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Involvement of Xeroderma Pigmentosum Group A (XPA) in Progeria Arising from Defective Maturation of Prelamin A

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Abstract

Cellular accumulation of DNA damage has been widely implicated in cellular senescence, aging, and premature aging. In Hutchinson-Gilford progeria syndrome (HGPS) and restrictive dermopathy (RD), premature aging is linked to accumulation of DNA double-strand breaks (DSBs) which results in genome instability. However, how DSBs accumulate in cells despite the presence of intact DNA repair proteins remains unknown. Here we report that the recruitment of DSB repair factors Rad50 and Rad51 to the DSB sites, as marked by γ -H2AX, was impaired in human HGPS and Zmpste24-deficient cells. Consistently, the progeria-associated DSBs appeared to be unreparable although DSBs induced by camptothecin were efficiently removed in the progeroid cells. We also found that these progeroid cells exhibited nuclear foci of XPA, a unique nucleotide excision repair protein. Strikingly, these XPA foci colocalized with the DSB sites in the progeroid cells. This XPA-DSB association was further confirmed, and found to be mediated by DNA, using a modified chromatin immunoprecipitation assay and co-immunoprecipitation. RNAi knockdown of XPA in HGPS cells partially restored DSB repair as evidenced by Western blot analysis, immunofluorescence and comet assays. We propose that the uncharacteristic localization of XPA to or near DSBs inhibits DSB repair, thereby contributing to the premature aging phenotypes observed in progeria arising from genetic defects in prelamin A maturation.

Keywords

XPA; Hutchinson-Gilford progeria syndrome; lamin A; Zmpste24; DNA double strand breaks and repair; premature aging; aging; DNA damage

INTRODUCTION

Hutchinson-Gilford progeria syndrome (HGPS) is a dominant premature aging disease caused by formation of a carboxyl-terminal truncated form (progerin) of the lamin A precursor, prelamin A (1, 2). Lamin A is an intermediate filament protein in the nuclear lamina, a scaffold underlying the inner nuclear membrane that structurally supports the nucleus and organizes chromatin (3). Homozygous deficiency of Zmpste24, an endoprotease essential for the proteolytic maturation of prelamin A (4), results in restrictive dermopathy (RD), which is a lethal perinatal progeroid disorder (5). Loss of Zmpste 24 proteinase activity arrests the processing of prelamin A at a stage similar to

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HGPS, although progerin instead of prelamin A is accumulated in HGPS cells. These two diseases have been suggested to be manifestations of the same cellular problem to different degrees (6). They have been grouped with other genetic diseases arising from mutations in the lamin A gene which are collectively referred to as “laminopathies”. We refer to RD and HGPS as “progeroid laminopathies”.

It has been recently shown that HGPS and RD cells exhibit DSB accumulation, impairment of DNA repair, and activation of the p53-dependent stress signaling pathway (7–9), suggesting that genome instability caused by HGPS and RD might contribute to premature aging. The DSB accumulation in HGPS and Zmpste24-deficient cells as well as in senescing and aging mammalian cells appears to be due to unreparable DSBs (10). However, the underlying mechanism of the repair defect is still poorly understood. We asked the question of how DNA repair is compromised in HGPS and RD cells even though there is no evidence of mutations in repair genes.

In the present study, using immunofluorescence microscopy and chromatin immunoprecipitation (ChIP) assay, we examined the localization of DNA repair proteins in relation to γ -H2AX, a molecular marker for DSBs, in the progeroid cells. We found that DSB repair proteins Rad51 and Rad50 were not localized to the DNA damage sites, while XPA, a unique nucleotide excision repair protein, largely colocalized with the DSBs formed in HGPS and RD cells. This suggests that the mislocalization of XPA to DSBs may be responsible for the lack of repair of DNA damage in the progeria cells. In support of this hypothesis, RNAi knockdown of XPA in HGPS cells significantly restored DSB repair.

MATERIALS AND METHODS

Cell cultures

Fibroblasts from a HGPS patient with the point mutation of 1824C $\rightarrow$ T were obtained from Coriell Cell Repository (no. AG11513A). Human RD fibroblasts were a gift from Dr. J.H. Miner (Washington University School of Medicine, St Louis, MO). The normal human fibroblasts, BJ cells, were purchased from American Type Culture Collection (ATCC, no. CRL-2522). All cultures were maintained in DMEM (for RD cells) or EMEM (for HGPS and BJ cells) supplemented with 10% FBS and antibiotics.

Immunofluorescence microscopy

Cells grown on coverslips were fixed with cold methanol (-20°C) or extracted with 0.5% IGEPAL CA-630 followed by fixation with methanol. The fixed cells were then incubated with primary antibodies against γ -H2AX (rabbit, Bethyl; or mouse, Stressgen), XPA (mouse, Kamiya; or rabbit, Santa Cruz), Rad51 (rabbit, Santa Cruz), Rad50 (rabbit, Bethyl), and XPC (rabbit, GeneTex). Secondary antibodies used include Alexa fluor 488-conjugated donkey anti-rabbit IgG and Alexa fluor 568-conjugated goat anti-mouse IgG (Molecular Probes). Cells were counterstained with DAPI to visualize nuclear DNA. Focus counting was performed by two blinded observers who randomly chose 50 cells for each experiment.

Western blotting

For immunoblotting of XPA and lamin proteins, whole-cell extracts were prepared from $\sim 10^7$ cells. The samples were separated by SDS-PAGE and immunoblotted with antibodies against XPA (GeneTex), prelamin A (11) or lamin A/C (Santa Cruz), or β -actin (Santa Cruz).

γ -H2AX association assay and co-immunoprecipitation

The γ -H2AX association assay used in this study was modified from the histone association assay described by Ricke et al. (12). Briefly, cells were treated with formaldehyde to crosslink interacting protein-DNA as well as protein-protein complexes. Nuclei were prepared by fractionation. The chromatin was sheared into 200–1500 bp fragments by sonication. The sheared chromatin was incubated with γ -H2AX antibody, followed by precipitation with protein G Sepharose beads. The immunoprecipitates were boiled for at least 30 minutes to reverse the crosslinks (12). Proteins that co-precipitate with chromatin were detected by Western blotting.

The co-immunoprecipitation was performed using a Nuclear Complex Co-IP kit (Active Motif, CA), following the manufacture's instructions.

Comet assay

The neutral comet assay was performed to assess DNA strand breaks in cells. The first layer of agarose on microscope slides were prepared by dipping the slides into 1% NMA followed by drying. 85 μ l of 0.5% LMA containing 4×10^5 cells was made by mixing 10 μ l cell suspension with 75 μ l LMA, and then poured onto the pre-coated slides. Slides were immersed in freshly prepared ice-cold buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, 1% Triton X-100, pH 10) to lyse the cells for at least 1 hr at 4 °C in the dark.. The slides were equilibrated in TBE buffer for 5 min twice followed by electrophoresis at 1 volt/cm in TBE buffer for 10 min. The slides were then dipped in 70% ethanol for 5 min and dried at room temperature for 1 hr. 50 μ l of 600 μ M DAPI was used for staining. All steps described above were conducted under dimmed light to prevent additional DNA damage. The quantification of the comets was conducted for randomly chosen 70–100 cells, and DNA damage was scored as the percentage of DNA in tail which is the relative DNA intensity (fluorescence-staining) of tail to the total DNA intensity (%DNA tail = $100 \times \Sigma I_p / \Sigma I_T$, where I_p is the product, the tail, intensity and I_T the total DNA intensity).

RESULTS

We first examined accumulation of DSBs in HGPS and RD fibroblasts by probing γ -H2AX focus formation using immunofluorescence (IF) microscopy. The normal human diploid fibroblasts, BJ cells, were used here as a control. The expression of wild-type prelamin A and/or progerin in HGPS and RD cells or normal lamin A in BJ cells was confirmed by Western blotting (Fig. 1a). Consistent with a previous report (13), prelamin A accumulates with increasing number of passages of HGPS cells. By using IF, we observed γ -H2AX foci in approximately 61% of RD cells and 53% of HGPS cells (passage 16), but only in about 8% BJ cells at the same passage. The number of γ -H2AX foci in RD (13.8 ± 3.3 per cell) and HGPS fibroblasts (10.5 ± 3.0 per cell) was significantly higher than that in the control BJ fibroblasts (0.4 ± 0.3 per cell) (Fig. 1b). Although γ -H2AX foci were also observed in human M-phase cells in the absence of DSBs and DNA damage signaling (14), these foci were locally condensed in nuclei. This pattern was very different from that of the discrete foci formed throughout the whole of the nucleus observed in response to DSBs (14). In the present study, only discrete foci have been considered. Importantly, our BJ cells control which had similar percentage (25–30%) of G2/M-phase population as HGPS and RD cells (data not shown and refs. (7, 8)) showed little discrete focus formation as compared to that of progeria cells. In HGPS and RD cells, discrete γ -H2AX foci appeared in more than half of the population. High levels of DSBs in HGPS cells was confirmed by comet assay (Fig. 1c) Using this method, we found that about 77% HGPS cells contained DSBs while only about 6% BJ cells show possible DSB formation. Since this is comparable to the DSB determination by formation of γ -H2AX, it supports the use of γ -H2AX foci as a DSB

marker. These results on the formation of DSBs in HGPS and RD cells are in agreement with the activated DNA damage responses in these progeria cells which we have previously reported (15). These findings indicate that defective lamin A maturation is correlated with DSB formation.

In an effort to determine the mechanism by which DSBs are resistant to being repaired, and, thus, accumulate in HGPS and RD cells, we examined the nuclear localization of several DNA repair proteins. We found that the HGPS and RD cells showing γ -H2AX foci also contained foci of the DSB repair protein Rad51 (Fig. 2a). The massive fluorescent staining of Rad51 in HGPS and RD cells indicates that some Rad51 protein may aggregate in the patient cells. However, colocalization of Rad51 and γ -H2AX was random (only about 5% and 2% for RD and HGPS cells, respectively) (Fig. 2a). This suggests that Rad51 was unable to localize to the DSB sites formed in the progeroid laminopathies, for DNA damage repair. In contrast, discrete Rad51 foci were formed and perfectly colocalized with DSBs as marked by γ -H2AX foci in BJ cells treated with camptothecin (CPT), a DSBs inducer (Fig. 2a). The same results were also obtained with Rad50 (Fig. 2b), a subunit of the Mre11/Rad50/Nbs1 (MRN) complex involved in DSB damage signaling and repair (16–19). These results suggest that the defective repair of DSBs in HGPS and RD fibroblasts is probably due to a disruption of recruitment of DSB repair factors such as MRN and Rad51 to the damage sites.

Further investigation led to the surprising finding that xeroderma pigmentosum group A (XPA), a unique nucleotide excision repair (NER) factor, formed a large number of nuclear foci in HGPS and RD cells. Strikingly, these foci colocalized very well with γ -H2AX foci (Fig. 2c). Specificity of the human XPA antibody (mouse) used in the detection was confirmed by Western blotting of whole cell extracts and by the similar immunofluorescence measurement with a different XPA antibody (rabbit) from a different company (Santa Cruz) (Fig. 2f). The specificity was also verified by siRNA knockdown experiments (Fig 5a). By contrast, other NER proteins, XPC (xeroderma pigmentosum group C) and RPA (replication protein A), showed no focus formation in HGPS and RD cells (Fig. 2d, and data not shown). XPA and XPC are DNA damage-recognition proteins in NER and have no role in DSB repair (16). RPA also is a DNA damage recognition protein and the main single-stranded DNA (ssDNA) binding protein in human cells (20). These results suggest that the colocalization of XPA and γ -H2AX was specific and NER-unrelated. This is consistent with the specificity of NER which neither processes DSBs nor generates DSB intermediates (16). Further support for the specificity of XPA- γ -H2AX colocalization in HGPS and RD cells came from the observation that XPA did not colocalize with CPT-induced γ -H2AX foci (arrowed green foci in the merged images in Fig. 2e) in BJ, RD or HGPS cells. This indicates that the DSBs induced by genotoxic agents are different from those formed upon accumulation of farnesylated and carboxymethylated prelamin A in the progeroid laminopathies. It should be noted that since it has been shown that the XPA in the nucleus is only a small portion of the total XPA in the cells (21), the good staining images of nuclear XPA shown for the BJ cells after cytoplasmic extraction were obtained by relatively long exposure of the staining.

To confirm the XPA localization to or near the DSB sites, the association of XPA with γ -H2AX in HGPS cells was examined using a modified ChIP (chromatin immunoprecipitation) assay. In the modified ChIP assay, cells were first treated with formaldehyde to crosslink protein-DNA and protein-protein complexes. After shearing the chromatin into 200–1500-bp fragments, the crosslinked chromatin-associated γ -H2AX in cell extracts was immunoprecipitated with anti- γ -H2AX antibody, followed by reversal of the cross-linking. As shown in Fig. 3a, Western blot analysis of the immunoprecipitates indicated that XPA was associated with γ -H2AX either through direct interaction or

mediated by DNA or other proteins. By contrast, no association between γ -H2AX and XPA was observed for BJ cells treated with CPT. Also interestingly, the lesser amount of XPA in HGPS cells than in BJ cells in the nuclear extract input is very likely due to the chromatin association of XPA which is resistant to extraction. This is supported by the fact that no difference in XPA level was observed when fully denaturing conditions were used (data not shown). To determine whether the colocalization was mediated by chromatin or a result of protein-protein interactions between γ -H2AX and XPA, co-immunoprecipitation (Co-IP) (without crosslinking) was performed after nuclease treatment using a Nuclear Complex Co-IP kit (Active Motif, CA). As shown in Fig. 3b, the nuclease digestion of DNA resulted in the loss of XPA- γ -H2AX association, suggesting that their association in these progeria cells was mediated by chromatin. As a positive control, XPA was efficiently co-immunoprecipitated with RPA (Fig. 3b).

To examine whether the accumulated XPA-localized DSBs in the patient cells are resistant to repair, we compared their repair rate to that of CPT-induced DSBs. As shown in Fig. 4a, the number of XPA- γ -H2AX colocalized foci had no substantial change in HGPS and RD cells 24 hrs after CTP treatment. By contrast, the number of CPT-induced γ -H2AX foci (not colocalized with XPA foci) was significantly reduced in the progeroid or BJ cells, although repair in the patient cells occurred at much slower rates. Further quantitative analysis of the DSB repair as a function of time confirmed these results (Fig. 4b), suggesting that the XPA-localized DSBs in the patient cells may be resistant to repair.

The appearance of XPA at unrepairable DSB sites may suggest a physiological role of XPA in the accumulation and persistence of DSBs in HGPS and RD cells. To test this notion, XPA was knocked down in HGPS cells using XPA-specific siRNA (Fig. 5a), followed by determination of the effect of the knockdown on accumulation of γ -H2AX in these cells using Western blotting. As shown in Fig. 5a, XPA knockdown consistently resulted in 37–45% reduction of γ -H2AX levels in HGPS cells as compared to that of GFP siRNA transfected HGPS cells. However, XPA knockdown had no effect on the formation of CPT-induced γ -H2AX in BJ cells (Fig. 5a). These results were confirmed by immunofluorescence. The number of γ -H2AX foci was significantly decreased after XPA knockdown (from 9.6 ± 1.6 to 5.5 ± 1.1 foci per cell in HGPS fibroblasts, $P = 0.03$; from 14.4 ± 2.5 to 8.0 ± 2.1 foci per cell in RD fibroblasts, $P = 0.03$) (Fig. 5b), while XPA knockdown had no effect on the formation of CPT-induced γ -H2AX foci in BJ cells (Fig. 5b). More direct evidence came from the comet assay of single cell electrophoresis which showed significant reduction in DNA double-strand breaks in HGPS cells with XPA knockdown as compared with the control HGPS cells transfected with GFP-siRNA (Fig. 5c). To confirm that the partial restoration of DSB repair was due to recovery of the recruitment of DSB repair proteins to the DSB sites in HGPS cells, a chromatin immunoprecipitation-based γ -H2AX association assay (see Materials and Methods) was performed. As shown in Fig. 6a, the recruitment of Rad50 and Ku70 to the DSB sites was significantly restored in HGPS cells upon XPA knockdown. Consistent results were also obtained from immunofluorescence determination of the nuclear focus colocalization between γ -H2AX and Rad51 (Fig. 6b). In addition, to determine whether the DSB repair machinery per se is functional in the progeria cells, HGPS cells with XPA knockdown were treated with CPT for production of non-progeria-related DSBs. Indeed, significantly more Rad50 and Ku70 were recruited to the γ -H2AX sites in the CPT-treated cells as compared with the cells without CPT treatment, suggesting that Rad50 and Ku70 were able to efficiently localize to CPT-induced γ -H2AX sites in HGPS cells. This implies that the inherent DSB repair system may function efficiently in HGPS cells, which is consistent with the results in Fig. 4, although we can not rule out the possibility that the repair system per se could be partially affected in the disease cells. Taken together, these results suggest that the

presence of XPA may inhibit DSB repair in HGPS cells and the depletion of XPA by siRNA may partially restore the repair.

DISCUSSION

Recent studies have shown that DSBs typically accumulate in HGPS cells and RD cells and the resultant genome instability might contribute to premature aging (7–9). The accumulation is likely due to a deficiency in DNA repair in these progeria cells. In an effort to address the important question of why and how DNA repair is compromised in HGPS and RD cells despite the presence of intact DNA repair proteins, we found that DSB repair proteins Rad51 and Rad50 were absent at the laminopathy-related DNA damage sites in the patient cells. Consistently, the laminopathy-induced DSBs were resistant to repair in the progeria cells, while the repair of CPT-induced DSBs appears to be effective but at a slower rate than that in BJ cells (Fig. 4b). Surprisingly, the inefficiency in DNA repair was correlated with the formation of nuclear foci of the NER protein XPA and the chromatin-mediated association of XPA with the DSBs. This uncharacteristic XPA activity occurred exclusively in progeria cells but not in normal fibroblasts. Our results further demonstrated that RNAi knockdown of XPA in HGPS cells partially restored DSB repair. The mislocalization of XPA to or near the laminopathy-induced DSB sites may play a role in denying the accessibility of the damage sites to DSB-repair factors, thus inhibiting DNA repair. On the other hand, Liu et al. (7) recently showed that *Zmpste24*^{-/-} mouse embryonic fibroblasts are not only hypersensitive to DSB-inducing agents, but also highly sensitive to UV irradiation which typically induces DNA damage exclusively removed by NER. Our results provide a potential mechanistic explanation for this observation, as it is possible that the XPA-DSB mislocalization may trap the protein at the DSB sites and thus reduces the number of XPA molecules available for NER. This may cause the activity loss or reduction of both DSB repair and NER with the consequence of massive accumulation of damaged DNA.

It is well known that XPA is a damage verifier of bulky DNA lesions in NER (22–24), and has no role in DSB repair. Our results in Fig. 3 indicate that the association of XPA with γ -H2AX appears to be mediated by chromatin. Since nuclear lamins directly interact with histone H2A (25), it is possible that disruption of the chromatin-supporting scaffolds formed with nuclear lamins in HGPS and RD cells may produce γ -H2AX marked broken chromosomes with unique DNA structures recognized by XPA. In support, we recently identified a novel activity of XPA for recognition of double-strand/single-strand DNA (ds/ssDNA) junctions with 3'- and/or 5'-ssDNA overhangs (26). The binding affinity of XPA for these sites is between one and two orders of magnitude higher than its ability to bind to damaged DNA. This type of DNA structure forms as intermediates during some DNA metabolic pathways including replication and DSB repair. Furthermore, a recent study demonstrated that XPA, but not NER-mediated damage processing, is required for UV-induced S-phase ATR checkpoint activation (27) which is replication-dependent. Interestingly, DSBs could result from replication fork stalling and collapse (28–30).

It has been reported that disruption of lamin organization by injecting lamin A mutant protein (Δ NLA) into mammalian cells resulted in redistribution of PCNA and RFC, thus blocking the elongation phase of DNA replication (31, 32). The replication blockage which may induce DSBs is likely due to the loss of replicative PCNA trapped in lamin A aggregates (31, 32). Therefore, a possible loss of PCNA at the collapsed replication forks in HGPS cells may leave the ds/ssDNA junctions of Okazaki fragments unprotected. We speculate that this may make the naked DNA junctions near DSBs at the stalled replication forks accessible to XPA for binding during progeria development. In support of this notion, PCNA formed discrete foci in early passages of HGPS cells in S-phase (33), while no foci

were observed in relatively later passages of HGPS cells (unpublished data). Consistently, our results indicated that XPA formed no foci in the early passage of S-phase HGPS cells with PCNA foci, but did form foci and colocalize with γ -H2AX in the later passage of HGPS cells (DSB formation also increases with passage number in HGPS cells). It is possible that XPA might localize to the ds/ssDNA junctions at collapsed replication forks unprotected by PCNA due to laminopathies. It is clear that details of the mechanism underlying the uncharacteristic XPA-DNA binding in the progeria cells needs to be defined in the future.

Findings presented in this study serve as the first step towards uncovering the underlying mechanism of DSB accumulation seen in the premature aging diseases HGPS and RD. Our results suggest a potential pathological role of XPA in development of the progeroid laminopathies in contrast to its indispensable role in NER. Recent studies showed that inhibiting farnesylation of progerin or prelamin A by farnesyltransferase inhibitors (FTIs) in HGPS and RD fibroblasts respectively could reverse the aberrant nuclear morphology caused by deficiency in lamin A maturation (34–37). FTI administration also showed amelioration of the disease in a mouse model of progeria (38). However, we recently found that FTI treatment could not reduce the accumulated DSBs in both HGPS and RD cells (15). This suggests that DNA damage accumulation and misshapen nuclei are probably two independent phenotypes caused by lamin A dysfunction in the progeroid laminopathies. Thus, inhibition of DNA damage accumulation and improvement of nuclear morphology would be potential goals in development of an effective strategy for treatment of progeroid syndromes.

DNA damage accumulation is believed to be one of the major causes of cellular senescence and normal aging (6, 39–44) and the similar unrepairability of DSBs has been reported in senescing human cells (10). Importantly, lamin A-dependent nuclear defects were recently found in normal human aging (45). Thus, it is of great interest to determine if XPA also plays a role in these processes.

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Figure 1.

Prelamin A and DSB accumulation in BJ, HGPS and RD fibroblasts. **(a)** Western blot analysis of prelamin A. Cells were grown for the indicated number of passages, and Western blot analyses of cell extracts were performed with antibodies specific for: *Top*, prelamin A, (specific for the extreme C terminus) and *Bottom*, lamin A/C (binds to both lamin A and lamin C). Symbols indicate the following: (*) = Prelamin A, (o) = mature Lamin A, (-) = LA 50 and (+) = Lamin C. **(b)** DSB formation as revealed by γ -H2AX foci. Cells were stained with anti- γ -H2AX antibody. Blue, DAPI; green, γ -H2AX. The percentage of cells containing γ -H2AX foci is shown on the left bottom of each subpanel; the number of γ -H2AX foci per cell is shown on the right bottom. Bar, 12 μ m. **(c)** DSB formation as measured by comet assay. 100 cells were randomly chosen and counted for the DNA comet tail formation of individual cells in HGPS and control BJ cells. Any cells that had 17% DNA tail were considered as comet-positive cells. About 77% HGPS cells were found to be comet-positive, while only 6% BJ cells were comet-positive. Bar, 85 μ m.

Figure 2.

Colocalization of γ -H2AX foci and the foci of DNA repair proteins in BJ, HGPS and RD fibroblasts. Nuclear focus localization of Rad51 (a), Rad50 (b), XPA (c and e), and XPC (d) relative to γ -H2AX foci were detected with corresponding antibodies by immunofluorescence microscopy. Uniform staining of the given protein throughout nucleus indicates homogenous distribution of the protein without foci formation. CPT treatment of cells was conducted by incubating the cells with 4 μ M CPT for 1 hour before fixation. The green foci in the Merge subpanel of (e) represent the CPT-induced DSBs (arrows) that were not colocalized with XPA foci. The nucleus is visualized by DAPI staining. (f) Left panel: Western blot analysis of whole cell extracts by human XPA antibody (mouse, Kamiya). XPA was visualized as a doublet of bands (46). Right panel: Formation of XPA foci in HGPS cells identified by human XPA antibody raised from rabbits (Santa Cruz). Photomicrographs were taken at 63X magnification. Bar, 25 μ m.

Figure 3.

Chromatin-mediated XPA- γ -H2AX interaction. **(a)** γ -H2AX association assay was performed as described in Materials and Methods. HGPS and BJ cells were treated with the crosslinking agent formaldehyde. BJ cells were treated with 4 μ M camptothecin for 1 hour before the formaldehyde fixation. Immunoprecipitated proteins were analyzed by Western blotting with indicated antibodies. IP with IgG was used as a negative control to demonstrate the specificity of γ -H2AX antibody. **(b)** Co-immunoprecipitation was performed with nuclease treatment. The nuclear extracts (Input) and precipitated proteins were analyzed by immunoblotting with indicated antibodies. IP with RPA32 antibody was carried out in parallel, which served as a positive control demonstrating that the Co-IP conditions used herein preserved protein-protein interactions.

Figure 4.

Laminopathy-induced DSBs are unrepairable. **(a)** Cells were treated with 4 μ M CPT and then harvested at the indicated times for immunofluorescence analysis. The γ -H2AX foci that did not colocalize with XPA were CPT-induced γ -foci. Photomicrographs were taken at 63X magnification. Bar, 25 μ m. **(b)** Focus counting was performed by two blinded observers who randomly chose 50 cells for each experiment. The number of foci was plotted against time post treatment with CPT.

Figure 5.

DSB repair in HGPS cells with XPA knockdown by RNAi. **(a)** Knockdown of XPA reduces DSB accumulation. For the knockdown of XPA by RNAi, the cells were transfected with XPA siRNA (Santa Cruz), or GFP siRNA as a control, using TransIT-TKO transfection reagent (Mirus) following manufacture's instruction. Western blotting was performed to analyze the knockdown efficiency 96 hours after transfection. XPA was shown as a known doublet of bands plus a weak third band due to phosphorylation (46). The amount of γ -H2AX was quantified against β -actin, the loading control, by densitometry. Standard deviation was generated from three independent experiments. The symbol * indicates $P < 0.05$. **(b)** Number of γ -H2AX foci in the XPA-siRNA or GFP-siRNA transfected cells. The symbol * indicates $P < 0.05$. **(c)** Comet assays were carried out with XPA-siRNA or GFP-siRNA transfected BJ cells and HGPS cells as described in Materials and Methods.

DNA damage was quantified and expressed as the percentage of DNA in tail which is the relative DNA intensity (fluorescence-staining) of tail to the total DNA intensity. Bar, 65 μ m.

a **b** **Figure 6.**

XPA depletion increases recruitment of DSB repair proteins to the DNA damage sites. **(a)** Restoration of Rad50 and Ku70 recruitments to DSB sites by XPA knockdown in HGPS cells. The γ -H2AX-Rad50 or γ -H2AX-Ku70 association in HGPS (with or without XPA knockdown) was determined using a modified chromatin immunoprecipitation assay. The IgG probing was used as a loading control. **(b)** Nuclear foci colocalization of Rad51 and γ -H2AX foci in HGPS cells with or without XPA depletion. 50 cells were randomly chosen and counted for the colocalization of γ -H2AX and Rad51 foci in GFP siRNA (control) or XPA siRNA transfected HGPS cells. 28% XPA siRNA-transfected HGPS cells were found to be colocalization-positive which have at least one γ -H2AX-Rad51 colocalized foci, while only 6% GFP siRNA-transfected cells were colocalization-positive.