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## SIRT6 promotes DNA repair under stress by activating PARP1

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### Abstract

SIRT6 is a mammalian homolog of the yeast Sir2 deacetylase that promotes longevity in yeast and invertebrates. Mice deficient for SIRT6 exhibit premature aging and genome instability. Here we show that in mammalian cells subjected to oxidative stress SIRT6 is recruited to the sites of DNA double-strand breaks (DSBs) and strongly stimulates both pathways of DSB repair, nonhomologous end joining and homologous recombination. We found that SIRT6 physically associates with PARP1 leading to stimulation of PARP1 poly-ADP-ribose polymerase activity. Mono-ADP-ribosylation activity of SIRT6 is sufficient for the activation of PARP1 *in vitro*, while both mono-ADP-ribosylation and deacetylation activities are required for the stimulation of DSB repair *in vivo*. Our results suggest that SIRT6 mono-ADP-ribosylates PARP1 on lysine 521 thereby stimulating PARP1 activity and enhancing DSB repair under oxidative stress. We propose that SIRT6 functions as a regulator integrating oxidative stress signaling and DNA damage response.

Silent chromatin regulator 2 (Sir2) is a histone deacetylase proposed to regulate yeast lifespan by suppressing the formation of ribosomal DNA circles (1) and stabilizing telomeric chromatin (2, 3). Sir2 has also been implicated in DNA repair (4) and caloric restriction (5). Sir2 is activated by changes in NAD<sup>+</sup>/NADH ratio, which may be triggered by oxidative stress, caloric restriction, and other adverse conditions. Sir2 has seven mammalian homologs known as Sirtuin (SIRT) proteins. Sirtuins function in multiple pathways, which are important for longevity, including stress response and genome maintenance. SIRT1 is activated in response to stress such as growth factor deprivation, heat shock, hypoxia, and oxidative stress (6–8). Furthermore, SIRT1 and SIRT6 deficient cells have a reduced ability to repair DNA double-stranded breaks (DSB) (9, 10) and SIRT6 knockout mice exhibit a premature aging phenotype associated with impaired base excision repair (BER) (11).

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### Supporting Online Material

Materials and Methods

Figures S1–S10

**Author Contributions** Z.M. performed DNA repair assays, Western blots, Co-IPs, *in situ* staining and microscopy, and *in vitro* assays of SIRT6 and PARP1 activities; C.H. performed ChIP assays; X.T. performed analysis of PARP1 mutants; M.V. analyzed the DNA damage foci; A.V. contributed to the ChIP assays; M.A. performed comet assays; Z.M., C.H., A.S., and V.G. designed the study, analyzed data and wrote the manuscript.

Furthermore, SIRT6 participates in HR by deacetylating CtIP (12). Repair of DNA DSBs is essential for longevity, and mutations in DSB repair genes lead to premature aging phenotypes (13). Due to the involvement of sirtuins in stress response and DNA repair we hypothesized that members of the Sir2 family may promote longevity by integrating stress signaling and DNA DSBs repair pathways.

To test this hypothesis we systematically examined the ability of nuclear localized mammalian SIRT proteins to promote repair of DNA DSBs under stress. For analysis of DSB repair we used two hTERT-immortalized, diploid human fibroblast cell lines containing chromosomally integrated GFP-based reporter constructs (14), which allow for the separate analysis of the two pathways of DSB repair, homologous recombination (HR) and nonhomologous end joining (NHEJ) (Supplementary Figure 1). A site-specific DNA break in these cell lines is induced by transient transfection with a plasmid encoding I-SceI endonuclease. In the NHEJ cell line the functional GFP gene is reconstituted upon a successful NHEJ event, and in the HR cell line GFP is reconstituted upon a successful repair by gene conversion, a predominant HR pathway in human cells. We overexpressed the four nuclear-localized human sirtuins SIRT1, SIRT2, SIRT6, or SIRT7 in the reporter cell lines (Figure 1A) and measured the efficiency of DSB repair. Overexpression of SIRT1 and SIRT2 had no effect on DSB repair, while overexpression of SIRT6 improved the efficiency of NHEJ by 3.3-fold and HR by 3.4-fold (Figure 1B). SIRT7 overexpression increased the efficiency of NHEJ by 1.5-fold and HR by 2.8-fold (Figure 1B). We next pre-treated the cells with paraquat prior to induction of DSBs to test the effect of oxidative stress in the DNA repair assay. The effects of SIRT1, SIRT2, and SIRT7 overexpression on DSB repair after oxidative stress were not significant (Figure 1B). In contrast, SIRT6 overexpression under stress led to a striking stimulation of both pathways of DSB repair. NHEJ was increased 6.7-fold, and HR was increased 6-fold relative to control paraquat-treated cells or 16-fold relative to untreated controls (Figure 1B). Addition of a sirtuin inhibitor, nicotinamide, which inhibits both deacetylase and mono-ADP-ribosylase activities, removed the stimulatory effect of SIRT6 overexpression (Figure 1B). We also observed stimulation of DSB repair by SIRT6 when a different inducer of oxidative stress, H<sub>2</sub>O<sub>2</sub> was used (Supplementary Figure 2). Furthermore, overexpression of SIRT6 accelerated clearance of  $\gamma$ H2AX foci in paraquat treated cells (Figure 1C), and accelerated repair of DSBs induced by neocarzinostatin, measured by neutral comet assay (Supplementary Figure 3). Oxidative stress strongly elevated endogenous SIRT6 levels (Figure 1D), indicating that ectopic overexpression enhances this physiological effect. These experiments show that increased levels of SIRT6 strongly stimulate DNA repair, especially in cells under stress.

We then tested whether depletion of SIRT6 compromises DNA DSB repair. SIRT6<sup>-/-</sup> MEFs had 2.6-fold lower NHEJ efficiency than the wild type MEFs (Supplementary Figure 4B). This difference increased to 4.3-fold when MEFs were pretreated with paraquat. Since the frequency of HR in MEFs was extremely low, we tested HR efficiency in human fibroblasts transfected with siRNA to SIRT6. SIRT6 depletion resulted in a 2.2-fold reduction in HR efficiency after paraquat treatment (Supplementary Figure 4C, D). Together these observations suggest that SIRT6 plays an important regulatory role in DNA damage response by stimulating DSB repair under oxidative stress.

In addition, we found that SIRT6 overexpression led to faster recruitment of 53BP1 to DNA damage sites after paraquat treatment (Supplementary Figure 5A) and enhanced the recruitment of NBS1 to DNA breaks (Supplementary Figure 5B). 53BP1 and NBS1 are involved in the early stages of DSB processing and the observed SIRT6-mediated changes may promote both NHEJ and HR. These experiments provide further evidence that SIRT6 regulates DSB repair.

To understand the mechanism by which SIRT6 stimulates DNA repair after oxidative stress we examined changes in SIRT6 intracellular localization upon DNA damage. SIRT6 is a chromatin-associated protein (11, 15) and has been shown to bind to sites of DNA DSBs (10). Our *in situ* analysis of SIRT6 distribution in untreated cells showed distinct aggregates within the nucleus that co-localized with heterochromatin protein HP1 $\beta$  (Figure 2A). However, we were unable to detect quantitative changes in SIRT6 distribution after DNA damage by *in situ* analysis. We therefore analyzed the kinetics of SIRT6 recruitment to damaged DNA after oxidative stress by chromatin immunoprecipitation (ChIP). SIRT6 was recruited to Alu elements after  $\gamma$ -irradiation (Figure 2B) and to the site-specific DSB generated by I-SceI (Figure 2C, and Supplementary Figure 6). In the absence of oxidative stress SIRT6 was recruited after 8 and 10 hours of  $\gamma$ -irradiation and I-SceI induced breaks respectively (the delay with I-SceI transfection (Figure 2C) is due to the time required for I-SceI expression). When the cells were pretreated with paraquat, we observed an additional, early wave of SIRT6 recruitment, within 30 min after induction of DSBs. Thus, we show that under normal conditions SIRT6 is recruited to DSBs relatively late, whereas preexisting oxidative stress results in early mobilization of SIRT6.

Sir2 family members have two enzymatic activities, deacetylase and mono-ADP-ribosyltransferase (16–18). SIRT6 has been shown to deacetylate histone H3 K9 and K56 residues (19–21), and CtIP (12). To determine which enzymatic activity, deacetylase or mono-ADP-ribosyltransferase, is important for the observed stimulation of DSB repair, we introduced point mutations in conserved amino acids of SIRT6. Three mutants were analyzed: S56Y lacking both histone deacetylase and mono-ADP-ribosyltransferase activities; G60A lacking mono-ADP-ribosyltransferase activity, and R65A lacking deacetylase activity (Figure 3A, B). All three mutations reduced the ability of SIRT6 to stimulate DSB repair suggesting that both activities are important for this function (Figure 3C). Histone deacetylase activity of SIRT6 has been intensely investigated (19–21) and changes in chromatin structure produced by histone deacetylation are likely to be important for promotion of both pathways of DNA repair, furthermore deacetylation of CtIP is important for HR (12). In contrast, the *in vivo* targets of mono-ADP-ribosyltransferase activity of SIRT6 are unknown, and the function of this activity has received little attention.

We next set out to identify the targets of SIRT6 mono-ADP-ribosyltransferase activity. *In vitro* SIRT6 did not show substrate specificity, and mono-ADP-ribosylated a variety of proteins including BSA and GST. Therefore, we developed an *in vivo* approach to identify the substrate of SIRT6 mono-ADP-ribosylation. We introduced biotin-labeled NAD, a substrate for mono-ADP-ribosyltransferase, into the wild type and SIRT6 knockout MEFs, which were pretreated with paraquat. Poly-ADP-ribosylated proteins were depleted from the cell extracts using anti-poly-ADP-ribose antibodies. The remaining mono-ADP-ribosylated



To confirm the role of SIRT6-mediated PARP1 activation in stimulation of DSB repair we measured the efficiency of NHEJ and HR after SIRT6 overexpression in the presence of the PARP1 inhibitors 3-ABA or PJ34. PARP1 inhibitors suppressed SIRT6-mediated activation of DSB repair (Figure 4F). From this result we conclude that SIRT6 promotes DSB repair by stimulating PARP1. Multiple evidence (28, 29) support the role of PARP1 in DSB repair and suppressing aberrant recombination events by stabilizing broken DNA ends (30, 31). Furthermore, PARP1 is required for alternative, DNA-PK<sub>cs</sub> independent, pathway of NHEJ (32, 33). Overexpression of SIRT6 in DNA-PK<sub>cs</sub> null MEFs upregulated NHEJ 1.7-fold, and in the wild type MEFs by 2.3-fold (Supplementary Figure 10), suggesting that SIRT6 stimulates alternative NHEJ pathway by activating PARP1.

Although SIRT6, like other sirtuins (18, 34), is best known as protein deacetylase, it was first described as ADP-ribosyltransferase (35) and only later found to have histone deacetylase activity (19–21). It was hypothesized that mono-ADP-ribosylase activity of sirtuins is important for DNA repair while deacetylase activity is important for gene silencing (16). However, *in vivo* targets of sirtuin mono-ADP-ribosylation activity remained unknown. In this study we identify PARP1 as the first *in vivo* target of SIRT6 ribosylation.

As PARP1 is involved in both BER and DSB repair (22, 23), the role of SIRT6 as an activator of PARP1 explains the phenotype of the SIRT6 knockout mice, which are characterized by deficient BER and genomic instability likely stemming from a defect in DSB repair (11). Importantly, in the absence of oxidative stress SIRT6 overexpression mildly induced repair, while under stress DNA repair was stimulated many fold. This observation suggests that SIRT6 plays a regulatory function in DNA repair by integrating DNA repair and stress signaling pathways. The theory of hormesis proposes that mild doses of stress may have beneficial effects on the organism by stimulating stress and survival pathways. This theory has been explored extensively by biogerontologists, and was also used to explain life extending effects of food restriction (36). We hypothesize that SIRT6 serves as a mediator of hormetic response, promoting longevity by stimulating DNA repair under stressful conditions. Furthermore, our finding that DSB repair can be improved or hindered by adjusting SIRT6 levels opens a possibility of using SIRT6 as a drug target for cancer therapy or possibly for life extension.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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**One sentence summary**

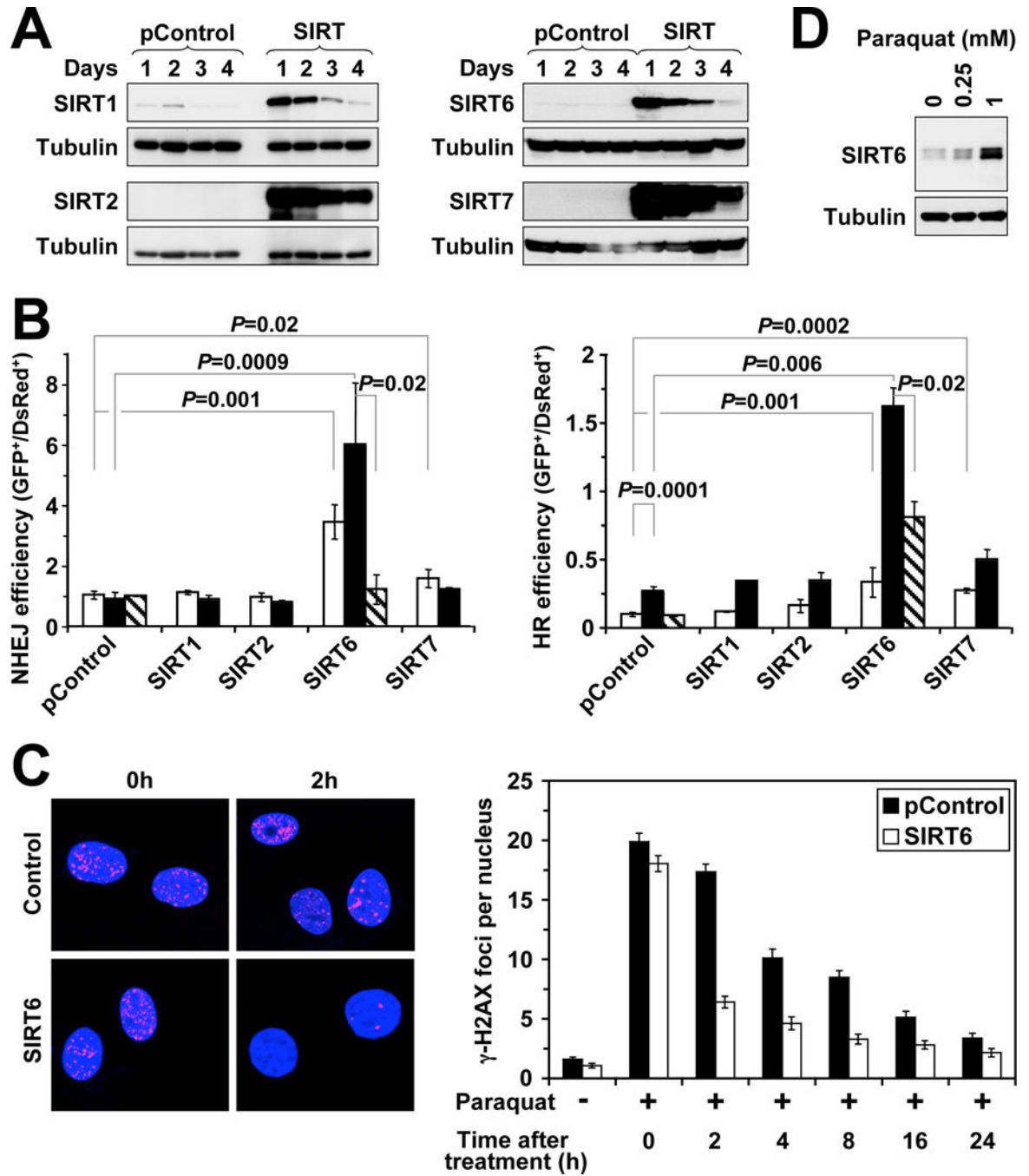
SIRT6 promotes double-strand break repair by stimulating PARP1 poly-ADP-ribose polymerase activity.

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**Figure 1. SIRT6 stimulates DSB repair**

**A**, Overexpression of SIRT1, 2, 6, and 7 in human fibroblasts. Western blot with sirtuin-specific antibodies after transfection with a control vector encoding HPRT mini gene (pControl) or SIRT-expressing vector. **B**, Effect of sirtuin overexpression on the efficiency of NHEJ and HR. Human fibroblasts containing chromosomally integrated reporter cassettes (Supplementary Figure 1) were co-transfected with I-SceI endonuclease to induce DSBs, SIRT expression vectors or pControl, and DsRedN-1 plasmid as transfection control. Prior to transfection the cells were untreated (open bars) or treated with 1 mM paraquat (filled bars)



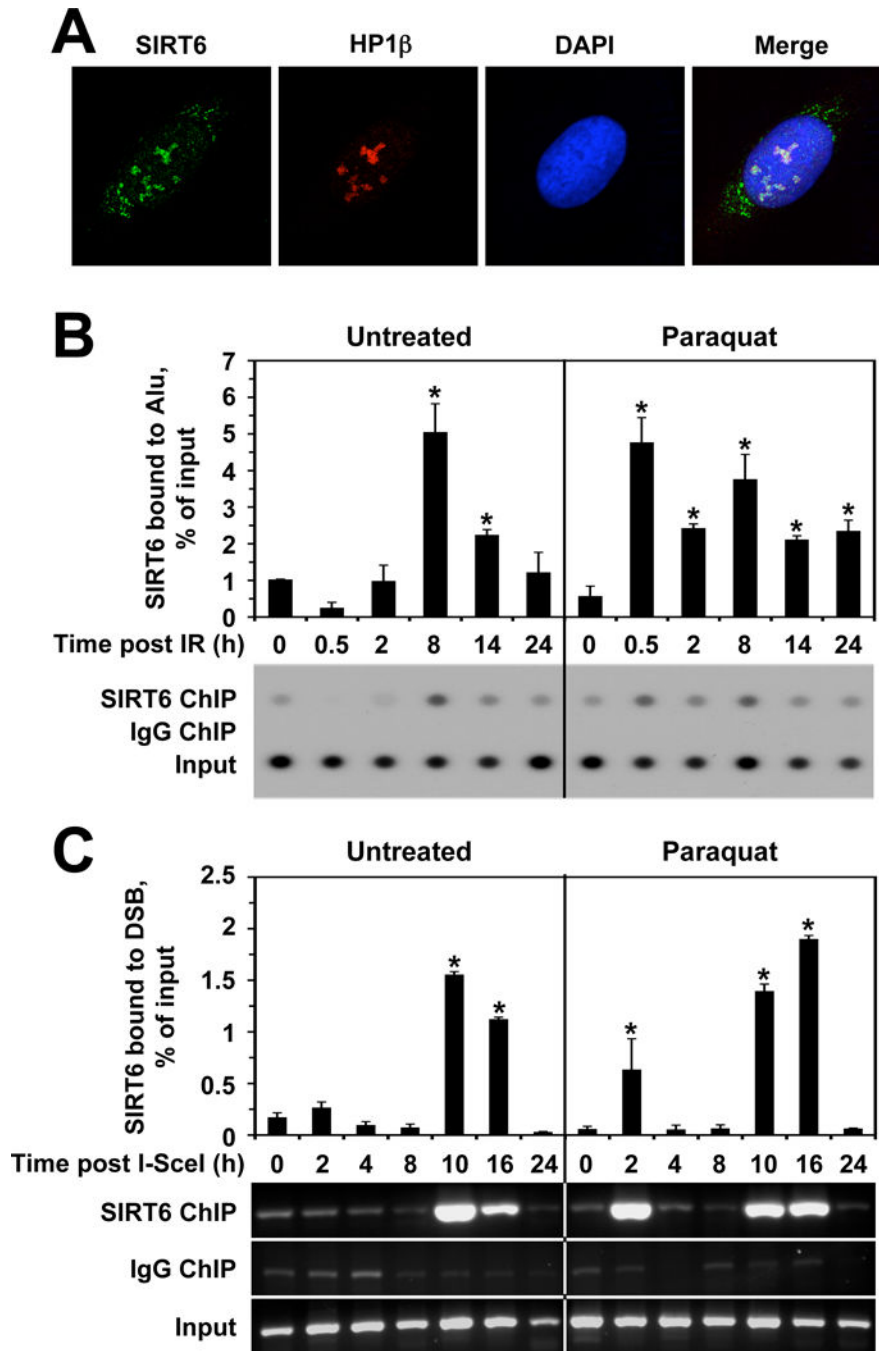
or paraquat plus 5 mM nicotinamide (striped bars) for 16 h. Values represent the ratio between the numbers of GFP+ cells corresponding to successful repair events to the DsRed+ transfection controls. Error bars indicate s.d.; n=8 (control and SIRT6); n=3 (other sirtuins). For some of the treatments error bars are too small to be visible. *P*-values were calculated by two-tailed Student's *t*-test. **C**, SIRT6 overexpression accelerates disappearance of  $\gamma$ H2AX foci after paraquat treatment. Cells were treated with 1 mM paraquat for 16h. After removal of paraquat cells were stained for  $\gamma$ H2AX foci at indicated time points. Data represents an average of at least 50 nuclei. Error bars indicate s.e.m. For some of the treatments error bars are too small to be visible. **D**, Induction of endogenous SIRT6 protein levels by oxidative stress. Human fibroblasts were treated with indicated doses of paraquat for 16 h.

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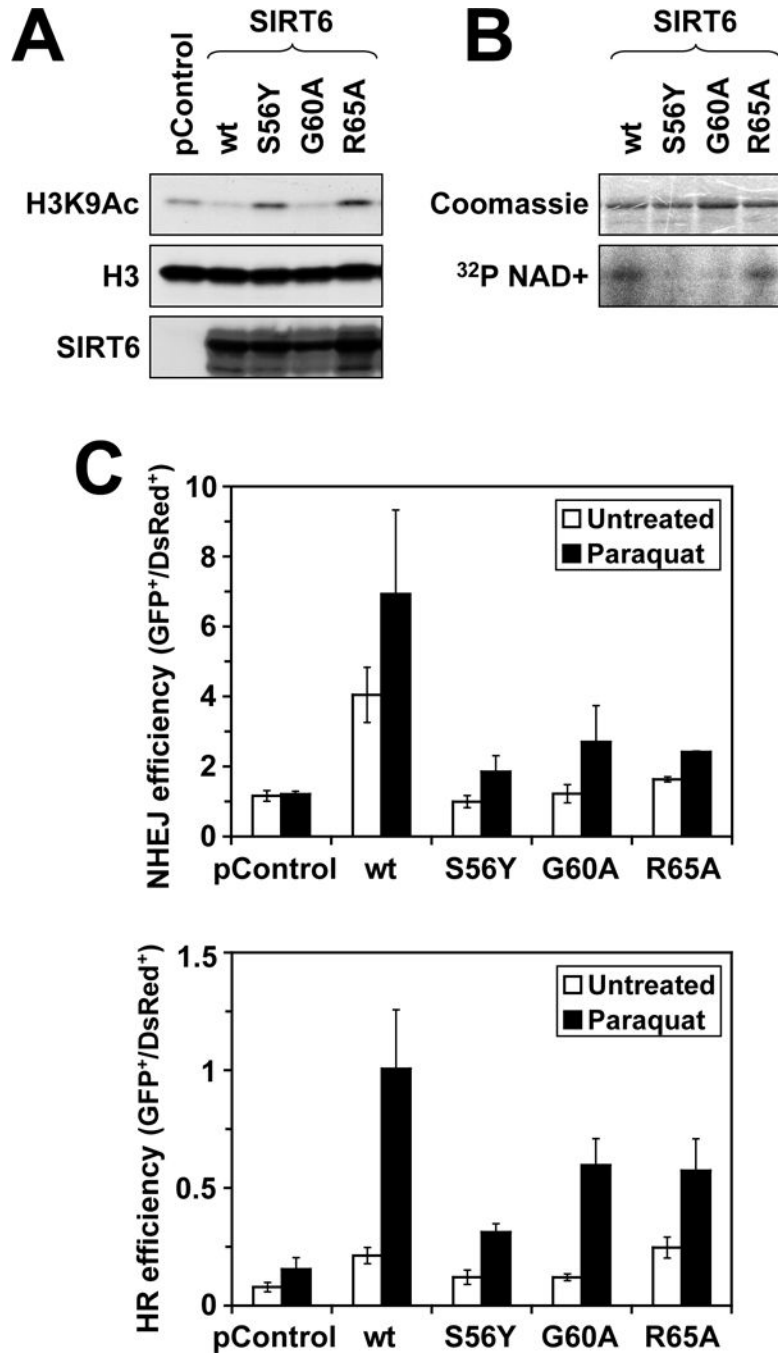
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**Figure 2. Oxidative stress results in early recruitment of SIRT6 to DNA breaks**

**A**, SIRT6 forms aggregates in the nucleus that co-localize with HP1β. *In situ* staining of human fibroblasts with SIRT6 and HP1β antibodies. **B,C** ChIP analysis showing kinetics of SIRT6 recruitment to Alu sequences following 8 Gy of  $\gamma$ -irradiation (**B**) and sequences flanking I-SceI-induced DSB after transfection with I-SceI expression vector (**C**). Control ChIP with SIRT6 knockout cells is shown in Supplementary Figure 6. Quantification of five ChIP experiments is shown. (\*) indicates values significantly ( $P < 0.05$ ) different from corresponding 0 time points. Error bars indicate s.d.



**Figure 3. Deacetylation and mono-ADP-ribosylation activities of SIRT6 are required for the stimulation of DNA repair**

**A**, Western analysis of H3K9 deacetylation activity of SIRT6 in human fibroblasts showing that S56Y and R65A mutations abolish the activity and possibly have a dominant negative effect. **B**, *In vitro* assay of mono-ADP-ribosylation activity of SIRT6 showing that S56Y and G60A mutations abolish the activity. **C**, SIRT6 mutants for deacetylation and/or ribosylation activities have reduced ability to stimulate NHEJ and HR. Prior to transfection with SIRT6 expressing vectors or pControl, cells were untreated (open bars) or treated with paraquat

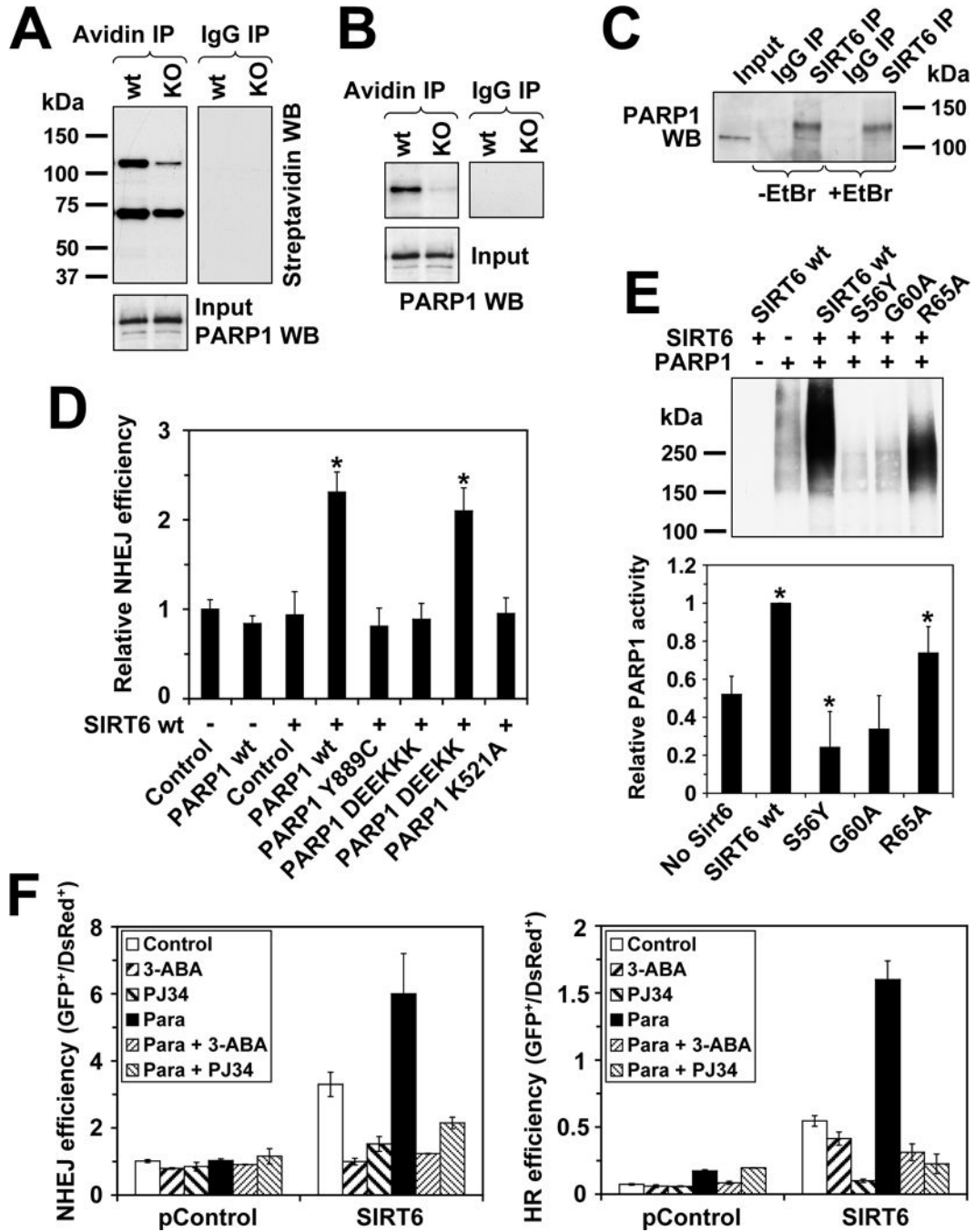
(filled bars). Quantification of four independent experiments is shown. Error bars indicate s.d.

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**Figure 4. SIRT6 interacts with PARP1 and stimulates its poly-ADP-ribosylation activity**

**A**, Analysis of mono-ADP-ribosylated proteins in the wild type and SIRT6 knockout MEFs stressed with paraquat for 16 h. Labeling with biotinylated NAD followed by depletion of poly-ADP-ribosylated proteins with PAR antibodies, and avidin immunoprecipitation of the mono-ADP-ribosylated proteins reveals two bands of 120 and 70 kDa. **B**, Western blot with PARP1 antibodies of the protein extract shown in (A). The 120 kDa band is recognized by PARP1 antibodies. **C**, Interaction of SIRT6 and PARP1 in human fibroblasts after treatment with 1 mM paraquat. Cell lysates were immunoprecipitated with SIRT6 antibodies in the

presence of 50 µg/ml Etidium bromide followed by Western analysis with PARP1 antibodies. **D**, PARP1 K521 is essential for activation of NHEJ by SIRT6. NHEJ assay was performed in PARP1 knockout MEFs containing integrated NHEJ reporter. Cells were transfected with SIRT6 and/or PARP1 or PARP1 mutants. Both SIRT6 and PARP1 are required for the stimulation of repair. PARP1 Y889C is a catalytically inactive PARP1. PARP1 DEEKKK contains mutations in the all six poly-ADP-ribosylation sites. PARP1 DEEKK contains mutations in the all poly-ADP-ribosylation sites, but not K521. Error bars show s.d. (\*) indicate values significantly different from control ( $P<0.01$ ). **E**, *In vitro* assay of PARP1 poly-ADP-ribosylation activity showing that PARP1 is stimulated by the addition of the wild type SIRT6 and SIRT6 R65A mutant that has mono-ADP-ribosylation activity only. The graph shows quantification of six independent experiments. Error bars show s.d. (\*) indicate values significantly different from control ( $P<0.01$ ). **F**, Stimulation of NHEJ and HR by SIRT6 is abolished by addition of PARP1 inhibitors 5 mM 3-ABA, or 20 µM PJ34. Quantification of four independent experiments is shown. Error bars indicate s.d.