Human CST Facilitates Genome-wide RAD51 Recruitment to GC-Rich Repetitive Sequences in Response to Replication Stress

Highlights

- STN1 is enriched at GC-rich repetitive sequences in response to replication stress
- STN1 suppression exacerbates the fragility of these sequences under replication stress
- CST interacts with RAD51 in an ATR-dependent manner
- CST deficiency diminishes RAD51 foci formation and recruitment to fragile sequences

In Brief

Chastain et al. find that under replication stress, the telomeric complex CST interacts with RAD51 and is enriched at GC-rich repetitive fragile sites. CST suppression inhibits RAD51 recruitment to fragile sites, resulting in genome instability.

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Human CST Facilitates Genome-wide RAD51 Recruitment to GC-Rich Repetitive Sequences in Response to Replication Stress

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SUMMARY

The telomeric CTC1/STN1/TEN1 (CST) complex has been implicated in promoting replication recovery under replication stress at genomic regions, yet its precise role is unclear. Here, we report that STN1 is enriched at GC-rich repetitive sequences genome-wide in response to hydroxyurea (HU)-induced replication stress. STN1 deficiency exacerbates the fragility of these sequences under replication stress, resulting in chromosome fragmentation. We find that upon fork stalling, CST proteins form distinct nuclear foci that colocalize with RAD51. Furthermore, replication stress induces physical association of CST with RAD51 in an ATR-dependent manner. Strikingly, CST deficiency diminishes HU-induced RAD51 foci formation and reduces RAD51 recruitment to telomeres and non-telomeric GC-rich fragile sequences. Collectively, our findings establish that CST promotes RAD51 recruitment to GC-rich repetitive sequences in response to replication stress to facilitate replication restart, thereby providing insights into the mechanism underlying genome stability maintenance.

INTRODUCTION

Faithful and complete duplication of chromosomal DNA is vital for avoiding detrimental replication errors and preserving genome stability. Replication stress, induced by exposure to environmental agents, oncogenic stress, or partial inhibition of DNA replication, results in fork stalling at fragile sites (FSs) that may lead to fork collapse, thereby generating DNA breaks that trigger unwanted repair or rearrangement activities and driving genome instability (Debacker and Kooy, 2007; Debatisse et al., 2012; Durkin and Glover, 2007; Tercero et al., 2003). FSs are frequently involved in sister chromatid exchanges, deletions, translocations, and intra-chromosomal gene amplifications (Durkin and Glover, 2007). Important genes, including certain tumor suppressors, have been identified within FSs (Arlt et al., 2006; Barlow et al., 2013; Debacker and Kooy, 2007; Durkin and Glover, 2007). Thereafter, pathways have evolved to prevent fork stalling and to facilitate the restart of stalled replication to preserve genome stability.

Successful rescue of stalled replication requires coordination of multiple proteins that stabilize stalled forks and promote reinitiation of DNA synthesis (Franchitto and Pichierri, 2014; Zeman and Cimprich, 2014). Crucial genome maintenance proteins, including RAD51, MRE11, XRCC3, SLX1-SLX4-MUS81-EME1, BLM, WRN, RTEL1, SMARCA1, and FANCD2, play important roles in this process (Betous et al., 2012; Bryant et al., 2009; Davies et al., 2007; Franchitto and Pichierri, 2004; Hanada et al., 2007; Hashimoto et al., 2010; Pepe and West, 2014; Petermann and Helleday, 2010; Petermann et al., 2010; Sarbajna et al., 2014; Schlacher et al., 2008; Sidorova et al., 2008; Tittel-Elm et al., 2009; Vannier et al., 2013). In addition, TIMELESS, TIPIN, CLASPIN, and AND1 form the replication protection complex that stabilizes stalled forks and keeps helicases connected to polymerases, thus preventing excessive DNA unwinding (Chini and Chen, 2003, 2004; Enrico et al., 2007, 2009; Gotter et al., 2007; Kemp et al., 2010; Kumagai and Dunphy, 2000; Lee et al., 2003; Unsal-Kacmaz et al., 2007; Zhu et al., 2007). Fork restart also requires reinitiation of DNA synthesis mediated by various replication factors, including MCM2-7, PCNA, CDC45, and POLβ (Heller and Marians, 2006), and the PrimPol primase that is important for priming DNA synthesis at stalled forks (Bianchi et al., 2013; Garcia-Gomez et al., 2013; Mouron et al., 2013).

The human CST complex (hCST), composed of the three proteins CTC1, STN1, and TEN1, has emerged as an important player in counteracting replication stress. CST is an RPA-like complex that binds non-specifically to single-stranded DNA (ssDNA) with high affinity (Miyake et al., 2009). Originally discovered as a telomere maintenance factor (described later), the hCST complex also promotes efficient replication of difficult-to-replicate sequences in the genome (Kasbek et al., 2013; Stewart et al., 2012). Deficiency in CST components reduces cell viability after exposure to reagents stalling replication forks,
including hydroxyurea (HU), aphidicolin (APH), methyl methanesulfonate (MMS), and camptothecin (Wang et al., 2014; Zhou and Chai, 2016). Mutations in CTC1 cause Coats plus disease, a complex disorder characterized by bilateral exudative retinopathy, retinal telangiectasias, growth retardation, intracranial calcifications, bone abnormalities, and gastrointestinal vascular ectasias, accompanied by common early-aging pathological features such as premature hair graying, anemia, and osteoporosis (Anderson et al., 2012; Armanios and Blackburn, 2012; Keller et al., 2012; Polvi et al., 2012). DNA fiber analysis shows that STN1 suppression decreases new origin firing following release from HU (Stewart et al., 2012), while other studies show that CST stimulates the priming activity of DNA polymerase α-primase (POLα) and primase-to-polymerase switching in vitro and increases the affinity of POLα for template DNA (Casteel et al., 2009; Lue et al., 2014; Nakaoa et al., 2012). However, evidence supporting the involvement of CST’s stimulatory effect on POLα in replication restart is lacking. The molecular mechanism underlying how CST facilitates replication restart at non-telomeric sites remains largely unknown; moreover, the interplay between CST and other key replication restart players remains unexplored.

The role of CST in telomere maintenance is better defined. The hCST complex interacts with the TPP1-POT1 shelterin complex (Chen et al., 2012; Wan et al., 2009). Mammalian CST promotes efficient replication of telomeric DNA, mediates C strand synthesis at telomere ends during late S/G2 phase, and inhibits telomerase access to telomeres to prevent excessive telomere lengthening (Chen et al., 2012; Huang et al., 2012; Kasbek et al., 2013; Stewart et al., 2012; Wang et al., 2014). A subset of CTC1 mutations identified in Coats plus patients induces accelerated telomere shortening and displays telomeric DNA replication defects (Anderson et al., 2012; Chen et al., 2013; Gu and Chang, 2013), leading to the conclusion that the pathogenesis of Coats plus may partly derive from telomere maintenance defects. CST-related proteins are also present in Saccharomyces cerevisiae, Schizosaccharomyces pombe, Tetrahymena thermophila, and Arabidopsis thaliana (Gao et al., 2007; Martin et al., 2007; Surovtseva et al., 2009; Wan et al., 2015). In budding yeast, the Cdc13-Strn1-Ten1 complex controls telomerase access to telomeres and is an essential component for capping telomere ends that prevent the C strand from degradation (reviewed in Giraud-Panis et al., 2010, and references therein). CST proteins in fission yeast and plants are also required for telomere capping (Martin et al., 2007; Song et al., 2008; Surovtseva et al., 2009), while human and mammalian CST lack the capping function (Boccardi et al., 2015; Gu et al., 2012; Huang et al., 2012). It appears that the telomere capping function of CST in yeasts and plants has been lost during evolution, but its functions in telomere replication and C strand synthesis have been preserved (Price et al., 2010).

Each CST subunit contains the oligonucleotide- or oligosaccharide-binding (OB)-fold domains formed by five-stranded beta-barrel motifs. The OB-fold domains are known to be frequently used in binding to ssDNA or single-stranded RNA and in mediating protein-protein interactions (Arcus, 2002; Flynn and Zou, 2010; Theobald et al., 2003). STN1 and TEN1 structures are highly conserved from yeast to humans, with conformational similarities to the two smaller subunits of the RPA complex RPA32 and RPA14 (Bryan et al., 2013; Sun et al., 2009; Wan et al., 2015). TEN1, the smallest subunit, contains a single OB fold that interacts with the N-terminal portion of STN1. Human STN1 contains an OB-fold domain in the N terminus and two wing-helix-turn-helix (wHTH) motifs at the C terminus (Bryan et al., 2013), and both domains are required for CTC1 and TEN1 interaction (Miyake et al., 2009). Although the structure of the largest subunit CTC1 is unavailable, its predicted structure contains multiple putative OB-fold domains, with the C-terminal OB fold interacting with STN1-TEN1 (Chen et al., 2013; Miyake et al., 2009). The hCST complex efficiently binds to 32 nt or longer single-strand oligonucleotides containing a variety of G- or C-rich sequences with high affinity (Miyake et al., 1996). In vitro binding assays reveal that the C-terminal half of CTC1 is important for DNA binding, whereas both the N and the C termini of CTC1 are required for telomeric DNA binding in vivo, suggesting that telomeric association of CST relies on DNA binding, complex formation, and interaction with POLα (Chen et al., 2013).

In this study, we investigate the molecular mechanism by which CST promotes the restart of stalled replication. We employ chromatin immunoprecipitation sequencing (ChIP-seq) to map STN1-binding sites in the human genome under HU-induced replication stress. We find that STN1 is significantly enriched at GC-rich repetitive sequences genome-wide after fork stalling. Fluorescence in situ hybridization (FISH) analysis reveals that these STN1-binding sites are prone to breakage under replication stress, and STN1 deficiency further elevates the frequency of instabilities at these sites, resulting in chromosome fragmentation. Next, we show that upon exposure to HU, CST proteins form distinct nuclear foci that colocalize with RAD51. In addition, replication stress markedly induces physical interaction of CST proteins with RAD51 in a DNA-independent manner, and the CST/RAD51 interaction depends on ATR. We observe that suppression of each CST protein drastically impairs HU-induced RAD51 foci formation. Furthermore, ChIP assays show that STN1 suppression reduces RAD51 recruitment to telomeres and non-telomeric GC-rich sequences. Collectively, our findings establish that CST is an important component for maintaining the stability of GC-rich repetitive sequences genome-wide in response to replication stress. We propose that CST facilitates RAD51 recruitment to these sequences when replication forks stall, promoting efficient replication restart and suppressing genome instability under replication stress.

**RESULTS**

**STN1 Is Enriched at Telomeric and Non-telomeric GC-Rich Repetitive Sequences Genome-wide in Response to Replication Stress**

Analysis of gene expression data from several databanks (Oncomine, 2012) reveals that human STN1 expression is universally
suppressed in multiple types of tumors, including colorectal, esophageal, breast, lung, cervical, brain, prostate, gastric, and head and neck cancers (Figure S1; Table S1). The reduced STN1 expression in tumor tissues suggests that STN1 may be important in tumor suppression. It has been shown that STN1 deficiency induces γ-H2AX-labeled DNA damage at non-telomeric sites (Huang et al., 2012) and CST promotes efficient replication restart after fork stalling (Stewart et al., 2012). Thus, we hypothesized that CST might play an important role in protecting the stability of difficult-to-replicate sequences in the genome under replication stress. To understand the nature of these sequences, we employed ChIP-seq to determine the STN1-binding sites genome-wide under replication stress. To overcome the unavailability of ChIP-quality STN1 antibody, we constructed HeLa cells stably expressing Myc-STN1 (HeLa-Myc-STN1) with retroviral transduction. Western blotting showed that the expression level of exogenous Myc-STN1 was comparable to that of endogenous STN1 (Figure 1A), minimizing possible artifacts induced by protein overexpression. We also noticed that expressing exogenous Myc-STN1 suppressed endogenous STN1 expression (Figure 1A), indicating that STN1 expression is likely regulated by a negative feedback mechanism.

HeLa-Myc-STN1 was then synchronized in a G1/S phase boundary with a double-thymidine block, released into S phase for 3 hr, and then treated with 2 mM HU for 3 hr to induce replication stress. Subsequently, ChIP assays were performed with anti-myc antibody (Figure 1B). Fluorescence-activated cell sorting (FACS) analysis showed that HU treatment significantly stalled replication progression, and most cells remained in mid-S phase (Figure 1C). To minimize possible effects caused by different cell-cycle stages, cells synchronized in mid-S phase (3 hr after double-thymidine release) were used as control in ChIP assays. Following ChIP, STN1-binding DNA was subjected either to slot blot to detect STN1 binding to telomeres or to Illumina Hi-Seq 2500 next-generation sequencing to detect its binding sites genome-wide.

Under unstressed conditions, components of the CST complex are associated with telomeres, which are naturally occurring FSs. However, such an association is weak in S phase, and telomeric association of CST is strongest in late S/G2 phase (Chen et al., 2012; Miyake et al., 2009). We confirmed the weak association of STN1 with telomeres in mid-S phase (Figure 1D). Upon HU treatment, STN1 association with telomeres increased (Figure 1D), suggesting additional STN1 recruitment to telomeres in response to replication stress.

Two independent HU treatments and ChIP-seq were performed, giving rise to 3,430 and 2,988 significant ChIP-seq peaks (p < 0.001), respectively. The two experiments showed high reproducibility of genome-wide STN1 association in response to HU, with Spearman correlation coefficient R² = 0.9852, suggesting a high confidence of ChIP-seq data. Snapshots of ChIP-seq peaks at various loci, including STN1-binding and non-binding sites, are shown in Figure 1E. To validate ChIP-seq results, we performed an independent ChIP assay followed by PCR to detect STN1 binding to representative non-telomeric sequences identified from ChIP-seq. Five ChIP-seq loci were analyzed. Upon HU treatment, STN1 was enriched at four of these sites but not at tubulin, GAPDH, or the SLITRK6 loci that were not identified by ChIP-seq (Figure S3), validating our ChIP-seq results. One ChIP-seq locus showed amplification in both Myc-vector-only control and Myc-STN1 ChIP, suggesting that a small portion of peaks might have resulted from non-specific binding of myc antibody during the ChIP-seq experiment.

Analysis of ChIP-seq reads revealed that STN1 was enriched at repetitive sequences, with nearly 90% of sequences containing repetitive features (Figure 2A). These repetitive sequences included long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs), as well as regions of low complexity (>100 nt stretch of >87% AT or 89% GC, and >30 nt stretch with >29 nt poly(N)n, where N denotes any nucleotide) and those containing simple repeats (short tandem repeats like (5’-TTAGGG-3’)x) (Figure 2A). STN1-binding sites also displayed higher G and C contents than chromosomal averages by ~8% GC (p = 1.14e−287,300, chi-square test) (Figure 2B). Many sequences contain G-rich repeats. Examples of STN1-binding sequences containing G-rich repeats are shown in Figure 2C and Figure S4. We also recovered telomeric sequences (sequencing reads with ≥12 tandem 5’-TTAGGG-3’ repeats) from unaligned sequences, confirming STN1 binding to telomeres. Interestingly, 1.3% of non-telomeric sequencing reads contained a 5’-TTAGGG-3’ sequence or sequences, with most of these reads (~98%) containing a single 5’-TTAGGG-3’ sequence in isolation and/or non-tandem 5’-TTAGGG-3’ repeats, corroborating the previously identified lack of sequence specificity of hCST (Miyake et al., 2009). After aligning sequencing peaks to the reference genome, we found that a great portion (73%) of peaks resided within known or predicted CpG islands (epigenetic score ≥ 0.5, p = 1.72e−2017409, chi-square test) (Figure 2D). Altogether, our data suggest that STN1 is preferentially localized at GC-rich repetitive sequences genome-wide.

**STN1-Binding Sites Exhibit Fragility upon Exposure to Replication Stress**

The sequence features of STN1-binding sites, including high GC contents, repetitiveness, and CpG dinucleotides, share similarities with early-replicating fragile sites (ERFSs) identified in mouse B lymphocytes (Barlow et al., 2013). We therefore examined the stability of STN1-binding sequences under replication stress. STN1 was knocked down with two short hairpin RNA (shRNA) sequences (Figure 3A), cells were treated with HU (2 mM), and FISH was performed on chromosome spreads to detect fragility of these sequences. Four representative sites were examined (HU-8, HU-9, HU-10, and HU-12), as well as two non-STN1-binding control sites (ACTIN and SLITRK6). In the absence of replication stress, the FISH signal at individual chromatids is normally represented as a single signal with an intensity that is roughly equal to that at the sister chromatid (Figure 3B). After HU exposure, increased fragility was observed at all four sites, characterized by increased DNA breakage, abnormal signal elongation, bridges, and signals spatially separated from the chromosome (Figure 3B). STN1 deficiency further elevated fragility of these sites (Figure 3C). In some cases, one chromatid arm was severed at the hybridization point and lost...
Control probes exhibited minimal fragility, regardless of STN1 depletion and replication stress (Figure 3C). Concurrently, we observed a marked increase of chromosome fragmentation in HU-treated STN1-deficient cells, suggesting a high level of chromosome instability (Figures 3D and 3E). Our results support that STN1-binding sites identified from ChIP-seq are likely bona fide FSs that are sensitive to replication stress and that functional STN1 is required for protecting the stability of these loci and chromosome integrity under replication stress.

(Figure 3B; HU-9, white circle indicating the missing chromatid arm). Control probes exhibited minimal fragility, regardless of STN1 depletion and replication stress (Figure 3C). Concurrently, we observed a marked increase of chromosome fragmentation in HU-treated STN1-deficient cells, suggesting a high level of chromosome instability (Figures 3D and 3E). Our results support that STN1-binding sites identified from ChIP-seq are likely bona fide FSs that are sensitive to replication stress and that functional STN1 is required for protecting the stability of these loci and chromosome integrity under replication stress.

Given that several STN1-binding sites were fragile after HU treatment (Figure 3), we analyzed whether STN1 might be enriched at known FSs that are prone to break upon replication stress. Human common fragile sites (CFSs) induced by low
doses of APH or oncogene expression have been defined cytogenetically in lymphocytes, fibroblasts, colon epithelial cells, and erythroid cells (Hosseini et al., 2013; Le Tallec et al., 2011, 2013; Miron et al., 2015). These sites are usually megabase-long chromosomal regions and associated with large genes (Helmrich et al., 2011), with CFS fragility being tissue dependent (Letessier et al., 2011). Because CFSs have not been mapped in HeLa cells, we used a group of 111 reported human CFS sequences identified in other cell types (Durkin and Glover, 2007; Fungtammasan et al., 2012; Schwartz et al., 2006) as putative CFSs in HeLa. Because CFSs have not been mapped in HeLa cells, we used a group of 111 reported human CFS sequences identified in other cell types (Durkin and Glover, 2007; Fungtammasan et al., 2012; Schwartz et al., 2006) as putative CFSs in HeLa. Aligning STN1 ChIP-seq peaks to the genome revealed that only a portion of STN1-binding peaks (~25%) overlapped with or were located near these putative CFSs (Figure S5). Despite ChIP-seq peaks mapping to CFSs at a greater frequency than expected for a random distribution (p = 0.017, chi-square test), a large portion of peaks resided outside putative CFSs (Figure S5). In addition, sequences used in Figure 3 (HU-8, HU-9, HU-10, and HU-12) that displayed fragility upon replication stress were all located outside these putative CFSs. Thus, it is likely that STN1 may protect the stability of a group of non-CFS sequences that are sensitive to HU-induced replication stress.

Replication Stress Induces CST Proteins to Form Distinct Foci that Partially Colocalize with RAD51

Because STN1 depletion increased fragility of its binding sequences under HU (Figure 3), we hypothesized that CST might be at stalled forks to promote replication restart. RAD51 is the key player that localizes at stalled forks and is required for stabilizing stalled forks and restarting replication (Art et al., 2006; Hashimoto et al., 2010; Petermann and Hellday, 2010; Petermann et al., 2010). Upon replication stress, RAD51 forms foci at stalled

Figure 2. Mapping STN1-Binding Sites Genome-wide upon HU Exposure

(A) Relative frequency of repetitive sequences among STN1-binding sites. Repetitive sequences were identified using RepeatMasker. LTR, long terminal repeat; DNA, repetitive transposable element; Simple, short tandem repeat; Low complexity, low-complexity repeat; RNA, RNA repeat, including tRNA, rRNA, small nuclear RNA, small cytoplasmic RNA, and signal recognition particle RNA.

(B) STN1-binding sequences contain higher G and C nucleotide content compared to the average of GC content of each human chromosome.

(C) One example of GC-rich repetitive genomic sequences identified from STN1 ChIP-seq. Gs and Cs are indicated in bold. Continuous Gs or Cs with at least 4 nt in length are underlined. CpG islands are in yellow. 5'-TTAGGG-3' and variants of 5'-TTAGGG-3' sequences are in green.

(D) Proportion of STN1-binding sites containing CpG islands.
A

shRNA: STN1, STN1-2, STN1-4
Actin

B

HU-8 chr 12
HU-9 chr 20
HU-10 chr 16
HU-12 chr 9

shLuc HU-
shLuc HU+
shSTN1 HU+

C

Frequency of Abnormalities

D

shLuc HU-
shSTN1-2 HU+
shSTN1-4 HU+

E

% Metaphase Spreads With Chromosome Fragmentation

(legend on next page)
forns. We then examined whether CST colocalized with RAD51 after stress. We constructed HeLa cells stably expressing FLAG-STN1 (HeLa-FLAG-STN1) using retroviral transduction. As shown in Figure 4A, STN1 showed weak and dispersed nuclear staining without replication stress. In striking contrast, it formed distinct foci following HU exposure (Figure 4A). Simultaneously, increased colocalization of FLAG-STN1 foci with RAD51 was observed (Figures 4B and 4C), suggesting that STN1 also localized at stalled sites. Not all STN1 foci colocalized with RAD51 in a given cell, indicating that STN1 may be involved in RAD51 independent pathways to facilitate the reinitiation of stalled replication.

Because two distinct pathways are used for restarting stalled forks in response to short and prolonged HU treatment (Petermann et al., 2010), we compared STN1/RAD51 colocalization in cells exposed to HU for a short time (3 hr) and a prolonged time (20 hr). Both treatments showed nearly identical STN1/RAD51 colocalization patterns (Figure S6), indicating that STN1 colocalizes with RAD51 regardless of HU treatment time and perhaps participates in replication restart in both pathways involving RAD51.

Next, we constructed HeLa cells stably expressing hemagglutinin (HA)-tagged TEN1 (HeLa-HA-TEN1) or Myc-tagged CTC1 (HeLa-Myc-CTC1) with retroviral transduction and exposed cells to HU overnight. Like STN1, TEN1 and CTC1 showed weak nuclear staining in the absence of replication stress (Figures 4D and 4G). Following HU treatment, both proteins formed distinct foci (Figures 4D and 4G). Similar to STN1, a significant portion of TEN1 and CTC1 foci colocalized with RAD51 (Figures 4D–4I). Altogether, our results suggest that CST proteins localize at stalled forks.

Replication Stress Induces Physical Interaction between CST and RAD51 in an ATR-Dependent Manner

In addition to CST/RAD51 colocalization, we observed physical interaction between CST proteins and RAD51 under replication stress. In communoprecipitation (coIP) assays, HeLa-Myc-STN1 cells were treated for 24 hr with HU (2 mM) or APH (0.2 μM) and then subjected to coIP with anti-myc antibody. Association between STN1 and endogenous RAD51 was detected in HU- or APH-treated cells, while such association was negligibly detectable in unstressed cells (Figure 5A). This explains why CST/RAD51 interaction was not observed previously under the unstressed condition (Miyake et al., 2009). Reciprocal coIP confirmed CTC1/RAD51 and TEN1/RAD51 interaction in cells treated with HU or APH (Figure 5B). The STN1/RAD51 interaction was likely not mediated by DNA, because STN1/RAD51 association remained unchanged after DNase I treatment (Figure 5C).

Similarly, CTC1 and TEN1 physically interacted with RAD51 in response to HU or APH treatment, while such interaction was minimal without replication stress (Figures 5D–5F). Immunoprecipitation (IP) of endogenous RAD51 pulled down all three components of CST after HU or APH exposure (Figure 5D). Reciprocal coIP corroborated CTC1/RAD51 and TEN1/RAD51 interaction in cells treated with HU or APH (Figures 5E and 5F). HU or APH treatment did not affect CST complex formation, because FLAG-CTC1 efficiently pulled down both Myc-STN1 and HA-TEN1 after HU or APH treatment (Figure 5E). Collectively, our results suggest that in response to HU or APH treatment, the CST complex and RAD51 are in proximity and likely in the same complex, further supporting that CST is localized at stalled forks.

ATR is the major kinase in response to replication stress (Flynn and Zou, 2011). Our results showed that CST/RAD51 interaction were drastically diminished upon ATR inhibition (Figure 5G). Thus, CST/RAD51 interaction is likely regulated by the ATR signaling pathway.

CST Deficiency Reduces RAD51 Foci Formation in Response to Replication Stress

Because RAD51 is the pivotal player in replication restart (Hashimoto et al., 2010, 2011; Petermann et al., 2010; Schlacher et al., 2012), we next investigated the impact of CST deficiency on RAD51 behavior in response to replication stress. We found that HU-induced RAD51 foci formation was drastically reduced after knocking down STN1, CTC1, or TEN1 (Figures 6A and 6B). Western blotting showed that RAD51 expression was unaltered by STN1 depletion in either unstressed or HU-treated samples (Figure 6C), excluding the possibility that the reduced RAD51 foci formation was caused by decreased protein expression. Due to the unavailability of high-quality TEN1 and CTC1 antibodies, western blotting was not performed in TEN1 and CTC1 knockdown cells.

CST Deficiency Impairs RAD51 Recruitment to Telomeres and FSs in Response to Replication Stress

The decreased RAD51 foci formation led us to hypothesize that CST might be important for recruiting RAD51 to fragile sequences upon fork stalling. To test this, we performed ChIP and examined whether STN1 deficiency affected RAD51 recruitment to telomeres and ChIP-seq identified fragile sequences. Consistent with previous reports (Badie et al., 2010; Verdun and Karlseder, 2006), we observed RAD51 binding to telomeres...
without HU treatment (Figures 7A and 7B). As expected, RAD51 loading to telomeres was increased upon HU treatment (Figures 7A and 7B), presumably because additional RAD51 was recruited to telomeres for restarting stalled replication. RAD51 recruitment to telomeres was significantly reduced by STN1 deficiency in both untreated and HU-treated cells (Figures 7A and 7B). Complementing RNAi-resistant STN1 cDNA resulted in near-complete rescue of RAD51 binding under both untreated and HU-treated conditions (Figures 7C and 7D, no HU; Figure 7E, with HU), indicating that the decreased RAD51 binding was
specific to STN1 knockdown. We also found that STN1 deficiency diminished RAD51 binding to telomeres in a different cell line, U2OS (Figure S7), implying that the effect of STN1 deficiency on RAD51 telomeric recruitment is not cell line specific.

Next, we tested whether RAD51 recruitment to non-telomeric fragile sequences was affected by STN1 deficiency. qPCR was performed following RAD51 ChIP on six representative STN1-binding sequences identified from ChIP-seq (HU-7, HU-8, HU-10, HU-12, HU-13, and HU-21). Again, we observed a marked increase of RAD51 binding to these sequences upon HU treatment (Figure 7F), in agreement with our conclusion from cytogenetic analysis that STN1-binding sequences were FSs (Figure 3). In contrast, non-STN1-binding sites such as actin, tubulin, and SLITRK6 sequences showed undetectable RAD51 binding (Figure 7F). STN1 deficiency resulted in a significant reduction in RAD51 binding to all six tested fragile sequences after HU treatment (Figure 7F). Altogether, our results support that CST facilitates RAD51 recruitment to GC-rich repetitive FSs genome-wide in response to HU-induced replication stress and dysfunctional CST impairs the recruitment of RAD51 to these sites, resulting in inefficient replication restart (Figure 7G).

**DISCUSSION**

Restart of stalled replication requires the assembly of multiple proteins at stalled sites, and their synergistic actions are needed to stabilize stalled forks and to ensure successful reinitiation of DNA synthesis (Sirbu et al., 2013). The CST complex has been shown to be important for reinitiating stalled DNA synthesis at both telomeric and non-telomeric sequences (Gu et al., 2012; Huang et al., 2012; Stewart et al., 2012; Wang et al., 2014). Although it has been proposed that CST promotes origin firing upon fork stalling (Stewart et al., 2012), the molecular mechanism underlying CST-mediated replication reinitiating is largely unclear. It is unknown how CST fits into the overall picture of replication restart and how the complex interplays with other replication restart factors. In this study, we map STN1-binding sites with ChIP-seq and find that STN1 is frequently enriched at GC-rich repetitive sequences after HU exposure. Representative STN1-binding sequences display fragility upon HU exposure, suggesting that these sequences are bona fide FSs. Fragility is exacerbated by STN1 deficiency, resulting in chromosome fragmentation. Thus, we provide the first line of evidence that functional CST is required for protecting the stability of these fragile loci in response to perturbed replication.

To date, most mapped FSs have been induced by low concentrations of the DNA polymerase inhibitor APH. These CFSs are usually AT-rich, transcriptionally repressed, and late replicating (Debatissé et al., 2012). Studies suggest that sequences sharing no obvious features with CFSs can also become sensitive to replication stress. For instance, oncogene expression induces an FS landscape that only partially overlaps with APH-induced CFSs (Miron et al., 2015). In mouse B lymphocytes, HU treatment induces a group of FSs termed ERFSs that are in general GC-rich, transcriptionally active, and located at CpG islands. These regions are associated with chromosome breakages and aberrant rearrangements (Barlow et al., 2013). Most STN1-binding sites reside outside putative CFSs (Figure S5), with sequence features being similar to ERFSs. Therefore, our study adds to the growing evidence for the complexity and diversity of FSs in the human and mammalian genome.

Upon fork stalling, ssDNA accumulates at stalled sites due to uncoupling of DNA unwinding and DNA synthesis. Failure to protect ssDNA leads to nucleolytic attack, compromising the integrity of nascent DNA at stalled forks and resulting in increased chromosomal aberrations in human precancerous lesions. Proteins binding to ssDNA have multiple roles in protecting ssDNA, mediating protein-protein interactions, and stimulating strand-exchange proteins and helicases (Richard et al., 2009). It has been shown that prompt restart of stalled replication relies on the ssDNA-binding protein RAD51. RAD51 protects nascent DNA from nuclease degradation at stalled forks (Hashimoto et al., 2010) and mediates two pathways for restarting stalled forks by facilitating new origin firing and/or by homologous recombination (Petermann et al., 2010). One report suggests that CST facilitates the restart of stalled replication through promoting origin firing (Stewart et al., 2012), but the underlying mechanism for such promotion is unknown. In this study, we provide evidence that in response to HU treatment, components of CST form distinct foci that colocalize with RAD51 and physically interact with RAD51 (Figures 4 and 5). We further show that CST deficiency significantly diminishes HU-induced RAD51 foci formation and reduces RAD51 recruitment to telomeres and non-telomeric fragile sequences under replication stress (Figures 6 and 7). We propose that CST may be particularly important for efficient RAD51 recruitment to these GC-rich repetitive sequences upon fork stalling (Figure 7G). Because single-stranded G-rich repetitive sequences, including telomeric repeats, are prone to forming secondary structures like G-quadruplexes, it is tempting to speculate that CST may bind to G-rich repetitive ssDNA and disfavor G-quadruplex formation, allowing efficient binding of RAD51 to ssDNA for replication restart. It is also possible that binding of CST to ssDNA may assist in recruiting helicases and/or stimulate helicase unwinding of G-quadruplexes. Alternatively, CST deficiency may disrupt the chromatin structure favoring RAD51 recruitment and nucleofilament formation, resulting in reduced RAD51 binding to ssDNA at stalled sites. Nonetheless, our findings provide novel mechanistic insights into how CST promotes replication restart, which is important for understanding how genome stability is protected under replication stress.

Results in this study do not exclude the possibility that CST may use RAD51-independent pathways to facilitate replication restart. In fact, a subset of CST protein foci did not share colocalization with RAD51 after HU treatment (Figure 4). It will be interesting to examine the relationship of CST with other replication restart proteins, which will provide needed insights into RAD51-independent pathways in which CST may be involved.

The CST complex interacts with POLζ and is able to stimulate the priming activity of POLζ and primase-to-polmerase switching (Casteel et al., 2009; Lue et al., 2014; Nakaoka et al., 2012). POLζ is an important replisome component at active replication forks and necessary for Okazaki fragment synthesis. It could be argued that its primase or polymerase activity might be used to reinitiate DNA synthesis during replication start. However, proteomics study fails to identify the enrichment of POLζ subunits at stalled replication forks (Sirbu et al., 2013). Mammalian cells...
Figure 5. CST Proteins Physically Interact with RAD51 under Replication Stress in an ATR-Dependent Manner

(A) HeLa-Myc-STN1 cells were treated with 2 mM HU (left panel) or treated with 0.2 μM APH (right panel) for 20 hr, followed by coIP with anti-myc antibody. Precipitates were analyzed by western blotting to detect endogenous RAD51 that was pulled down by Myc-STN1.

(B) Reciprocal coIP. IP was performed with HeLa-Myc-STN1 cells with anti-RAD51 antibody, followed by western blotting to detect Myc-STN1 pulled down by RAD51.
encode a different primase, PrimPol, which displays both primase and polymerase activities (García-Gómez et al., 2013). PrimPol is able to reprim DNA synthesis at forks stalled by HU or UV irradiation and facilitate fork progression (Bianchi et al., 2013; Mourón et al., 2013), raising the question of whether POLα is an active component in fork rescue. Thus, it remains a subject of debate whether the enhancing effect of CST on POLα activity plays a significant role in rescuing stalled replication. However, it seems that CST/POLα interaction may be important for telomere maintenance. CTC1 mutations that abolish POLα interaction show reduced telomere association, accompanied by a telomere replication defect, suggesting that

Figure 6. Deficiency in CST Reduces RAD51 Foci Formation after HU Treatment

(A) HU-induced RAD51 foci formation was significantly decreased after CTC1, STN1, or TEN1 knockdown. HeLa cells stably expressing shLuc, shCTC1, shSTN1, or shTEN1 were treated with 2 mM HU for overnight and then fixed for RAD51 immunofluorescent staining (red).

(B) Quantification of relative RAD51 foci intensity in CST knockdown cells. **p ≤ 0.001.

(C) RAD51 expression detected by western blotting in the presence or absence of HU in STN1 knockdown cells.

(D–F) CTC1 and TEN1 physically interact with endogenous RAD51 under replication stress. FLAG-CTC1, Myc-STN1, and HA-TEN1 were coexpressed in HEK293T cells and treated with 2 mM HU or 0.2 μM APH for 20 hr, followed by reciprocal coIP. (D) IP was performed with anti-RAD51 recognizing endogenous RAD51, followed by western blotting to detect FLAG-CTC1, Myc-STN1, and HA-TEN1 in immunoprecipitates. (E) IP was performed with anti-FLAG, followed by western blotting to detect RAD51, Myc-STN1, and HA-TEN1 in the immunoprecipitates. An asterisk indicates the RAD51 band. (F) IP was performed with anti-HA, followed by western blotting to detect RAD51 in the immunoprecipitates. An asterisk indicates the RAD51 band.

(G) CST/RAD51 interaction is dependent on ATR activity. FLAG-CTC1, Myc-STN1, and HA-TEN1 were coexpressed in HEK293T cells and treated with 2 mM HU or 0.2 μM APH in the presence or absence of ATR inhibitor (ATRi) for 20 hr. IP was performed with anti-RAD51 recognizing endogenous RAD51, followed by western blotting to detect FLAG-CTC1, Myc-STN1, and HA-TEN1 in immunoprecipitates.
Figure 7. STN1 Deficiency Diminishes RAD51 Recruitment to Telomeres and STN1-Binding Sites upon Replication Stalling

(A) Representative slot blot of RAD51 ChIP at telomeres in STN1-deficient cells. HeLa cells expressing shLuc or shSTN1 were treated with HU (2 mM) or untreated, followed by crosslinking and ChIP. ChIP DNA was loaded on slot blot and hybridized to telomere probe.

(B) Quantitation of RAD51 binding to telomeric DNA. Results are represented as percentage of input. The p values were calculated with two-tailed t test from three independent experiments.

(C) The effect of STN1 deficiency on RAD51 binding to telomeric DNA is specific. RNAi-resistant STN1 (r-STN1) was expressed in STN1 knockdown cells, treated with HU or untreated, and crosslinked, and ChIP was performed. Representative slot blot of RAD51 ChIP at telomeres is shown.

(D and E) Quantitation of RAD51 binding to telomeric DNA without HU (D) and with HU (E). The p values were calculated with two-tailed t test from three independent experiments.

(F) STN1 deficiency reduced RAD51 recruitment to FSs after HU treatment. Quantitation of RAD51 recruitment to representative STN1-binding sites from ChIP in STN1-deficient cells and control cells with qPCR. Two-tailed t tests were used to calculate p values from three independent ChIP assays, with qPCR assays being performed in duplicates in each ChIP experiment. Results are represented as percentage of input. *p < 0.05, **p < 0.01.

(G) Model for CST in promoting replication restart. Upon fork stalling at GC-rich repetitive sequences, ssDNA accumulates. CST binds to ssDNA and recruits RAD51 to stalled sites. In the absence of CST, the GC-rich repetitive ssDNA at stalled sites may form secondary structures that prevent efficient binding of RAD51, resulting in fork collapse and genome instability.

Error bars, SEM.
CST/POLα interaction may facilitate telomeric DNA replication (Chen et al., 2013). CST stimulation on POLα activity could also be important for filling the telomeric C strand gap that remains after telomere replication and/or telomerase elongation (Chen et al., 2013; Huang et al., 2012).

Mutations in CTC1 cause Coats plus, a disease sharing overlapping pathological phenotypes with the telomere defect disease known as dyskeratosis congenita (DC) (Anderson et al., 2012; Keller et al., 2012; Polvi et al., 2012). Because a subset of Coats plus patients show markedly shortened telomeres (Anderson et al., 2012), Coats plus has been considered a telomere maintenance disorder, and the pathogenesis of Coats plus may partly derive from telomere defects (Savage, 2012). However, a few pathological CTC1 mutations display no obvious telomere maintenance defects (Polvi et al., 2012).

Moreover, CST binds to ssDNA in a sequence-independent manner, and Coats plus patients display neurological manifestations distinct from DC. These observations suggest that the pathogenesis of Coats plus may also be related to non-telomeric CTC1 dysfunction. Our findings that CST is important for maintaining the stability of non-telomeric GC-rich repetitive sequences potentiates the role of FS instabilities in Coats plus disease development. Further studies will be needed to test this possibility and to determine whether pathological CTC1 mutations induce FS instability in the genome. Results will allow for a more accurate understanding of the pathogenesis of Coats plus and aid in designing more effective therapeutic approaches.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures are provided in Supplemental Information. Cell culture, shRNA, ChiP-seq, qPCR, and immunofluorescence (IF) were performed using standard protocols. FISH on metaphase chromosomes, ChiP-seq analysis, and qPCR quantification are described in detail in Supplemental Information.

ACCESSION NUMBERS

The accession number for the ChiP-seq data reported in this paper is GEO: GSE82123.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.077.

AUTHOR CONTRIBUTIONS

M.C., Q.Z., O.S., P.J., C.H., X.D., M.F., and W.C. performed experiments and analyzed data. M.C. analyzed all ChiP sequences, obtained metaphase FISH data, performed IF experiments and data analysis, and assembled figures. Q.Z. obtained RAD51 ChiP results, performed qPCR and data analysis, participated in IF and figure assembly. O.S. obtained STN1 ChiP results. M.F.M. contributed to the initial conception of the project, gave technical advice, and performed initial ChiP-seq analysis, initial motif discovery experiments, and oncomine Str1 expression analysis. Q.Z., P.J., C.H., and X.D. contributed to co-IP analysis. L.W. processed ChiP-seq reads. P.Y. directed ChiP-seq reads processing study. W.C. conceived the project; directed the study; participated in cell line establishment, FISH, IF, and co-IP experiment execution; and wrote the manuscript.

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