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Timeless functions independently of the Tim-Tipin complex to promote sister chromatid cohesion in normal human fibroblasts

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Abbreviations: ATR, ataxia telangiectasia mutated and Rad3-related; hTERT, human telomerase reverse transcriptase; NHF, normal human fibroblast; NTC, non-targeting control; RFPC, replication fork protection complex; RPA, replication protein A; SCC, sister chromatid cohesion; siRNA, small interfering RNA; Tim-Tip, Timeless-Tipin

The Timeless-Tipin complex and Claspin are mediators of the ATR-dependent activation of Chk1 in the intra-S checkpoint response to stalled DNA replication forks. Tim-Tipin and Claspin also contribute to sister chromatid cohesion (SCC) in various organisms, likely through a replication-coupled process. Some models of the establishment of SCC posit that interactions between cohesin rings and replisomes could result in physiological replication stress requiring fork stabilization. The contributions of Timeless, Tipin, Claspin, Chk1 and ATR to SCC were investigated in genetically stable, human diploid fibroblast cell lines. Whereas Timeless, Tipin and Claspin showed similar contributions to UVC-induced activation of Chk1, siRNA-mediated knockdown of Timeless induced a 100-fold increase in sister chromatid discohesion, whereas the inductive effects of knocking down Tipin, Claspin and ATR were 4–20-fold. Knockdown of Chk1 did not significantly affect SCC. Consistent findings were obtained in two independently derived human diploid fibroblast lines and support a conclusion that SCC in human cells is strongly dependent on Timeless but independent of Chk1. Furthermore, the 10-fold difference in discohesion observed when depleting Timeless versus Tipin indicates that Timeless has a function in SCC that is independent of the Tim-Tipin complex, even though the abundance of Timeless is reduced when Tipin is targeted for depletion. A better understanding of how Timeless, Tipin and Claspin promote SCC will elucidate non-checkpoint functions of these proteins at DNA replication forks and inform models of the establishment of SCC.

Introduction

The ataxia telangiectasia-mutated and Rad3-related (ATR)-dependent intra-S checkpoint is activated when excess replication protein A (RPA)-coated ssDNA is generated at replication forks that are stalled by DNA damage or by chemicals that inhibit DNA synthesis.[1] The checkpoint is enforced by ATR phosphorylation of the transducer kinase, Chk1. Phosphorylation of Chk1 is associated with suppression of new origin firing, slowed fork displacement and the stabilization of stalled replication forks.[2] The phosphorylation of Chk1 by ATR is spatially and temporally regulated by proteins that recruit ATR to stalled replication forks, activate its kinase activity and mediate ATR interaction with Chk1.[1] Timeless, Tipin and Claspin are mediators of ATR phosphorylation of Chk1 in response to DNA damage and replication stress.[3-8]

Timeless and Tipin form a heterodimer and are components of the "replication fork protection complex" (RFPC), named for its role in stabilizing stalled DNA replication forks and protecting cells from genomic instability.[9,10] SiRNA-mediated reduction of Timeless, Tipin or Claspin proteins attenuates DNA damage-induced phosphorylation of Chk1 and compromises ultraviolet (UV) light-induced activation of the intra-S checkpoint in HeLa cells.[3,6,8,11] Tim-Tipin and Claspin likely work together to mediate ATR activation of Chk1 by Tipin-RPA recruitment of Claspin-Chk1 through a Tipin-Claspin interaction.[8] Furthermore, Tim-Tipin and Claspin orthologs/analogs associate with chromatin, interact with replisome components and appear to travel with replication forks in the absence of exogenously applied DNA damage,[9,10,12-16] and DNA synthesis is compromised when human cells are depleted of Timeless, Tipin or Claspin.[3,6,17]

Timeless, Tipin and Claspin orthologs/analogs also contribute to sister chromatid cohesion (SCC) in various organisms.[9,18-22] SCC is required for identical partitioning of duplicated genomes to daughter cells. The cohesin complex, a multi-subunit ring, creates physical linkages between sister chromatids. The loading of

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cohesin onto chromatin, the establishment of SCC and the maintenance and dissolution of cohesion are cell cycle-regulated processes.[23] Defective SCC is associated with developmental genetic disorders, including Roberts syndrome, Cordelia de Lange syndrome and Warsaw breakage syndrome,[24,25] and mutations in SCC genes have been detected in cancers.[26]

Large-scale genetic screens in yeast have revealed an intricate network of interactions among DNA replication, checkpoint activation and SCC.[27-29] The establishment of SCC, the process of generating a subset of cohesin complexes that will hold sister chromatids together until the metaphase-anaphase transition, occurs during S phase.[30] The mechanism of the establishment of SCC has yet to be fully defined. Recent advances suggest that the cohesion that persists until the separation of sister chromatids in metaphase may be the outcome of pro- and anti-establishment reactions that could occur at replication forks.[30] Reduction of Tim-Tipin and Claspin orthologs/analogs in various systems results in premature separation of sister chromatids in metaphase, a phenotype that could arise from incomplete establishment of cohesion during S phase or precocious removal of cohesins in metaphase. Studies using the *Xenopus laevis* egg extract system demonstrated that Tim1 specifically was required during replication for full paring of sister chromatids in metaphase,[19] which, taken together with other observations, indicates a role for Timeless orthologs in the establishment of SCC.

The interaction of cohesin rings with replisomes is a key feature of models that describe the events necessary for the establishment of SCC.[30] Some models posit that the association of cohesin rings with replisomes may require pausing of replication forks[23,30] an event that would require replisome stabilization through Tim-Tipin and Claspin.[19,20,31] The studies described here examined whether Timeless, Tipin and Claspin contribute to SCC in genetically stable, diploid human fibroblasts as part of, or independently of, their interactions with the checkpoint kinases ATR and Chk1. Even though Timeless and Tipin are known to form a heterodimeric complex,[3] targeting Timeless by siRNA-mediated knockdown produced a 10-fold greater increase in defective SCC compared to targeting Tipin. Furthermore, targeting of ATR and Claspin produced ~4–20-fold increases in discohesion, whereas Chk1-depleted cells were not statistically different from controls. The results indicate that the requirement for Timeless, Tipin and Claspin in SCC in human cells is independent of ATR-dependent intra-S checkpoint signaling.

Results

The involvement of Timeless, Tipin, Claspin, ATR and Chk1 in SCC was investigated by using siRNA to deplete hTERT-expressing normal human fibroblasts (NHF) of these proteins and examining metaphase preparations for aberrant pairing between sister chromatids.

At 48 h after introduction of siRNAs, levels of targeted protein were reduced by ≥ 95% compared to NHF1-hTERT that were electroporated with the non-targeting control (NTC) siRNA (**Fig. 1A**). Similar to previous reports using cancer cell lines, depletion of Timeless reduced the stability of Tipin and vice versa.[3,6,9,14] When NHF1-hTERT were electroporated with siRNAs targeting Chk1, Timeless, Tipin or Claspin, the targeted protein was reduced, but the levels of the other three proteins also decreased by 40–50%. These proteins may regulate each other's expression, and indeed, Chk1 has been shown to regulate the stability of Claspin.[32] However, the abundance of these proteins is higher during S phase in normal human fibroblasts and other cell lines.[4,11,12,33,34] Equivalent amounts of protein were loaded per well for SDS-PAGE, but the S-phase fraction in NHF1-hTERT cultures depleted of Chk1, Timeless, Tipin or Claspin was reduced (data not shown), which could account for the lower levels of these proteins when any one of them was targeted by siRNA. It remains to be formally examined whether Chk1 and Tim-Tipin regulate each other's stability.

To demonstrate functional depletion of checkpoint proteins, ATR-dependent phosphorylation of Chk1 at S345 was examined one hour after exposure to 2.5 J/m^2 (**Fig. 1A**). It has been shown that inhibition of DNA replication following exposures to low fluences of UVC results from active signaling as opposed to passive obstruction of replication forks.[35] Depletion of ATR reduced UVC-induced activation of Chk1 similar to depletion of Chk1 itself. Targeting of Timeless, Tipin, or Claspin attenuated UVC-induced activation of Chk1, consistent with previous reports in cancer cell lines that these proteins mediate ATR-dependent activation of Chk1 in response to DNA-damaging agents.[3,6-8] Despite ≥ 95% depletion of mediator proteins, the UVC-induced P-Chk1 S345 could not be fully attenuated, on average, by targeting of Timeless, Tipin or Claspin or by co-targeting of Timeless and Claspin (**Fig. 1B**), perhaps reflecting the contribution of other mediators to ATR-dependent intra-S checkpoint signaling.[2]

Giemsa-stained metaphase spreads were prepared to examine whether NHF1-hTERT that were depleted of checkpoint proteins would exhibit defects in SCC. At metaphase, sister chromatids were fully paired in cells electroporated with NTC siRNA (**Fig. 2A**). Timeless-depleted cells, however, exhibited striking defects in SCC (**Fig. 2B**). A range of defects was observed, from partial separation of sister chromatids to complete premature anaphase. In metaphases exhibiting partial separation of sister chromatids, some chromatids were still normally paired whereas others showed loss of both arm and centromeric cohesion. Fluorescence in situ hybridization (FISH) probes to centromere 9 and the CDKN2A locus were used to confirm that what was observed by Giemsa staining was, indeed, loss of centromeric cohesion (**Fig. 2C and D**). Chromosome 9 centromeric sequences and CDKN2A loci were detected with green or red fluorescent probes, respectively. Chromosome 9 centromeres were fully cohered in 100% of metaphases from NHF1-hTERT electroporated with NTC siRNA. In contrast, the fluorescent signals of the centromeres of chromosome 9 were separated in 38% of metaphases from Timeless-depleted cells.

Defective SCC was observed in 27 ± 5.4% of Giemsa-stained metaphases prepared from NHF1-hTERT depleted of Timeless, representing a 100-fold increase over the control level of 0.23 ± 0.17% (**Fig. 3**). However, Tipin siRNA did not produce the same effect on SCC as Timeless siRNA despite the dependency of the two proteins on each other for their levels

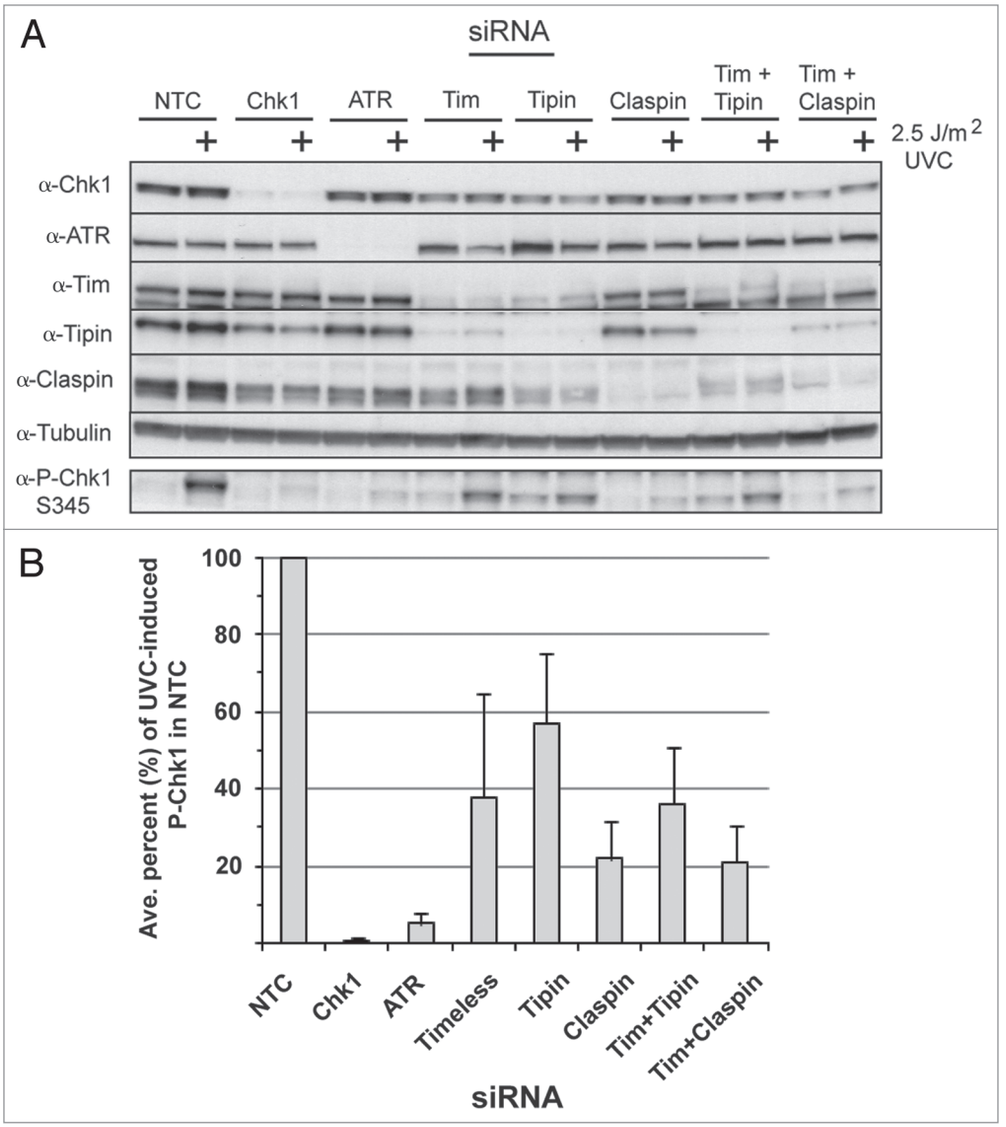

Figure 1. Depletion of checkpoint/RFPC proteins attenuates UVC-induced phosphorylation of Chk1 in NHF1-hTERT. Forty-eight h after electroporation with siRNAs, NHF1-hTERT were exposed to 0 or 2.5 J/m^2 UVC. Cells were harvested 1 hour after exposure. (A) A representative western blot from among three independent experiments depicting siRNA-mediated protein depletion and UVC-induced phosphorylation of Chk1 at S345. (B) Quantification of the attenuation of UVC-induced P-Chk1 S345 in NHF1-hTERT depleted of checkpoint proteins. Graph depicts average percents and standard deviations from three independent experiments.

of expression. Only 2 ± 2% of metaphases exhibited defective SCC when Tipin was targeted by siRNA. Similar to depletion of Tipin, 4 ± 1.5% of metaphases exhibited defective SCC when cells were depleted of Claspin. Although the percentage of metaphases with defective SCC was not as dramatic with depletion of Tipin or Claspin compared to depletion of Timeless, loss of Tipin or Claspin produced statistically significant ~10–20-fold increases in defective SCC compared to the control, respectively. Co-targeting of Timeless and Tipin produced defective SCC in 24 ± 2.5% of metaphases, a result that was not different from targeting of Timeless alone. Additionally, 22 ± 5.3% of metaphases showed defective SCC with co-targeting of Timeless and Claspin. NHF1-hTERT that were depleted of ATR or Chk1 exhibited 1 ± 1% or 0.7 ± 0.7% of metaphases with defective SCC, respectively; these results were not statistically different from the control. The percent of metaphases exhibiting partial discohesion versus complete premature anaphase for each knockdown is reported in **Supplemental Figure 1**.

One possibility for the 10-fold difference in defective SCC when targeting either Timeless (27%) or Tipin (2%) by siRNA could have been off-target effects of the Timeless siRNA. Tim-05 or Tim-06 siRNAs (designated by their Dharmacon catalog numbers) were equally effective at knocking down Timeless and reducing Tipin. Depletion of Timeless by either siRNA was accompanied by attenuation of UVC-induced Chk1 phosphorylation at S345 in NHF1-hTERT (**Fig. 4A**). Importantly, 37 ± 4.8% of metaphases from cells that were electroporated with Tim-06 siRNA exhibited defects in SCC (**Fig. 4B**), similar

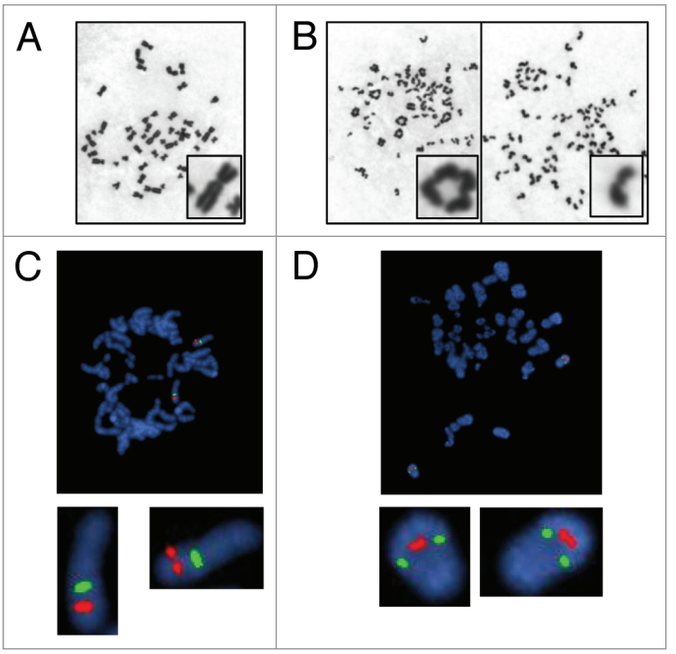

Figure 2. Metaphases from Timeless-depleted NHF1-hTERT display defective sister chromatid cohesion. (A) Metaphase from NHF1-hTERT 48 h after introduction of NTC siRNA. (B) Metaphases from Timeless-depleted NHF1-hTERT depicting range of discohesion phenotypes from partial sister chromatid separation (left panel) to complete premature anaphase (right panel). (C) Metaphase from NHF1-hTERT electroporated with NTC siRNA and analyzed by FISH. A green centromere 9 probe and a red CDKN2A probe are shown with DAPI counter-stain. (D) Premature centromere separation in a metaphase from NHF1-hTERT electroporated with Timeless siRNA and analyzed by FISH.

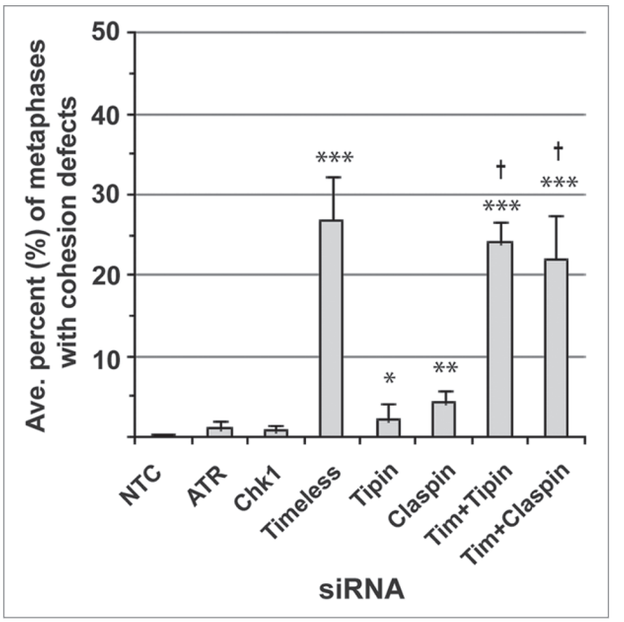

Figure 3. Metaphases from checkpoint/RFPC-depleted NHF1-hTERT display defective sister chromatid cohesion. NHF1-hTERT metaphases were prepared 48 h after electroporation with siRNAs. Error bars represent standard error of the mean (S.E.M.) from 25–50 determinations each from a minimum of three independent experiments per targeting siRNA and a total of 13 independent experiments (800 metaphases) for NTC siRNA (experiments were performed in randomized sets of knockdowns). * $P < 0.05$, ** $P < 0.002$, *** $P < 0.0001$, † = not statistically different from Timeless siRNA result.

to the degree of defective SCC observed with Tim-05 siRNA (**Fig. 3** and FISH result).

Lastly, to determine whether disruption of SCC by depletion of Timeless was a stereotypic response in normal human diploid fibroblasts, the analysis was repeated in a second line, NHF10-hTERT (**Fig. 5**). The depletion of targeted proteins and the attenuation of UVC-induced P-Chk1 S345 in NHF10-hTERT were similar to that observed in NHF1-hTERT (data not shown). Defective SCC was observed in 56 ± 2.8% of Giemsa-stained metaphases prepared from NHF10-hTERT depleted of Timeless, representing a 100-fold increase over the control level of 0.45 ± 0.18%. When accounting for the higher baseline of discohesion in NHF10-hTERT, all knockdowns (except for ATR) showed results that were highly similar to those obtained from NHF1-hTERT (**Sup. Fig. 2**).

Discussion

The establishment of sister chromatid cohesion occurs through a replication-coupled process.[30,36] Some models of replication-coupled cohesion suggest that fork uncoupling may take place during interactions between replisomes and cohesin rings, an event that would activate Chk1.[19,20,28,30] Experiments described herein have replicated in genetically stable human fibroblast lines the observation that Tim-Tipin and Claspin contribute to ATR-dependent activation of Chk1 in response to fork uncoupling by DNA damage, namely UVC-induced photoproducts (**Fig. 1A and B**). Timeless, Tipin and Claspin were also shown to contribute to SCC in NHF lines (**Figs. 3 and 5**). If Chk1 was required for replication-coupled SCC, it would be expected that depletion of Chk1 would be at least equivalent to loss of Tipin or Claspin in the magnitude of the discohesion phenotype. However, whereas UVC-induced P-Chk1 was nearly undetectable in cells depleted of Chk1, the same degree of knockdown did not significantly affect SCC. Depletion of ATR produced an 18-fold increase in discohesion in NHF10-hTERT that was similar to loss of Tipin or Claspin (7- to 18-fold) (**Sup. Fig. 2**). In NHF1-hTERT, the effect of ATR depletion on SCC was not as evident. Taken together, these results suggest that the contribution of Tim-Tipin and Claspin to SCC is separate from ATR-dependent activation of Chk1 in human cells. This finding is in agreement with the observation that the checkpoint-activation function of Swi3, the *S. pombe* ortholog of Tipin, could be separated from the role of Swi3 in SCC.[37]

Although Timeless and Tipin contribute to SCC, Timeless appears to have a role in SCC that is separate from Tipin. This result, documented in two NHF lines, is novel compared to observations made in HeLa cells and other organisms and is difficult to interpret, as expression or stability of Timeless and Tipin orthologs are interdependent. Differences between NHF lines and other experimental models may be attributable to the sensitivity of the assays available to detect sister chromatid discohesion and

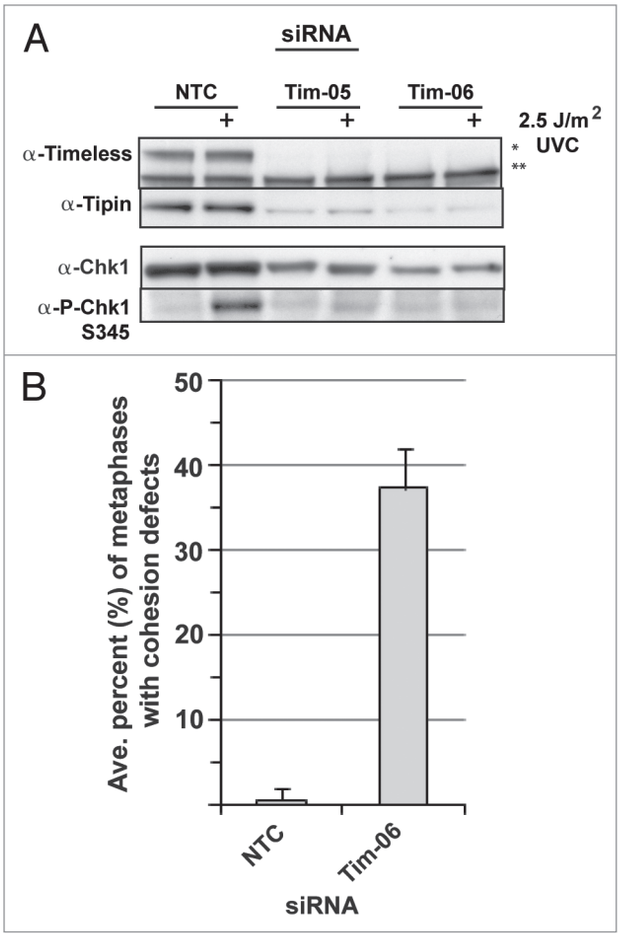

Figure 4. Defective sister chromatid cohesion in Timeless-depleted NHF1-hTERT is not an off-target effect of Timeless siRNA. (A) Comparison of Timeless depletion by two different siRNAs (Tim-05 or Tim-06). Forty-eight h after electroporation of siRNAs, cells were sham treated or exposed to 2.5 J/m^2 UVC and harvested 1 h later. *Timeless, **non-specific band. (B) NHF1-hTERT depleted of Timeless by Tim-06 siRNA display defective sister chromatid cohesion. Error bars represent standard error of the mean (S.E.M.) from 25–50 determinations each from three independent experiments.

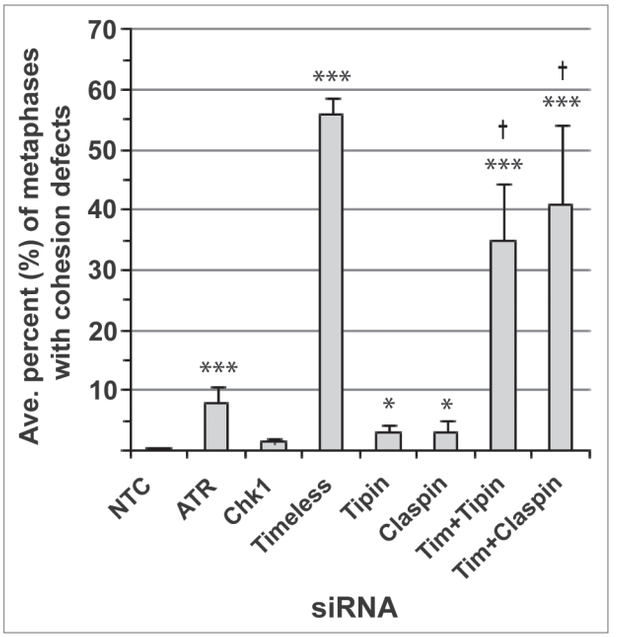

Figure 5. Metaphases from checkpoint/RFPC-depleted NHF10-hTERT display defective sister chromatid cohesion. NHF10-hTERT metaphases were prepared 48 h after electroporation with siRNAs. Error bars represent standard error of the mean (S.E.M.) from 25–50 determinations each from a minimum of three independent experiments per targeting siRNA and a total of 16 independent experiments (852 metaphases) for NTC siRNA (experiments were performed in sets of randomized knockdowns). * $P < 0.05$, *** $P < 0.0001$, † = not statistically different from Timeless siRNA.

the degree to which various models rely on Timeless and Tipin for SCC. Due to low baselines, it was possible to detect ~100-fold increases in discohesion in two different NHF lines depleted of Timeless. When Timeless or Tipin are targeted by siRNA, the remaining partner is dramatically reduced but tends to be present in greater amounts than the targeted partner (**Fig. 1A, 4A and Sup. Fig 3**). One possibility to account for the difference in discohesion between Timeless- versus Tipin-depleted NHFs is if the remaining Timeless in a Tipin-siRNA targeted cell is properly localized and functional. If this is the case, the remaining Timeless indeed has a function specific to SCC, as the efficiency of UVC-induced Chk1 activation was reduced similarly whether Timeless or Tipin were targeted individually or together by siRNA (**Fig. 1B**). The Tipin-independent function of Timeless in SCC could be mediated by Timeless itself or through the effect of Timeless on other cohesion-related proteins.

HeLa cells also exhibit a similar relationship with regard to levels of Timeless and Tipin when one or the other is targeted by siRNA.[6,9,14] Leman et al (2010)[9] found that Timeless interacted with cohesin ring subunits in the presence or absence of DNA, but that the interaction of Tipin with cohesins was DNA-dependent, suggestive of different contributions to SCC from Timeless versus Tipin. However, depletion of Timeless or Tipin produced equivalent (four-fold) increases in discohesion in HeLa cells. A high baseline (6–7%) of spontaneous discohesion in HeLa cells could have obscured phenotypic detection of an independent role of Timeless in SCC.

This report is the first to show a role for Claspin in SCC in normal human cell lines, as predicted by studies of the Claspin analog, Mrc1, in yeast.[28,38] Similar to Swi3, the contribution of Mrc1 to SCC was independent of its checkpoint function.[38,39] In *S. cerevisiae*, TOF1-CSM3 (orthologs of Timeless and Tipin, respectively) and MRC1 belong to different SCC epistasis groups.[21] It will be of interest to determine whether Tim-Tipin and Claspin interact separately or together to promote SCC in human cells.

Presently, little is known with regard to the mechanism by which Timeless, Tipin and Claspin orthologs/analogs contribute to replication-coupled SCC. In various models, Timeless and Tipin orthologs have been shown not only to interact with cohesin ring subunits and to promote their association with chromatin, but also to interact with other replisome-associated proteins that

contribute to normal SCC. These proteins include AND-1,[18,19] which has similarities to Tim-Tipin, including interaction with replicative DNA polymerases and promotion of normal DNA synthesis,[40] and ChlR1,[9] a helicase involved in lagging strand maturation,[41] that is associated with Warsaw breakage syndrome, a cohesinopathy.[24]

During the course of DNA replication, replisomes are remodeled when polymerization is stalled or blocked by exogenous and endogenous challenges. Fork uncoupling, translesion synthesis, template switching and fork reversal are examples of replisome remodeling. Replication forks also may undergo modification when interacting with cohesin rings to establish SCC. Present models suggest that cohesin rings might open and close at replisomes as part of the establishment reaction of SCC (reviewed in Sherwood, 2010).[30] This report suggests that Timeless, Tipin, Claspin and possibly ATR promote normal SCC, but not through Chk1, indicating that the kind of replisome alterations that could take place when cohesins and replisomes interact does not necessarily result in fork uncoupling. Analysis of the role of Tim-Tipin and Claspin in SCC will serve not only to advance understanding of the functions of these proteins in DNA metabolism but will also inform models of how establishment of SCC takes place during replication.

Materials and Methods

Normal human fibroblast cell lines and culture. NHF1-hTERT and NHF10-hTERT cell lines, immortalized by ectopic expression of the catalytic subunit of human telomerase,[35,42-44] were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum (culture reagents from Sigma Chemical Co.). Cell lines were grown at 37°C in a humidified atmosphere of 5% CO_2 and were determined to be free of mycoplasma contamination using the Gen-Probe Mycoplasma Tissue Culture, NI Rapid Detection kit (Gen-Probe Inc.).

Electroporation of siRNAs. The siRNA duplexes targeting the following proteins were purchased from Dharmacon: ATR (L-003202), Chk1 (J-003255), Claspin (J-005288), Timeless (J-019488-05 and J-019488-06) and Tipin (J-020843). Non-targeting control siRNA (D-001210-02) also was purchased from Dharmacon. The siRNA duplexes were delivered into NHF lines by electroporation with the Normal Human Dermal Fibroblast (NHDF) nucleofection kit VPD-1001 (Lonzo). The total amount of siRNA introduced into cells for single versus double depletions was held constant at 200 pmol siRNA per 1 x 10^6 cells.

UVC exposure, western blots and antibodies. Forty-eight hours after introduction of siRNAs, medium was reserved, NHF1-hTERT were washed with warmed PBS and were exposed to 2.5 J/m^2 UVC. Reserved medium was added back, and cells were incubated for 1 h before harvest for protein analysis. Sham-treated cells were handled similarly, except that they were not exposed to UVC. Cell lysates from NHF lines were prepared for protein electrophoresis and immunoblotting as described in Heffernan et al. (2002).[35] The protein concentration of cell lysates was determined using the BioRad Dc Protein Assay in order to load equivalent amounts of protein per well for SDS-PAGE. The degree of protein depletion was determined by using Image J to obtain the pixel density of protein bands from exposed film. Protein levels were first normalized against the anti-α-Tubulin loading control and then expressed as the percent of the NTC protein level. The P-Chk1 S345 signal was normalized to Chk1 levels. The UVC-induced component of P-Chk1 S345 was determined by subtracting background P-Chk1 S345 for each of the knockdowns and comparing the level of induction to cells electroporated with NTC siRNA in order to normalize across experiments.

The following antibodies were used for immunoblotting: goat anti-ATR (Santa Cruz), mouse anti-Chk1 (Santa Cruz), rabbit anti-phospho-Chk1 S345 (Cell Signaling), rabbit anti-Claspin (Santa Cruz), rabbit anti-Timeless (Bethyl), mouse anti-Timeless (Abnova), rabbit anti-Tipin (Bethyl) and rabbit anti-α-Tubulin (Cell Signaling). Western blots were analyzed using Image J software (Rasband, WS, Image J US National Institute of Health, Bethesda, MD, rsb.info.nih.gov/ij/, 1997-2009). Depletion of targeted proteins was verified by western blot for every experiment.

Giemsa-stained metaphase preparations. Cells were incubated with 100 ng/ml colcemid for 1 h, harvested, swelled in 75 mM KCl and fixed with a -20° C solution of 3:1 methanol: acetic acid. After fixation, cells were dropped onto slides that had been chilled in de-ionized distilled H2O (ddH2O). Metaphase spreads were aged for three days before staining. Slides were heated at 80° C for 10 min and immersed into a 3.5% solution of Kayro-MAX Giemsa stain (Gibco) in ddH2O for 2.5 min. Metaphases were examined using a 100x oil objective on an Olympus BH-2 Brightfield Microscope. Digital pictures were obtained using a Spot camera with Spot Imaging Software (Diagnostic Instruments, Inc). Twenty-five to 50 metaphases were evaluated per treatment. The experimenter was blind to treatment during the acquisition and scoring of metaphase data.

Fluorescence in-situ hybridization (FISH). NHF1-hTERT were harvested at 48 h after electroporation with siRNAs and were fixed and dropped onto slides using the same procedure as for metaphase preparations. Fluorescence in-situ hybridization was performed using the Vysis LSI p16 (9p21) SpectrumOrange/CEP 9 SpectrumGreen Dual Color Probe (Vysis LSI DNA Probes) according to the manufacturer's protocol. Two experimenters blind to treatment evaluated 50 metaphases per treatment for a total of 100 determinations per treatment. Pictures were obtained using a Zeiss Axioplan 2 microscope fitted with the appropriate filters and cooled CCD camera (CCD-1300DS; VDS Vosskuehler, Osnabruck, FRG).

Statistical analysis. The number of metaphases with discohesion was analyzed to determine whether the numbers varied significantly across different treatments. The generalized linear model framework was used to handle standard data analysis for estimating various parameters of interest with appropriate 95% confidence intervals and hypothesis testing. Specifically, Poisson regression was used to model the discohesion counts with the total metaphases counts as the OFFSET variable. Likelihood

Ratio Statistic was used to determine the statistical significance of the difference across treatments. All statistical analyses were performed using SAS 9.2 (SAS Institute Inc.).

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Note

Supplemental materials can be found at:
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