CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

Summary

The DNA Damage Response (DDR) is an inducible barrier to tumorigenesis at its earliest stages of development. Recent evidence from cell culture, animal models, and clinical samples reveal DDR activation in response to activation of oncogenes and inactivation of tumor suppressors. These genetic alterations promote replication stress and DNA damage that activates DDR signaling pathways. The DDR restrains the growth of cells with mutated or unstable genomes by preventing cell cycle progression, promoting DNA repair, and also inducing senescence or apoptosis. Several genetic alterations that activate DDR pathways have been identified, and recent sequencing efforts indicate hundreds of genes are mutated in breast or colorectal tumors. The heterogeneous basis of cancer suggests there are many genes that can contribute to the process of tumorigenesis. Thus, an important goal of cancer research is to understand the genetic basis of cancer, as well as the mechanisms through which genome instability arises and contributes to disease.

I identified seventy-four genes with genome maintenance activity using a functional and biologically relevant assay that monitors the ability of gene-silencing siRNAs to induce DDR activation. Additional analyses of these genes identified thirty-five that may possess replication-dependent genome maintenance functions, which I

confirmed for two genes of interest after further characterization. I demonstrated that DDB1 maintains genome integrity as part of an ubiquitin-ligase complex that mediates the S phase-dependent degradation of the replication-licensing factor CDT1. Proper regulation of CDT1 is critical to prevent re-replication and subsequent DSB formation that can threaten genome integrity. In collaboration with Xin Xu, I also demonstrated that the genome maintenance function of CINP involves regulation of ATR-dependent DDR signaling. CINP promotes ATR-mediated phosphorylation of CHK1 and maintenance of the G2 checkpoint, in addition to facilitating CDK2-dependent phosphorylation of ATRIP. The seventy-four genome maintenance genes I identified are excellent candidates for the gene function defects that may promote genome instability and DDR activation in pre-cancerous lesions. Further characterization of these genes may provide a greater understanding of the gene functions and cellular pathways that are critical for tumorigenesis.

Further Discussion and Future Directions

The DDR is an inducible barrier to tumorigenesis

Identifying novel proteins and post-translational modifications that regulate the kinases at the apex of the DDR is critical to understand how these proteins function to maintain the integrity of the genome. The biological significance of the DDR is exemplified by the fact that disruption of these pathways results in developmental defects, genetic disorders, and a predisposition to various forms of cancer [1, 2]. The DDR is not only activated by exogenous and endogenous sources of DNA damage (such

as IR, UV, HU, ROS), but also responds to tumorigenic gene function defects that promote genome instability [136, 137].

The hypothesis of DDR activation in response to tumorigenic insults was initially formulated from observations of DNA damage and constitutive DDR activation in cancer cell lines, as well as chronically active checkpoints in clinical samples from breast and lung carcinomas [216]. Reinforcing this hypothesis were additional observations that the overexpression or activation of oncogenes, as well as the inactivation of some tumor suppressor genes, induces DDR activation in various cell types and animal models [210, 217-220]. Additional analyses of a larger panel of human tumors from various stages of cancer progression have also revealed constitutive DDR signaling in pre-cancerous lesions [209, 210]. These early lesions show evidence of senescence and apoptosis, suggesting that the DDR is functioning to maintain genome integrity by restricting the growth of aberrant cells. The progression to a malignant disease is associated with suppression of DDR signaling to relieve the restraint on cellular proliferation, often through mutations in the effector protein p53 [209, 210, 216]. These reports have led to a model whereby the ATM/ATR-mediated DDR serves as an inducible barrier to restrain tumor development at it earliest stages by restricting the proliferation of cells with mutated or unstable genomes (Figure 6.1) [136, 137].

DNA replication stress is a source of genome instability in pre-cancerous lesions

An important question arising from these studies pertains to the mechanistic basis of the DNA damage caused by the gene function defects in pre-cancerous lesions. The evidence thus far suggests that replication stress is a key component of the genome

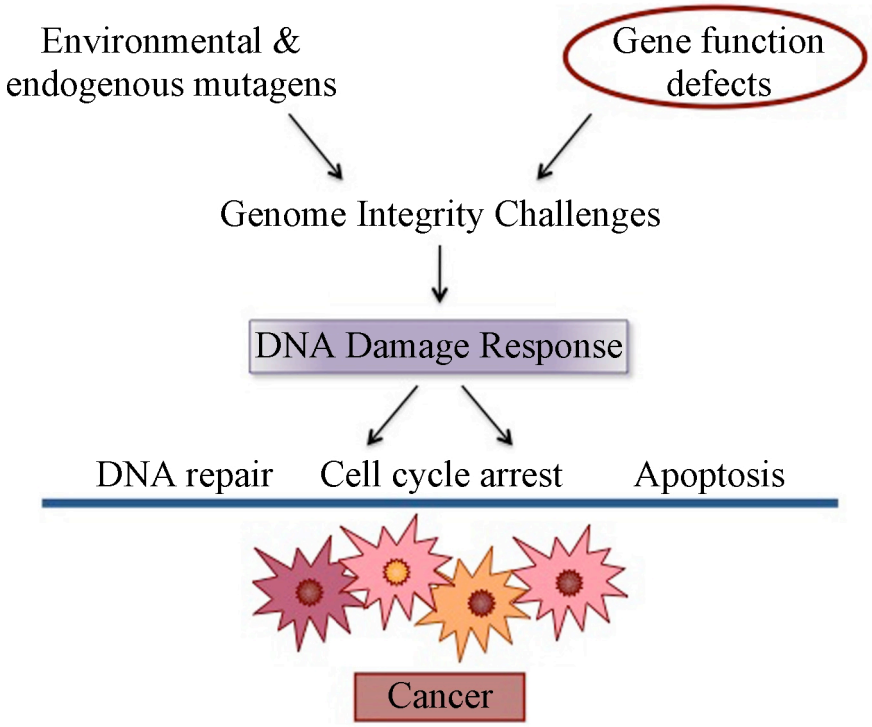

Figure 6.1. The DDR functions as a barrier to tumorigenesis. The DDR is activated by environmental and endogenous mutagens, as well as gene function defects that promote genome instability. The activated DDR functions to restrict the proliferation of cells with mutated and unstable genomes by preventing cell cycle progression, initiating DNA repair, and inducing apoptosis. These genome maintenance functions of the DDR are important to prevent the progression to a malignant disease.

destabilizing events that activate the DDR in pre-cancerous lesions. Activation of the replication stress response kinase ATR initially suggested the presence of aberrant DNA replication [209, 210]. Additional studies have demonstrated the co-localization of DDR foci with sites of DNA replication, premature termination of replication forks, preferential DNA breakage at common fragile sites, and a requirement for progression through S phase for the generation of DNA damage [209, 210, 218, 219]. These findings do not exclude additional sources of genome instability, such as telomere erosion or the generation of ROS, from promoting DDR activation in these early lesions. However, telomere attrition was not observed in a model of hyperplasia that did mimic pre-cancerous lesions by displaying DDR activation [209]. Furthermore, antioxidant treatment only marginally repressed DDR activation in transformed and senescent cells [210]. The extent that each mechanism contributes to genome instability in human tumors remains to be determined, but these observations suggest that replication stress is the predominant contributor to genome instability in early lesions.

This relatively new model of an inducible barrier to tumorigenesis caused by gene function defects highlights the need to identify the genetic alterations that can contribute to genome instability and DDR activation in pre-cancerous lesions, particularly by promoting replication stress. The heterogeneous basis of most cancers suggests there are many gene function defects that can contribute to this phenotype. A critical avenue of investigation is to identify the specific genes and cellular pathways that are important for maintaining genome integrity, as this information can potentially allow for more accurate diagnoses and effective treatments of this disease.

Identification of genome maintenance genes

Using DDR activation as a reliable indicator of genome instability, I performed a functional RNAi screen to identify genetic defects that may have important roles in promoting tumorigenesis at its earliest stages by generating genome instability. This screen identified seventy-four genes that reproducibly display genome instability and DDR activation with at least three RNAi molecules. The constitutive DDR activation observed in pre-cancerous lesions from a variety of human tissues suggests that the DDR barrier is a biologically relevant tumor suppressive mechanism across cell types [209, 210, 261-263]. In agreement with these observations, I observed DDR activation in two distinct cell types and with two markers of DDR activation. This suggests that the genome instability created by silencing of these gene products is not restricted to the cell type or DDR marker examined, a necessary phenotype for the general promotion of genome instability. Additionally, as validation of the screening methodology, several gene products with recognized genome maintenance activities were identified, including *BRIP1*, *CHK1*, *DDB1*, and *RPA1* [221, 228, 252, 264]. The identification of these seventy-four genome maintenance genes begins to provide necessary insight into our limited understanding of the mechanisms that promote tumorigenesis.

Recent sequencing efforts have identified hundreds of genes that are mutated in breast and colorectal cancer genomes; however, the impact these mutations have on the process of tumorigenesis remains unknown [222, 230]. The combination of our functional analyses with these cancer genome sequencing efforts can identify the genes that are both mutated in cancers and capable of promoting genome instability. These represent the highest priority genes that are most likely to play a causative role in

tumorigenesis. This RNAi screen can provide functional information regarding the mutations observed in six specific genes identified in these cancer genome sequencing efforts (*ANAPC*, *CENTB1*, *CNTN4*, *ERCC6*, *OTOF*, and *TBX22*). Our data suggest mutations inhibiting the function of these gene products may facilitate tumorigenesis by promoting genome instability.

Gene products that regulate cell cycle progression have established roles in the maintenance of genome integrity, as is likely the case for the anaphase promoting complex (*ANAPC*). ERCC6 functions in transcription-coupled nucleotide excision repair (TCR). Mutations in TCR genes cause Cockayne's syndrome (CS), which is characterized by slow growth, photosensitivity, and neurologic degeneration. However, CS patients with defective TCR do not have an increased risk of cancer, suggesting ERCC6 may have genome maintenance functions that are independent of its role in this repair process [265]. The genome maintenance activities of the remaining four genes are also unclear, as these gene products have functions in diverse cellular processes such as cytoskeletal and membrane trafficking (*CENTB1*), cell adhesion (*CNTN4*), transcriptional repression (*TBX22*), and membrane vesicle fusion (*OTOF*). The function of *OTOF* may be important for its genome maintenance activity since two additional genes with connections to vesicle trafficking were also identified as genome maintenance genes in this screen (*SCFD1* and *HERC2*). The vesicle fusion function of OTOF could potentially serve an important role in mitosis, where the last stage of cell division requires deposition of membrane vesicles in the cleavage furrow. Consistent with a failure to complete cytokinesis, multinucleated cells were observed following *OTOF* silencing (Figure 4.3A).

These six genome maintenance genes are mutated in breast and colorectal cancer genomes, and additional genes identified in this screen are clearly relevant for the process of tumorigenesis because they display tumor suppressor phenotypes (Appendix A, Table 3). Further sequencing efforts of a broader range of tumors could also reveal additional mutations among these seventy-four genome maintenance genes in human cancers. The identification of specific gene mutations that are observed in cancer genomes and capable of promoting genome instability could prove useful as biomarkers for the early detection of cancerous lesions.

Perhaps as important as the identification of individual genes that function to maintain genome integrity, is the identification of pathways that can regulate the course of tumorigenesis. Disruption of a pathway could be accomplished by mutations in any one of several components required for the signaling cascade, and thus mutation of the specific genome maintenance gene identified in our screen would not be necessary if the relevant pathway was inactivated by alternative mutations. Multiple siRNAs targeting gene products involved in DNA replication, DNA repair, cell cycle checkpoints, mitotic control, and chromatin regulation induced DDR activation (Figure 4.11), highlighting the importance of these processes to the maintenance of genome integrity. Additional gene function defects that compromise these prominent genome maintenance pathways will likely be important for tumor progression.

Replication-dependent genome maintenance activities

Importantly, fifteen of the seventy-four genes could not be placed into the prominent genome maintenance categories listed above. Further characterization of these

gene products may reveal additional cellular processes that contribute to genome maintenance, or perhaps reveal novel roles for these gene products in the established genome maintenance pathways described above. Those with replication-dependent genome maintenance activities are of particular interest since replication stress is proposed to be a key contributor to the genome instability and DDR activation observed in pre-cancerous lesions [209, 210, 218, 219].

To distinguish gene products with potential replication-dependent genome maintenance activities I performed a series of assays to identify genes that regulate cell sensitivity to replication stress. Based on observations in *ATR*-deleted cells, we hypothesized that cells with compromised replication-dependent processes would be particularly sensitive to further replication interference. Sensitivity to replication stress was assessed after RNAi silencing by an increase in the percentage of cells with DNA damage following aphidicolin treatment, reduced viability after release from HU arrest, and an inability to complete DNA synthesis and progress into mitosis following release from HU arrest. Many gene products with established roles in DNA replication displayed sensitivity to these replication stress agents (*CHK1*, *POLA*, *POLB*, *RPA1*, *RRM1*), suggesting these assays have the ability to identify replication-dependent genome maintenance activities [6, 228].

Thirty-five of the genome maintenance genes displayed sensitivity to replication stress agents in at least one of the assays examined, suggesting they may have critical functions in replication-dependent processes. Further characterization of these gene products will be necessary to confirm that they possess replication-dependent genome maintenance activities. The identification of gene function defects that promote genome

instability through replication stress is important not only because of the potential role these gene function defects may play in the process of tumorigenesis, but the characterization of these replication-dependent activities may also provide insight into the mechanisms by which DNA replication stress is induced. Gene function defects may promote replication stress by generating excessive amounts of normal replication intermediates. With a greater number of replication forks present, there would be an increased liklihood of fork stalling and potential fork collapse, resulting in recombinogenic DSBs. The deregulation of gene products required for DNA replication may also produce abnormal replication structures that impede replication fork progression and increase the abundance of stalled replication forks.

An interesting and potentially informative observation is that genetic alterations to replication-dependent gene products are not equal in their ability to induce DDR activation. While disruption of the RB pathway, and overexpression of E2F1 or cyclin E induces DDR activation, overexpression of cyclin D1 or loss of p16Ink4A are unable to activate the DDR [210, 266-268]. The strength of the oncogenic signal could very well be an important aspect in the ability of these genetic alterations to induce genome instability and DDR activation, which may or may not be related to the specific mechanisms by which these genetic alterations promote replication stress. Identifying the diverse genetic alterations that can promote genome instability through replication stress, and understanding the mechanisms by which replication stress induces DNA damage, is necessary to understand what distinguishes cancer cell cycles from normal cell cycles.

Silencing of DDB1 promotes replication stress through aberrant origin firing

One gene that exhibited sensitivity to replication stress in the RNAi screen was *DDB1*. Silencing of this gene prevented cells from reaching mitosis after release from replication arrest, suggesting an inability to accurately complete DNA replication. Further characterization of *DDB1* confirmed this gene product is required for maintaining genome stability in human cells. Silencing of *DDB1* results in an accumulation of DNA damage and activation of cell cycle checkpoints. DDB1 protects the integrity of the genome as part of an E3 ubiquitin ligase complex containing CUL4A. One target of this complex that must be degraded to preserve genome integrity is the replication licensing factor CDT1. While multiple levels of CDT1 regulation exist in human cells, my data suggest these mechanisms possess unique and necessary roles in the regulation of CDT1, to properly restrain this protein and prevent the adverse cellular consequences associated with its misregulation.

DDB1-dependent regulation of CDT1 is not sufficient to explain all the genome instability that arises from silencing of *DDB1*. Co-silencing of *CDT1* with *DDB1* eliminates re-replication; however, it does not completely prevent DNA damage or checkpoint activation. Additional genome maintenance functions of DDB1 likely have cell cycle-dependency, since the loss of *DDB1* from G_0 arrested cells does not cause DNA damage (Figure 3.13). Identifying other substrates of DDB1-CUL4A whose degradation is necessary to prevent genetic instability will be important. Greatly facilitating this effort is the identification of numerous proteins that function as adaptor molecules for DDB1-CUL4A, and target this ubiquitin ligase complex to its substrates [269]. The list of additional DDB1-CUL4A substrates is now rapidly growing, and

includes the NER proteins DDB2 and XPC, the cell cycle regulators p27 and p21, the transcription factors c-Jun and STAT1, the tumor suppressors TSC2 and Merlin, as well as CHK1 and multiple histones [150, 151, 155, 161, 192, 270-275]. However, it is not clear if increased levels of these target proteins may contribute to the replication-dependent damage observed after silencing of *DDB1*.

Interestingly, deletion of the budding yeast orthologs of DDB1 and CUL4, Mms1 and Rtt01, results in sensitivity of replication forks to drug-induced DNA lesions [276]. Deletion of Rtt101 also results in spontaneous DNA damage, G2 checkpoint activation, and hyper-recombination at stalled replication forks. The viability of Rtt101 deletion strains required a DNA helicase that participates in displacement of protein complexes at replication pause sites [277]. These results suggest that Mms1-Rtt101, and possibly DDB1-CUL4, promote replication fork progression through obstacles such as DNA lesions and protein/DNA complexes by an ubiquitin-mediated mechanism.

CINP is important for ATR-mediated checkpoint signaling

CDK2-interacting protein (*CINP*) was also identified in the RNAi screen for genome maintenance activities. Silencing of *CINP* results in DNA damage, activation of DDR pathways, and sensitization of cells to replication stress. Further characterization of CINP's genome maintenance activities was facilitated by the identification of CINP as an ATRIP-interacting protein in a yeast 2-hybrid screen performed in the laboratory. Another graduate student, Xin Xu, and I discovered a novel role for CINP in regulating ATR checkpoint signaling. We demonstrated that CINP associates with the ATR-ATRIP complex through an interaction with the ATRIP coiled-coil domain. Additionally, we

found that CINP is required for ATR-mediated CHK1 phosphorylation and maintenance of the G2 checkpoint in response to DNA damaging agents.

The genome maintenance activities of CINP thus include regulation of ATR checkpoint signaling. One possible mechanism of ATR regulation was suggested by the observation that silencing of *CINP* compromises CDK2-mediated phosphorylation of ATRIP S224 (Figure 5.5). This phosphorylation event is mediated by CDK2 and is reported to be important for maintenance of the G2 checkpoint response [142]. The ability of CINP to interact with both CDK2 and ATR-ATRIP, the defect in ATRIP S224 phosphorylation after silencing of *CINP*, and the shared G2 checkpoint defect observed with silencing of *CINP* or expression of ATRIP S224A, correlate well to present a model where CDK2-dependent phosphorylation of ATR-ATRIP, mediated by CINP, promotes checkpoint signaling in response to DNA damaging agents [142, 256].

CDK2-mediated phosphorylation of ATRIP S224 is cell cycle regulated and not inducible by DNA damage [142]. Together with the observation that S224 phosphorylation is slow to decay, this suggests that CDK2-dependent phosphorylation of ATRIP S224 is not a mechanism to facilitate a rapid response to DNA damaging agents. Instead, the CINP-mediated, CDK2-dependent phosphorylation of ATRIP S224 may potentiate ATR activation during a phase of the cell cycle when its activity is essential for genome maintenance.

Despite the similarity in phenotypes observed with expression of ATRIP S224A and silencing of *CINP*, additional data suggests CINP may promote ATR signaling independently of regulating ATRIP S224 phosphorylation by CDK2 (discussed below). By interacting with the ATR-ATRIP complex, CINP could promote interactions with

additional proteins that are required for the activation of ATR or the phosphorylation of downstream target proteins. The lack of functional domains in CINP severely limits the speculation as to how these interactions are facilitated, but a broader examination of ATR-dependent signaling events may reveal useful information about where in the signaling pathway CINP functions.

We have demonstrated that CINP is important for ATR-dependent phosphorylation of CHK1, but the involvement of CINP in the phosphorylation of additional ATR target proteins is unclear. A requirement for CINP in ATR-mediated phosphorylation of multiple target proteins would suggest that CINP functions upstream of ATR-ATRIP to promote activation of this complex. Alternatively, a requirement for CINP specifically in the phosphorylation of CHK1 suggests that CINP functions downstream of ATR-ATRIP to promote phosphorylation of this particular target protein. The placement of CINP either upstream or downstream of ATR-ATRIP can then suggest protein-protein interactions that may be important for the regulation of ATR-ATRIP by CINP. Silencing of *CINP* disrupts maintenance of the G2 checkpoint, but has no observable effect on the ability of cells to resume DNA synthesis after replication fork arrest (data not shown). Since CINP is not required for all ATR-mediated events, I therefore favor a model that places CINP downstream of ATR-ATRIP to facilitate interactions that are necessary for specific genome maintenance activities of this complex.

Additional genome maintenance activities of ATR-ATRIP should also be examined to determine if CINP has a unique role in the maintenance of the G2 checkpoint, or whether it regulates additional genome maintenance functions of the ATR-

ATRIP complex. One interesting possibility is that CINP provides a connection between ATR-ATRIP and the regulation of replication origin firing. In addition to mediating the G2 checkpoint response, ATR-ATRIP also functions to maintain genome integrity through inhibition of late origin firing in the presence of replication stress or DNA damage. The mechanism by which this is accomplished is unclear, but involves inhibition of the S-phase kinases CDK and DDK [21, 44]. The initial and only publication on CINP suggests this protein has a role in replication initiation [256]. This potential function of CINP may provide an avenue through which ATR-ATRIP may regulate origin firing.

CINP may mediate CDK-dependent phosphorylation of additional ATR-ATRIP sites

The published data on ATRIP S224 phosphorylation correlate well with my observations of compromised CHK1 phosphorylation and defective G2 checkpoint maintenance in *CINP*-silenced cells. However, I was unable to reproduce the G2 checkpoint defect that was previously observed with inhibition of ATRIP S224 phosphorylation [142]. In two independent experiments, I found that cells expressing ATRIP S224A complemented the loss of endogenous *ATRIP* as well as a wild type construct, with both significantly reducing the percentage of cells entering mitosis in the presence of DNA damage. Thus, the G2 checkpoint defect observed in *CINP*-silenced cells may not be due to inhibition of ATRIP S224 phosphorylation alone if this phosphorylation site is dispensable for G2 checkpoint integrity.

One possibility is that CINP mediates CDK2-dependent phosphorylation of multiple sites in ATR-ATRIP, which cumulatively are important for ATR signaling

events. There are two phosphorylation sites that would be of particular interest to examine. Phosphorylation of ATRIP S239 is a possible candidate for regulation by CINP. This phosphorylation event is present in cells prior to treatment with DNA damaging agents, and is reported to be important for maintenance of the G2 checkpoint by mediating an interaction between ATRIP and the BRCT domains of BRCA1 [260]. The kinase responsible for ATRIP S239 phosphorylation has not been identified, but this proline-directed phosphorylation site is consistent with the consensus site of CDK2. The presence of a CDK2 consensus phosphorylation site and a requirement for this phosphorylation event in maintaining G2 checkpoint integrity suggest that the involvement of CINP in mediating ATRIP S239 phosphorylation should be examined.

There is controversy surrounding the functional relevance of this phosphorylation site as well though. While a single report has identified ATRIP S239 phosphorylation as important for G2 checkpoint maintenance, studies within our laboratory have observed no functional defect resulting from inhibition of ATRIP S239 phosphorylation. Further examination of both ATRIP S224 and S239 is necessary to determine the functional significance these phosphorylation sites may have individually and in concert for the regulation of ATR checkpoint signaling.

Additional phosphorylation sites in ATR or ATRIP have also been reported, but the impact of these post-translational modifications on the regulation of ATR activity is unknown. Two damage-inducible and ATR-mediated phosphorylation sites in ATRIP have been identified (Ser 68 and Ser 72). However, loss of these phosphorylation events has no adverse effect on ATR-ATRIP localization to sites of DNA damage or on the phosphorylation of downstream target proteins [278]. Three large-scale proteomic

studies on undamaged cells have identified several putative phosphorylation sites in both ATR and ATRIP, but these phosphorylation events have not been confirmed or explored further for functional relevance [279-281]. One of these sites, ATR S428, may be of particular interest as a CINP-mediated CDK2 phosphorylation event. Of the fourteen potential proline-directed serine or threonine phosphorylation sites in ATR, only S428 and S776 conform well to a CDK2 consensus phosphorylation site in having a basic residue at the +2 position. Furthermore, phosphorylation of ATR S428 was identified in a mass spectrometry analysis of mitosis and S phase-arrested HeLa cells [280]. The potential cell cycle-dependent phosphorylation of ATR S428 in cells and the presence of a CDK2 consensus phosphorylation site suggest that further analysis of ATR S428 is warranted to determine whether it is phosphorylated in a CDK2-dependent manner, whether CINP functions to mediate this phosphorylation event, and what significance ATR S428 phosphorylation may have in the regulation of ATR checkpoint signaling.

ATR-independent genome maintenance activities of CINP

The genome maintenance functions of CINP include regulation of ATR-dependent CHK1 phosphorylation and maintenance of the G2 checkpoint. Whether this regulation is the result of CINP-mediated CDK2 phosphorylation of the ATR-ATRIP complex is unclear. CINP may also function as a mediator protein to facilitate interactions between ATR-ATRIP and additional checkpoint proteins that are required for CHK1 phosphorylation.

In addition to the CHK1 phosphorylation and G2 checkpoint maintenance defects, *CINP*-silenced cells also display sensitivity to replication stress. Inhibition of ATRIP

S224 phosphorylation does not cause marked sensitivity to replication stress, suggesting that the requirement for CINP in promoting resistance to replication stress is independent of its role in mediating CDK2-dependent phosphorylation of ATRIP S224. As mentioned previously, CINP may mediate multiple CDK2-dependent phosphorylation events in the ATR-ATRIP complex that when inhibited individually have no obvious effect, but when inhibited collectively result in sensitivity to HU. More extensive analysis of CDK2-mediated ATR-ATRIP phosphorylation is necessary to determine the functional significance of these phosphorylation sites individually and collectively, and what role CINP may have in mediating them.

The HU sensitivity of *CINP*-silenced cells could still be attributed to compromised CHK1 phosphorylation and activation, but the contribution of additional ATR-independent genome maintenance activities cannot be excluded. The sensitivity of *CINP*-silenced cells to replication stress could also arise if CINP had another replication-dependent genome maintenance function. In fact, the only publication on this relatively novel protein proposes a role for CINP 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 [256]. Interfering with replication initiation could produce the HU sensitivity phenotype observed in *CINP*-silenced cells. Further analysis is necessary to confirm this putative role for CINP in DNA replication initiation and to determine how the replication-dependent activities of CINP may contribute to its genome maintenance functions.

Finally, the decrease in CDK2-mediated ATRIP S224 phosphorylation after *CINP*-silencing suggests that CINP may function to mediate the CDK2-dependent

phosphorylation of ATR-ATRIP. It will be important to determine whether CINP functions as an accessory protein specifically for the phosphorylation of ATR-ATRIP, or whether CINP functions to promote the phosphorylation of multiple CDK2-dependent targets. The HU sensitivity phenotype of *CINP*-silenced cells may also be attributable to defects in the phosphorylation of additional CDK2 substrates.

Further characterization of CINP

Further characterization of CINP's cellular functions will be necessary to gain a better understanding of its complete genome maintenance activities. Many cell cycle checkpoint and DNA replication proteins have homologues in yeast that have provided essential information about the function of the protein in higher eukaryotes. While CINP has been identified in many metazoans, there is no significant similarity to any protein in *S. cerevisiae* or *S. pombe*. There are also no identifiable domains in CINP that provide insight into its cellular functions, aside from a coiled-coil region. CINP lacks any recognizable ATM/ATR consensus phosphorylation site, suggesting it is not a substrate of these DDR kinases. However, CINP does contain a CDK2 consensus site that is conserved in rats, mice, and frogs. Phosphorylation of CINP in cells has not been examined, however, I have observed phosphorylation of CINP by CDK2-cyclin A in multiple *in vitro* kinase assays (Figure 6.2). It will be necessary to determine whether CINP is a substrate of CDK2 *in vivo*, and whether this phosphorylation event has any effect on the ability of CINP to mediate CDK2-dependent phosphorylation of ATR-ATRIP, facilitate ATR-mediated signaling events, regulate the phosphorylation of additional CDK2 substrates, or influence the activity of CINP in DNA replication

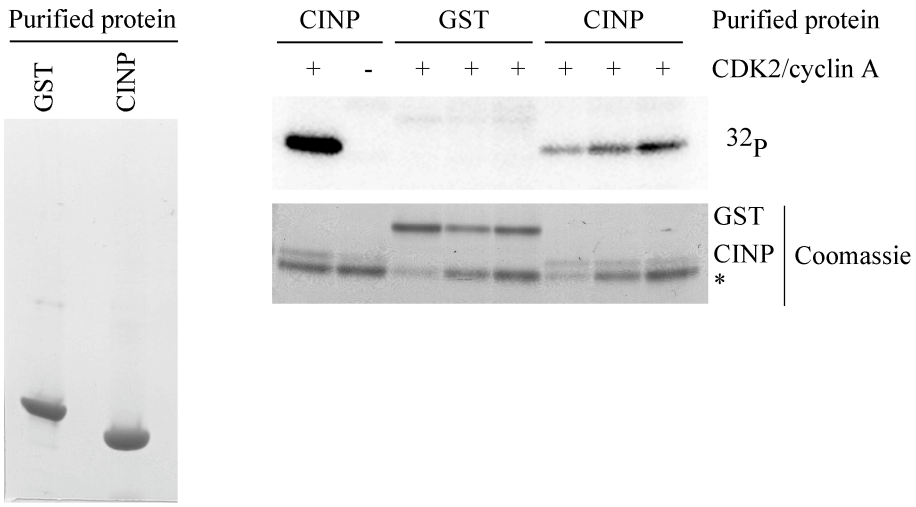

Figure 6.2. CINP is a substrate of CDK2/cyclin A in vitro. (A) Coomassie stained gel of GST and full-length CINP purified from bacterial cells. **(B)** Phosphorylation of CINP by CDK2-cyclin A complexes *in vitro*. Kinase reactions were separated by SDS-PAGE and phosphorylation was monitored by ^{32}P-ATP incorporation using a phospho-imager. The coomassie stained gel shows the addition of GST or CINP to each kinase reaction. The asterisk denotes the IgG light chain. The purification of GST and CINP from bacterial cells was performed by Gloria Glick.

processes. The identification of additional proteins that interact with CINP will greatly facilitate these efforts to better understand the cellular functions and genome maintenance activities of CINP.

Early detection and targeted approaches to cancer treatments

The identification of gene function defects that promote genome instability can suggest new ways to diagnose and treat cancer. Activation of the DDR is a functionally relevant assay for identifying the presence of genome instability, and it has been successfully used to identify many gene function defects that promote genome instability in cell culture models. Importantly, DDR activation has also been observed prior to the occurrence of mutations in tissue samples from a variety of pre-cancerous lesions [209, 210]. The development of an immunohistochemical assay for DDR activation may therefore be useful in the clinical setting to identify tumorigenic lesions at the earliest stages of development. Furthermore, the DDR appears to be activated preferentially in pre-cancerous lesions that are more likely to show progression to a malignant disease [267]. The ability to detect DDR activation may not only facilitate the diagnosis of early lesions, but may provide information on the prognosis of individual tumors as well [136].

Replication stress as a dominant cause of genome instability in pre-cancerous lesions may also be exploited for diagnostic purposes. Preferential breakage at common fragile sites are often observed in response to agents that cause replication stress, and have also been observed in early lesions and xenograft models of hyperplasia [209, 210]. Thus, in addition to DDR activation, the loss of heterozygosity at common fragile sites may also provide a means of identifying tumorigenic lesions at their earliest stages [282].

The identification of gene function defects and the mechanisms by which they promote genome instability could also facilitate the selection of a treatment that will be most beneficial on an individualized basis. Poly (ADP-ribose) polymerase 1 (PARP1) inhibitors demonstrate exceptional promise in pre-clinical and early phase clinical trials in the treatment of tumors with defects in DSB repair [283, 284]. PARP1 is required for efficient repair of DNA single strand breaks by base excision repair; therefore, inhibition of PARP1 results in the persistence of single strand gaps [285-287]. When these gaps are encountered by the replication machinery they can arrest replication fork progression and generate DSBs [288].

The repair of these DNA breaks is dependent on homologous recombination, which requires the activities of BRCA1 and BRCA2 [289-291]. In the absence of functional BRCA proteins, the stalled replication forks collapse and must be repaired by alternative error prone mechanisms of DSB repair [292]. This results in increased chromosome breaks and aberrant chromosome structures that arrest cell cycle progression and ultimately reduce the viability of the cells. Inhibition of ssDNA break repair would be minimally toxic to cells that have efficient homology-directed repair pathways, thus PARP inhibitors may be selectively lethal to tumors lacking BRCA1 or BRCA2 activity. The effectiveness of PARP inhibitors may be extended to additional tumors based on their genetic and functional profiling, and are likely to be beneficial in the treatment of tumors that display defects in homology-directed DSB repair.

The combined effectiveness of PARP inhibition and BRCA deficiency is an example of synthetic lethality, where disruption of either gene individually has no adverse effect but the combined disruption of both gene functions results in loss of

viability. Additional synthetic relationships with important implications for cancer treatment have been documented as well. Inactivation of pRB is observed in many cancers, and results in the transcription of S phase promoting genes by E2F1, including topoisomerase II. While activation of E2F1 can promote proliferation by stimulating cell cycle progression, this unscheduled entry into S phase can also trigger apoptosis [293-295]. Tipping this balance between proliferation and apoptotic stimuli may selectively inhibit tumors that harbor mutations in the pRB pathway. Consistent with this hypothesis, treatment of E2F1 overexpressing cells with etoposide, a topoisomerase II inhibitor that induces DNA damage, selectively sensitizes these cells to apoptosis [296, 297]. Additionally, mutant oncoproteins such as BRAF and EGFR have an increased requirement for HSP90 function, thus selectively sensitizing these cells to HSP90 inhibitors [298-301].

The concept of synthetic lethality is important for cancer treatment because it implies that targeting one gene product when another is defective should be selectively lethal to tumor cells with relatively low toxicity to normal cells. The identification of genome maintenance genes and the mechanisms through which they preserve genome integrity may thus facilitate the selection of a proper treatment that will be most effective in specifically targeting the tumor. This would be a significant improvement over the chemotherapeutic agents routinely used today, which have low therapeutic indices and narrow therapeutic windows.