CHAPTER V

THE GENOME MAINTENANCE ACTIVITIES OF CINP INCLUDE REGULATION OF ATR CHECKPOINT SIGNALING

Introduction

The RNAi screen described in the previous chapter identified gene products critical for the maintenance of genome integrity via the ability of gene-silencing shRNA and siRNA molecules to activate the DDR. The development of a series of secondary assays allowed us to further characterize gene products that may have genome maintenance activities related to DNA replication. The DDR activation and sensitivity to replication stress agents is not only indicative of defects in DNA replication-dependent processes, but is also consistent with defects in ATR signaling pathways. The loss of ATR activity causes the formation of replication fork-associated DSBs, presumably due to the inability of ATR-depleted cells to stabilize or recover stalled forks [43]. Cells in which *ATR* has been silenced or deleted accumulate DSBs that activate the ATM-mediated DDR pathway. Therefore, the RNAi screen may identify novel ATR regulators and effectors, as well as additional genome maintenance activities.

CDK2-interacting protein (CINP) was identified as a genome maintenance gene in the RNAi screen, and was also previously identified in the lab as a candidate ATRIP-interacting protein in a yeast two-hybrid screen. Little is known about the cellular function of CINP, since there is only a single publication identifying this novel gene as a CDK2-interacting protein in a yeast two-hybrid screen [256]. The authors speculate that

CINP has a role in transforming the pre-replication complex into the initiation complex at the onset of DNA replication by functioning as a docking protein to target the CDK2 and DDK kinases to origins of replication. Activation of the replicative helicase and recruitment of the replisome require phosphorylation events mediated by these S phase kinases, resulting in origin firing and initiation of DNA synthesis.

While a role for CINP in DNA replication has been suggested, there is presently no evidence that CINP functions in DDR pathways. The presence of genome instability after loss of *CINP*, combined with the potential interaction of CINP with ATRIP, prompted us to characterize this potential ATR signaling protein further. Our results indicate CINP is a novel checkpoint protein that is important for ATR-mediated CHK1 phosphorylation and maintenance of the G2 checkpoint.

Results

CINP is required for genome maintenance and resistance to replication stress

CINP is one of the seventy-four genome maintenance genes identified in the RNAi screen described in Chapter IV. DDR activation and sensitivity to replication stress were observed with multiple RNAi molecules targeting *CINP*. Silencing of *CINP* causes KAP1 phosphorylation in HeLa cells (3 of 5 shRNAs), γH2AX foci formation in U2OS (3 of 4 siRNAs), and sensitizes cells to HU treatment (4 of 4 siRNAs) (Figure 5.1 and Appendix A, Tables 1, 2, and 4). The DDR activation and HU sensitivity observed after silencing of *CINP* is consistent with a defect in ATR signaling or DNA replication.

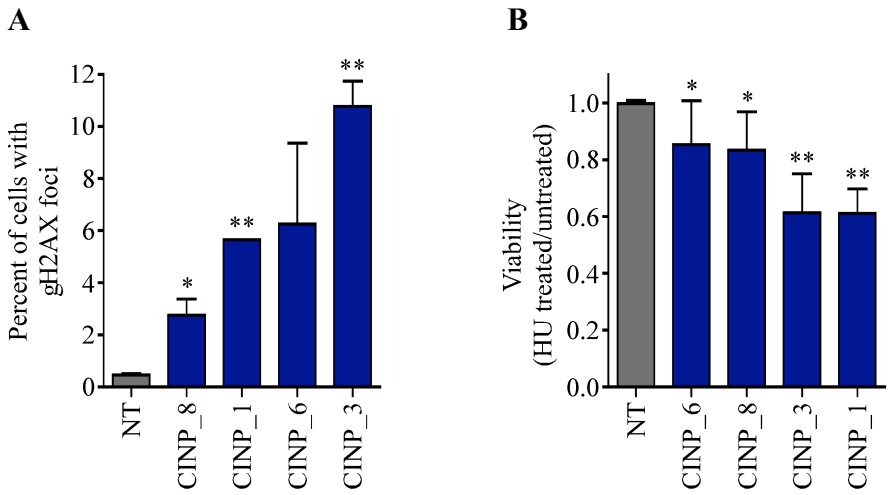

Figure 5.1. CINP displays genome maintenance activity and is required to maintain cellular viability in response to replication stress. γH2AX foci formation **(A)** and cell viability following HU treatment **(B)** are shown for the four CINP siRNA oligonucleotides screened, as well as the non-targeting control. (* = $p \leq 0.05$; ** = $p \leq 0.01$). Error bars represent standard error (n=3).

CINP interacts with the ATR-ATRIP complex

Although *CINP* was not one of the strongest hits in any of the assays associated with the RNAi screen, it was distinctive because we previously identified CINP as a candidate ATRIP-interacting protein. A yeast two-hybrid screen using full length ATRIP as bait identified eleven cDNAs encoding interacting proteins, two of which were full length CINP. We therefore confirmed the association of CINP with ATR-ATRIP complexes by co-immunoprecipitation of exogenously expressed, epitope-tagged proteins from human cells. Immunoprecipitation of Flag-ATR or Flag-ATRIP from HEK293 cells co-precipitates HA-CINP (Figure 5.2A). The reciprocal purification indicates that Flag-CINP co-immunoprecipitates ATRIP. An interaction between endogenous ATR-ATRIP complexes and CINP was also observed in both HeLa and HEK293 cells (Figure 5.2B), but is sub-stoichiometric and likely to be transient in the cell. The co-immunoprecipitation of CINP with ATR-ATRIP confirms the yeast two-hybrid data and indicates that these proteins interact in cells.

The interacting surfaces between ATRIP and CINP were further mapped using a two-hybrid assay. Full length CINP fused to the GAL4 DNA binding domain was used as bait to screen a library of thousands of random ATRIP fragments fused to the GAL4 activation domain. Sequencing of the recovered ATRIP fragments revealed that CINP binds to the N-terminal half of the predicted ATRIP coiled-coil domain, containing the minimum amino acids 118-156 (Figure 5.3A).

The interaction between CINP and the ATRIP coiled-coil domain was confirmed by co-immunoprecipitation. Purification of Flag-CINP co-immunoprecipitates

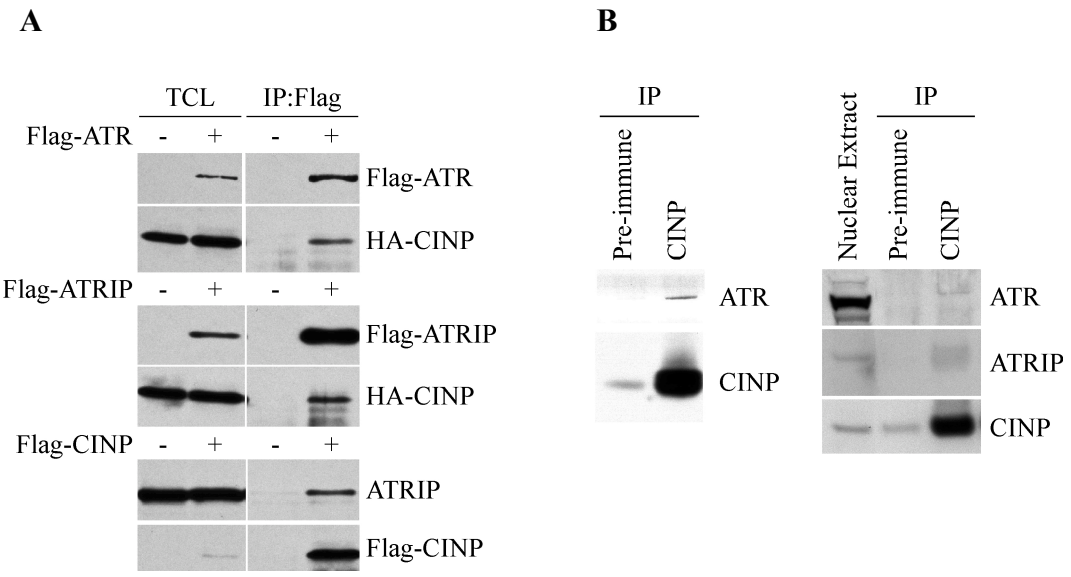

Figure 5.2. CINP interacts with ATR-ATRIP. **(A)** Flag-epitope-tagged ATR or ATRIP was co-expressed with HA-epitope-tagged CINP, or Flag-CINP was expressed in HEK293 cells, as indicated. Protein complexes were isolated by Flag purification, and co-precipitating proteins were identified by immunoblotting with Flag, HA, and ATRIP antibodies. **(B)** Endogenous CINP was immunoprecipitated from HeLa whole cell extracts (left panel) and HEK293 cell nuclear extracts (right panel). The co-precipitation of endogenous ATR and ATRIP was examined by immunoblotting co-purified proteins. The co-precipitation of exogenous proteins was obtained by Xin Xu, and the co-precipitation of endogenous proteins in HeLa extracts was obtained by Gloria Glick.

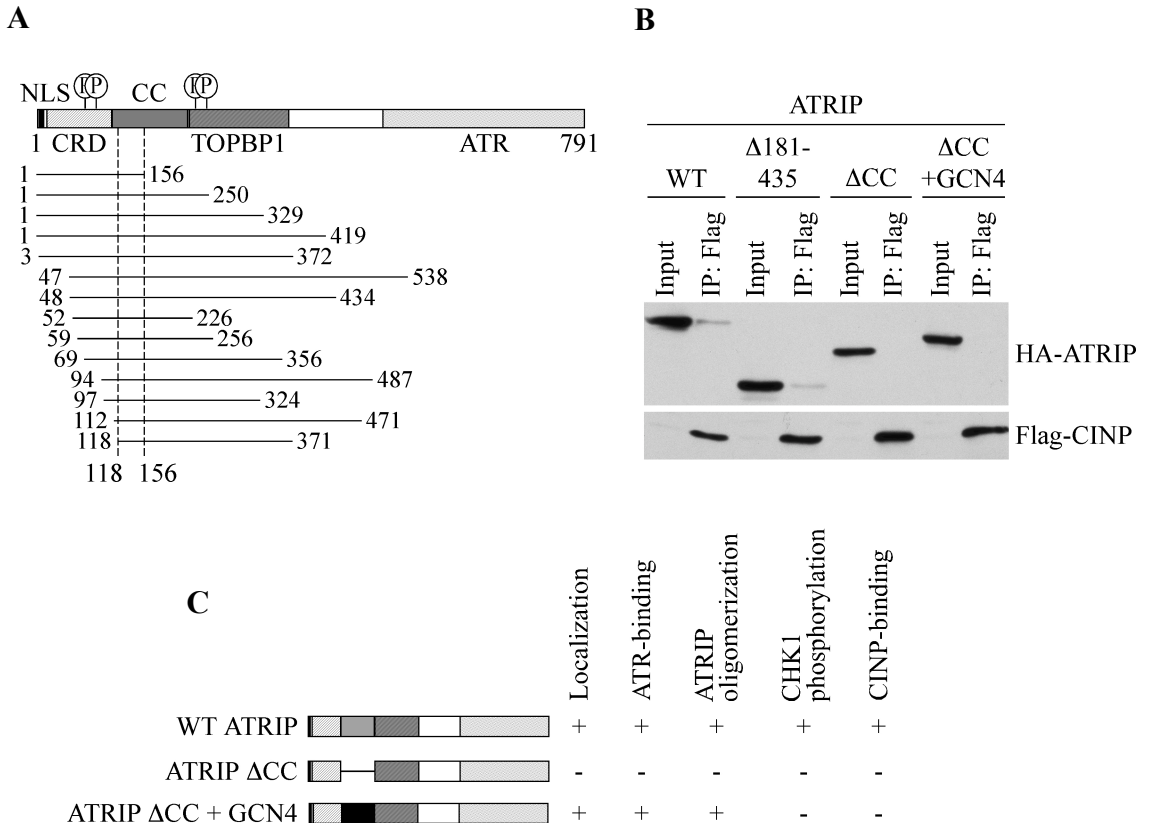

Figure 5.3. CINP interacts with the ATRIP coiled-coil domain. **(A)** A schematic diagram of ATRIP indicating the location of the nuclear localization signal (NLS), checkpoint recruitment domain (CRD), coiled-coil domain (CC), TopBP1-binding domain (TopBP1), ATR-binding domain (ATR), and phosphorylation sites. ATRIP fragments found to interact with full-length CINP in a yeast two-hybrid assay are shown with their starting and ending amino acid numbers. **(B)** HA-tagged wild-type or deletion mutants of ATRIP were expressed in HEK293 cells with Flag-tagged full length CINP. CINP was purified using the Flag-epitope, and co-precipitating ATRIP proteins were analyzed by immunoblotting with HA. ATRIPΔCC+GCN4 replaces the ATRIP coiled-coil domain with the GCN4 coiled-coil domain [141]. **(C)** Summary of the loss of function phenotypes associated with deletion of the ATRIP coiled-coil domain, and replacement of the ATRIP coiled-coil domain with that from GCN4. The yeast two-hybrid was performed by Gloria Glick, and the co-precipitation experiment was performed by David Cortez.

HA-tagged wild type ATRIP and an ATRIP deletion that retains amino acids 118-156. However, deletion of the coiled-coil domain (ΔCC) abrogates the ATRIP-CINP interaction (Figure 5.3B).

We have previously shown that the ATRIP coiled-coil domain is required for ATRIP oligomerization, stable ATR binding, accumulation of ATRIP at DNA lesions, and ATR-dependent checkpoint signaling [141]. Replacement of this domain with the coiled-coil dimerization domain of the *S. cerevisiae* GCN4 transcription factor restores all of these ATRIP functions except ATR-dependent CHK1 phosphorylation [141], suggesting there may be an activity of the coiled-coil domain in addition to promoting ATRIP oligomerization that is important to regulate ATR signaling. This activity may be binding of CINP, since replacement of the ATRIP coiled-coil domain with the GCN4 coiled-coil domain also fails to restore the interaction between ATRIP and CINP (Figures 5.3B and C).

CINP is important for ATR checkpoint signaling

Consistent with the interaction between CINP and the ATRIP coiled-coil domain, and the requirement for this domain in checkpoint signaling, RNAi silencing of *CINP* impairs ATR-dependent CHK1 phosphorylation in response to both UV and IR (Figures 5.4A and B). Thus, CINP is required for efficient phosphorylation of CHK1 by ATR in response to DNA damaging agents. Consistent with the ATR signaling defect, I also observed a defect in the ability of ATR to maintain a G2 checkpoint arrest in *CINP*-silenced cells. The G2 checkpoint is active and maintained in cells transfected with a non-targeting siRNA, as indicated by the low percentage of mitotic cells following

irradiation (Figure 5.4C). In comparison, the percentage of cells escaping the G2 checkpoint following IR treatment is greatly increased in *CINP*-silenced populations (Figure 5.4C).

CINP promotes CDK2-dependent phosphorylation of ATRIP

We have previously shown that ATRIP S224 is phosphorylated *in vivo* by CDK2 [142]. The ability of CINP to interact with both ATR-ATRIP and CDK2 suggests that CINP may have a role in CDK2-mediated phosphorylation of ATRIP S224. To test this hypothesis, we examined ATRIP S224 phosphorylation in hTERT-immortalized epithelial cells transfected with non-targeting or *CINP* siRNAs. G_0 and S-phase synchronized populations were obtained by release from contact inhibition. G_0-arrested cells lack ATRIP S224 phosphorylation, while cells transfected with a non-targeting siRNA that are released into S phase show an increase in ATRIP S224 phosphorylation (Figure 5.5A). This is consistent with the increased activity of CDK2 during S phase. In comparison, *CINP*-silenced cells show markedly reduced phosphorylation of ATRIP S224, without altering the percentage of cells entering S-phase (Figures 5.5A and B). This suggests CINP promotes CDK2-dependent phosphorylation of ATRIP S224.

The genome maintenance activities of CINP are not likely attributable to defects in ATRIP phosphorylation

The relevance of post-translational modifications to the activation of other PIKKs, and the observations that silencing of *CINP* disrupts ATRIP S224 phosphorylation and ATR-mediated CHK1 phosphorylation, suggest that ATRIP S224 phosphorylation may be important for ATR activation or signaling. To examine this possibility, I assessed the

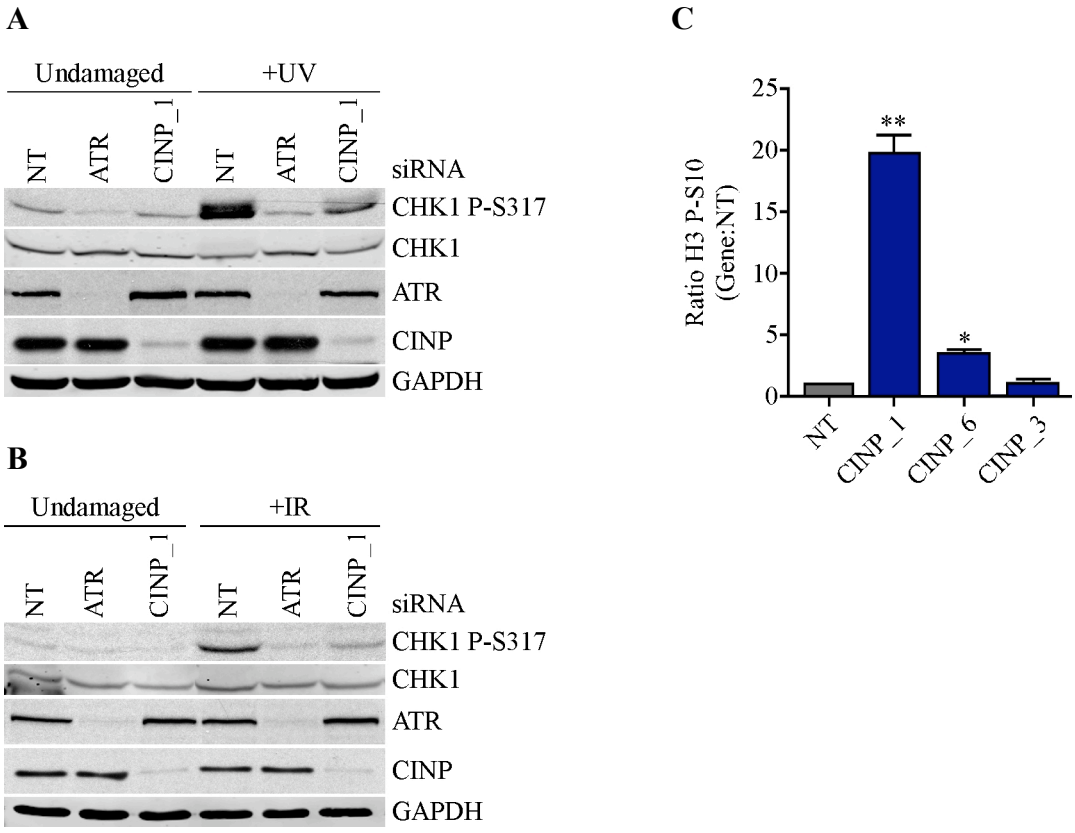

Figure 5.4. CINP regulates ATR-ATRIP checkpoint signaling. (A and B) U2OS cells were transfected with non-targeting (NT), ATR, or CINP siRNA oligonucleotides and irradiated with 30J/m^2 UV **(A)** or 5Gy IR **(B)**. Two hours after UV treatment and six hours after IR treatment, whole cell extracts were prepared and ATR activation was monitored by immunoblotting lysates with a phospho-peptide specific antibody to CHK1 S317. **(C)** The integrity of the G2 checkpoint was examined after silencing of *CINP*. U2OS cells were irradiated with 5Gy IR and cells entering mitosis were trapped by the addition of nocodazole. Mitotic cells were quantified by flow cytometry analysis using a phospho-peptide specific antibody to histone H3 S10. Error bars represent standard error (n=3). CINP siRNAs causing a statistically significant increase in the percentage of mitotic cells relative to the NT control are designated by asterisks (2-tailed, unpaired t test, * = $p \leq 0.05$, ** = $p \leq 0.01$).

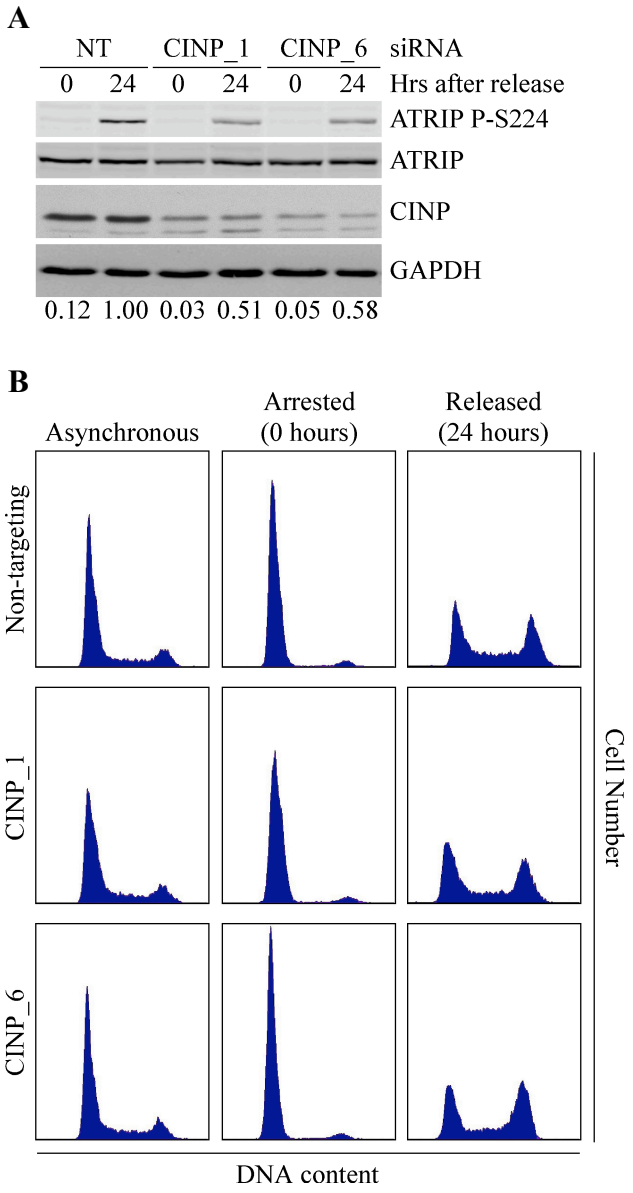

Figure 5.5. CINP is important for ATRIP S224 phosphorylation. **(A)** RPE-hTERT cells transfected with non-targeting (NT) or CINP siRNAs were arrested by contact inhibition. Cells were released and harvested at 0hrs or 24hrs, when the cells had reached S phase. CDK2-mediated phosphorylation of ATRIP was examined using a phospho-peptide specific antibody to S224 [142]. Quantitative analysis of ATRIP S224 phosphorylation is indicated below the immunoblots, and is normalized to the amount of ATRIP S224 phosphorylation observed in cells transfected with the NT siRNA at 24 hours. **(B)** The DNA content of the asynchronous, arrested, and released cells was measured after siRNA transfection by flow cytometry analysis of propidium-iodide stained cells to ensure that silencing of CINP did not affect cell cycle re-entry after contact inhibition. This data was obtained by Xin Xu.

requirement for ATRIP S224 phosphorylation in the resistance to replication stress and in G2 checkpoint maintenance, two checkpoint functions compromised by silencing of *CINP*. To determine if these phenotypes are due to a defect in ATRIP S224 phosphorylation, we examined the functional consequences of mutating this amino acid to an unphosphorylatable alanine residue (S224A). U2OS cell lines stably expressing an empty vector or siRNA-resistant HA-ATRIP constructs (wild type or S224A) were generated by retroviral infection. The endogenous ATRIP protein levels were depleted using siRNA, and the HU sensitivity and G2 checkpoint integrity phenotypes were examined as described previously.

Cells expressing an empty vector display significantly reduced viability (41%) following HU treatment when endogenous ATRIP levels are depleted, consistent with previous data and the role for ATR-ATRIP in the maintenance and recovery of stalled replication forks (Figure 5.6). Cell viability is markedly improved when cells retain wild type ATRIP expression from the exogenous plasmid, with 91% of cells surviving HU treatment. Similarly, 94% of cells expressing ATRIP S224A are viable following HU treatment, demonstrating this mutant complements the HU sensitivity normally observed following silencing of endogenous ATRIP (Figure 5.6). CDK2-mediated phosphorylation of ATRIP S224 is thus not critical for ATR-ATRIP responses to replication stress, suggesting that the HU sensitivity resulting from silencing of *CINP* is not due to a defect in ATRIP S224 phosphorylation.

I next examined whether a defect in ATRIP S224 phosphorylation had any adverse effect on the integrity of the G2 checkpoint. U2OS cells stably expressing an empty vector, wild type ATRIP, or ATRIP S224A were transfected with ATRIP siRNAs

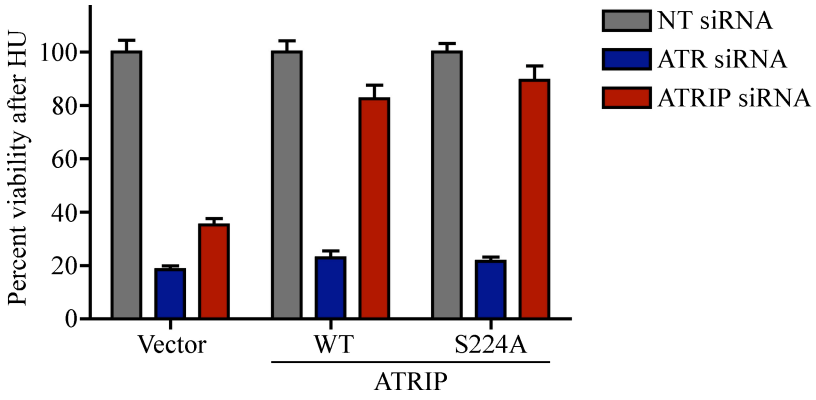

Figure 5.6. ATRIP S224 phosphorylation is not required for resistance to replication stress. U2OS cells expressing siRNA-resistant cDNAs for HA-ATRIP (WT), HA-ATRIP S224A, or no cDNA (Vector) were transfected with non-targeting (NT), ATR, or ATRIP siRNA. Transfected cells were split into four plates, two of which were treated with 3mM HU for 24hrs. The HU was removed and replaced with standard growth media for an additional 24h before cell viability was quantified using the WST-1 cell proliferation reagent. Error bars represent standard error (n=6).

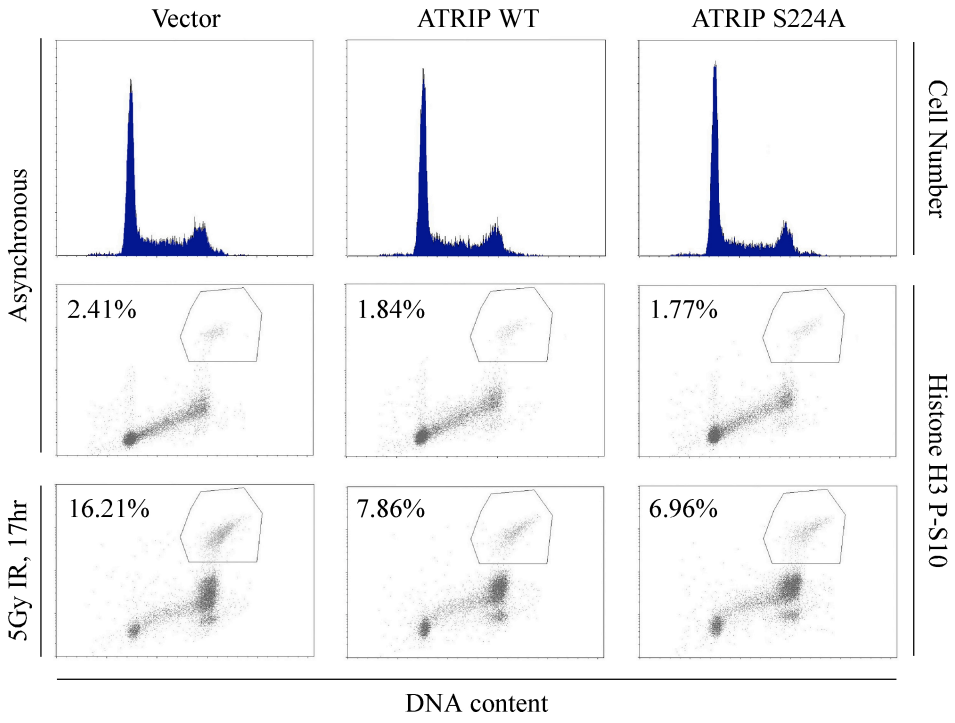

Figure 5.7. ATRIP S224 phosphorylation is not required for G2 checkpoint maintenance. U2OS cells expressing siRNA-resistant cDNAs for HA-ATRIP (WT), HA-ATRIP S224A, or no cDNA (Vector) were transfected with ATRIP siRNA. Three days after transfection, cells were irradiated with 5Gy IR and those entering mitosis were trapped by the addition of nocodazole. DNA content was monitored by propidium iodide staining, and the percentage of mitotic cells in each population was determined by immunostaining with a phospho-peptide-specific antibody to histone H3 S10.

to deplete endogenous protein levels. Cells were treated with IR, and those entering mitosis in the presence of damage were captured by the addition of nocodazole. Mitotic cells were then quantified by flow cytometry analysis of histone H3 phospho-S10 and propidium iodide staining. Maintenance of the G2 checkpoint is compromised in cells expressing the empty vector after silencing of endogenous ATRIP, as evidenced by the increased percentage of cells entering mitosis in the presence of damage (Figure 5.7). The expression of wild type ATRIP complements the loss of endogenous ATRIP and significantly reduces the percentage of mitotic cells after IR, indicating effective maintenance of the G2 checkpoint. The percentage of mitotic cells is similarly reduced in cells expressing ATRIP S224A, indicating that this mutant retains the ability to induce a G2 checkpoint in response to IR (Figure 5.7). All three cell lines have indistinguishable cell cycle profiles and percentages of cells in mitosis prior to irradiation. Thus, the G2 checkpoint defect observed following silencing of *CINP* is also unlikely to result from a defect in ATRIP S224 phosphorylation.

Discussion

Our data identify CINP as a novel regulator of ATR-mediated checkpoint signaling. CINP forms a complex with ATR-ATRIP through the ATRIP coiled-coil domain, is required for efficient phosphorylation of CHK1 after DNA damage, and is required for maintenance of the G2 checkpoint. CINP also mediates CDK2-dependent phosphorylation of ATRIP S224. The identification of CINP as a mediator of ATRIP

S224 phosphorylation suggests a mechanism through which a non-cyclin accessory protein promotes CDK-dependent regulation of the ATR-ATRIP complex.

While the damage-induced regulation of cell cycle progression by DDR pathways is accomplished through inhibition of CDK activity, there are several reports also suggesting that the cell cycle and CDKs regulate ATR. The recruitment of ATR-ATRIP to chromatin and the phosphorylation of ATR substrates are primarily restricted to S phase cells [33, 257, 258]. CDK activity is critical for generating the ATR-activating structure at DSBs by promoting end resection and formation of RPA-coated ssDNA [132, 133]. Furthermore, CDK phosphorylation of the PCNA-like clamp protein RAD9 is important for DDR signaling [62, 65, 259]. Additional mechanisms likely exist to restrict or enhance ATR activation specifically in S phase. The presence of multiple CDK consensus phosphorylation sites in both ATR and ATRIP suggest that CDKs may directly regulate the activity of this essential complex.

CDK2 phosphorylation of ATRIP S224 is unaffected by DNA damaging agents and is not rapidly turned over in cells [142]. ATR-ATRIP complexes containing a S224A mutant retain the ability to bind RPA, localize to sites of DNA damage, and have *in vitro* kinase activity similar to that of wild type complexes [142]. Thus, the phosphorylation of ATRIP S224 by CDK2 does not appear to be a mechanism of rapid regulation of the ATR-ATRIP complex. Furthermore, mutation of ATRIP S224 to alanine does not cause hypersensitivity to replication stress or a defect in G2 checkpoint maintenance, suggesting that the damage-induced activation of ATR-ATRIP is not severely affected by loss of this phosphorylation site. The phosphorylation of ATR-ATRIP in S and G2 phases by CDKs may instead potentiate ATR-ATRIP activation, or

specific activities of this complex, during a phase in the cell cycle when its activity is essential.

Cells have not evolved to respond to the high doses of irradiation that we apply to facilitate our study of these damage-responsive pathways. ATR-ATRIP is activated during every cell cycle to respond to endogenous replication stress. The extent of damage generated endogenously as a consequence of respiration and DNA metabolism is likely magnitudes less than what is used in these assays measuring sensitivity and checkpoint integrity. The phosphorylation of ATR-ATRIP by CDKs could potentially serve to sensitize this complex to the low level of endogenous stress generated during DNA replication. By lowering the threshold of damage required for activation, ATR-ATRIP could be more responsive to genome integrity challenges during S phase, when the genetic information is increasingly vulnerable.

Silencing of *CINP* adversely affected phosphorylation of the ATR substrate CHK1, cell viability in response to replication stress, and G2 checkpoint integrity. However, these effects appear to be independent of the role for CINP in mediating CDK2-dependent phosphorylation of ATRIP S224. The ATRIP S224A mutant protein displays no significant sensitivity to replication stress, and although I have also observed no defect in G2 checkpoint maintenance, previous results suggest that the ATRIP S224A mutant is compromised in maintenance of the G2 checkpoint response [142]. Additional CDK consensus phosphorylation sites are present in both ATR and ATRIP. The phenotypic effects of *CINP*-silencing could be attributed to the loss of multiple CDK-dependent phosphorylation events in the ATR-ATRIP complex. One such candidate is ATRIP S239, a conserved, proline-directed site that is phosphorylated *in vivo* in the

presence and absence of DNA damage [142, 260]. The kinase responsible for this phosphorylation event has not been identified, but significantly, ATRIP S239 phosphorylation may have a role in maintenance of the G2 checkpoint [260]. The potential involvement of ATRIP S239 phosphorylation in the G2 checkpoint response, and the observation that this represents a conserved CDK consensus site, suggests that a potential requirement for CINP in mediating this phosphorylation event should be examined. However, without a phospho-peptide specific antibody to ATRIP S239 at this time, we are unable to determine whether CINP promotes S239 phosphorylation in addition to S224 phosphorylation.

The phenotypic consequences of *CINP*-silencing cannot be accounted for solely by loss of ATRIP S224 phosphorylation. If CINP is functioning as a mediator protein to facilitate CDK-dependent phosphorylation of ATRIP S224, it may also promote the phosphorylation of additional CDK target proteins. Considering the important role that CDK2 has in the initiation and progression of DNA synthesis, defective phosphorylation of other CDK2 substrates could very well contribute to the HU sensitivity of *CINP*-silenced cells. Furthermore, based on the proposed interactions between CINP and several replicative proteins, the requirement for CINP in the resistance to replication stress may also arise from a role in replication-dependent processes that are independent of mediating CDK phosphorylation events.

While replication-dependent functions of CINP may exist and contribute to the HU sensitivity observed after silencing of *CINP*, this phenotype is also likely due, at least in part, to deficient checkpoint signaling. Phosphorylation of the ATR effector protein CHK1 is required for replication stress responses [228], consistent with the data in

Chapter IV demonstrating that silencing of *CHK1* results in hypersensitivity to HU treatment (Appendix A, Table 4). The defects in damage-induced phosphorylation of CHK1 likely contribute to the HU sensitivity of *CINP*-silenced cells, as well as the loss of G2 checkpoint integrity. The mechanism by which CINP promotes phosphorylation of CHK1 by ATR-ATRIP is unclear. CINP lacks any recognizable functional domains, but protein-protein interactions could be facilitated through the CINP coiled-coil domain. CINP may promote interactions between ATR-ATRIP and additional checkpoint proteins that are necessary for phosphorylation of CHK1.

The myriad of possibilities for the mechanisms by which CINP promotes resistance to replication stress and checkpoint arrest in response to damage underscores how little we know about the functions of this protein. The identification of additional CINP-interacting proteins and further characterization of the potential role for this protein in DNA replication will be necessary to fully understand its genome maintenance activities.