Functional genomic screens identify CINP as a genome maintenance protein

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Edited by Titia de Lange, The Rockefeller University, New York, NY, and approved September 28, 2009 (received for review August 17, 2009)

The DNA damage response (DDR) has a critical role in maintaining genome integrity and serves as a barrier to tumorigenesis by promoting cell-cycle arrest, DNA repair, and apoptosis. The DDR is activated not only by genotoxic agents that induce DNA damage, but also during aberrant cell-division cycles caused by activated oncogenes and inactivated tumor suppressors. Here we use RNAi and cDNA overexpression screens in human cells to identify genes that, when deregulated, lead to activation of the DDR. The RNAi screen identified 73 genes that, when silenced in at least two cell types, cause DDR activation. Silencing several of these genes also caused an increased frequency of micronuclei, a marker of genetically unstable cells. The cDNA screen identified 97 genes that when overexpressed induce DDR activation in the absence of any exogenous genotoxic agent, with an overrepresentation of genes linked to cancer. Secondary RNAi screens identified CDK2-interacting protein (CINP) as a cell-cycle checkpoint protein. CINP interacts with ATR-interacting protein and regulates ATR-dependent signaling, resistance to replication stress, and G2 checkpoint integrity.

ataxia telangiectasia-mutated and Rad3-related | ATR-interacting protein | checkpoint | DNA damage response

The DNA damage response (DDR) is regulated primarily by the activation of the ataxia telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) kinases, which have unique and overlapping functions in promoting genome maintenance (1, 2). While ATM responds primarily to DNA double-strand breaks (DSBs), ATR responds during every cell division cycle to replication stress and is essential for the viability of replicating somatic cells (3, 4). The loss of ATR activity causes the formation of replication-associated DSBs, presumably because of the inability of ATR-depleted cells to stabilize stalled forks (5).

ATR signaling is regulated by several mechanisms, including protein-protein interactions, localization, and posttranslational modifications (2). Recruitment of ATR to ssDNA generated at stalled forks is largely mediated by an interaction between its obligate binding partner, the ATR-interacting protein (ATRIP), and the heterotrimeric ssDNA binding protein replication protein A (RPA) (4, 6, 7). An evolutionarily conserved RPA-binding surface in ATRIP, termed the "checkpoint protein-recruitment domain," binds an N-terminal domain of RPA70 (8).

ATR activation also requires topoisomerase binding protein 1 (TopBP1), which is a direct activator of ATR (9). TopBP1 binds to the ATR-ATRIP complex primarily through a binding surface on ATRIP, with some contribution from a PIKK regulatory domain in ATR (10). TopBP1 recruitment and positioning at the site of DNA damage is dependent on an interaction with the RAD9 subunit of a checkpoint clamp complex composed of RAD9-RAD1-HUS1 (9–1-1) (11–13). TopBP1 also functions upstream of the 9–1-1 complex because 9–1-1 recruitment to sites of replication stress is dependent on TopBP1 (14). Thus, multiple protein interactions promote the assembly of two checkpoint complexes (ATR-ATRIP and 9–1-1-TopBP1) at ssDNA gaps formed as a consequence of many types of DNA lesions.

Finally, there are additional modes of ATR-ATRIP regulation that remain poorly defined. ATRIP phosphorylation regulates the G2 checkpoint through an as yet undefined mechanism (15, 16). Furthermore, ATRIP orthologues contain a coiled-coil region that has at least two functions. First, it allows ATRIP to form homodimers (17, 18). ATRIP dimerization is required for stable association with ATR, and is therefore critical for proper ATR localization and signaling (17). Second, an ATRIP mutant containing a heterologous coiled-coil dimerization domain restores the ability of ATRIP to form a stable complex with ATR, restores proper localization of the ATR-ATRIP complex to sites of replication stress and DNA damage, and can bind to TopBP1. However, it does not support efficient ATR-dependent checkpoint signaling when introduced into cells (17). These data suggest there is an unidentified function for the ATRIP coiled-coil domain in regulating ATR signaling.

ATR and other DDR pathways are not only activated by endogenous and exogenous sources of DNA damage (such as radiation and reactive oxygen species), but also respond to tumorigenic gene-function defects that promote genome instability (19). The overexpression or activation of oncogenes and inactivation of some tumor suppressors induces DDR activation in premalignant lesions, presumably because of aberrant cell-division cycles and replication stress (20–24). These findings suggest a model whereby the ATM/ATR-mediated DDR serves as a barrier to constrain tumor development by limiting cellular proliferation, inducing apoptosis, and promoting DNA repair (19).

Several oncogenes and tumor suppressors that induce DDR activation when deregulated have been identified; however, the heterogeneity of cancer suggests there are many others that challenge genome integrity and promote tumorigenesis. We reasoned that we could identify cancer genes whose deregulation challenge genome integrity by monitoring DDR activation in cDNA overexpression and RNAi loss-of-function screens in otherwise untreated cells. Furthermore, we expected that the loss-of-function screen would identify previously unrecorded genome-maintenance genes, including those linked to the ATR-dependent replication checkpoint, as loss of ATR causes hyperactivation of the ATM signaling pathway. Indeed, we report the identification of over 170 genes that, when deregulated, cause an increase in DDR activity. These include an overrepresentation of cancer genes and a new regulator of the ATR checkpoint pathway: CDK2-interacting protein (CINP).

Results

RNAi Screen. To identify novel genome-maintenance activities, as well as genes whose deregulation may cause the DDR activation observed in tumor cells, we completed functional genomic screens

Author contributions: C.A.L., X.X., C.E.B., and D.C. designed research; C.A.L., X.X., C.E.B., G.G.G., R.Z., B.M.S., and L.C.T. performed research; C.A.L., X.X., C.E.B., F.Y., B.M.S., Y.S., and D.C. analyzed data; and C.A.L. and D.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0909345106/DCSupplemental.

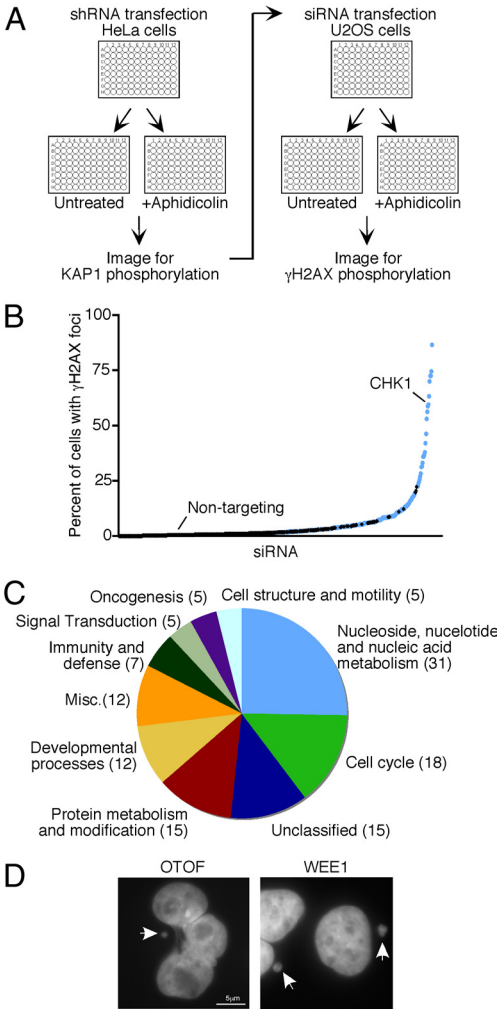

Fig. 1. An RNAi screen identifies genome maintenance genes. (*A*) Schematic of the RNAi screen. (*B*) U2OS cells were transfected with four siRNAs targeting each of the candidate genes identified in the HeLa shRNA screen. The percentage of cells containing γH2AX was determined by immunofluorescence staining. Each data point represents the mean of four replicas from a single siRNA oligonucleotide. Gene-silencing siRNAs causing a significant increase ($P < 0.05$) in γH2AX foci compared to the nontargeting control are highlighted in blue. (*C*) Biological classifications of genes reproducibly activating the DDR after RNAi silencing. Classifications were assigned using PANTHER. (*D*) Representative images of micronuclei (*arrows*). Incomplete mitotic segregation is also evident after silencing of the putative tumor suppressor OTOF.

for genes that, when silenced or overexpressed, increase DDR signaling. Starting from a genome-wide shRNA library (25), we created a sublibrary of 6,386 RNAi molecules targeting 2,287 genes. Genes containing protein-domain architectures associated with nuclear regulatory activities were selected, thus increasing the possibility they may function in genome-maintenance pathways. HeLa cells were transfected with one shRNA vector per well in 96-well plates, and successfully transfected cells were identified by expression of GFP from the shRNA plasmids. DDR activation resulting from RNAi-mediated gene silencing was assayed in the absence and presence of a low dose of a replication stress agent (0.1 μM aphidicolin) by immunofluorescence staining using a phosphopeptide-specific antibody to the ATM substrate KAP1 (KRAB domain-associated protein 1) (26) [Fig. 1*A* and Fig. S1*A*].

The primary RNAi screens identified 130 candidate genes whose silencing activated the DDR. To confirm this phenotype, eliminate off-target effects, and ensure the results were not cell-type or DDR-marker specific, we analyzed four individual siRNA oligonucleotides targeting each of the candidate genes. DDR activation was monitored in U2OS cells by examining phosphorylation of H2AX S139 (γH2AX) (Fig. 1*B* and Fig. S1*B*). γH2AX foci were observed in ≈1% of cells transfected with a nontargeting siRNA. In contrast, silencing of the ATR substrate checkpoint kinase 1 (CHK1) causes DDR activation in 59% of cells, consistent with previous observations (27). The measurement of γH2AX foci as a high-throughput screening assay for DDR activation is robust, with the analyses of both untreated and low-dose aphidicolin-treated samples producing excellent Z-factor scores (0.61 and 0.59, respectively). The analysis of γH2AX identified 37 genes that met stringent statistical criteria, and an additional 36 genes that are strong candidates for possessing genome-maintenance activities (Table S1 and Table S2). This phenotype is unlikely to be off-target because at least three independent RNAi molecules targeting each gene activate the DDR (minimally, one shRNA and two siRNA molecules). Furthermore, DDR activation in two distinct cell types with two DDR markers indicates these gene products are not cell type-specific activities and do not regulate a specific ATM/ATR substrate.

Most genes identified in the RNAi screen increased DDR activation both in the absence and presence of aphidicolin. Of the 73 genes identified in the RNAi screen, silencing of 63 caused γH2AX phosphorylation in untreated U2OS cells and silencing of 72 genes caused γH2AX phosphorylation in the cells treated with low doses of aphidicolin; 62 genes were common to both datasets (Fig. S1*C*).

As expected, gene products involved in nucleic acid metabolism and the cell cycle were prominent among those that exhibited genome-maintenance defects and DDR activation after RNAi silencing (Fig. 1*C*). Additionally, 17 of the genes have been suggested to function as tumor suppressors (Table S3), including 6 that were identified as breast and colorectal cancer genes by cancer genome-sequencing efforts (28, 29). In addition to activating the DDR, three of these putative tumor-suppressor genes also caused micronuclei formation after RNAi silencing (*CNTN4*, *S100A11*, and *OTOF*) (Fig. 1*D*, FigS1 *D* and *E*, Table S4), a phenotype frequently used as a marker for chromosome breaks and genome instability.

cDNA Overexpression Screen. A three-step methodology was used to identify genes that, when overexpressed, cause activation of the DDR in the absence of any added genotoxic agents (Fig. 2*A*). First, pools of three cDNAs were cotransfected into HeLa cells with a GFP expression vector to identify successfully transfected cells. A total of 5,796 cDNAs expressed from the pCMV-SPORT6 vector were analyzed. Two days after transfection, cells were monitored for DDR activation by immunofluorescence staining for KAP1 phosphorylation. Second, the vectors from cDNA pools that caused KAP1 phosphorylation were deconvoluted and the KAP1 phosphorylation assay was repeated. Finally, individually positive cDNA vectors were transfected into a distinct cell type (U2OS) and DDR activation was monitored by immunofluorescence analysis of γH2AX.

A total of 97 genes were identified that caused DDR activation when overexpressed in both HeLa and U2OS cells (Fig. 2*B* and Table S5). Gene products with biological functions linked to gene expression, cell-cycle regulation, nucleic acid metabolism, and cancer were strongly overrepresented when compared to the biological functions present within the cDNA screening library (Fig. 2 *C* and *D*). Among the genes linked to cancer (see Table S3) are several ets family transcription factors that act as oncogenes (30). We confirmed that their overexpression activates the DDR even in untransformed epithelial cells (Fig. 2 *E* and *F*). Other oncogenes that induce DDR activation when overexpressed include *DEK*, *ZBTB16* (*PZLF*), and *ELAVL1* (*HuR*).

In some cases, both overexpression and silencing of a gene causes

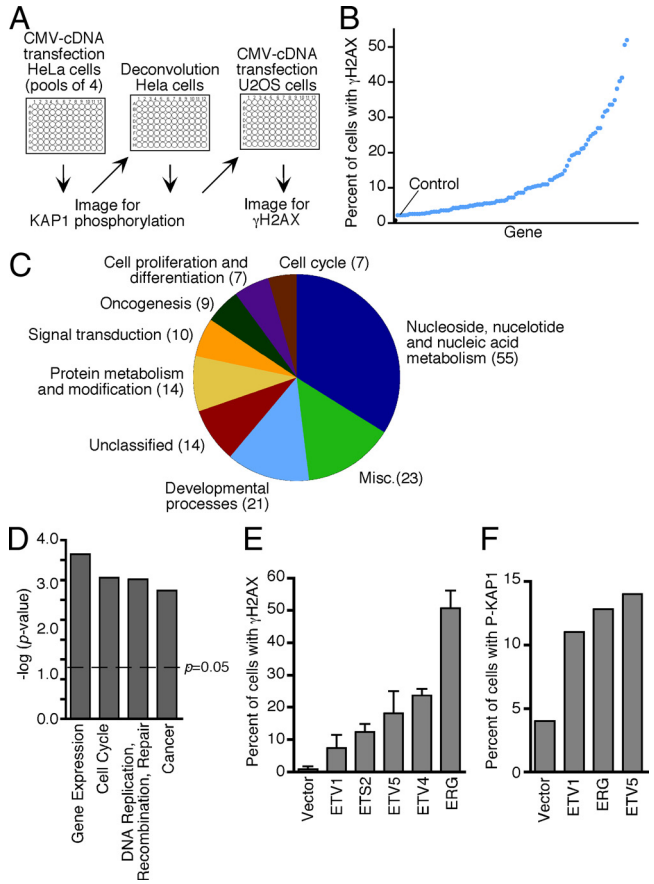

Fig. 2. A cDNA overexpression screen identifies genes that cause DDR activation. (*A*) Schematic of the cDNA overexpression screen. (*B*) The average of the γH2AX count from each of the 97 positive cDNAs is graphed and compared to the control empty vector. (*C* and *D*) Biological classifications of genes reproducibly activating the DDR after cDNA overexpression. Classifications were assigned using (*C*) PANTHER or (*D*) Ingenuity pathways. (*E* and *F*) Activation of the DDR by overexpression of ets family members was monitored in U2OS cells (*E*) or hTERT-immortilized retinal epithelial cells (*F*). Error bars are standard deviation ($n = 3$).

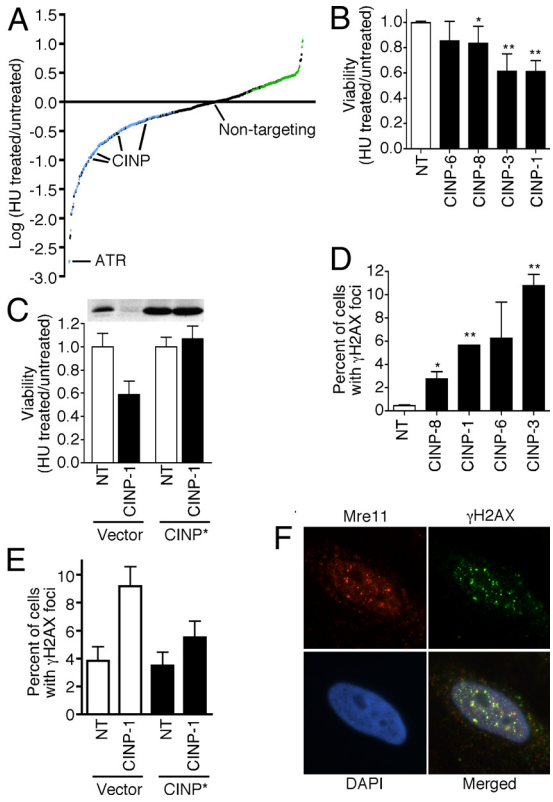

Fig. 3. CINP-silenced cells accumulate DNA damage and are hypersensitive to replication stress. (*A*) Gene-silencing siRNAs causing a significant sensitivity (negative values) or resistance (positive values) to HU treatment compared to the nontargeting siRNA are highlighted in blue and green, respectively ($P < 0.05$). Each data point represents the average of three independent experiments for a single siRNA. The HU-sensitivity of CINP-silenced cells is shown in (*B*). NT, nontargeting; *, $P < 0.05$; **, $P < 0.01$. (*C*) An siRNA resistant CINP cDNA (CINP*) or empty vector was introduced into U2OS cells via retroviral infection before performing the HU-sensitivity assay. The immunoblot shows the expression of CINP in each condition. (*D*) The percentage of cells containing γH2AX foci was scored following transfection with the indicated siRNAs. (*E*) γH2AX staining was scored in cells transfected with an empty vector or siRNA resistant CINP cDNA and either nontargeting or CINP_1 siRNA. (*F*) CINP-silenced cells were stained with antibodies to γH2AX and Mre11.

DDR activation. For example, we identified *SMARCAL1* and members of the *H2AFY* histone family in both the siRNA and cDNA overexpression screens. *SMARCAL1* encodes an annealing helicase that functions to maintain genome integrity at stalled replication forks (31). We also found that overexpression of the mitotic kinase PLK1 activated the DDR, while silencing *PLK1* was previously reported to cause DNA damage (32, 33).

To further understand the functional relationships between the genes identified in both the RNAi and cDNA overexpression screens, we performed an extensive bioinformatics analysis using published literature and functional annotation programs. This analysis placed many of the genes into four major functional groups: the ATM/ATR-related DDR, mitosis, chromatin regulation, and RNA metabolism (Fig. S2).

CINP Is a Checkpoint Gene. To characterize gene products from the RNAi screens that may be involved in ATR signaling, we developed a secondary assay for cellular sensitivity to hydroxyurea (HU) following RNAi silencing. The assay was optimized using silencing of *ATR* and validated by the results of the internal positive control *CHK1*, which cause a 70% and 50% reduction, respectively, in cell viability compared to the control (Fig. 3*A*; $P < 0.001$). Of the 73 genes identified in the RNAi screens, silencing 20 produced a significant HU-sensitivity score with at least two of four siRNAs ($P < 0.05$) (Table S6).

One gene of particular interest is *CINP*. Silencing of *CINP* causes KAP1 phosphorylation in HeLa cells (three of five shRNAs), γH2AX foci formation in U2OS cells (three of four siRNAs), and sensitizes cells to HU treatment (three of four siRNAs) (Fig. 3 *B–D*, and see Table S1, Table S2, and Table S6). Complementation of the HU-sensitivity and γH2AX phenotypes with an RNAi-resistant CINP cDNA indicates they are not off-target effects (Fig. 3 *C* and *E*). The γH2AX foci observed in CINP-silenced cells are likely to be sites of DNA damage because they colocalize with DNA repair proteins including Mre11 (Fig. 3*F*).

CINP is distinctive among the genome maintenance genes because we also identified it as a candidate ATRIP-interacting protein. A yeast two-hybrid screen using full-length ATRIP as bait identified 11 cDNAs encoding interacting proteins, 2 of which were full-length CINP. The potential interaction of CINP with ATRIP, combined with the DNA damage and HU-sensitivity phenotypes resulting from loss of CINP, prompted us to characterize this potential ATR pathway protein further.

CINP Interacts with ATR-ATRIP Through the ATRIP Coiled-Coil Domain. We first confirmed the association of CINP with ATR-ATRIP complexes by coimmunoprecipitation of exogenously expressed

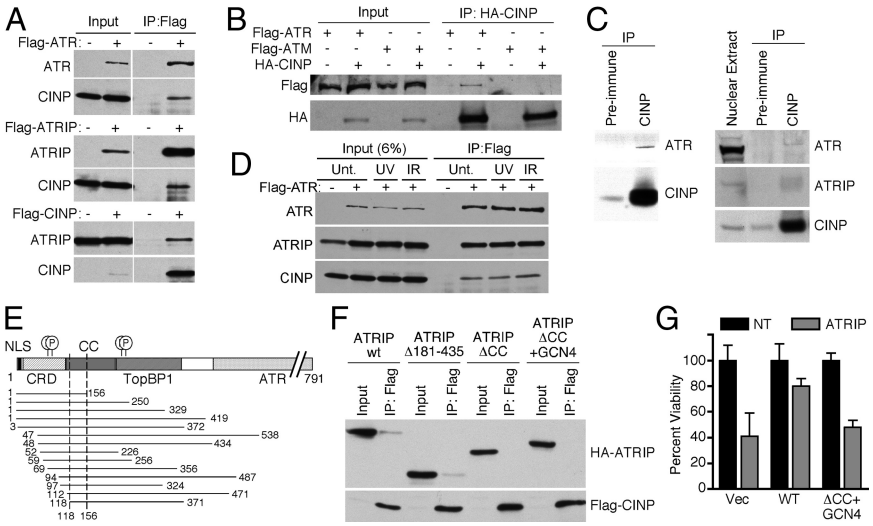

Fig. 4. CINP interacts with ATR-ATRIP. (*A*) Flag-ATR or Flag-ATRIP was coexpressed with HA-CINP, or Flag-CINP was expressed in HEK293 cells, as indicated. Protein complexes were isolated by Flag immunopurification, and coprecipitating proteins were identified by immunoblotting with Flag, HA, and ATRIP antibodies. (*B*) Flag-ATR or Flag-ATM was coexpressed with HA-CINP. CINP was immunoprecipitated with anti-HA antibodies and coprecipitating proteins were identified by immunoblotting. (*C*) Endogenous CINP was immunoprecipitated from HeLa whole cell extracts (*Left*) and HEK293 cell nuclear extracts (*Right*). The coprecipitation of endogenous ATR or ATRIP was examined by immunoblotting copurified proteins. (*D*) Myc-ATRIP and HA-CINP were expressed in cells with or without Flag-ATR as indicated. The cells were left untreated (Unt.) or treated with IR or UV radiation. Following Flag-immunoprecipitation, protein complexes were immunoblotted with the indicated antibodies. (*E*) 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. (*F*) 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 coprecipitating ATRIP proteins were analyzed by immunoblotting with HA. ATRIPΔCC+GCN4 replaces the ATRIP coiled-coil domain with the GCN4 coiled-coil domain (17). (*G*) U2OS cells stably expressing an empty vector (Vec), siRNA-resistant wild-type ATRIP (WT), or siRNA-resistant ATRIPΔCC+GCN4 were transfected with nontargeting (NT) or ATRIP siRNA. An HU-sensitivity assay was performed as shown in Fig. 3*A*.

proteins. Immunoprecipitation of Flag-ATR or Flag-ATRIP from HEK293 cells coprecipitates HA-CINP (Fig. 4*A*). The reciprocal immunoprecipitation indicates that Flag-CINP binds endogenous ATRIP. In contrast, we did not detect any interaction between CINP and ATM (Fig. 4*B*). An interaction between endogenous ATR-ATRIP and CINP was also observed (Fig. 4*C*). However, the interaction is likely to be transient or low affinity, as only a small percentage of these proteins were coimmunoprecipitated. The CINP-ATR-ATRIP interaction is not stimulated by DNA damage or replication stress (Fig. 4*D*). Furthermore, subcellular localization studies on CINP showed pan-nuclear staining without any concentration into ATRIP-containing foci in response to DNA damage. These data suggest CINP may regulate ATR-ATRIP via transient interactions, and is not likely to be part of the active signaling complex at sites of DNA damage.

To further understand how CINP functions, we commissioned a two-hybrid screen to identify CINP-interacting proteins. To accomplish this, 75.5 million prey clones were screened using a full-length CINP bait. ATRIP was identified 12 times and was the only high-confidence CINP-interacting protein identified. All 12 ATRIP clones identified contained its coiled-coil domain. To further map the CINP binding surface on ATRIP, we used a two-hybrid prey library of thousands of random ATRIP fragments (7) in a targeted screen with full-length CINP as bait. Sequencing of the ATRIP-interacting fragments revealed that CINP binds to the N-terminal half of the predicted ATRIP coiled-coil domain containing the minimum amino acids 118 to 156 (Fig. 4*E*). The interaction between CINP and the ATRIP coiled-coil domain was confirmed by coimmunoprecipitation. Flag-CINP coimmunoprecipitates HA-ATRIP and an ATRIP deletion mutant that retains amino acids 118 to 156; however, deletion of the ATRIP coiled-coil domain (ΔCC) eliminates the interaction with CINP (Fig. 4*F*).

The ATRIP coiled-coil domain is required for ATRIP dimerization, stable ATR binding, accumulation of ATRIP at DNA lesions, and ATR-dependent checkpoint signaling (17, 18). Replacement of this domain with the coiled-coil dimerization domain of the *Saccharomyces cerevisiae* GCN4 transcription factor restores all of these ATRIP functions except ATR-dependent CHK1 phosphorylation (17), 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, because replacement of the ATRIP coiled-coil domain with the GCN4 coiled-coil domain also fails to restore the interaction between ATRIP and CINP (see Fig. 4*F*). Consistent with this interpretation, we found that cells expressing the ATRIPΔCC+GCN4 mutant in place of wild-type ATRIP are hypersensitive to HU (Fig. 4*G*). Despite its functional defects in cells, the ATRIPΔCC+GCN4 protein does support TopBP1-dependent activation of ATR in vitro (Fig. S3*A*). Similarly, CINP is not required for the TopBP1-activation of ATR in vitro (Fig. S3 *C* and *D*).

To gain further evidence for a function of CINP in the ATR-ATRIP pathway, we examined whether CINP silencing impairs ATR-dependent signaling in response to genotoxic agents. Silencing of CINP significantly reduces CHK1 phosphorylation after both IR and UV radiation, although not to the extent that ATR silencing does (Figs. 5 *A* and *B*). ATR-dependent SMC1 phosphorylation is also impaired, indicating that CINP is required for efficient ATR signaling to multiple effector proteins. The CINP-dependency for ATR substrate phosphorylation is not the result of an indirect cell-cycle effect. CINP-depleted cells continue to proliferate. Cell-cycle analysis indicates there is a slight decrease in the percentage of G1-phase cells, an increase in G2-phase cells, and a similar percentage of S-phase cells in CINP-silenced cells compared to control (Fig. 5*C*). These results are consistent with the mild checkpoint activation induced by CINP silencing in untreated cells.

Finally, we investigated whether CINP regulates the ATR-

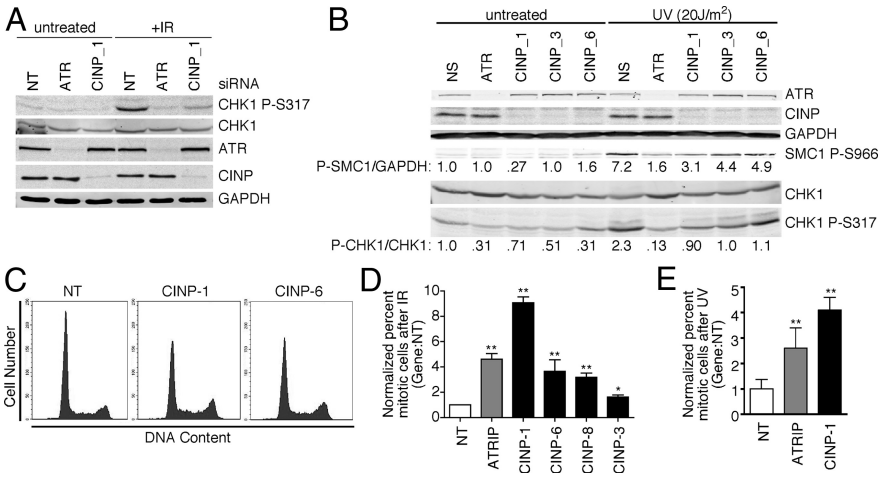

Fig. 5. CINP regulates ATR-ATRIP signaling. (*A* and *B*) U2OS cells were transfected with nontargeting (NT), ATR, or CINP siRNA oligonucleotides and irradiated with 5 Gy IR (*A*) or 20 J/m^2 UV (*B*). Six hours after IR and 2 h after UV, whole-cell extracts were prepared and ATR activation was monitored by immunoblotting cell lysates with the indicated antibodies. Detection and quantitation was performed on an Odyssey instrument. (*C*) DNA content of control (NT), CINP_1-, or CINP_6-silenced cells was examined by flow cytometry. (*D* and *E*) G2 checkpoint integrity after IR (*D*) or UV (*E*) radiation was examined in cells transfected with the indicated siRNAs. Mitotic cells were quantified by flow-cytometry analysis using a phospho-peptide specific antibody to histone H3 S10. Error bars represent standard error from three independent experiments. ATRIP and CINP siRNAs causing a statistically significant increase in the percentage of mitotic cells relative to the nontargeting control (NT) are designated by asterisks (two-tailed, unpaired *t*-test; *, $P < 0.05$, **, $P < 0.01$).

dependent checkpoint response. The G2 checkpoint is active and maintained in cells transfected with a nontargeting siRNA, as indicated by the low percentage of mitotic cells following irradiation (Fig. 5 *D* and *E*). In comparison, the percentage of cells escaping the G2 checkpoint following IR and UV treatment is increased in *CINP*-silenced populations, a phenotype also observed when *ATRIP* is silenced.

Discussion

Maintenance of genome integrity is critical for cancer prevention. We exploited markers of active DDR signaling to identify gene products important for maintaining genome integrity. We found that RNAi-mediated silencing of 73 genes, and the overexpression of 97 genes, increase DDR signaling.

Genome Maintenance and Cancer. As expected, many of the genes identified are suspected or known tumor suppressors or oncogenes (see Table S3). The cDNA overexpression screen found several oncogenes known to induce genetic instability when overexpressed, including *PLK1*, which causes chromosomal instability because of its function in centrosome and mitotic control (34). Another interesting example is the *DEK* oncogene. DEK regulates chromatin and DNA topology, and was originally identified in a translocation in acute mylogenous leukemia (35). DEK overexpression also suppresses the phenotypic defects of an ataxia-telangiectasia cell-line defective in ATM activity (36). This suppression is cell-line specific and unique to a relatively mild ATM mutation (deletion of amino acids 2427 and 2428). Thus, DEK overexpression may cause chromatin changes that increase the activity of the mutant ATM protein, leading to partial suppression of the DNA damage-sensitivity phenotype.

Our data indicate several ets family transcription factors cause DDR activation when overexpressed. Because these proteins are frequently overexpressed and amplified in human tumors (30), they may contribute to tumorigenesis in part by promoting genome instability (19). This is also true for other known or putative oncogenes identified in the overexpression screen. In some cases, gene overexpression may directly perturb genome maintenance activities, such as DNA repair. For example, HOXB7 is linked to DNA repair through interactions with PARP and DNA-PK proteins (37), and its overexpression is associated with melanoma. Overexpression of the DNA repair proteins DCLRE1A and MLH1 may cause DNA damage via dominant negative effects because these proteins function as part of protein complexes.

Other major categories of genes identified in the screens include replication, mitotic control, and chromatin regulation. POLD1, POLA1, RBMS1, RFC5, RPA1, RPA2, RRM1, PRIM1, POLE2, and POLB are replication proteins whose silencing may cause increased replication stress. A group of genes that encode regulators of mitosis, including PLK1, WEE1, and APC subunits, may cause misregulated mitotic entry that could yield DNA damage either as a consequence of incomplete DNA replication or perhaps during the subsequent cell cycle. Several chromatin regulatory proteins, including multiple histones, HDAC7A, SETDB2, DEK, and ZNHIT4 were identified. In some cases the effect on genome maintenance may be indirect through transcriptional changes. In other cases, the chromatin regulation may directly affect DNA repair. For example, ZNHIT4 is an INO80 complex protein that is targeted to sites of DSBs. Chromatin regulation and DNA repair were also prominent categories of genes found in an *S. cerevisiae* screen that used increased spontaneous Rad52 foci as an assay (38).

We also identified several proteins phosphorylated by ATM or ATR in response to DNA damage but with unknown functions, including PLEKHO2, SCFD1, MED13L, COPZ1, RBBP5, and PPP1R12C (39). Our data confirm their placement in a DDR pathway.

Finally, in some cases it is unclear why deregulation of the genes we identified would lead to DDR activation. This is particularly the case in the cDNA overexpression screen. For example, a large number of RNA binding proteins not previously linked to DNA metabolism were found in the overexpression screen. In some of these cases, the effect may be indirect or could be because of induction of apoptosis. We excluded obvious apoptotic cells in our analysis, and the siRNA screen did not enrich for genes with known functions in apoptosis. We also have repeated the γH2AX screen using an apoptosis siARRAY library of RNAi molecules from Dharmacon and failed to identify any positive hits. However, in the cDNA overexpression screen we did identify *CIDEC*, *CASP10*, and *CRADD* that participate in apoptotic pathways. Thus, in some cases the γH2AX staining could be a result of the initiation of early stages of an apoptotic program.

CINP. CINP was originally identified in a two-hybrid screen for CDK2-interacting proteins (40). However, nothing has been reported about its cellular function and we have not observed an interaction between CINP and CDK2 in either our two-hybrid or coimmunoprecipitation experiments. Our data identify CINP as a regulator of ATR-mediated checkpoint signaling. CINP forms a complex with ATR-ATRIP through ATRIP, promotes cell viability in response to replication stress, is required for efficient ATR-dependent signaling after DNA damage, and is required for maintenance of the G2 checkpoint. CINP was also identified as a genome-maintenance protein in a recently published functional genomics screen (41).

The need for CINP for full ATR signaling likely explains the defects associated with replacing the ATRIP-coiled-coil domain

with a heterologous coiled-coil domain (17). The ATRIP-ΔCC-GCN4 protein is defective in promoting ATR-dependent CHK1 phosphorylation and G2 checkpoint control, yet it is capable of localizing the ATR complex to foci (17) and supporting TopBP1-dependent ATR activation in vitro (see Fig. S3). CINP binds the ATRIP coiled-coil domain and CINP-silencing mimics the functional defects of the ATRIP-ΔCC-GCN4 mutant. Therefore, CINP binding may be the second required activity of the ATIP coiled-coil domain, in addition to dimerization, that is important for ATR checkpoint signaling.

CINP is a small protein with no described domain structure beyond a predicted coiled-coil motif. Because CINP does not accumulate in DNA-damage foci with ATRIP, it may not be an integral component of the active ATR signaling complex. Furthermore, CINP is not needed to localize ATR-ATRIP to damage-induced foci or for the TopBP1-stimulated in vitro kinase activity of ATR-ATRIP complexes (see Fig. S3). We did observe a 50% reduction in ATRIP S224 phosphorylation in CINP-silenced cells (see Fig. S3 *E* and *F*). S224 is phosphorylated by CDK2 (15), suggesting that CINP regulates ATR signaling in part by controlling the cell-cycle-dependent phosphorylation of ATRIP. However, abolishing phosphorylation on this site completely using a S224A mutant does not fully mimic the phenotypes caused by CINP silencing, including the γH2AX induction and HU-hypersensitivity. Therefore, there are likely additional mechanisms by which CINP regulates ATR checkpoint function and genome maintenance.

Methods

RNAi, cDNA, and Yeast Two-Hybrid Screens. Details of these methodologies can be found in the *SI Text*.

HU Sensitivity Screen. U2OS cells were transfected with siRNA and split into four 96-well plates the day after transfection. Three days after transfection, cells were incubated in media with (two plates) or without (two plates) 3 mM HU for 24 h, followed by 24 h in fresh growth media. Cell viability was quantified using the WST-1 cell-proliferation reagent (Roche). Sensitivity to HU was calculated by two methods. First, the ratio of the 450 nM absorbance for the mean of the two HU-treated wells to the mean of the two untreated wells was determined. Ratios for each gene-silencing siRNA were then normalized to the mean ratio of the nontargeting siRNAs within each plate to control for plate-to-plate variation. The $\log_2$ of these viability ratios was calculated for determination of statistical significance. Second, the effect of siRNA silencing on HU sensitivity was calculated as an index of antagonism or sensitivity (see *SI Text*), taking into account the individual effects of the siRNA and the HU on cell viability (42). The mean viability ratio and sensitivity-index values were calculated from three independent transfections, and statistical significance was determined using a two-tailed, unpaired *t*-test comparing each gene-silencing siRNA to the nontargeting siRNA.

ACKNOWLEDGMENTS. This work was supported by National Cancer Institute Grants R21CA132010 and R01 CA102729 (to D.C.) and a Susan G. Komen for the Cure grant (to D.C.). Additional support came from the Vanderbilt-Ingram Cancer Center SPORE in Breast Cancer (P50 CA098131), the Robert J. Kleberg Jr. and Helen C. Kleberg Foundation, the Ingram Charitable Fund, and the Vanderbilt Center for Molecular Toxicology (P30 ES000267), and Department of Defense Breast Cancer Research Program Predoctoral Fellowship W81XWH-06–1-0528 (to C.A.L.).

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