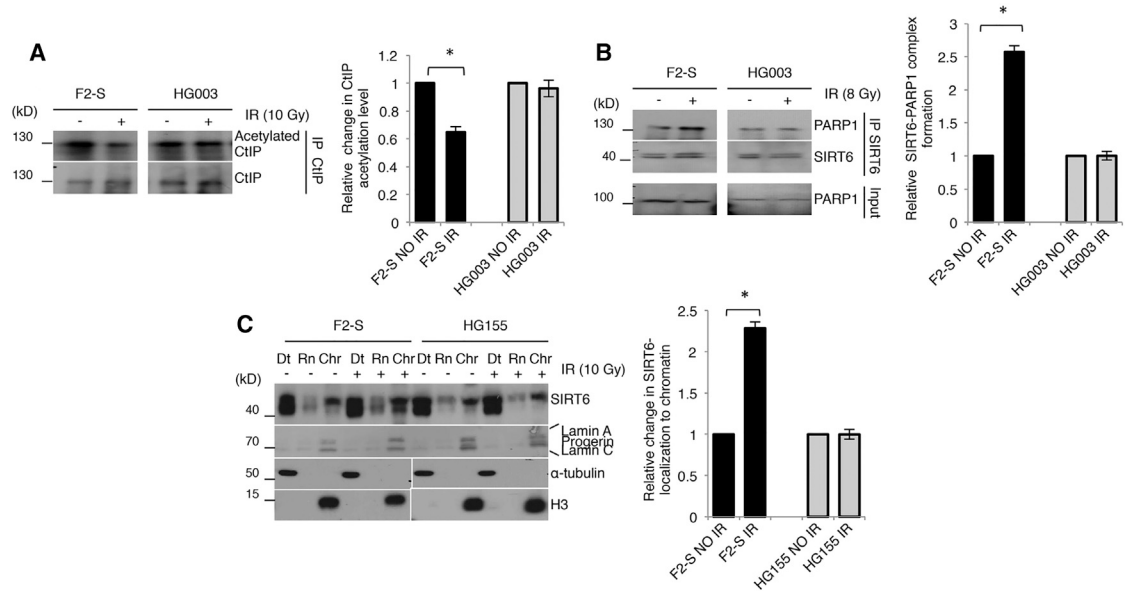

Figure 6. Defective SIRT6 Functions in HGPS

(A) Acetylation of CtIP in normal human dermal fibroblasts (F2-S) and fibroblasts derived from HGPS patients (HG003) in response to DNA damage (10 Gy gamma irradiation) analyzed by western blotting. Immunoprecipitated CtIP was probed with anti-acetyl lysine antibodies. Quantified data represent mean ± SEM (n = 3) relative to corresponding non-irradiated controls. *p < 0.05.

(B) Levels of SIRT6-mediated PARP1 mono-ADP ribosylation in F2-S and HG003 fibroblast cells in response to DNA damage (8 Gy gamma irradiation). Endogenous SIRT6 was immunoprecipitated by specific antibodies and SIRT6 interacting mono-ADP ribosylated PARP1 was detected by western blotting. Quantified data represent mean ± SEM (n = 3). *p < 0.05 relative to corresponding non-irradiated controls.

(C) Localization of SIRT6 on chromatin in F2-S fibroblasts and HGPS fibroblasts upon DNA damage (10 Gy gamma irradiation). Cell lysates were fractionated to determine SIRT6 levels by western blotting. Dt, detergent-extractable portion; Rn, RNase-extractable portion; Chr, purified chromatin portion. Bar graph shows quantified data of SIRT6 relative to corresponding non-irradiated controls, representing mean ± SEM (n = 3). *p < 0.05.

See also Figures S4–S6.

overexpression resulted in increased SIRT6 retention in the pellet fraction (depicted by PE fraction) and reduced localization at the chromatin fraction (depicted as ChrE fraction) as compared to lamin A-overexpressing cells.

Defective SIRT6 Functions in HGPS

To further address the importance of lamin A as an endogenous activator of SIRT6, we investigated whether the presence of progerin impacts SIRT6 functions in HGPS. Since HGPS fibroblasts exhibit defective DNA damage repair (Liu et al., 2005), we hypothesized that such defect might be resulted from dysfunctional SIRT6-mediated CtIP deacetylation or PARP1 mono-ADP ribosylation. To test this, we examined and compared these functions of SIRT6 between HGPS dermal fibroblasts and normal dermal fibroblasts, i.e., F2-S cells. Several lines of HGPS fibroblasts were used for experimentation (Table S1). As shown in Figure 6A, we observed a significant impairment of CtIP deacetylation upon DNA damage in HGPS fibroblasts as compared to F2-S cells (data from three independent experiments, *p < 0.05). Similarly, impairment in CtIP deacetylation upon DNA damage was observed in three other HGPS fibroblast cell lines (Figure S4A). The increased SIRT6-PARP1 complex formation in response to DNA damage was also abolished in the HGPS fibroblasts (Figures 6B and S4B). In addition, the increased chromatin localization of SIRT6 upon DNA damage was hampered in HGPS fibroblasts (Figures 6C, S4C, and S6F), as compared with F2-S controls (data from three independent experiments, *p < 0.05). Consistent with these, delayed localization of SIRT6 to DNA damage foci (ɣ-H2AX foci) was observed in HGPS cells as compared to that in WT controls (Figure S4D). Taken together, the data observed in HGPS cells clearly showed defects in a multitude of SIRT6 activities that are considered crucial in DNA DSB repair through HR and NHEJ pathways. These findings suggest that impaired SIRT6 functions contribute to defective genomic maintenance and accelerated aging in HGPS.

Since functioning of SIRT6 was found to be impaired in the HGPS cells, we tested whether overexpression of SIRT6 could rescue the abnormalities found in HGPS cells, such as distorted nuclear morphology and impaired 53BP1 foci formation (Liu et al., 2005). Overexpression of SIRT6 in two independent HGPS cell lines did not result in any significant rescue of cellular morphology (Figure S5A). Also, ectopic expression of SIRT6 was unable to rescue the impaired foci formation of 53BP1 in the HGPS cells, both before and after gamma IR-induced DNA damage (Figure S5B).

DISCUSSION

With the emerging functions of SIRT6 in a range of biological processes, there is increased interest in identifying its modulators that could be employed in developing effective therapeutics

(Beauharnois et al., 2013). Although several novel inhibitors of SIRT6 have been identified (Parenti et al., 2014), potential endogenous activators of this histone deacetylase have not been reported so far. A recent study claimed that long-chain fatty acids stimulate SIRT6 activity (Feldman et al., 2013). However, no data have been shown for any endogenous substrates. Here, we have identified lamin A as a direct interacting partner and an endogenous activator of SIRT6. Although progerin exhibited seemingly stronger interaction with SIRT6, it did not result in significant enhancement of SIRT6 activities. On the contrary, the presence of progerin compromised SIRT6 chromatin localization and SIRT6-dependent downstream events upon DNA damage. This phenomenon is reminiscent of DBC1's strong interaction with SIRT1 only to inhibit its downstream functioning (Kim et al., 2008). In line with the increased binding between progerin and SIRT6, it is plausible that the observed tethering of SIRT6 to nuclear skeleton by progerin jeopardizes SIRT6 mobilization to chromatin upon DNA damage. In addition, the binding may mask the enzyme from its substrates or alter its conformation in a way that the enzymatic activities are compromised. Alternatively, progerin's interaction with SIRT6 might facilitate other protein complexes to bind to SIRT6 and impair its activity.

Sirt6-deficient mice exhibit severe defects such as extensive lymphopenia, loss of subcutaneous fat, muscular dystrophy, and genomic instability. However, no nuclear architectural defects have been reported in the mutant MEF cells (Mostoslavsky et al., 2006). Interestingly, several of those defects have been also observed in lamin A-deficient mice. For example, *Lmna*$^{-/-}$ mice have smaller lymphoid organs and exhibit serious defects in lymphocyte development (Hale et al., 2010). Additionally, dystrophy of muscular fat and loss of white adipose tissues have also been reported in the *Lmna*$^{-/-}$ mice (Sullivan et al., 1999). Above all, both *Sirt6*$^{-/-}$ and *Lmna*$^{-/-}$ mice develop normally at birth, display growth retardation at ~2–3 weeks of age, and exhibit progeroid phenotypes (Mostoslavsky et al., 2006; Sullivan et al., 1999). Reduced lamin A expression has been associated with human ovarian cancer (Zhou et al., 2014) and breast cancer (Wazir et al., 2013), whereas loss of SIRT6 was reported to induce tumorigenesis (Sebastián et al., 2012). Thus, these observations also suggest a potential functional link between lamin A and SIRT6.

DSBs are one of the most critical DNA lesions, and SIRT6 is one of the earliest factors to localize to the damage foci and regulate the repair process (Liu et al., 2014; Toiber et al., 2013). SIRT6 is a weak deacetylase, and its association with nucleosomes considerably decreases the levels of H3K9 and H3K56 acetylation (Gil et al., 2013; Pan et al., 2011; Liszt et al., 2005). In this study, we have provided evidence that lamin A activates SIRT6 toward a variety of its substrates. However, we did not observe any posttranslational modification of lamin A mediated by SIRT6. Lamin A-dependent SIRT6 enrichment on chromatin upon DNA damage provides a mechanistic explanation for similar defects observed in DNA repair in SIRT6-deficient cells and HGPS cells. It is reported that HGPS patient-derived cells contain both lamin A and progerin, but progerin exhibits a dominant-negative effect resulting in impaired genomic stability (Goldman et al., 2004; McCord et al., 2013). Indeed, ectopic SIRT6 did not significantly restore nuclear architecture or DNA-damage repair in current study. The existence of progerin also jeopardizes the functioning of SIRT6 toward DNA damage repair. Hence, overexpression of SIRT6 in the presence of progerin was unable to significantly rescue the nuclear architectural abnormalities in HGPS cells. However, we cannot exclude the possibility that SIRT6-mediated DNA-damage repair may be partially rescued in HGPS cells if much higher level of ectopic SIRT6 is present.

Our data support a model wherein lamin A promotes recruitment of SIRT6 to the chromatin in response to DNA damage and facilitates SIRT6-dictated downstream events critical for DNA damage repair. It is plausible that lamin A helps SIRT6 to target its specific catalytic activities to different substrates on chromatin. On the other hand, the inability of progerin to augment chromatin localization of SIRT6 and its downstream functioning upon DNA damage provides mechanistic explanation not only for the compromised genomic maintenance in HGPS but also for the similar phenotypes observed in laminopathy-based premature aging and in *Sirt6*-deficient mice. Hence, defective SIRT6 localization to chromatin contributes to the delayed recruitment of checkpoint response/damage repair proteins in HGPS cells in response to DNA damage, leading to defective DSB repair pathways, thus partly explaining the genomic instability observed in the HGPS cells.

Given the momentous roles of SIRT6 in a multitude of biological processes like cell proliferation, genome maintenance, and transcriptional regulation, identification of lamin A as its endogenous activator can have a profound impact on our understanding of the importance of lamin A-mediated regulation of SIRT6 activities in a variety of physiological and pathological processes, including metabolic diseases and tumorigenesis. In addition, it provides prospective research directions in elucidating molecular mechanisms underlying laminopathy-based premature aging and in developing effective therapeutic strategy for HGPS.

EXPERIMENTAL PROCEDURES

Cell Lines, Constructs, Recombinant Proteins, Reagents, and Antibodies

Information regarding cell lines, constructs, recombinant proteins and antibodies can be found in Supplemental Experimental Procedures.

Whole-Cell Lysate Collection, Western Blotting, and Co-immunoprecipitation

Detailed procedures used for transfection, whole-cell lysate extraction, western blotting, and co-immunoprecipitation can be found in the Supplemental Experimental Procedures.

Immunofluorescence Staining

Detailed procedures used for immunofluorescence staining can be found in Supplemental Experimental Procedures.

Acid Extraction of Histones

Histones were extracted with 0.2 N HCl using the protocol from Abcam. Briefly, cells were harvested and washed with PBS containing sodium butyrate. Cells were then resuspended in Triton extraction buffer (PBS supplemented with 0.5% Triton X-100 [v/v], 2 mM PMSF, 0.02% NaN_3 [v/v]) and lysed on ice for 10 min followed by centrifugation at 4°C. Cells were washed again with TEB (Triton extraction buffer) and centrifuged, and the pellet was resuspended in 0.2 N HCl followed by overnight acid extraction at 4°C. Samples were

centrifuged for 10 min at 4°C, and supernatant containing the extracted histones was removed and stored at −80°C as aliquots.

Cellular Fractionation and Chromatin Purification

Cellular fractionation and chromatin purification were performed as described in Drouet et al. (2005) and McCord et al. (2009) to obtain stringent chromatin extraction. Detailed procedures are provided in Supplemental Experimental Procedures.

In Vitro SIRT6 Deacetylation Assay

SIRT6 deacetylase activity was assayed on its endogenous substrate (histone H3) using the assay buffer as described in the SIRT6 direct fluorescent screening assay kit (Cayman Chemical). Purified acetyl histones were incubated with recombinant human SIRT6 (rhSIRT6) and NAD^+ for 45 min at 37°C, in the presence or absence of recombinant lamin A (rhlamin A), recombinant progerin (rhprogerin), or nicotinamide. Acetylation levels of histone H3 at lysine 9 and 56 were detected using antibodies against H3K9Ac (Millipore) and H3K56Ac (Upstate).

Comet Assay

The neutral comet assay was performed as previously described in Olive and Banáth (2006). Detailed procedures are provided in Supplemental Experimental Procedures.

Statistical Analysis

Statistical significance was analyzed using two-tailed t tests with error bars representing SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.10.006.

AUTHOR CONTRIBUTIONS

S.G. and Z.Z. designed the experiments. S.G. performed the experiments. Y.W. and Q.H. provided the human SIRT6 recombinant protein. S.G., B.H., and Z.Z. analyzed the data. S.G. and Z.Z. wrote the manuscript.

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