Poly(ADP-ribose) polymerase 1 (PARP1) promotes oxidative stress–induced association of Cockayne syndrome group B protein with chromatin

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ABSTRACT

Cockayne syndrome protein B (CSB) is an ATP-dependent chromatin remodeler that relieves oxidative stress by regulating DNA repair and transcription. CSB is proposed to participate in base-excision repair (BER), the primary pathway for repairing oxidative DNA damage, but exactly how CSB participates in this process is unknown. It is also unclear whether CSB contributes to other repair pathways during oxidative stress. Here, using a patient-derived CS1AN-sv cell line, we examined how CSB is targeted to chromatin in response to menadione-induced oxidative stress, both globally and locus-specifically. We found that menadione-induced, global CSB–chromatin association does not require CSB’s ATPase activity and is, therefore, mechanistically distinct from UV-induced CSB–chromatin association. Importantly, poly(ADP-ribose) polymerase 1 (PARP1) enhanced the kinetics of global menadione-induced CSB–chromatin association. We found that the major BER enzymes, 8-oxoguanine DNA glycosylase (OGG1) and apurinic/apyrimidinic endodeoxyribonuclease 1 (APE1), do not influence this association. Additionally, the level of γ-H2A histone family member X (γ-H2AX), a marker for dsDNA breaks, was not increased in menadione-treated cells. Therefore, our results support a model whereby PARP1 localizes to ssDNA breaks and recruits CSB to participate in DNA repair. Furthermore, this global CSB–chromatin association occurred independently of RNA polymerase II–mediated transcription elongation. However, unlike global CSB–chromatin association, both PARP1 knockdown and inhibition of transcription elongation interfered with menadione-induced CSB recruitment to specific genomic regions. This observation supports the hypothesis that CSB is also targeted to specific genomic loci to participate in transcriptional regulation in response to oxidative stress.

Cockayne syndrome is a devastating recessive disorder characterized by features of premature aging, extreme sun sensitivity, and neurological and developmental abnormalities (1,2). The majority of Cockayne syndrome cases are the result of mutations within the gene encoding Cockayne syndrome protein B (CSB), an ATP-
PARP1 targets CSB to chromatin upon oxidative stress

dependent chromatin remodeler (3-5). CSB plays a role in transcription regulation (6-11) and is essential for transcription-coupled nucleotide excision repair (TC-NER) (3,12-17). CSB also contributes to the relief of oxidative stress by regulating DNA repair as well transcription (18-20); however, mechanisms underlying these activities remain elusive. Cells deficient in CSB show increased sensitivity to oxidizing agents (20-22), accumulate oxidative DNA damage (22,23) and display increased levels of intracellular reactive oxygen species (ROS) (21).

The major repair pathway for oxidative DNA damage is base excision repair (BER) (24). BER is initiated by a substrate-specific DNA glycosylase that removes the oxidized base. This is followed by cleavage of the sugar-phosphate backbone and excision of the remaining apurinic-apyrimidinic site (AP site) by apurinic-apyrimidinic endonuclease 1 (APE1), or in some cases glycosylases with inherent endonuclease activity. The resulting nicked DNA is recognized by and activates poly(ADP-ribose) polymerase 1 (PARP1), which uses NAD+ to catalyze the addition of poly(ADP-ribose) (PAR) polymers to itself as well as other proteins. PARP1 is hypothesized to recruit proteins important for DNA repair, such as the scaffold protein XRCC1. PARP1 may also serve to stabilize nicked DNA, preventing degradation of single strand breaks into double strand breaks (24-28). The remaining gap is filled by DNA polymerase β and ligation is performed by DNA ligase IIIα (Lig3). An alternative pathway, long-patch BER, is initiated by blocked 5'-ends during nick repair.

Evidence for a role of CSB in BER has been provided by several groups, which report that cellular extracts from CSB null cells demonstrate reduced incision activity of oxidative DNA lesions in vitro (18,22,29-31). Recent findings by Menoni et al. (2012) provide support for the notion that CSB functions in the repair of oxidized DNA, by demonstrating that CSB accumulates at sites of locally induced oxidative DNA damage in cells (32). CSB has also been shown to physically and functionally interact with several key BER proteins, such as OGG1 and APE1 (33,34). Additionally, CSB associates with PARP1, and PARP1 has been shown to poly(ADP-riboseylate) CSB (35). Recently, Scheibye-Knudsen et al. (2014) demonstrated PARylated PARP1 is required for retaining CSB at sites of oxidative DNA damage and hypothesized that CSB participates in PARP1 displacement from damaged DNA to facilitate repair (36).

Under replicative cell growth conditions, CSB interacts with chromatin very dynamically, and only ~10% of CSB stably associates with chromatin (37). In response to UV DNA damage, where CSB is employed for TC-NER, the situation is reversed, and ~90% of CSB can become stably associated with chromatin. Recently, we demonstrated that oxidative stress also stabilizes the association of CSB with chromatin on a global level (20). In addition, we found that oxidative stress induces the occupancy of CSB at specific genomic loci, including loci containing the binding motif for the chromatin architectural protein CCCTC-binding transcription factor (CTCF) (20). Importantly, we found that CSB and CTCF reciprocally regulate each other’s site-specific, chromatin association in response to oxidative stress and that these two proteins directly interact (20). These observations suggest a role for CSB in regulating higher-order chromatin structure during oxidative stress.

In this study, we further characterize the mechanisms by which CSB stably associates with chromatin, both globally and locus-specifically, in response to oxidative stress.

**Results**

**Oxidative stress induces stable CSB-chromatin association**

CSB interacts with chromatin dynamically. During replicative cell growth, ~10% of CSB co-fractionates with chromatin (Fig. 1A-B) (20). However, when cells are treated with menadione, which creates oxidative stress by producing reactive oxygen species (38), a substantial increase in CSB-chromatin association is observed (Fig. 1A-B) (20). This observation suggests that enhanced CSB-chromatin association results from oxidative stress created by menadione; however, we cannot rule out the possibility that enhanced chromatin association could be associated with another biological consequence of menadione treatment, especially at a relatively high menadione dose (100 µM). To
dissect the mechanisms by which menadione induces the global association of CSB with chromatin, we used the patient derived, CSB-deficient CS1AN-sv cell line that has been stably reconstituted with wild-type CSB (CS1AN-CSB<sup>WT</sup>) (Fig. S1A-B). CSB’s expression level in CS1AN-CSB<sup>WT</sup> cells is within two-fold of that of the human fibroblast cell line MRC5 (Fig. S1A) (39). We examined the time dependence of CSB-chromatin association in CS1AN-CSB<sup>WT</sup> cells treated with 100-µM menadione and found that ~90% of CSB co-fractionates with chromatin within 30 minutes (Fig. 1A-B). As previously demonstrated, the partitioning between soluble and chromatin fractions of another ATP-dependent chromatin remodeler, BRG1, was not grossly altered by menadione treatment and, therefore, BRG1 was used as a protein loading control for normalization (Fig. 1A) (20). Acetylated histones H3 as well as Ponceau S staining of total histone proteins were used as controls to examine chromatin fractionation efficiency (Fig. 1A). Additionally, as expected, the active form of RNA polymerase II was in the chromatin fraction, while GAPDH was in the soluble fraction. The CTCF protein, which was previously shown to increase its association with CSB in response to menadione treatment (20), was chromatin-associated regardless of menadione treatment (Fig. 1A).

We next examined how two other DNA repair proteins behaved in this fractionation assay (Fig. 1A). Menadione treatment induced the chromatin association of XRCC1, a scaffolding protein involved in DNA repair (Fig. 1A-B). We found that PARP1 was present in both the soluble and chromatin fractions and its partitioning between these two fractions was not significantly changed by menadione treatment (Fig. 1A-B). In addition, we did not observe an apparent change in the levels of the classic marker for DNA double-strand breaks, γ-H2AX, after menadione treatment (Fig. 1A).

To further demonstrate that oxidative stress increases CSB-chromatin association, we performed anti-CSB chromatin immunoprecipitation (ChIP) followed by western blot analysis, using an antibody against histone H3. We found a more than five-fold increase of histone H3 co-immunoprecipitating with CSB in cells treated with menadione than in untreated cells, demonstrating that menadione treatment increases CSB’s association with chromatin (Fig. 1C).

**ATP hydrolysis by CSB is dispensable for menadione-induced chromatin association**

Stable CSB-chromatin association can also be induced by UV irradiation, and this association requires ATP hydrolysis by CSB to relieve auto-repression (37). We next determined if menadione-induced stable CSB-chromatin association is also ATP-dependent. To accomplish this, we used the CSB-deficient CS1AN-sv cell line reconstituted with a CSB protein harboring a patient derived mutation, CSB<sup>R670W</sup>, which is devoid of ATPase activity (Figs. 1D and S1A-B) (37). In sharp contrast to UV-induced CSB-chromatin association, menadione-induced stable association of CSB<sup>R670W</sup> with chromatin was kinetically similar to CSB<sup>WT</sup>. This result reveals that ATP hydrolysis by CSB is dispensable for global CSB-chromatin association in response to menadione treatment.

**Oxidative stress-induced global CSB-chromatin association is initiated by the N- and C-terminal regions and sustained through the ATPase domain and C-terminal region**

To dissect further the mechanism by which CSB becomes stably associated with chromatin in response to oxidative stress, we analyzed a set of CSB deletion-derivatives (Fig. 2). All mutant proteins were stably expressed in CS1AN-sv cells and nuclear localized (Fig. S1A-B) (37). CSBAN<sup>Δ</sup>, which is devoid of its N-terminal region but has intact ATPase and C-terminal domains, co-fractionates with chromatin, even in the absence of UV irradiation (37). However, unlike UV-induced CSB-chromatin association, menadione treatment resulted in a further increase in the association of CSBAN<sup>Δ</sup> with chromatin (Fig. 2B). This result suggests that CSB responds to oxidative stress through its ATPase and/or C-terminal domains.

Deletting the last 484 amino acids of CSB (CSB<sup>A</sup>δC) abolishes the ability of CSB to associate with chromatin in response to UV irradiation (37). In contrast, CSB<sup>A</sup>δC still responds to menadione treatment, however, the fraction of CSB<sup>A</sup>δC associating with chromatin was lower at the 20- and 30-minute time points as
compared to full-length CSB (Fig. 2C), supporting the hypothesis that the C-terminal region contributes to chromatin binding, similar to UV-induced CSB-chromatin association. Increased menadione treatment increased the amounts of CSB-N (CSB1-507) co-fractionating with chromatin, indicating that CSB-N can respond to oxidative stress (Fig. 2D). However, CSB-N showed an overall lower chromatin association as compared to CSBΔC (Fig. S1C), supporting the notion that the CSB-ATPase domain contributes to stable CSB-chromatin association upon oxidative stress, similar to UV-induced CSB-chromatin association. However, CSB-C alone did not bind chromatin as efficiently as full-length CSB when cells were within the first 10 minutes of menadione treatment. Nonetheless, CSB-C eventually bound to a level similar to that of full-length CSB, suggesting that CSB-C can also respond to oxidative stress and bind to chromatin, albeit not as efficiently as the full-length protein (Fig. 2E). Together these findings support a model in which oxidative stress-induced global CSB-chromatin association is initiated by the N- and C-terminal regions and sustained through the ATPase domain and C-terminal region. Moreover, the results reveal that menadione-induced chromatin association of CSB does not rely upon ATP-dependent relief of auto-repression.

Menadione-induced, global CSB-chromatin association does not require active transcription by RNA polymerase II

Another key factor underlying UV-induced CSB-chromatin association is active transcription. Inhibition of RNA polymerase II (RNA pol II) transcription elongation by 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) prevents stable CSB-chromatin association induced by UV irradiation (37). We, therefore, examined whether CSB-chromatin association induced by menadione treatment also requires active RNA pol II transcription. CS1AN-CSBWT cells were exposed to DRB or a DMSO control for 1 hour prior to treatment with menadione for 20 minutes. As demonstrated in Fig. 3A-B, DRB did not significantly alter the stable association of CSB with chromatin that is induced by menadione on a global level. However, as previously observed, similar DRB treatment prevented UV-induced CSB-chromatin association (Fig. 3C). This finding indicates that stable CSB-chromatin association resulting from oxidative stress is regulated by a mechanism that is distinct from UV-induced association.

APE1 and OGG1 are dispensable for global menadione-induced CSB-chromatin association

CSB has been suggested to relieve oxidative stress by both facilitating base-excision repair and regulating transcription of specific genes. Therefore, the menadione-induced global CSB-chromatin association would be expected to represent, to a large degree, sites of oxidized DNA. Accordingly, we used the chromatin-fractionation assay to dissect the mechanism by which menadione induces CSB-chromatin association. As CSB directly interacts with the major apurinic/apyrimidinic endonuclease APE1 (34), we hypothesized that APE1 may recruit CSB to sites of APE1-mediated DNA strand breaks to facilitate APE1 activity in cells treated with menadione. If this hypothesis were correct, we expect to find less CSB co-fractionating with chromatin in cells with decreased APE1 levels. To test this hypothesis, we reduced the level of the APE1 protein using shRNA and determined its consequence on the amount of CSB co-fractionating with chromatin (Fig. 4A-D). As shown in Fig. 4A, we were able to reduce APE1 protein levels to less than 30%; however, we did not observe a significant change in menadione-induced CSB-chromatin association. This result suggests that APE1 is unlikely to be essential for global CSB recruitment to chromatin when cells are treated with menadione (Fig. 4B-D).

OGG1, a glycosylase, initiates the base excision repair of 7,8-dihydro-8-oxoguanine (8-oxoG), the major oxidized DNA lesion. Given that CSB has been reported to be in complex with OGG1, we next tested if the global recruitment of CSB to chromatin is mediated by OGG1. To accomplish this, we reduced OGG1 protein levels using shRNA-targeting OGG1 (Fig. 4E) and determined its impact on the levels of CSB co-fractionating with chromatin in response to menadione treatment (Fig. 4F-H). Reduction of OGG1 levels to ~10% did not significantly reduce the level of CSB co-fractionating with
PARP1 targets CSB to chromatin upon oxidative stress

PARP1 facilitates CSB-chromatin association induced by menadione treatment

Another candidate protein for targeting CSB to chromatin in response to oxidative stress is PARP1, as it not only interacts with CSB but also poly(ADP)ribosylates CSB (35). Therefore, we examined CSB-chromatin association following control or PARP1 shRNA knockdown (Figs. 5 and S2-3). Fig. 5A is a representative western blot showing the level of PARP1 knockdown, which was routinely about 90%. We found that PARP1 knockdown significantly reduced the kinetics of CSB-chromatin association following menadione treatment, although ~90% of CSB eventually associates with chromatin after a 1-hour menadione treatment (Fig. 5B-D). To confirm this finding, we repeated the experiments in control and PARP1 knockdown cells, either untreated or treated with menadione for 20 minutes (average ~96% knockdown, n=11). We found a drop from ~40% CSB co-fractionating with chromatin in cells treated with control shRNA to ~17% in cells treated with PARP1 shRNA (Fig. S2A). A difference was observed whether or not we used BRG1 to normalize protein levels (compare Fig. S2A to S2B). Together, these results indicate that PARP1 enhances the kinetics of menadione-induced CSB-chromatin association.

PARP1 could facilitate menadione-induced CSB-chromatin association through its ability to directly interact with CSB. Alternatively, PARP1 might do so through its enzymatic activity. To determine the contribution of PARP1’s enzymatic activity in menadione-induced CSB-chromatin association, we treated cells with the potent PARP inhibitor, KU-0058948 (Fig. 5E-H). Cells treated with KU-0058948 had less poly(ADP-ribosyl)ation activity, as demonstrated by western blot analysis using an anti-PAR antibody (Fig. 5E). However, we did not observe a significant change in the kinetics of CSB-chromatin association induced by menadione treatment, suggesting that PARP1 may influence CSB-chromatin recruitment through direct protein-protein interaction.

We also examined if CSB played any role in the global recruitment of PARP1 to chromatin (Fig. S4); however, we did not observe any change of PARP1-chromatin association in cells with or without CSB.

PARP1 facilitates the recruitment of CSB to specific genomic loci induced by menadione treatment

ChIP-seq experiments revealed that menadione treatment also increases the occupancy of CSB at specific genomic loci (20). To determine if PARP1 participates in recruiting CSB to these loci in response to oxidative stress, we used ChIP-qPCR to examine CSB occupancy at four of these sites (chrX-1, chrX-2, chr17-1, and chr19-2) in cells treated with shRNA targeting PARP1 (Figs. 6A and S5). These loci are the four highest CSB occupancy sites induced by menadione. Chr12-7 was used as a control locus that represents a CSB occupancy site that is independent of menadione treatment (20). These loci lie in introns (chr17-1 and chr19-2), a promoter (chrX-2), or an intergenic region, (chrX-1). When the PARP1 protein was reduced to ~15% of its normal level, the menadione-induced occupancy of CSB at these loci was significantly reduced (Figs. 6A and S5) (20). On the other hand, the occupancy of CSB at the control locus, chr12-7, was not altered by a decrease in PARP1 protein levels (Figs. 6A and S5). Together these results indicate that PARP1 plays a key role in facilitating the recruitment of CSB to specific genomic loci in response to oxidative stress.
PARP1 targets CSB to chromatin upon oxidative stress

oxidative stress, in addition to playing a role in influencing the kinetics of global CSB-chromatin association following oxidative stress.

We next determined if the locus-specific CSB occupancy relies upon the enzymatic activity of PARP1. As shown in Fig. 6B, after treating cells with KU-0058948, we observed a significant decrease in CSB occupancy at chrX-1 and chrX-2, but not chr17-1 and chr19-2. These results indicate that the enzymatic activity of PARP1 contributes to the recruitment of CSB to specific loci, but only at a subset of its occupied sites. Interestingly, we found that treating cells with the transcription inhibitors DRB or α-amanitin significantly decreased menadione-induced site-specific CSB occupancy at all four loci, further supporting the notion that CSB functions in transcription regulation at these loci when cells are under oxidative stress (Fig. 6C-D).

Discussion

In this study, we demonstrated that the global chromatin association of CSB induced by oxidative stress does not require ATP-dependent relief of auto-repression (Fig. 1) and, therefore, is distinct from the mechanism by which UV irradiation induces CSB-chromatin association for its essential function in TC-NER (37). Our structure-function studies indicate that the N and C terminal regions of CSB are required to respond to oxidative stress, and that the ATPase domain and C-terminal domain sustain menadione-induced CSB occupancy on a global level (Fig. 2). Importantly, we find that PARP1, a CSB binding protein, which responds to both single-strand and double-strand DNA breaks (35,40), enhances the kinetics of global CSB-chromatin association induced by oxidative stress (Fig. 5). Since we observed no apparent increase in the level of γ-H2AX, a marker for DNA double-strand breaks, in cells treated for 30 minutes with menadione (Fig. 1A), these results together support the notion that PARP1 functions in the recruitment of CSB to single-strand DNA breaks upon oxidative stress (Fig. 7A). The majority of single-strand breaks that CSB responds to are unlikely the product of BER, as menadione-induced global CSB-chromatin association remains unchanged when the BER proteins OGG1 and APE1 are reduced by approximately 90% and 70%, respectively (Fig. 4). However, we cannot exclude completely the possibility that the remaining protein participates in CSB recruitment. These observations, therefore, suggest that PARP1 may enhance the recruitment of CSB to sites of single-strand DNA breaks directly generated by reactive oxygen species through menadione treatment (Fig. 7A) (38). Accordingly, we would like to propose that one major function of CSB in cells exposed to oxidative stress is to cooperate with PARP1 in single-strand DNA break repair.

This model shown in Fig. 7A is consistent with the observation of Menoni et al. (2012), where OGG1 was not required for the recruitment of CSB to locally induced oxidative DNA damage generated by photo-activation of Ro-19-8022 (32). Furthermore, as we found that PARP1’s enzymatic activity is not required for the global CSB-chromatin association induced by menadione, this result suggests that the enhanced chromatin association kinetics mediated by PARP1 is likely the result of direct protein-protein interaction (Fig. 5).

The global CSB-chromatin association induced by menadione treatment differs from UV-induced CSB-chromatin association (37) in that the later requires ATP hydrolysis by CSB, inducing a conformational change in CSB that exposes a chromatin-interacting domain in the C-terminal region. Our results are consistent with a model in which the association of PARP1 with CSB leads to the exposure of a chromatin-binding domain within the C-terminal region, which occurs in an ATP-independent manner. In vitro binding assays by Thorslund et al. identified two regions of CSB that interact with PARP1; one lies between residues 2-341 and the other lies between 953-1204 (35). CSB<sup>2-341</sup> is part of the N-terminal region, and CSB<sup>953-1204</sup> spans part of the ATPase domain and the C-terminal regions. Given that the ATPase domain and C-terminal regions contain DNA binding surfaces (37), our results are consistent with a model in which PARP1 brings CSB to chromatin via direct protein-protein interaction, and that CSB uses its ATPase domain and C-terminal region to further stabilize its association at sites of single-stranded DNA damage created by menadione treatment. In agreement with this model, CSB-C lacks one of the PARP1 binding regions, which may account
PARP1 targets CSB to chromatin upon oxidative stress

for the delayed kinetics of chromatin association. While sufficient to bind to PARP1 in vitro (ref.35) CSB-N lacks chromatin-binding domains, and thus fails to associate with chromatin upon menadione treatment (Fig. 2D). Moreover, our observation that DRB does not affect menadione-induced global CSB-chromatin association (Fig. 3) suggests that sites of DNA lesions where CSB binds upon oxidative stress are independent of transcription regulation. This is in sharp contrast to the essential function of CSB in TC-NER, where CSB is delivered to bulky DNA lesion-stalled transcription (41). In the case of TC-NER, when cells are treated with DRB, CSB is not recruited to chromatin after UV irradiation (Fig. 3C).

Repair of single-strand DNA breaks occurs rapidly, within minutes (42,43). The study by Bryant et al. revealed that there are two components of single-strand DNA repair, an initial fast repair phase with a \( t_{1/2} \) of 5-6 min, followed by a slow repair phase, which was proposed to be the repair of ssDNA breaks generated by base-excision DNA repair (44). On average, one PARP1 molecule scans approximately 10 nucleosomes of chromatin. This rapid scanning function is believed to enable PARP1 to quickly detect DNA damage (45). Therefore, a delay of 10-15 minutes in CSB recruitment would be significant, relative to PARP1 function in single-stranded DNA repair.

Based on the work from Aherne and O'brien (2000), treating Caco-2 cells with 10 uM menadione for 30 minutes creates 348 ± 8 single-strand DNA breaks, as determined by the comet assay (38). One possible reason that we do not see increased menadione-induced PARP1-chromatin association is that the fraction of PARP1 binding to single-strand breaks generated by menadione is small relative to the total number of PARP1 molecules performing additional functions. It is also important to note that PARP1 binds to chromatin through multiple domains. For example, PARP1 binds to nucleosomes through its affinity to core histones via its C-terminal region, while PARP1 binds to DNA lesions through its zinc fingers.

We also found that CB245-365 (CSBΔN1), a CSB derivative that is devoid of any chromatin remodeling activity (39), cannot complement the menadione sensitivity of CSB-deficient cells (Fig. 7B). This indicates that the chromatin remodeling activity of CSB is required for CSB’s function in the repair of menadione-induced DNA damage or the transcriptional response to oxidative stress, or both. In the case of DNA repair, CSB may function to displace PARP1 to facilitate single-strand DNA break repair, as proposed by Scheibye-Knudsen et al. (2014) (36). Additionally, our results with CSBΔN1 (Fig. 7B) suggest that CSB may facilitate single-strand break repair by opening up chromatin structure. Indeed, prior studies have shown that PARP1 can recruit other chromatin-remodeling complexes, such as ALC1, CHD2 and SNF2h, to facilitate DNA repair (46-49).

Previously, we had shown that menadione treatment promotes the occupancy of CSB at specific loci throughout the genome, with a significant enrichment in promoters and sites containing the binding motifs of the CTCF transcription factor, and this site-specific occupancy likely reflects a role that CSB plays in mounting a transcriptional response to oxidative stress (20). Here, we have shown that decreasing PARP1 protein levels can significantly decrease the menadione-induced enhancement of site-specific CSB occupancy (Figs. 6A and S5). PARP1 has been suggested to regulate transcription through multiple mechanisms (50,51); therefore, decreased PARP1 levels may reduce transcription at specific loci, and this may lead to decreased CSB occupancy at these sites (Figs. 6A and S5). This hypothesis is supported by our observation that inhibiting RNA pol II transcription elongation with DRB or α-amanitin also decreases the enhancement of site-specific CSB occupancy induced by menadione (Fig. 6C-D). Interestingly, the enzymatic activity of PARP1 is only required at a subset of the loci examined (Fig. 6B). PARP1 has recently been found to regulate transcription elongation, in part, by ADP-ribosylating and, thus, inhibiting negative elongation factor (NELF) (45). Our results are consistent with the notion that the requirement for PARP1 activity in transcription is context dependent (Fig. 6A) (46) and suggests that PARP1 likely enhances CSB occupancy on chromatin through both activity-dependent and -independent mechanisms.

Together, we would like to propose that, in addition to DNA repair, as assayed by global
chromatin association, CSB likely functions together with PARP1 and CTCF to regulate transcription upon oxidative stress (Fig. 7C). Both PARP1 and CTCF can facilitate locus-specific CSB-chromatin association in cells treated with menadione (Figs. 6A and S5), and these two proteins have been shown to work together to regulate long-range chromatin structure and transcription regulation (52). Therefore, the menadione-induced locus-specific CSB-chromatin association may represent sites where CSB functions with PARP1 and CTCF to regulate long-range chromatin interactions to facilitate menadione-induced transcription regulation.

We previously demonstrated that CSB and CTCF can reciprocally regulate each other’s occupancy at specific genomic loci upon oxidative stress, and we hypothesized that CSB may cooperate with CTCF by altering 3-D genome organization to facilitate the relief of oxidative stress (20). While the role of this 3-D genome reorganization may be to regulate gene expression, this study also opens up the possibility that 3-D chromatin reorganization mediated by CTCF and CSB may facilitate the formation of hubs for the repair of single-strand DNA breaks identified by PARP1.

**Experimental procedures**

**Cell culture and treatment protocol**

CS1AN-sv cells and CS1AN-sv cells stably expressing CSB or mutant CSB proteins were maintained in DMEM-F12 supplemented with 10% FBS (10,39). 293T cells were maintained in DMEM supplemented with 10% FBS. All cells were cultured at 37°C in 5% CO₂. CS1AN-sv cells stably expressing CSB, CSB<sup>R670W</sup>, CSBΔN, CSBΔC were expressed as previously described (37). CS1AN cells stably expressing CSB-N and CSB-C were generated by transfecting cells with CSB-N or CSB-C expression plasmids and selecting with 600 µg/mL G418 (37). Oxidative stress was induced by treating cells with 100-µM menadione (MP Biomedicals, 102259). The PARP inhibitor, KU-0058948 hydrochloride (Axon Medchem, 2001), was used at a final concentration of 1 µM for 1 hour (53). RNA pol II transcription elongation was inhibited by treating cells with 50 µM 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, Sigma-Aldrich, D1916) for one hour prior to treatment with menadione (37). Cells were treated with transcription inhibitor α-amanitin (Cayman Chemical Co, 17898) at 1 µg/ml for 1 hour before to menadione treatment. Menadione was added directly to the DRB-, α-amanitin- or KU-0058948-containing medium. For the UV control experiment, cells were treated with 50 µM DRB for 1 hour and then irradiated with 100 J/m² UV (245 nm) using a Stratalinker (37). Cells were allowed to recover for one hour prior to processing.

**Protein fractionation and western blotting**

Equal numbers of cells were seeded onto 60 mm dishes and allowed to grow overnight to ~80% confluence. Media was changed on all plates and cells were left untreated or treated with 100 µM menadione for indicated times. Cells were lysed and proteins were fractionated as described previously (20,37). Briefly, cells were rinsed with PBS, collected in 200 µl buffer B (150 mM NaCl, 0.5 mM MgCl₂, 20 mM HEPES (pH 8.0), 10% glycerol, 0.5% Triton X-100, 1 mM DTT) on ice and centrifuged at 15,000 rpm for 20 minutes at 4°C. 150 µl supernatant was added to 50 µl 4x SDS sample buffer (soluble fraction, ‘s’) and 200 µl 1xSDS sample buffer was added to the pellet, which was sonicated for 10 seconds at 25% amplitude with a Branson 101-135-126 Sonifer (chromatin-enriched fraction, ‘c’), 1.3 times more concentrated than ‘s’). Proteins were run on a NuPAGE<sup>™</sup> 4-12% Bis-Tris protein gel (Invitrogen, NP0323BOX) with the BenchMark<sup>™</sup> pre-stained protein ladder (Invitrogen, 10748-010) and gels are labeled with molecular weight markers (kDa). The loading ratios between the ‘s’ and ‘c’ fractions was 1:1.25, if unspecified. Western blots were developed using SuperSignal West Pico or Dura chemiluminescent substrate (ThermoFisher Scientific, 34580 and 34075), and imaged with a Fujifilm ImageQuant LAS-4000 imager or developed using a Kodak Processor M35A. To determine the percentage of CSB co-fractionated with chromatin, images were scanned and quantified using ImageJ. Determination of percent CSB co-fractionated with chromatin was calculated as previously described by
normalizing respective to BRG1 and adjusting for the 1.25-fold more concentrated chromatin-enriched fraction if not specified (20).

**Lentiviral shRNA knockdown**
Mission shRNA targeting OGG1 (TRCN0000314740), APE1 (TRCN0000007958), PARP1 (TRCN0000007932) (54,55), and a non-targeting shRNA (SHC002) were from Sigma-Aldrich. Virus was produced as previously described (20). Briefly, virus was produced by co-transfecting 293T cells with shRNA and the third generation lentiviral packaging plasmids pMGLg-RRE, pRSV-REV, and pMD2.G/VSV. Media was changed 24 hours after transfection and virus-containing medium was collected 24 hours later. The target cell confluence at time of infection was ~20%. Media was changed 24 hours after infection, and cells were harvested at 72 hours (PARP1, APE1) or 96 hours (OGG1) post-infection.

**ChIP-western and ChIP-qPCR analyses**
Chromatin immunoprecipitation (ChIP) was carried out as previously described (10,20). Briefly, approximately 4 million cells were collected after treatment, fixed, and processed for sonication. Fixed chromatin was sonicated on ice for 12 cycles (30 sec on, 90 sec off) with 40% amplitude, using a Branson Sonifier 150T. In general, the size range of sonicated chromatin is between 200 bps and 1 kb with a peak of 500 bps (see Fig. S6 for a representative gel showing DNA fragmentation size range). 5 µl monoclonal anti-CSB antibody (1B1) (10) and 5 µl protein-G agarose beads (Invitrogen, 15920010) were used in each ChIP. Real-time PCR was done using a 7900HT Fast Real-Time PCR System (Applied Biosystems) and SensiFAST™ Sybr Hi-Rox Mix (Bioline, BIO-92020) following manufacturer instructions. Primers are listed in Table S1. Real-time PCR data were analyzed using ∆∆Ct method (56). For ChIP-western analysis, ChIP was conducted as above following treatment with 100 µM menadione for 30 minutes. Samples were reverse cross-linked in 1xSDS sample buffer at 95°C for 30 minutes and immediately ran on a gel (39).

**Antibodies**
Antibodies used for western blot analysis were rabbit polyclonal anti-CSB antibodies to the N-terminus (Jasmine) or C-terminus (Libra) (both used at 1:2000) (provided by Dr. Weiner, University of Washington) (37), rabbit polyclonal anti-BRG1 (1:2000) (provided by Dr. Kingston, MGH) (37), rabbit polyclonal anti-XRCC1 (1:1000) (Cell Signaling Technology, 2735), rabbit polyclonal anti-PARP1 (1:1000) (Cell Signaling Technology, 9542), rabbit polyclonal anti-γ-H2A.X (1:1000) (Cell Signaling Technology, 2595), rabbit polyclonal anti-CTCF (1:2000) (Millipore, 07-729), mouse monoclonal anti-RNA polymerase II (1:500) (Covance, H5), rabbit polyclonal anti-acetyl-Histone H3 (1:1000) (Millipore, 06-599), rabbit polyclonal anti-histone H3 (1:2000) (Cell Signaling Technology, 9715), mouse monoclonal anti-GAPDH (1:10,000) (Millipore, MAB374), rabbit polyclonal anti-OGG1 (1:10,000) (Abcam, ab124741), rabbit polyclonal anti-APE1 (Cell Signaling Technology, 4128S), HRP-conjugated goat anti-rabbit IgG (1:10,000) (Pierce, 31460), and HRP-conjugated goat anti-mouse (1:10,000) (Jackson Laboratory, 115-035-044). ChIP was performed using the N-terminal anti-CSB antibody 1B1 (10). Poly ADP-Ribose (PAR) was analyzed using mouse monoclonal anti-PAR (1:1000) (Tulip BioLabs, #1020/N) and peroxidase-conjugated AffiniPure goat anti-mouse IgG, Fcγ Subclass 3 Specific (1:2000) (Jackson ImmunoResearch Laboratories, Inc., 115-035-209).

**Menadione sensitivity assay**
Approximately 100,000 cells were seeded onto 35 mm dishes in DMEM/F12 medium supplemented with 10% FBS and allowed to grow for 24 hours at 37°C. Cells were then given fresh media and left untreated or treated with indicated concentrations of menadione for 1 hour. After 1 hour, menadione-containing medium was removed and fresh medium without menadione was added. Cells were cultured for an additional 24 hours at which point cell viability was determined by trypan blue exclusion using a hemocytometer. Percent survival was calculated as the ratio of treated cells to untreated cells (20).
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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.
REFERENCES


PARP1 targets CSB to chromatin upon oxidative stress


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The abbreviations used are: CSB, Cockayne syndrome protein B; TC-NER, transcription-coupled nucleotide excision repair; ROS, reactive oxygen species; BER, base excision repair; AP site, apurinic-apyrimidinic site; APE1, apurinic-apyrimidinic endonuclease 1; PARP1, poly(ADP-ribose) polymerase 1; PAR, poly(ADP-ribose); polβ, DNA polymerase β; Lig3, DNA ligase IIIα; OGG1, oxoguanine glycosylase 1; 8-oxoG, 8-oxo-guanine; CTCF, CCCTC-binding transcription factor; RNA pol II, RNA polymerase II; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; SDS, sodium dodecyl sulfate; ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; BRG1, brahma-related gene-1; XRCC1, X-ray repair cross-complementing protein 1; shRNA, short hairpin RNA.
Figure 1. The association of CSB with chromatin in response to menadione treatment occurs independently of ATP hydrolysis. (A) Protein fractionation assay in CS1AN-CSB<sup>WT</sup> cells following treatment with 100 µM menadione for times indicated. Western blots were probed with antibodies listed. BRG1 was used as a loading control. Acetylated histone H3 and total core histones (visualized by Ponceau S staining) were used as markers for the chromatin-enriched fraction. GAPDH was used as a marker for the soluble fraction. (B) Quantification of percent CSB<sup>WT</sup> (n=5), PARP1 (n=4), XRCC1 (n=4), and CSB<sup>R670W</sup> (n=2) in the chromatin-enriched fraction as a function of time, normalized to BRG1. Error bars represent SEM. (C) CSB ChIP-western analysis in CS1AN-CSB<sup>WT</sup> cells untreated (-) or treated with 100 µM menadione for 30 minutes (+). Numbers at the bottom show fold change in histone H3 normalized to CSB (n=2). (D) Protein fractionation assay in CS1AN-CSB<sup>R670W</sup> cells following treatment with 100 µM menadione for times indicated (n=2). Shown is a representative western blot probed with antibodies to CSB and BRG1, and stained with Ponceau S.
PARP1 targets CSB to chromatin upon oxidative stress

Figure 2. The association of CSB with chromatin in response to menadione treatment is largely mediated through its ATPase domain and C-terminal region. (A) Schematic representation of the CSB protein and CSB deletion constructs used in the protein fractionation assays. Grey boxes represent the seven conserved helicase motifs, thin black boxes represent the two putative nuclear localization signals (NLS), and the thick black box represents the ubiquitin binding domain (UBD). (B-E) Protein fractionation assays demonstrating chromatin association as a function of time after 100 µM menadione treatment, in CS1AN-sv cells reconstituted with the indicated CSB derivatives: CSBWT (n=5) (from Fig. 1A) and (B) CSBΔN (n=2), (C) CSBΔC (n=3), (D) CSB-N (n=2), and (E) CSB-C (n=4). Shown are representative western blots probed with the indicated antibodies and stained with Ponceau S for histones. Plots show quantification of the western blot data with CSB signals normalized to BRG1 signals. Error bars represent SEM. Paired t-tests compare CSB-derivative enrichment to CSBWT (* p<0.05, ** p<0.01, *** p<0.001).
Figure 3. Inhibiting transcription elongation of RNA pol II by DRB does not alter menadione-induced CSB-chromatin association. (A) Protein fractionation assay in CS1AN-CSB\textsuperscript{WT} cells. Cells were treated with 50 µM DRB or DMSO for 1 hour followed by a 100 µM menadione treatment for 20 minutes. Representative western blot probed with antibodies listed. “S” denotes the soluble protein fraction and “C” denotes the chromatin-enriched protein fraction. (B) Quantification of CSB chromatin co-fractionation data in (A) normalized to BRG1. Shown are means ± SEM, and paired t-test compares enrichment in cells with DMSO versus DRB treatment (n=3, ns: not significant). (C) Protein fractionation assay in CS1AN-CSB\textsuperscript{WT} cells treated with 50 µM DRB or DMSO for 1 hour, followed by 100 J/m\textsuperscript{2} of UV irradiation. Cells were analyzed 1 hour after UV treatment.
Figure 4. APE1 or OGG1 are dispensable for menadione-induced global CSB-chromatin association.

(A) Representative western blot revealing the extent of APE1 knockdown (average knockdown ~72%, normalized to GAPDH). (B-C) Protein fractionation assay revealing CSB-chromatin association as a function of time after menadione treatment in CS1AN-CSB\textsuperscript{WT} cells expressing a control (ctrl) or APE1 shRNA. Representative western blot probed with antibodies listed and stained with Ponceau S. (D) Quantification of data in B and C showing percent CSB co-fractionating with chromatin. Error bars represent SEM. Paired \textit{t}-test comparing CSB enrichment in control vs APE1 knockdown ($n=4$) revealed no significant differences in association kinetics. (E) Representative western blot revealing the extent of OGG1 knockdown (average knockdown ~90%, normalized to GAPDH). (F-G) Protein fractionation assay revealing CSB-chromatin association as a function of time after menadione treatment in CS1AN-CSB\textsuperscript{WT} cells expressing a control (ctrl) or OGG1 shRNA. Representative western blot probed with antibodies listed and stained with Ponceau S. (H) Quantification of data in F and G showing percent CSB co-fractionating with chromatin. Error bars represent SEM. Paired \textit{t}-test comparing CSB enrichment in control to OGG1 knockdown ($n=4$, * $p \leq 0.05$).
Figure 5. The PARP1 protein, but not its enzymatic activity, is required for efficient, global CSB-chromatin association in response to menadione treatment. (A) Representative western blot revealing the extent of PARP1 knockdown (average knockdown ~89%, normalized to GAPDH). (B-C) Protein fractionation assay revealing CSB-chromatin association as a function of time after menadione treatment in CS1AN-CSB<sup>WT</sup> cells expressing a control (ctrl) or PARP1 shRNA. Representative western blot probed with antibodies listed and stained with Ponceau S (the loading ratio of soluble to chromatin is 1: 2.2). (D) Quantification of data in B and C showing percent CSB co-fractionating with chromatin. Error bars represent SEM. Paired t-test comparing CSB enrichment in control vs PARP1 knockdown (n=4, * p<0.05). (E) Western blot probed with an anti-PAR antibody demonstrating PARP1 inhibition by KU-0058948. (F-G) Protein fractionation assay of CS1AN-CSB<sup>WT</sup> cells treated with DMSO (vehicle control) or KU-0058948, followed by the addition 100 µM menadione for the indicated times. Representative western blots probed with antibodies listed and stained with Ponceau S (the loading ratio of soluble to chromatin is 1: 1.25). (H) Quantification of data in F and G showing percent CSB co-fractionating with chromatin. Error bars represent SEM. Paired t-test comparing CSB enrichment in DMSO vs KU-0058948 treated cells (n=5) revealed no significant difference.
Figure 6. PARP1 and active transcription contribute to menadione-induced CSB occupancy at specific genomic loci. ChIP-qPCR analyses of CSB recruitment to specific genomic loci in response to menadione treatment. Shown are four loci where CSB occupancy is significantly enhanced by oxidative stress (chrX-1, chrX-2, chr17-1, and chr19-2) and a control locus where CSB occupancy is not changed by oxidative stress (chr12-7). (A) CSB ChIP-qPCR analyses of CS1AN-CSB<sup>WT</sup> cells expressing a control (ctrl) or PARP1 shRNA. Shown are means ± SEM, n=3. (B) CSB ChIP-qPCR analyses as above except that cells were exposed to KU-0058948 (PARP1 i) or DMSO for 1 hour prior to menadione treatment. Shown are means ± SEM, n=2. (C) CSB ChIP-qPCR analyses of cells exposed to DRB or DMSO for 1 hour prior to menadione treatment. Shown are means ± SEM, n=2. (D) ChIP-qPCR analyses of CSB enrichment at specific genomic loci in cells with (aA) or without (mock) α-amanitin treatment, prior to menadione treatment. Cells were treated with 1 mg/ml of α-amanitin for 1 hour before to menadione treatment for 20 min. Shown are means +/- SEM, n=2. Paired t-test comparing CSB enrichment (* p<0.05, ** p<0.01, *** p<0.001).
PARP1 targets CSB to chromatin upon oxidative stress

Figure 7. Models for CSB’s functions during oxidative stress. (A) Single-stranded DNA breaks generated by reactive oxygen species are recognized by PARP1. Localization of PARP1 to single-stranded breaks facilitates the recruitment of CSB. CSB binds chromatin through its ATPase domain. Upon oxidative stress, PARP1 binds to CSB’s N- and C-terminal regions, and this interaction exposes a chromatin interaction surface in the C-terminal region of CSB that stabilizes CSB-chromatin association. CSB may function to make the chromatin landscape more permissible for DNA repair and/or to regulate repair-protein retention at sites of repair. (B) Menadione sensitivity assays. The chromatin remodeling deficient CSBΔN1 derivative does not complement the menadione sensitivity of CS1AN-sv cells. Paired t-test comparing CS1AN-CSBWT to CS1AN-CSBΔN1 (n=5, * p≤0.05, *** p≤0.001). (C) Menadione-induced CSB occupancy at specific genomic loci is dependent on PARP1 (this study) and CTCF (20). These proteins may likely organize higher-order chromatin structure to mount a transcriptional response to oxidative stress.
Poly(ADP-ribose) polymerase 1 (PARP1) promotes oxidative stress–induced association of Cockayne syndrome group B protein with chromatin

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Figure S1. CSB and CSB derivative expression in CS1AN-sv cells. (A) Western blots showing CSBWT and mutant CSB protein expression levels. Values report GAPDH normalized mutant protein levels relative to GAPDH normalized CSBWT level. The second western blot at the top shows CSBWT expression level in CS1AN fibroblasts relative to endogenous CSB in the MRC5 fibroblast cell line. The arrow indicates CSB-N, while other bands in that blot are background, present in both cell lines. (B) Immunofluorescence images demonstrating that CSBWT and all CSB mutant constructs localize to the nucleus of CS1AN cells (CSB green, DAPI blue). (C) Comparison of chromatin association of CSBΔC with CSB-N in response to menadione treatment. Protein fractionation assays of chromatin association in CS1AN-sv cells reconstituted with the indicated CSB derivatives, following treatment with 100 µM menadione. CSBΔC (n=3), and CSB-N (n=2). Error bars represent SEM. Paired t-test compares construct enrichment to CSBWT (* p<0.05).
Figure S2. PARP1 decreases CSB-chromatin co-fractionation regardless of BRG1 normalization.

Eleven replicate experiments are depicted comparing the impact of control shRNA (ctrl) and PARP1 shRNA on CSB-chromatin co-fractionation following treatment with 100 µM menadione for 20 minutes. (A) Values were normalized to BRG1. (B) Values are not normalized. Shown are means ± SEM. Paired t-test compares enrichment in cells with control vs PARP1 shRNA (n=11, * p<0.05, ** p<0.01, *** p<0.001).
Figure S3. PARP1 enhances CSB-chromatin association upon menadione treatment. (A) Western blot analysis to determine the extents of PARP1 KD using a second shRNA targeting PARP1. CS1AN-sv reconstituted with CSB were treated with control shRNA or PARP1 shRNA (#2) for 96 hours. (B) shRNA treated cells were incubated with menadione for the times indicated, and protein fractionation was carried out (the loading ratio of soluble to chromatin is 1:2.2). Western blots were probed with antibodies against CSB, GAPDH, and stained with Ponceau S to show histone proteins. (C) Quantification of data in B showing percent CSB co-fractionating with chromatin. Error bars represent SEM (n=3).
Figure S4. **CSB does not contribute to global PARP1-chromatin association upon menadione treatment.** CS1AN-sv and CS1AN-sv reconstituted with CSB were treated with 100 uM menadione. Protein fractionation was carried out at the indicated times (the loading ratio of soluble to chromatin is 1:4.4). Western blots were probed with antibodies against CSB, PARP1, and stained with Ponceau S to reveal histone proteins.
Figure S5. PARP1 contributes to menadione-induced locus-specific CSB binding. ChIP-qPCR analyses of CSB recruitment to specific genomic loci in response to menadione treatment. Shown are four loci where CSB occupancy is significantly enhanced by oxidative stress (chrX-1, chrX-2, chr17-1, and chr19-2) and a control locus where CSB occupancy is not changed by oxidative stress (chr12-7). (A) Western blot analysis showing PARP1 protein levels in cells expressing PARP1 shRNA (TRCN0000007929) for 96 hours. (B) CSB ChIP-qPCR analyses of CS1AN-CSBWT cells expressing a control (ctrl) or the second PARP1 shRNA. Shown are mean +/- SEM, n=2.
Figure S6. A representative gel showing DNA fragmentation size range of sonicated chromatin. CS1AN-CSBWT cells were fixed, and processed for sonication using the protocol described in Experimental Procedures. Sonicated chromatin was reverse-crosslinked overnight at 65°C, and ~500 ng of input DNA was resolved on a 1% agarose gel.
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