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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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Abstract

The telomeric CTC1/STN1/TEN1 (CST) complex has recently been implicated in promoting replication recovery under replications 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 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 between 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.

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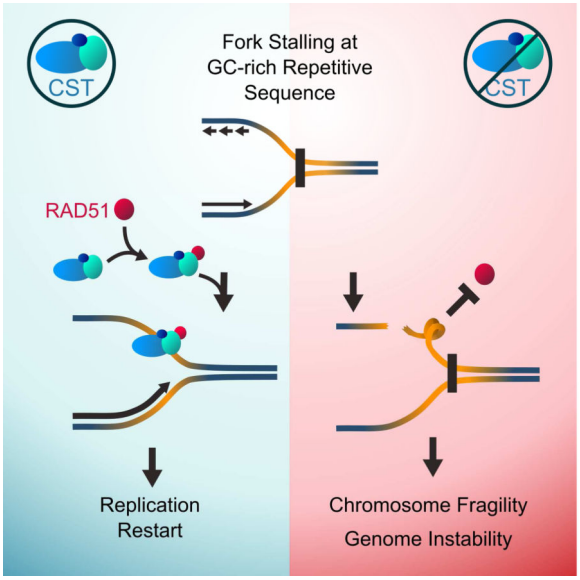
Author contributions:

MC, QZ, OS, PJ, CH, XD, MF, WC performed experiments and analyzed data. MC analyzed all ChIP-seq sequences, obtained metaphase FISH data, performed IF experiments and data analysis, assembled figures. QZ obtained RAD51 ChIP results. OS obtained STN1 ChIP results. QZ, PJ, CH, and XD contributed to co-IP analysis. LG processed ChIP-seq reads. PY directed ChIP-seq reads processing study. WC conceived the project, directed the study, participated in cell line establishment, FISH, IF, co-IP experiment execution, and wrote the manuscript.

Conflict of interest:

We declare no conflicts of interest.

Graphical Abstract



Keywords

CTC1/STN1/TEN1; replication stress; RAD51; genome stability

Faithful and complete duplication of chromosomal DNA is vital for avoiding detrimental replication errors and preserving genome stability. Replication stress, induced by either 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/rearrangement activities and driving genome instability (Debacker and Kooy, 2007; Debatisse et al., 2012; Durkin and Glover, 2007; Tercero et al., 2003). Indeed, 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; Ozeri-Galai et al., 2012). Therefore, pathways have evolved to prevent fork stalling and to facilitate the restart of stalled replication in order to preserve genome stability.

Successful rescue of stalled replication requires coordination of multiple proteins that stabilize stalled forks and promote re-initiation of DNA synthesis (Franchitto and Pichierri, 2014; Zeman and Cimprich, 2013). Crucial genome maintenance proteins, including RAD51, MRE11, XRCC3, SLX1-SLX4-MUS81-EME1, BLM, WRN, RTEL1, SMARCAL1, 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., 2012; Sidorova et al., 2008; Tittel-Elmer 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; Errico et al., 2009; Errico et al., 2007; 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 re-initiation of DNA synthesis mediated by various replication factors including MCM2-7, PCNA, CDC45, POLδ (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, composed of 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 ssDNA with high affinity (Miyake et al., 2009). Originally discovered as a telomere maintenance factor (see below), the human CST 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 HU, aphidicolin (APH), methyl methanesulfonate (MMS) and camptothecin (Wang et al., 2014; Zhou and Chai). Mutations in CTC1 cause Coats Plus disease, a complex disorder characterized by bilateral exudative retinopathy, retinal telangiectasias, growth retardation, intracranial calcifications, bone abnormalities, gastrointestinal vascular ectasias, accompanied by common early-aging pathological features like premature hair graying, anemia, and osteoporosis (Anderson et al., 2012; Armanios and Blackburn, 2012; Keller et al., 2012; Polvi et al., 2012). Early study using 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; Nakaoka et al., 2011). However, evidence supporting the involvement of CST's stimulatory effect on POLα in replication restart is lacking. Presently, the molecular mechanism underlying how CST facilitates replication restart at non-telomeric sites remains largely unknown, and moreover, the interplay between CST and other key replication restart players remains unexplored.

In contrast, the role of CST in telomere maintenance is better defined. Human CST 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 the 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 induce accelerated telomere shortening and display 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 in part 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-Stn1-Ten1 complex controls telomerase access to telomeres and is an essential component for "capping" telomere ends that protects 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 lacks 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/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 single-stranded DNA/RNA and also 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 ss oligonucleotides containing a variety of G- or C-rich sequences with high affinity (Miyake et al., 2009). Unlike the Cdc13-Stn1-Ten1 complex in budding yeast, which binds specifically to telomeric DNA with subnanomolar affinity (Nugent et al., 1996), hCST lacks sequence specificity. While hCST preferentially binds to G-rich versus C-rich oligonucleotides, such preferred binding disappears as oligo lengths increase (Chen et al., 2012). *In vitro* binding assays reveal that the C-terminal half of CTC1 is important for DNA binding, whereas both the N- and C-terminus 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 set out to investigate the molecular mechanism by which CST promotes the restart of stalled replication. We employ 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. 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 co-localize with RAD51. In addition, replication stress markedly induces physical interaction between CST proteins with RAD51 in a DNA-independent manner, and the CST/RAD51 interaction depends on ATR. We observe that suppression of each individual CST proteins 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 fork stalls, therefore 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) 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 (Oncomine, 2012) (Fig. S1, Table S1). The reduced STN1 expression in tumor tissues suggests that STN1 may be important in tumor suppression. Previously 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 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 (Fig. 1A), therefore minimizing possible artifacts induced by protein overexpression. We also noticed that expressing exogenous Myc-STN1 suppressed endogenous STN1 expression (Fig. 1A), indicating that STN1 expression is likely regulated by a negative feedback mechanism.

HeLa-myc-STN1 was then synchronized in G1/S phase boundary with double-thymidine block, released into S phase for 3 hrs, then treated with 2 mM HU for 3 hours to induce replication stress. Subsequently, ChIP assays were performed with anti-myc antibody (Fig. 1B). FACS analysis showed that HU treatment significantly stalled replication progression, and the majority of cells remained in the mid-S phase (Fig. 1C). In order to minimize possible effects caused by different cell cycle stages, cells synchronized in mid-S phase (3 hrs 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 condition, components of the CST complex are associated with telomeres, which are naturally occurring FSs. However, such association is weak is the S phase and telomeric association of CST is the 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 (Fig. 1D). Upon HU treatment, STN1 association with telomeres increased (Fig. 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: $R^2 = 0.9852$, 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. A total of 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 (Fig. S3), validating our ChIP-seq results. One ChIP-seq locus showed amplification in both the Myc-vector only control and in Myc-STN1 ChIP, suggesting that a small portion of peaks might have resulted from non-specific DNA 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 (Fig. 2A). These repetitive sequences included LINEs and 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$(N)_n$, N denotes any nucleotide) and those containing simple repeats (short tandem repeats like $(TTAGGG)_n$) (Fig. 2A). STN1 binding sites also displayed higher G and C contents than chromosomal averages by ~8% GC (p=1.14e-287,300, chi-squared test) (Fig. 2B). Many sequences contain G-rich repeats. Examples of STN1-binding sequence containing G-rich repeats are shown in Fig. 2C and Fig. S4. We also recovered telomeric sequences (sequencing reads with ≥12 tandem TTAGGG repeats) from unaligned sequences, confirming STN1 binding to telomeres. Interestingly, 1.3% of non-telomeric sequencing reads contained TTAGGG sequence(s), with the majority of these reads (~98%) containing a single TTAGGG sequence in isolation and/or non-tandem TTAGGG repeats, corroborating the previously identified lack of sequence specificity of hCST (Miyake et al., 2009). After aligning sequencing peaks to 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-squared test) (Fig. 2D). Together, 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 shRNA sequences (Fig. 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 (Fig. 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 (Fig. 3B). STN1 deficiency further elevated fragility of these sites (Fig. 3C). In some cases, one chromatid arm was severed at hybridization point and lost (Fig. 3B, HU-9, white circle indicates the missing chromatid arm). Control probes exhibited minimal fragility, regardless of STN1 depletion and replication stress (Fig. 3C). Concurrently, we observed a marked increase of chromosome fragmentation in HU-treated STN1 deficient cells, suggesting a

high level of chromosome instability (Figs. 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 (Fig. 3), we then 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; Le Tallec et al., 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). Since 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 genome revealed that only a portion of STN1 binding peaks (~25%) overlapped with or located near these putative CFSs (Fig. S5). Despite ChIP-seq peaks mapping to CFSs at a greater frequency than expected for a random distribution (p=0.017, chi-squared test), a large portion of peaks resided outside putative CFSs (Fig. S5). In addition, sequences used in Figure 3 (HU-8, −9, −10, −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

Since STN1 depletion increased fragility of its binding sequences under HU (Fig. 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 (Arlt et al., 2006; Hashimoto et al., 2010; Petermann and Helleday, 2010; Petermann et al., 2010). Upon replication stress, RAD51 forms foci at stalled forks. We then examined if CST co-localized with RAD51 after stress. We constructed HeLa cells stably expressing FLAG-STN1 (HeLa-FLAG-STN1) using retroviral transduction. As shown in Fig. 4A, STN1 showed weak and dispersed nuclear staining without replication stress. In striking contrast, it formed distinct foci following HU exposure (Fig. 4A). Simultaneously, increased co-localization of FLAG-STN1 foci with RAD51 was observed (Figs. 4B, 4C), suggesting that STN1 also localized at stalled sites. It is noteworthy that not all STN1 foci colocalized with RAD51 in a given cell, indicating that STN1 may be involved in RAD51 independent pathways to facilitate the re-initiation of stalled replication.

Since two distinct pathways are used for restarting stalled forks in response to short and prolonged HU treatment, respectively (Petermann et al., 2010), we then compared STN1/RAD51 colocalization in cells exposed to HU for short time (3 hrs) and prolonged time (20 hrs). Both treatments showed nearly identical STN1/RAD51 colocalization patterns (Fig.

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 HA-tagged TEN1 or Myc-tagged CTC1 with retroviral transduction and exposed cells with HU for overnight. Like STN1, TEN1 and CTC1 showed weak nuclear staining in the absence of replication stress (Figs. 4D, 4G). Following HU treatment, both proteins formed distinct foci (Figs. 4D, 4G). Similar to STN1, a significant portion of TEN1 and CTC1 foci colocalized with RAD51 (Figs. 4D to 4I). Together, 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 also observed physical interaction between CST proteins and RAD51 under replication stress. In co-IP assays, HeLa-Myc-STN1 cells were treated for 24 hrs with HU (2 mM) or APH (0.2 μM), and then subjected to co-IP 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 (Fig. 5A). This explains why CST/RAD51 interaction was not observed previously under unstressed condition (Miyake et al., 2009). Reciprocal co-IP confirmed STN1/RAD51 interaction in cells treated with HU or APH (Fig. 5B). The STN1/RAD51 interaction was unlikely mediated by DNA, since STN1/RAD51 association remained unchanged after DNase I treatment (Fig. 5C).

Similarly, CTC1 and TEN1 physically interacted with RAD51 in response to HU or APH treatment, while such interaction was minimal without replication stress (Figs. 5D, 5E, 5F). IP of endogenous RAD51 pulled down all three components of CST after HU or APH exposure (Fig. 5D). Reciprocal co-IP confirmed CTC1/RAD51 and TEN1/RAD51 interaction in cells treated with HU or APH (Figs. 5E and 5F). Noticeably, HU or APH treatment did not affect CST complex formation, since FLAG-CTC1 efficiently pulled down both Myc-STN1 and HA-TEN1 after HU or APH treatment (Fig. 5E). Collectively, our results suggest that in response to HU or APH treatment, the CST complex and RAD51 are in close 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 (Fig. 5G). Thus, CST/RAD51 interaction is likely regulated by the ATR signaling pathway.

CST deficiency reduces RAD51 foci formation in response to replication stress

Since RAD51 is the pivotal player in replication restart (Hashimoto et al., 2010; Hashimoto et al., 2012; 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 (Fig. 6A, 6B). Western blotting showed that RAD51 expression was unaltered by STN1 depletion in either unstressed or HU-treated samples (Fig. 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 (Figs. 7A, 7B). As expected, RAD51 loading to telomeres was increased upon HU treatment (Figs. 7A, 7B), presumably because additional RAD51 was recruited to telomeres for restarting stalled replication. Remarkably, RAD51 recruitment to telomeres was significantly reduced by STN1 deficiency in both untreated and HU-treated cells (Figs. 7A, 7B). Complementing RNAi-resistant STN1 cDNA resulted in near complete rescue of RAD51 binding under both untreated and HU-treated conditions [Figs. 7C, 7D (no HU), 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 (Fig. 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. Quantitative PCR (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, HU-21). Again, we observed a marked increase of RAD51 binding to these sequences upon HU treatment (Fig. 7F), in agreement with our conclusion from cytogenetic analysis that STN1-binding sequences were FSs (Fig. 3). In contrast, non-STN1 binding sites such as actin, tubulin, and SLITRK6 sequences showed undetectable RAD51 binding (Fig. 7F). STN1 deficiency resulted in a significant reduction in RAD51 binding to all six tested fragile sequences after HU treatment (Fig. 7F). Taken together, 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 (Fig. 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 re-initiation 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 re-initiating 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 further 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 DNA polymerase inhibitor, APH. These CFSs are usually AT-rich, transcriptionally repressed, and late replicating (Debatisse et al., 2012). Recent studies suggest that sequences sharing no obvious features with CFSs can also become sensitive to replication stress. For instance, oncogene expression induces 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). Interestingly, the majority of STN1-binding sites reside outside putative CFSs (Fig. 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, as RAD51 protects nascent DNA from nuclease degradation at stalled forks (Hashimoto et al., 2010) and also mediates two pathways for restarting stalled forks by facilitating new origin firing and/or by homologous recombination (Petermann et al., 2010). Previous 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 (Figs. 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 (Figs. 6 and 7). We propose that CST may be particularly important for efficient RAD51 recruitment to these GC-rich repetitive sequences upon fork stalling (Fig. 7G). As 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 helicases 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 (Fig. 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 that CST may be involved in.

The CST complex interacts with POLα and is able to stimulate the priming activity of POLα and primase-to-polymerase switching (Casteel et al., 2009; Lue et al., 2014; Nakaoka et al., 2011). POLα is an important replisome component at active replication forks and necessary for Okazaki fragment synthesis. It could be argued that its primase/polymerase activity might be used to reinitiate DNA synthesis during replication start. However, proteomics study fails to identify the enrichment of POLα subunits at stalled replication forks (Sirbu et al., 2013). Noticeably, mammalian cells encode a different primase PrimPol, which displays both primase and polymerase activities (Garcia-Gomez et al., 2013). PrimPol is able to reprime DNA synthesis at forks stalled by HU or UV irradiation and facilitate fork progression (Bianchi et al., 2013; Mouron 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. On the other hand, it seems that CST/POLα interaction may be important for telomere maintenance. CTC1 mutations that abolish POLα interaction show reduced telomere association, accompanied by telomere replication defect, suggesting that CST/POLα interaction may facilitate telomeric DNA replication (Chen et al., 2013). CST stimulation on POLα activity could also be important for filling the terminal 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 dyskerotosis congenita (DC) (Anderson et al., 2012; Keller et al., 2012; Polvi et al., 2012). Since a subset of Coats Plus patients show markedly shortened telomeres (Anderson et al., 2012), Coats Plus has been considered as a telomere maintenance disorder, and the pathogenesis of Coats Plus may in part 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 potentiate 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 IF were performed using standard protocols. FISH on metaphase chromosomes, ChIP-seq analysis, and qPCR quantification were described in detail in Supplemental Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- STN1 is enriched at GC-rich repetitive sequences in response to replication stress
- STN1 suppression exacerbates 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

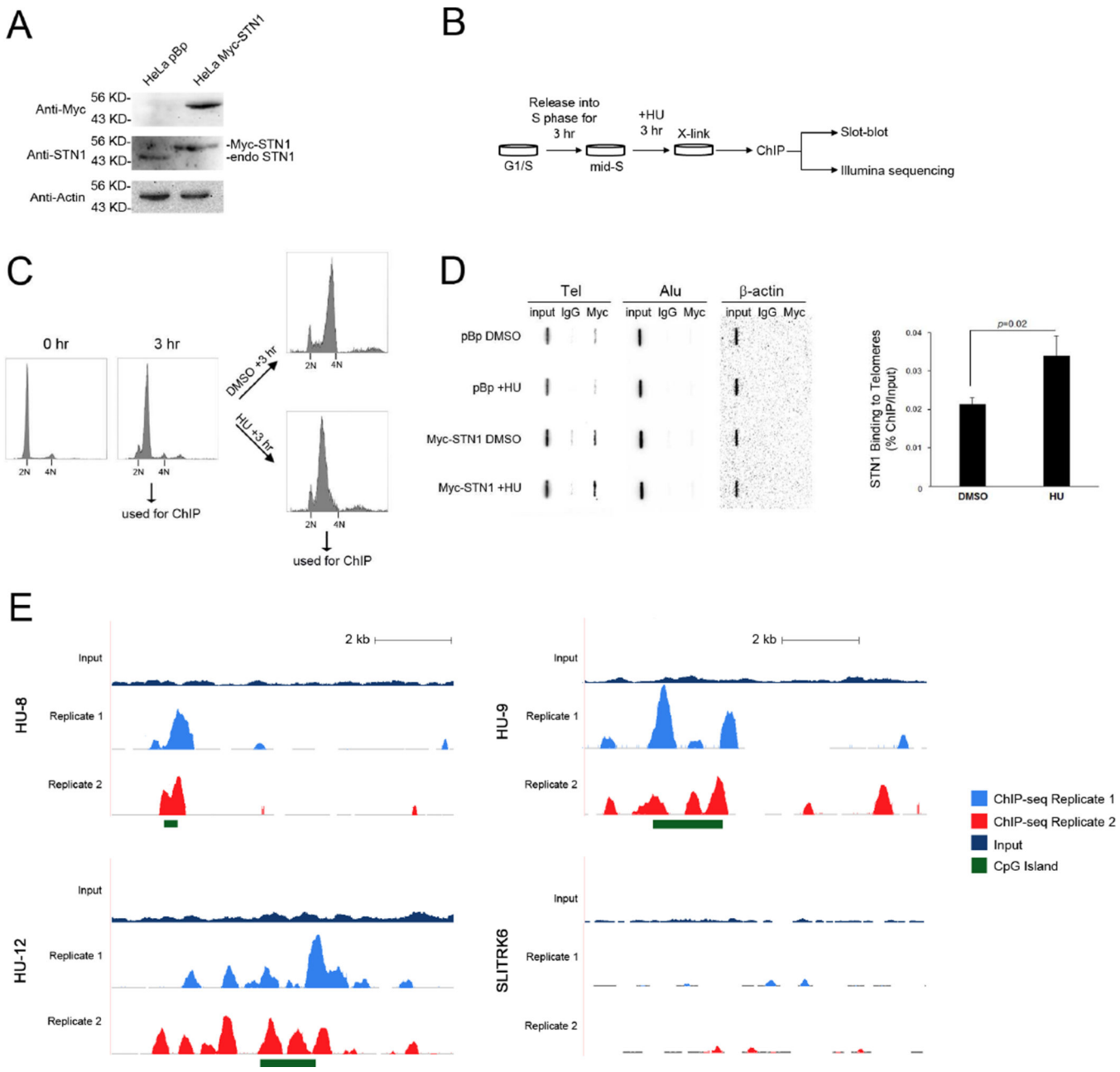

Figure 1. Enrichment of STN1 at telomeres and non-telomeric sequences in response to replication stress

(A) Myc-STN1 was expressed in HeLa cells at near endogenous level. Myc-STN1 was stably expressed in HeLa using retroviral transduction. STN1 expression was detected by western blotting with anti-STN1 or anti-Myc antibody. Exogenous (exo) Myc-STN1 was expressed at a similar level to endogenous (endo) STN1. Expression of exogenous Myc-STN1 also suppressed endogenous STN1 expression. HeLa pBp: HeLa cells expressing pBabe-puro vector. **(B)** Experimental plan of sample preparation for ChIP experiments. HeLa-myc-STN1 were synchronized with double-thymidine block, released into S phase for 3 hrs, and then treated with HU (2 mM) for 3 hrs, followed by crosslinking. ChIP DNA was

used both for slot-blot to detect STN1-bound telomeric DNA and for Illumina sequencing. **(C)** FACS analysis of DNA content of DMSO and HU treated cells. Untreated sample 3 hr after double-thymidine release was used as control in ChIP assays. **(D)** ChIP of myc-STN1 binding to telomeric DNA after fork stalling. ChIP DNA was loaded onto slot-blot and telomeric DNA was detected by hybridization with a ^{32}P-$(TTAGGG)_3$ probe. The membrane was then stripped and rehybridized to Alu repeat probe. Lastly, the membrane was stripped again and hybridized to a probe recognizing β-actin sequence. Quantification of ChIP results were from three independent ChIP experiments. *P* value was calculated with two-tailed *t*-test. Error bars: SEM. **(E)** Genome browser tracks of three STN1 binding sites HU-8, HU-9, HU-12 and non-binding site SLITRK6 in two ChIP-seq replicates. CpG islands are labeled with green bars below the tracks.

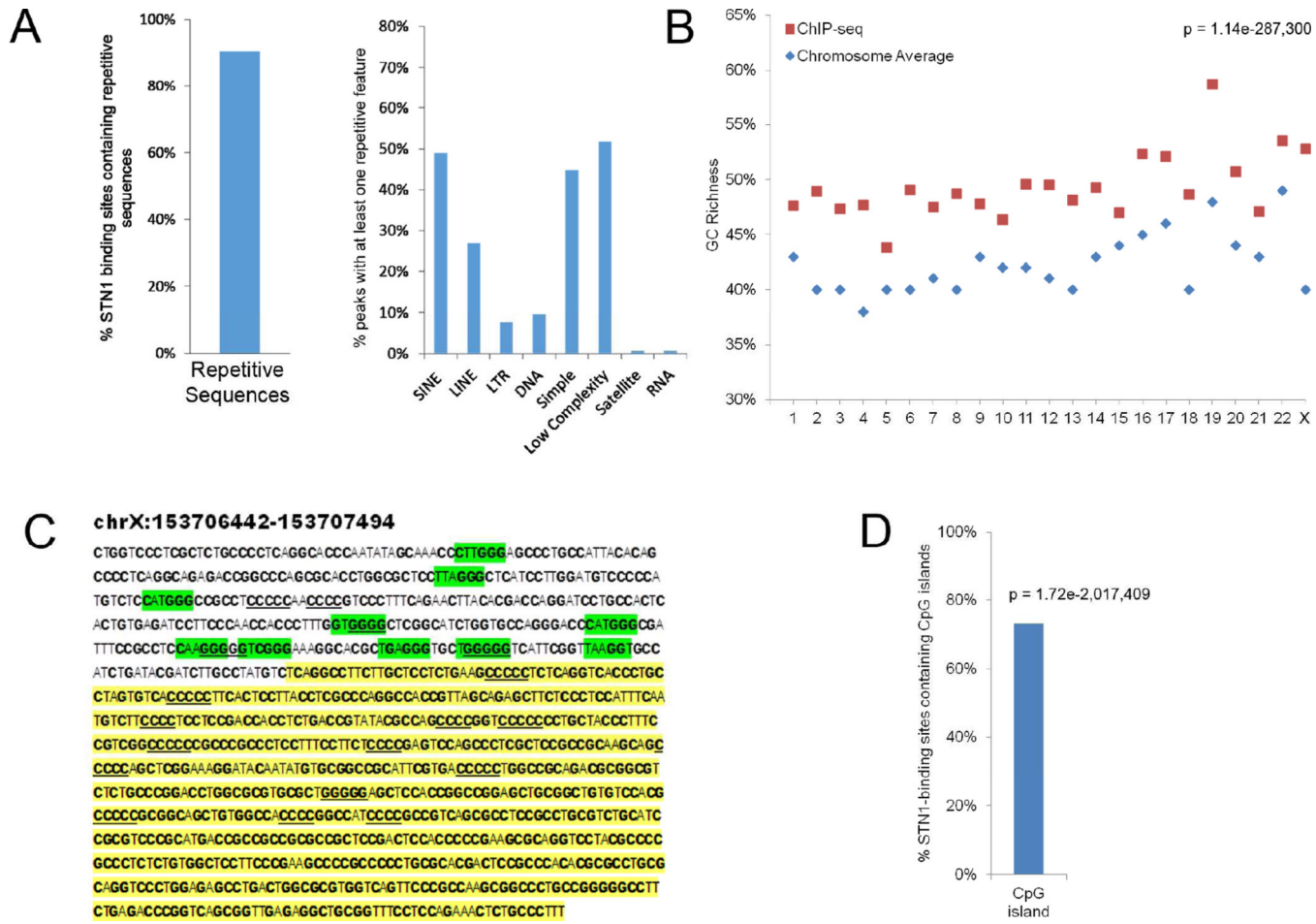

Figure 2. Mapping STN1 binding sites genome-wide upon HU exposure

(A) Relative frequency of repetitive sequences among STN1-binding sites. Repetitive sequences were identified using RepeatMasker. LINE: Long interspersed elements. SINE: Short interspersed nuclear elements. LTR: Long terminal repeats. DNA: repetitive transposable elements. Simple repeats: short tandem repeats. Low complexity: low complexity repeats. RNA: RNA repeats including RNA, tRNA, rRNA, snRNA, scRNA, srpRNA. **(B)** STN1 binding sequences contain a 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. TTAGGG and variants of TTAGGG sequences are in green. **(D)** Proportion of STN1-binding sites containing CpG islands.

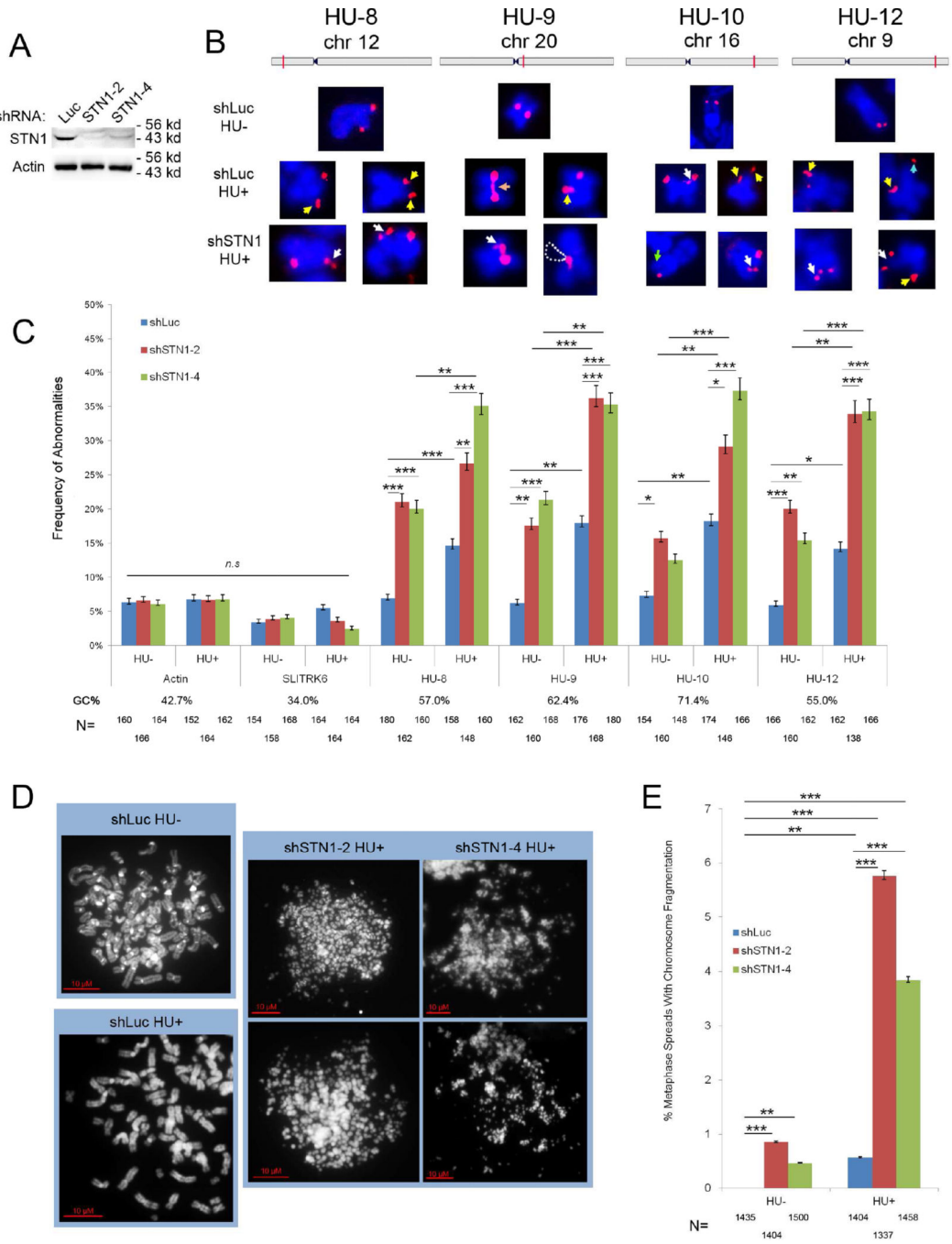

Figure 3. Fragility of STN1 binding sequences in the presence of HU

(A) Western blot showing STN1 knockdown. Two shRNA sequences were used to independently knockdown STN1. **(B)** Examples of aberrant structures at STN1-binding loci HU-8, HU-9, HU-10 and HU-12 in metaphase cells after HU treatment. FISH probes are red. Locations of HU-8, −9, −10 and −12 on individual chromosomes are indicated as red bars above the images. Different types of fragilities are indicated by arrows. White arrows: fragile site breakage. White circle: missing chromosome arm. Yellow arrows: elongated signals. Orange arrows: signals on two sister chromatids are connecting to each other. Green

arrows: missing signals. Blue arrows: signals spatially separated from chromosome. **(C)** Quantification of frequency of abnormalities at each locus in untreated and HU-treated cells. Two non-STN1 binding sites, actin and SLITRK6, were used as negative controls. GC content for each locus is indicated. *N*: the number of chromatids analyzed in each sample. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. **(D)** Representative images showing chromosome fragmentation in STN1 knockdown cells after HU exposure. **(E)** Percentage of metaphase spreads with chromosome fragmentation. *n* denotes the number of total metaphase spreads measured in each sample. Binomial z-statistic pairwise comparison was used to calculate statistical significance. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

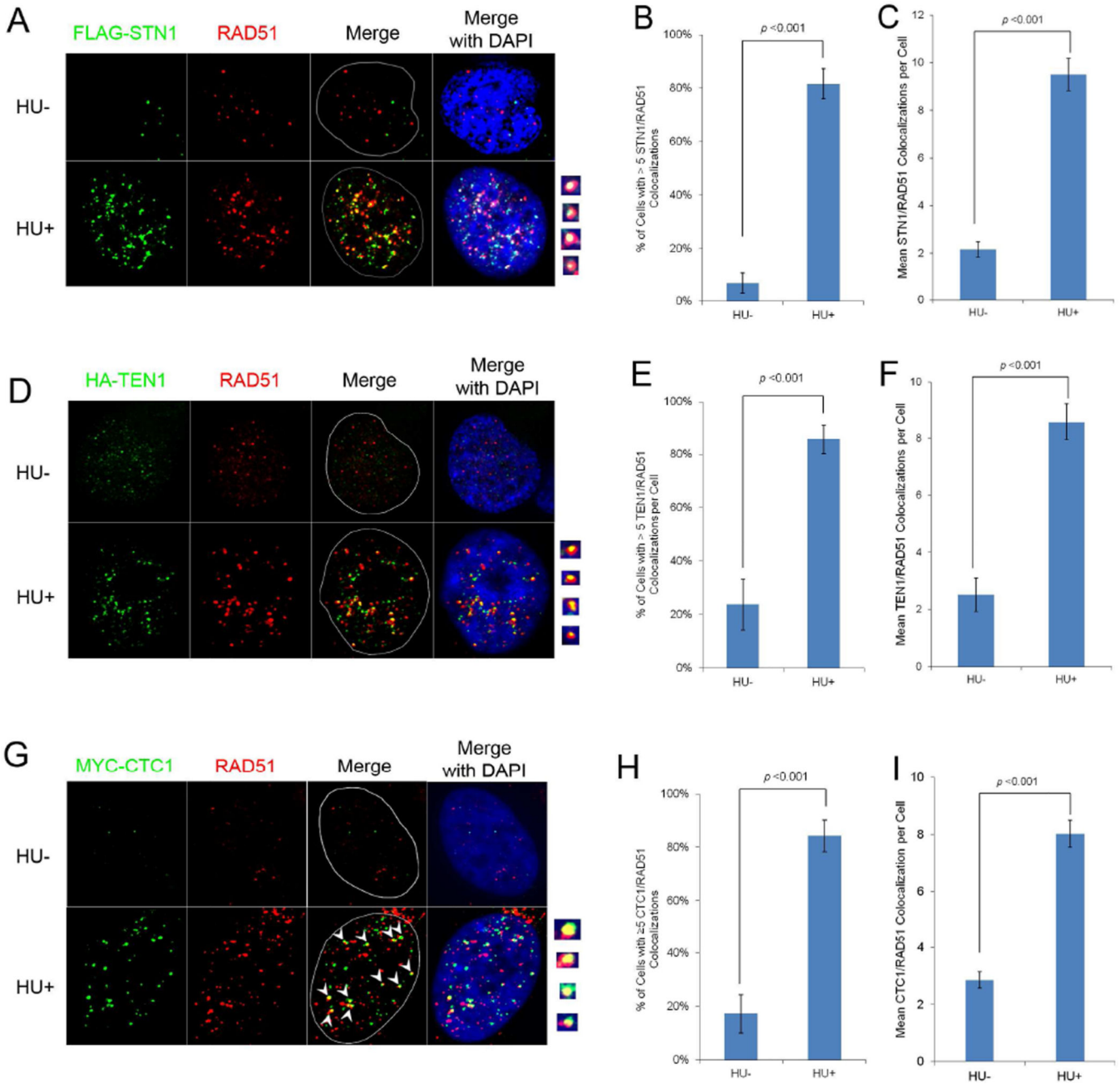

Figure 4. CST proteins colocalize with RAD51 after fork stalling

Asynchronized HeLa-FLAG-STN1, HeLa-FLAG-TEN1, or HeLa-Myc-CTC1 cells were treated with 2 mM HU overnight, fixed, and stained with the indicated antibodies. **(A), (D), and (G):** Colocalization of FLAG-STN1, HA-TEN1, Myc-CTC1 with RAD51 after HU treatment. **(B), (E), and (H):** Percentage of cells containing ≥5 CTC1/STN1/TEN1 proteins and RAD51 colocalizations. Binomial z-statistic pairwise comparison was used for statistical analysis. Error bars: SEM. **(C), (F), and (I):** Mean number of CST/RAD51 colocalization per cell. Two-tailed *t*-tests were used for statistical analysis. Error bars: SEM.

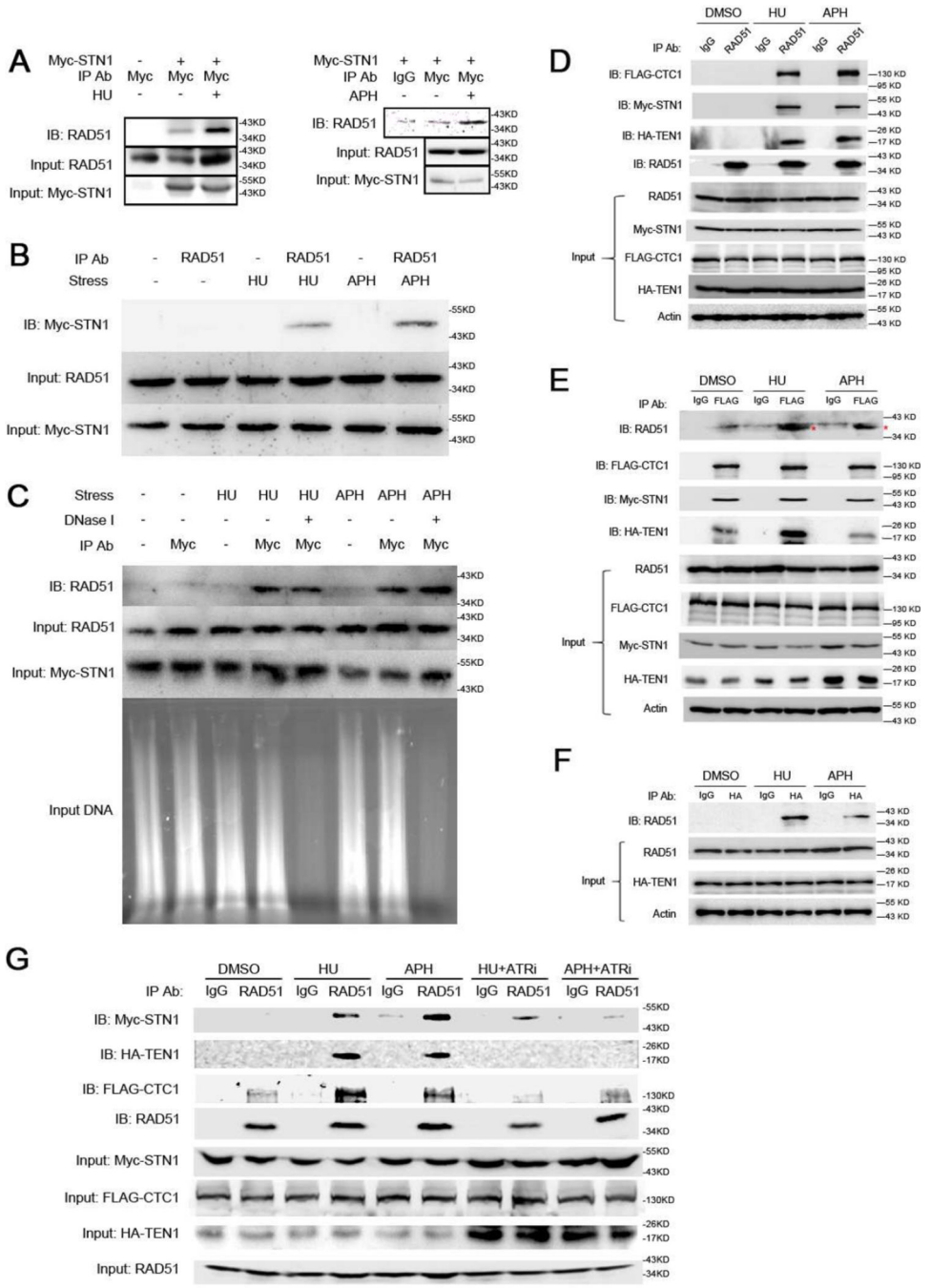

Figure 5. CST proteins physically interacts with RAD51 under replication stress in an ATR-dependent manner

(A) HeLa cells stably expressing Myc-STN1 were treated with or without 2 mM HU (left panel) or 0.2 μM APH (right panel) for 20 hrs, followed by co-IP with anti-myc antibody. Precipitates were analyzed by western blotting to detect endogenous RAD51 that was pulled down by Myc-STN1. **(B)** Reciprocal co-IP. IP was performed with HeLa-myc-STN1 cells with anti-RAD51 antibody, followed by western blotting to detect Myc-STN1 pulled down by RAD51. **(C)** STN1/RAD51 interaction is independent of DNA. Whole cell lysates were

treated with or without DNase I prior to IP. Following +/− DNase I treatment, co-IP was performed with anti-myc antibody and precipitates were analyzed by anti-RAD51 western blotting to detect RAD51 that was pulled down. DNA from treated and untreated lysates was purified by phenol extraction, separated on agarose, and stained with ethidium bromide. The agarose gel image (bottom panel) shows the removal of DNA by DNase I treatment. **(D), (E), (F):** CTC1 and TEN1 physically interacts with endogenous RAD51 under replication stress. FLAG-CTC1, Myc-STN1 and HA-TEN1 were co-expressed in HEK293T cells and treated with 2 mM HU or 0.2 μM APH for 20 hrs, followed by reciprocal co-IP. **(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. * indicates the RAD51 band. **(F)** IP was performed with anti-HA, followed by western blotting to detect RAD51 in the immunoprecipitates. **(G)** CST/RAD51 interaction is dependent on ATR activity. FLAG-CTC1, Myc-STN1 and HA-TEN1 were co-expressed in HEK293T cells and treated with 2 mM HU or 0.2 μM APH in the presence or absence of ATRi for 20 hrs. IP was performed with anti-RAD51 recognizing endogenous RAD51, followed by western blotting to detect FLAG-CTC1, Myc-STN1, and HA-TEN1 in immunoprecipitates.

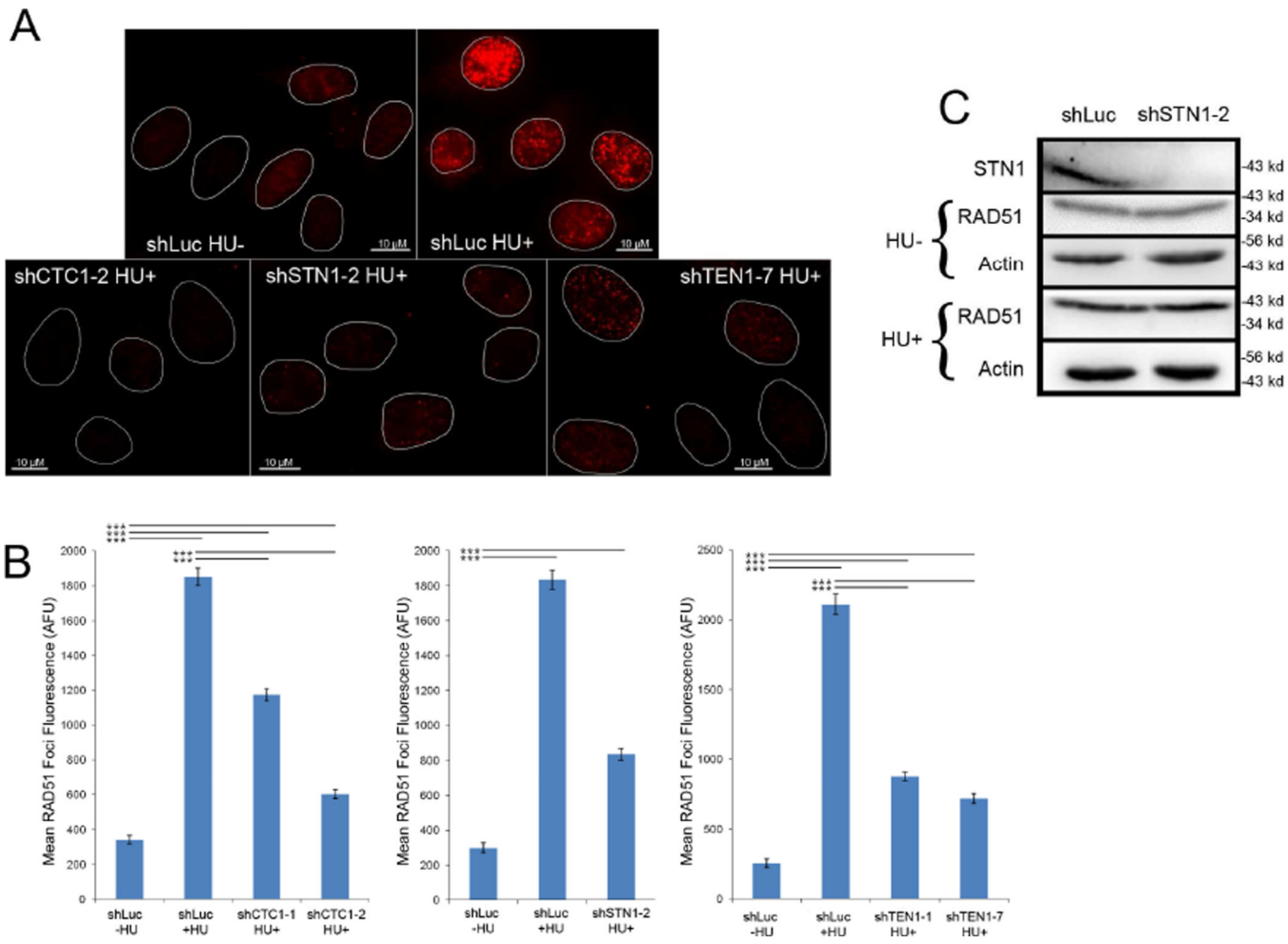

Figure 6. Deficiency in CST reduces RAD51 foci formation after HU treatment

(A) HU-induced RAD51 foci formation was significantly decreased after CTC1, STN1 or TEN1 knockdown. HeLa stably expressing shLuc, shCTC1, shSTN1, 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. ***: $p \leq 0.001$. **(C)** RAD51 expression detected by western blotting in the presence or absence of HU in STN1 knockdown cells.

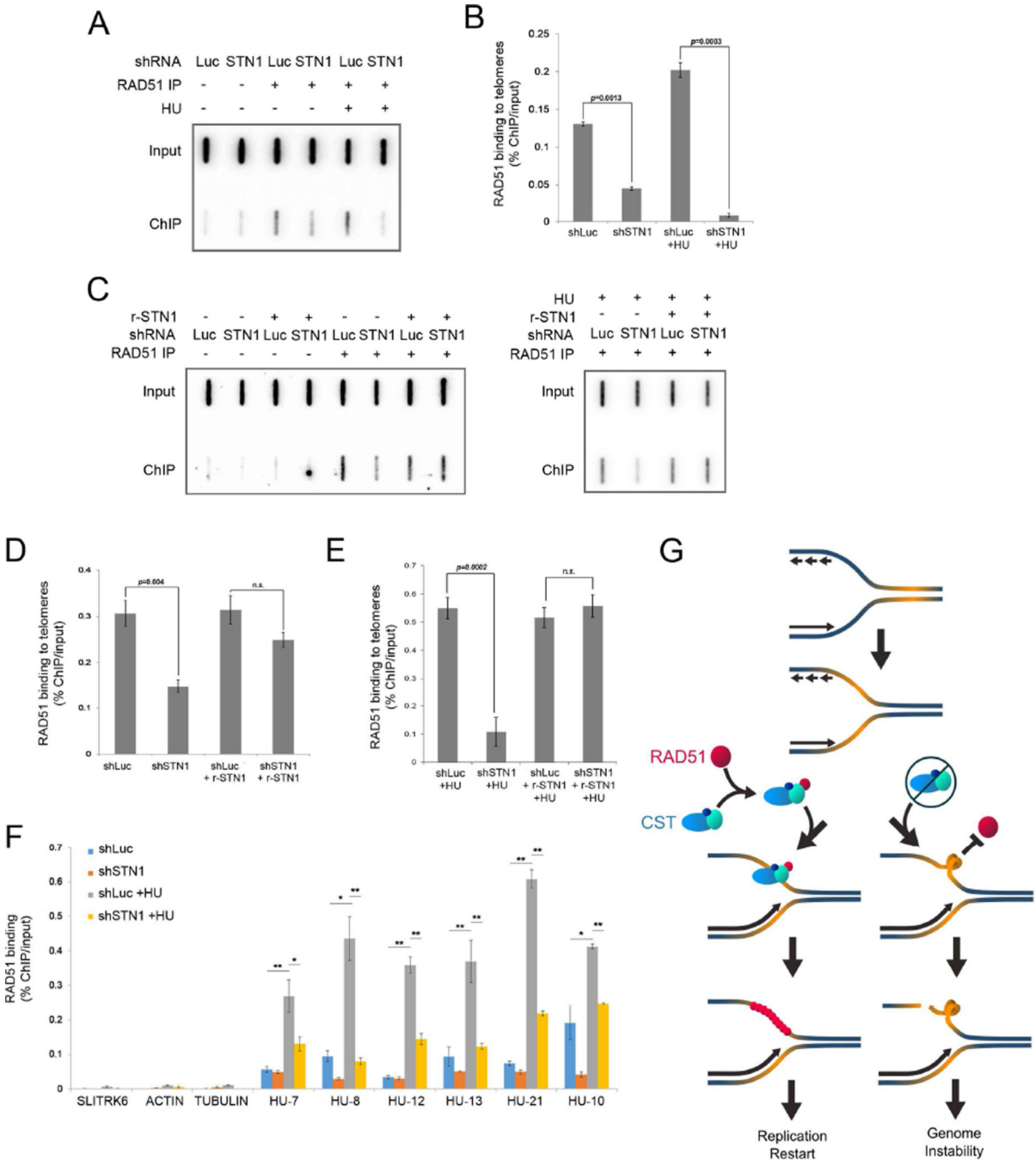

Figure 7. STN1 deficiency diminishes RAD51 recruitment to telomeres and STN1-binding sites upon replication stalling

(A) Representative slot-blot of RAD51 ChIP at telomeres in STN1-deficient cells. HeLa expressing shLuc or shSTN1 were treated with or without HU (2 mM), 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. *P* values were calculated with two-tailed *t*-test from three independent experiments. Error bars: SEM. **(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 or without HU, 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). *P* values were calculated with two-tailed *t*-test from three independent experiments. Error bars: SEM. **(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. Error bars: SEM. *: $p<0.05$. **: $p<0.01$. **(G)** Model for CST in promoting replication restart. Upon fork stalling at GC-rich repetitive sequences, ssDNA is accumulated. 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.