CHAPTER IV

A FUNCTIONAL RNAi SCREEN IDENTIFIES NOVEL REGULATORS OF GENOME INTEGRITY[2]

Introduction

Defects in genome maintenance are the underlying cause of many human diseases, most notably cancer [212] [2]. Genome instability is considered critical for cancer development, because it would otherwise be difficult for a normal cell to accumulate the number of mutations necessary for transformation to a cancer cell [213]. An important goal of cancer research has been to understand the genetic basis of cancer. The identification of gene products and biological processes that function to maintain genome integrity is critical to advance our understanding of the initiation and progression of this disease.

Replication stress and the generation of DNA double strand breaks (DSBs) are common features of many genome-destabilizing events [214]. These events activate DDR pathways, which function to preserve genome integrity by arresting cell cycle progression, initiating repair of DNA lesions, and stabilizing stalled replication forks. The DDR can also induce cellular senescence or apoptosis to prevent the proliferation of

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[2]Portions of this chapter have been submitted for publication. Bansbach CE, Lovejoy CA, Zhao R, Glick GG, Cappel DA, Ye F, Titus LC, Shyr Y, & Cortez D. (2009) The annealing helicase SMARCAL1 maintains genome integrity at replication forks.

cells with mutated or unstable genomes [215]. Two kinases at the apex of these DDR signaling pathways are ATM and ATR, which have unique and overlapping functions in promoting genome maintenance. While ATM is nonessential and responds primarily to infrequently occurring double-strand breaks, ATR responds during every cell division cycle to replication stress and is essential for the viability of replicating somatic cells [41, 42]. The loss of ATR activity causes replication fork-associated double strand breaks to form, presumably as a consequence of the inability of ATR-depleted cells to stabilize or recover forks that stall at ubiquitous DNA lesions or regions of the chromosomes that are particularly difficult to replicate (i.e. fragile sites) [43].

The DDR is not only activated by endogenous and exogenous sources of DNA damage (such as IR, UV, HU, and ROS), but also responds to tumorigenic gene function defects that promote genome instability [136, 137]. DNA damage and constitutive DDR activation are observed in cancer cell lines, as well as chronically active checkpoints in clinical samples from breast and lung carcinomas [216]. Furthermore, overexpression or activation of oncogenes, as well as the inactivation of some tumor suppressors, induces DDR activation in various cell types and animal models [210, 217-220].

Constitutive DDR activation is also observed in pre-cancerous lesions prior to the occurrence of mutational events [209, 210]. These lesions display 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 inactivation of DDR signaling, often through mutations in p53 [209, 210].

The current evidence suggests oncogene-induced replication stress is a key component of the genome destabilizing events that activate the DDR in pre-cancerous lesions. Activation of the ATR-mediated DDR pathway initially suggested the presence of aberrant DNA replication [209, 210]. Additional studies have observed preferential DNA breakage at common fragile sites, co-localization of DDR foci with replication foci, a requirement for progression through S phase for the generation of DNA damage, and oncogene-induced premature termination of replication fork progression [209, 210, 218, 219]. These observations further support the hypothesis that replication stress induces genome instability in pre-cancerous lesions. Additional sources of genome instability, such as telomere attrition or the generation of ROS, cannot be excluded from promoting DDR activation in early lesions. However, xenograft models of human hyperplasias and additional studies from cell culture models indicate telomere attrition and the generation of reactive oxygen species have minimal contributions to the DDR activation observed in early lesions [209, 210].

These findings suggest a model whereby the ATM/ATR-mediated DDR serves as an inducible barrier to constrain tumor development at its earliest stages by limiting cellular proliferation [136]. Several gene function defects capable of causing DDR activation have been identified, and could potentially promote the genome instability and replication stress observed in pre-cancerous lesions. Our finding that silencing of *DDB1* causes replication-dependent DNA damage and DDR activation is one example [221]. However, the genetic alterations underlying the genome instability in these pre-cancerous lesions are unknown in most cases, and the heterogeneous basis of cancer suggests there are many genes contributing to this phenotype.

I have exploited DDR activation as a reliable and biologically relevant indicator of genome instability, and developed an RNAi screen to monitor DDR activation following gene silencing. We identified seventy-four gene products that function to maintain genome integrity, as evidenced by the presence of DNA damage and activated DDR pathways after gene silencing. Furthermore, secondary assays suggest thirty-five of these gene products may have replication-dependent genome maintenance activities. Our data provide insight into the breadth of gene products and cellular processes that function to maintain genome integrity, and identify those which may contribute to the replication stress and genome instability observed in pre-cancerous lesions.

Results

Selection of the RNAi library

Starting from a genome-wide shRNA library [139], we created an RNAi sublibrary enriched for targets that are associated with nuclear regulatory activities. Gene targets were selected based on protein domain architectures of interest, including helicase, exonuclease, breast cancer carboxy-terminal domains (BRCT), forkhead associated domains (FHA), bromo, chromo, tudor, hect, ubiquitin ligase (Ubox), and ubiquitin specific protease domains (DUSP). Proteins containing these domains have prominent functions in DNA replication and repair pathways, the DNA damage response, the regulation of chromatin structure and gene expression, the recognition of histone modifications, or mediate phospho-peptide interactions with proteins that are phosphorylated by serine/threonine kinases, such as ATM and ATR. The association of

these domains with processes that are critical for genome maintenance suggests that additional proteins with these domains may also have important but unidentified roles in the maintenance of genome integrity.

In addition to the selection of gene targets based on protein domain architectures, we also included gene targets predicted to function in DNA metabolic processes, cell cycle regulation, the DNA damage response, or ubiquitin pathways based on gene ontology. Two recent reports have identified gene products that are mutated at a significant frequency in breast and colon cancer genomes [222], or that are phosphorylated after DNA damage on serine or threonine residues that represent consensus phosphorylation sites for the DDR kinases ATM and ATR [96]. These potential tumor suppressors and targets of the DDR were also included in our shRNA library based on their predicted association with genome maintenance activities. Finally, in addition to our selected shRNA library, we screened the apoptosis and cell cycle regulation siGENOME libraries produced by Dharmacon. In total, we analyzed 6,386 RNAi molecules targeting 2,287 genes.

Identification of candidate genome maintenance genes

HeLa cells were transfected with one shRNA or siRNA molecule per well in 96-well plates. Successfully transfected cells were identified by expression of GFP from the shRNA plasmids, and the transfection efficiency of the siRNA molecules was estimated to be >80% based on the knockdown achieved with control siRNAs. Genome integrity challenges resulting from RNAi-mediated gene silencing were examined in the absence of any exogenous stress by monitoring activation of the DDR (Figures 4.1A and B).

DDR activation was assayed by immunofluorescence staining using a phospho-peptide-specific antibody to the ATM/ATR substrate KAP1 (KRAB domain associated protein 1; Figure 4.1B). The damage-induced phosphorylation of this transcriptional co-repressor is necessary for ATM-mediated chromatin relaxation following DSB formation [223]. KAP1 phosphorylation is absent from cells transfected with a non-targeting RNAi molecule. In contrast, treatment with a high dose of HU induces DDR activation and phosphorylation of KAP1, as does RNAi silencing of the large subunit of replication protein A (*RPA1*), a gene product critical for both DNA replication and ATR activation (Figure 4.1B).

In addition to examining the ability of RNAi silencing alone to induce genome instability and DDR activation, we examined DDR activation resulting from the combined effects of RNAi silencing and a low dose of replication stress (0.1μM aphidicolin). This low dose of aphidicolin does not induce KAP1 phosphorylation in HeLa cells transfected with a non-targeting RNAi molecule, and was used to augment genome integrity defects resulting from RNAi inhibition that alone may be insufficient to cause DDR activation above the threshold of detection. Gene depletions causing sensitivity to this low level of replication stress could also provide useful information about the genome maintenance activity of the gene target, potentially implicating the gene product in replication-dependent cellular processes. For example, low levels of aphidicolin combined with RNAi to *ATR* or other replication checkpoint genes causes a synergistic induction of genome instability in the form of fragile site breakage [224].

A

RNAi transfection

↓

Untreated   +Aphidicolin

↓

DDR Activation

B
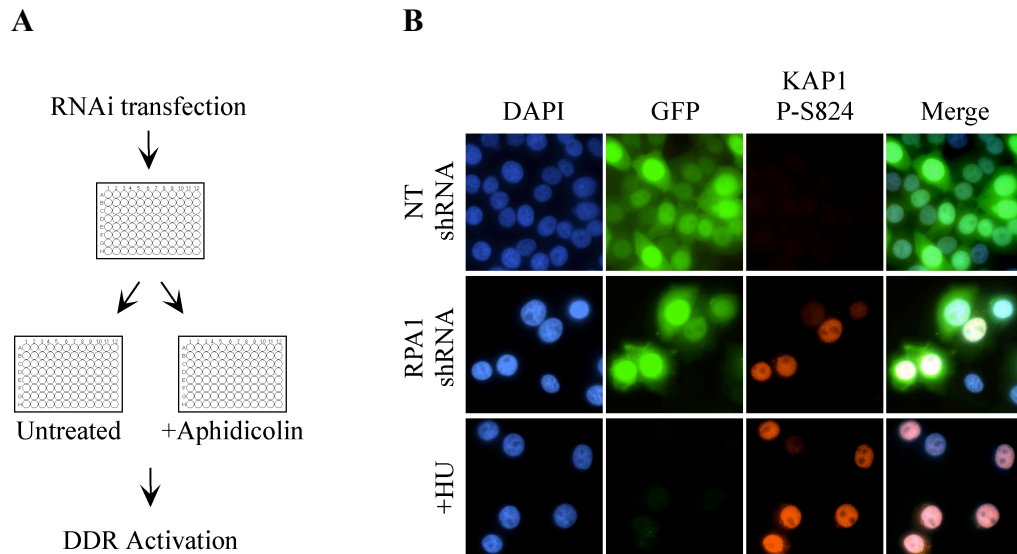
Figure 4.1. An RNAi screen identifies genome maintenance genes. **(A)** Schematic of the RNAi screen. HeLa or U2OS cells were transfected with one RNAi molecule/well in 96-well dishes. The transfected cells were split into two plates, one of which was treated with 0.1μM aphidicolin for 24hrs prior to the assessment of DDR activation. **(B)** DDR activation was monitored by immunofluorescence staining using a phospho-peptide-specific antibody to the ATM/ATR substrate KAP1. Nuclei are distinguished by DAPI staining, and cells successfully transfected with the shRNA molecules are identified by GFP expression. Silencing of RPA1 or treatment with a high dose of HU activates the DDR, as indicated by robust, pan-nuclear staining of KAP1 P-S824.

*Confirmation of RNAi-induced genome maintenance defects*

These primary screens identified 130 candidate genes whose deregulation by RNAi, in the absence or presence of aphidicolin, cause genome maintenance defects that activate DDR pathways. To confirm this phenotype, an additional marker of DDR activation was examined using a second source of RNAi in a cell type distinct from that used for the primary screen. Four individual siRNA oligonucleotides were obtained for each of the candidate genes, and DDR activation was monitored by examining H2AX S139 phosphorylation (γH2AX) in U2OS cells (Figure 4.2A and B). The PIKK family of kinases phosphorylate γH2AX at sites of double strand breaks, resulting in the formation of discrete, nuclear foci [225, 226]. γH2AX foci were observed in ~1% of cells transfected with a non-targeting siRNA. In contrast, silencing of the ATR substrate checkpoint kinase 1 (*CHK1*) caused DDR activation in 59% of cells, consistent with previous observations [227]. The measurement of γH2AX foci as a high-throughput screening assay for DDR activation is robust, with the analyses of both untreated and aphidicolin-treated samples producing excellent Z-factor scores (0.6102 and 0.5939, respectively).

The examination of γH2AX in U2OS cells confirmed that silencing of 74 genes caused DDR activation with two or more siRNAs (Figure 4.2C and Appendix A, Tables 1 and 2). The DDR activation resulting from RNAi-mediated inhibition of these genes is unlikely to be off-target because at least three independent RNAi molecules targeting each gene product result in DDR activation (minimally, one shRNA and two siRNA molecules). Furthermore, DDR activation in two distinct cell types and with two markers
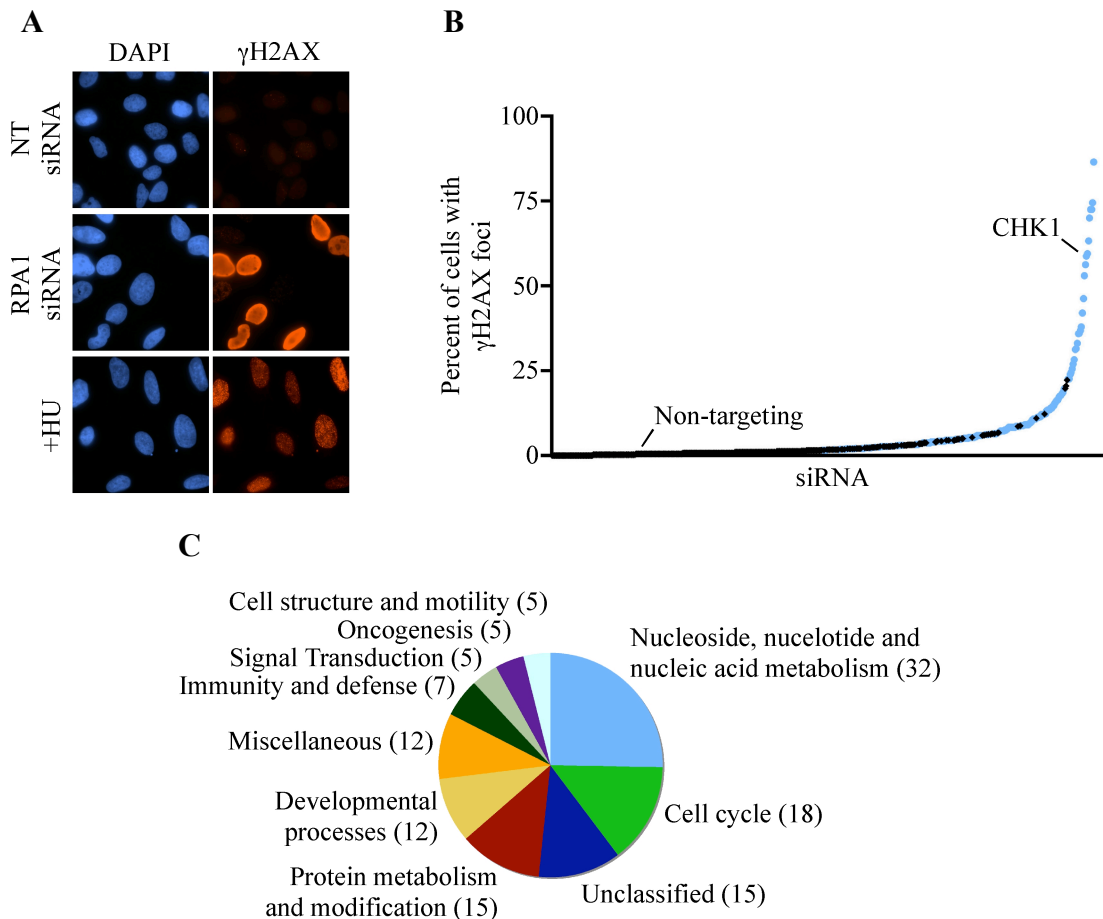
Figure 4.2. DDR activation is confirmed with second marker in a distinct cell type.
(A) U2OS cells were transfected with one RNAi molecule/well, and three days after transfection DDR activation was monitored by immunofluorescence staining using a phoshpo-peptide-specific antibody to the ATM/ATR substrate γH2AX. Nuclei are distinguished by DAPI staining. **(B)** γH2AX staining was quantitated with four siRNAs targeting each of the candidate genes identified in the primary screens. The percentage of cells containing γH2AX was determined by immunofluorescence staining. Each data point represents the mean of 4 replicas from a single siRNA. Gene silencing siRNAs causing a significant increase ($p \leq 0.05$) in γH2AX foci compared to the non-targeting (NT) control are highlighted in blue. **(C)** Biological classifications of genes reproducibly activating the DDR after RNAi silencing. Classifications were assigned using PANTHER (www.pantherdb.org). The number of genes classified in each category are indicated in parentheses, and genes may be assigned to multiple biological processes by this program. The siRNA transfections for γH2AX analysis were accomplished with assistance from Runxiang Zhao, and foci were counted with assistance from David Cortez and Laura Titus.

A

OTOF

B

C
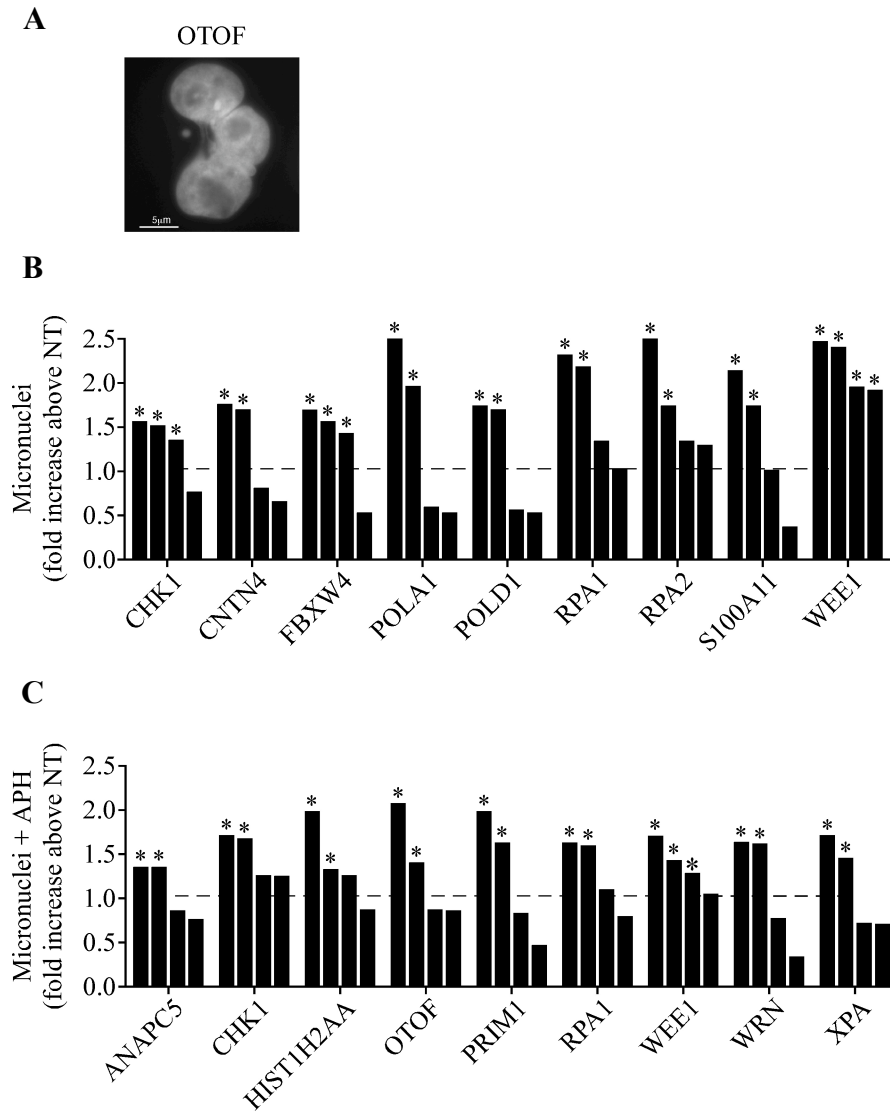
Figure 4.3. Micronuclei formation provides additional evidence of genome instability. (A) Micronuclei formation and incomplete mitotic segregation is evident after silencing of the putative tumor suppressor OTOF. Gene targets that reproducibly displayed DDR activation with at least two independent siRNAs, and additionally demonstrated an increase in micronuclei formation with at least two independent siRNAs are shown in the absence (B) and presence (C) of the genotoxic agent aphidicolin (0.1μM). All four siRNAs examined are shown for each gene target, and those producing a statistically significant increase in micronuclei formation relative to the non-targeting siRNA control are denoted by an asterisk ($p \leq 0.05$). Micronuclei were counted by David Cortez and Gloria Glick.

of DDR activation indicates these gene products are not cell type-specific activities and do not regulate a specific PIKK substrate.

In addition to activating DDR pathways, RNAi silencing of 15 genes caused a significant increase in micronuclei formation with at least two independent siRNA oligonucleotides (Figure 4.3). Micronuclei result from chromosomes or acentric chromosome fragments being excluded from the nucleus during cell division, resulting in a loss of genetic information. This phenotype is frequently used as a marker for chromosome breaks and genomic instability. The presence of micronuclei after RNAi silencing reinforces our conclusion that these gene-silencing siRNAs induce genome instability.

*Defects in DNA replication promote genome instability and DDR activation*

Genetic disruptions to DNA replication-associated processes caused the greatest amount of DNA damage. Gene-silencing siRNAs targeting *CHK1*, *POLA*, *RPA1*, and *RRM1* caused more than 50% of cells to display γH2AX foci in the absence of any exogenous genotoxic agent (Appendix A, Table 2). Twenty-one of the genome maintenance genes identified in this screen have recognized or putative roles in DNA metabolism, replication, or repair. The activation of the DDR by silencing of numerous genes involved in replication, as well as the extent of DNA damage induced by many of these gene-silencing siRNAs, underscores the significance of DNA replication-associated processes to the maintenance of genome integrity. Importantly, 15 genes failed to be classified into any biological process (Figure 4.2C). Further characterization of these gene products may reveal additional cellular processes that impact genome integrity, or

identify previously unrecognized roles for these gene products in the prominent genome maintenance pathways previously described. Of particular interest are those with genome maintenance activities during DNA replication, since replication stress is a key contributor to the genetic instability observed in pre-cancerous lesions [136, 137].

Identification of replication-dependent genome maintenance activities by increased DDR activation after aphidicolin

We performed three assays to facilitate the identification of gene products with replication-associated genome maintenance activities. First, we assessed whether low doses of aphidicolin combined with the RNAi silencing of each gene causes a synergistic activation of the DDR, as might be expected if the gene product functions in DNA replication or a replication stress response. Treatment with $0.1\mu M$ aphidicolin for 24 hours does not cause a substantial increase in the percentage of control cells with DNA damage. U2OS cell populations transfected with a non-targeting siRNA have 0.70% of aphidicolin-treated cells displaying foci compared to 0.46% of untreated cells (Appendix A, Table 2).

Several gene disruptions did display a notable increase in the percentage of cells with DDR activation after treatment with aphidicolin, suggesting sensitivity to this replication stress (Figure 4.4). Consistent with the hypothesis of synergistic DDR activation resulting from the combined application of exogenous replication stress and RNAi targeting replication genes, silencing of DNA polymerases delta, epsilon, and alpha, as well as *RPA2* and primase, display sensitivity to aphidicolin treatment. The mean percentage of cells with γH2AX foci increased 10-50% in the presence of this replication stress with at least two independent siRNAs targeting these gene products.
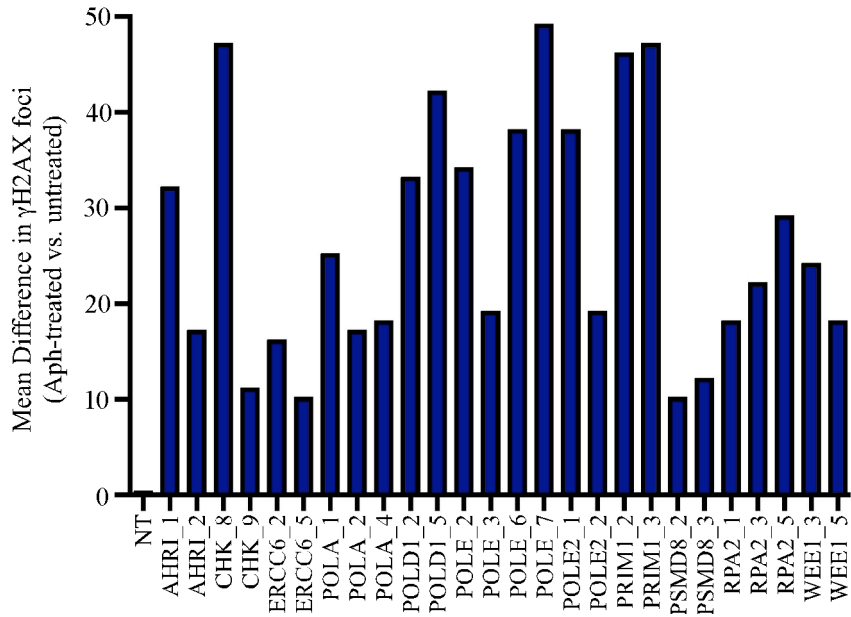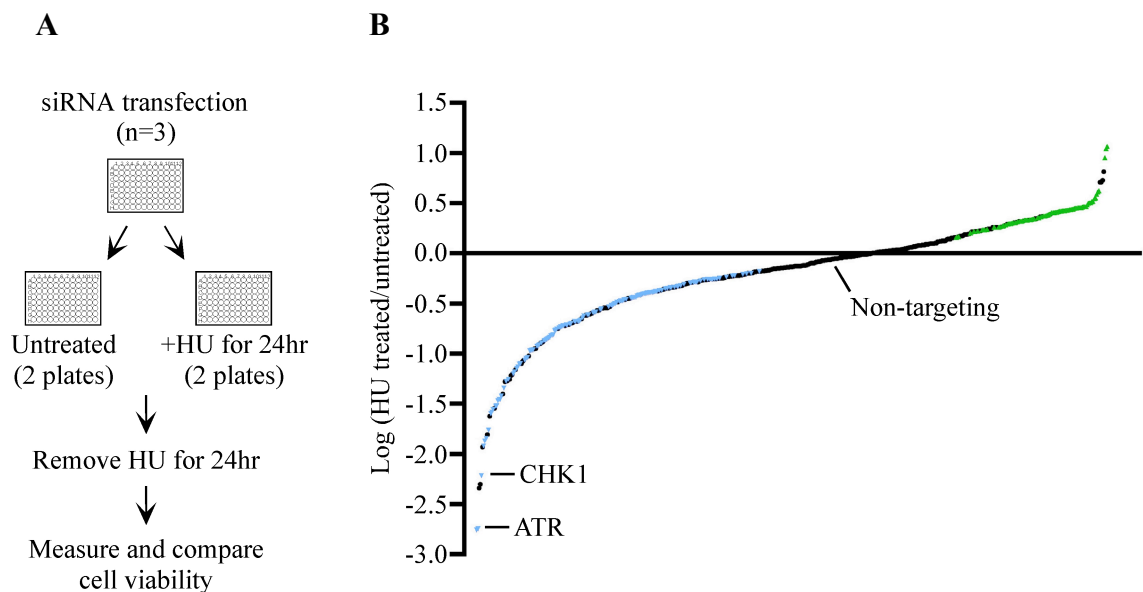
Figure 4.4 Replication genes display increased DDR activation after aphidicolin treatment. γH2AX foci formation was examined following silencing of all genome maintenance genes with four independent siRNAs in the absence and presence of 0.1μM aphidicolin (24hr). Gene targets showing an increase in the mean percentage of cells with γH2AX foci after aphidicolin treatment, with at least 2 independent siRNAs, are represented.

Silencing of two other genes, *DIRASS (AHRI)* and *ERCC6,* are noteworthy in their sensitivity to this low dose of aphidicolin since neither have reported roles in DNA replication. Two siRNAs targeting each of these genes also cause an ~10-30% increase in the mean percentage of cells with γH2AX foci after aphidicolin treatment.

Identification of replication-dependent genome maintenance activities by reduced viability after HU

The initial experiment examining sensitivity to replication stress identified gene-silencing siRNAs that caused an increase in the percentage of cells with DDR activation (γH2AX foci) following exposure to a low dose of aphidicolin. The second approach to facilitate the identification of genes with replication-dependent genome maintenance activities examined cell viability after exposure of RNAi-silenced cells to HU. The sensitivity to HU was determined using a colorimetric cell viability assay that compares treated and untreated populations (Figure 4.5A). In addition to measuring an alternative outcome, this experiment differs from the first in that cells were treated with a dose of HU sufficient to arrest DNA replication, and were then allowed to recover in the absence of replication stress for a period of 24 hours.

The HU viability 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 after HU treatment compared to the control ($p < 0.001$; Figure 4.5B). Both ATR and CHK1 have essential roles in DNA replication, and are required for the maintenance and recovery of stalled replication forks [228]. The ability of this assay to identify replication-dependent genome maintenance activities is

A

siRNA transfection
(n=3)

Untreated   +HU for 24hr
(2 plates)   (2 plates)

Remove HU for 24hr

Measure and compare
cell viability

B

Figure 4.5. Replication and replication stress response genes display reduced viability after HU treatment. (A) Schematic for the identification of gene targets causing HU sensitivity. U2OS cells were transfected with four siRNAs targeting each of the candidate genes identified in the primary screens. 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 24hrs before cell viability was quantified using the WST-1 cell proliferation reagent. **(B)** Gene silencing siRNAs causing a significant sensitivity (negative values) or resistance (positive values) to HU treatment compared to the non-targeting siRNA are highlighted in blue and green, respectively ($p \leq 0.05$). Each data point represents the average of three independent experiments for a single siRNA. The ratio of treated/untreated values was normalized to the non-targeting control ratio and the $\log_2$ of the ratios are presented. The HU sensitivity assay was developed by David Cortez.

A

B
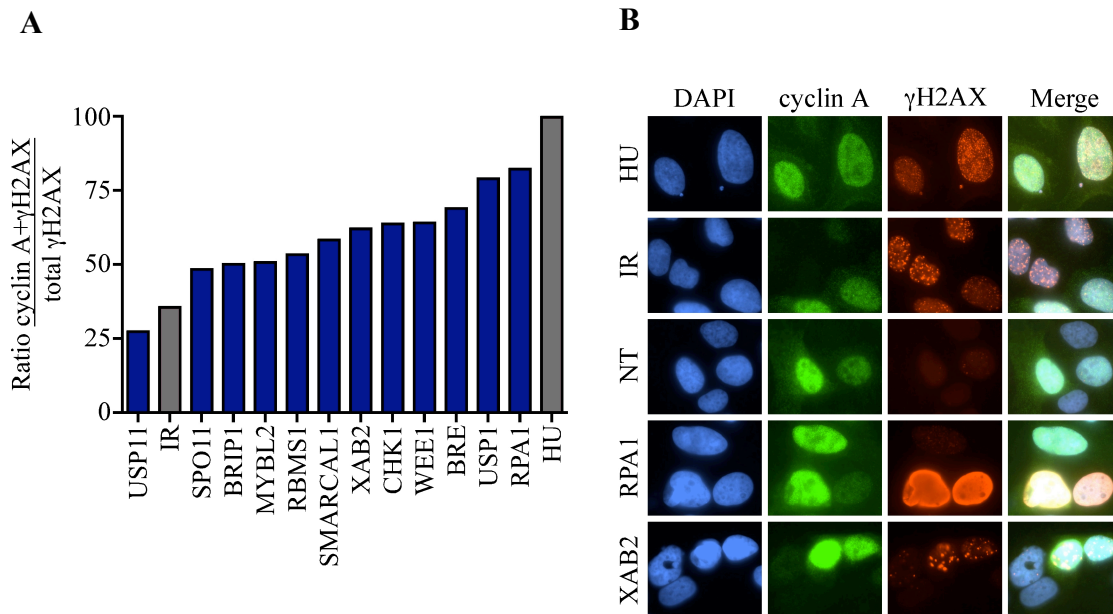
Figure 4.6. DDR activation is selective for S and G2 phase cells after siRNA silencing of genome maintenance genes. **(A)** The cell cycle dependency of DDR activation was examined in U2OS using immunofluorescence by co-staining with γH2AX and cyclin A, a protein expressed in S and G2 phase cells, for gene-silencing siRNAs causing γH2AX foci in >20% of cells. Ionizing radiation serves as a negative control, because this DNA damaging agent causes DDR activation independent of cell cycle progression. Thus, by chance, γH2AX foci are observed in approximately 35% of cyclin A-positive cells. Hydroxyurea serves as a positive control for the cell cycle dependency of DDR activation, with this replication-dependent genotoxic stress causing γH2AX foci in nearly 100% of cyclin A-positive cells. The majority of gene-silencing siRNAs examined show greater γH2AX staining in cyclin A-positive cells than what was observed with IR, suggesting a cell cycle dependency to the DNA damage that resulted in DDR activation. This increased percentage of γH2AX in S and G2 phase cells is consistent with the DNA damage occurring in a replication-dependent manner. **(B)** Representative pictures of cyclin A and γH2AX immunofluorescence staining. Cells transfected with a non-targeting siRNA show little γH2AX staining. Silencing of *RPA1* causes replication-dependent DNA damage, and accordingly, the γH2AX positive cells are also largely cyclin A-positive. Silencing of *XAB2*, like *RPA1*, shows increased γH2AX foci in cyclin A positive cells. Cell nuclei are distinguished by DAPI staining.

demonstrated by the reduced viability of several established replication genes, such as *CHK1, POLB*, *RFC5*, and *RPA2* (Appendix A, Table 4). Gene products essential for DNA replication could potentially demonstrate resistance to HU treatment and increased cell viability as well. Efficient silencing of gene products critical for DNA replication could prevent entry into S phase, or cause sufficient DNA damage during replication such that the majority of cells have arrested in G2 prior to HU treatment at 72 hours after siRNA transfection. Cell populations in which the majority of cells are no longer cycling would thus be unaffected by a replication-dependent genotoxic agent. Consistent with this hypothesis, silencing of the essential DNA replication genes *POLA* (2 siRNAs) and *RPA1* (4 siRNAs) resulted in HU resistance (Appendix A, Table 4).

Of the 74 genes causing DDR activation after RNAi inhibition, silencing of 20 resulted in sensitivity to HU treatment, as evidenced by a reduction in cell viability with at least two independent siRNAs (Figure 4.5B and Appendix A, Table 4). Consistent with a replication-dependent genome maintenance activity, we observed that the DNA damage resulting from silencing of several HU-sensitive genes is selective for S and G2 phase cells (including *CHK1*, *SPO11*, *SMARCAL1*, *USP1*, *WEE1*, and *XAB2*; Figure 4.6). This suggests that the DNA damage observed in these RNAi-silenced populations may be due to genome integrity challenges experienced during DNA replication.


Identification of replication-dependent genome maintenance activities by defective replication recovery after HU

The third assay used to identify genes with genome maintenance functions during DNA replication was an examination of DNA replication recovery following a transient challenge with HU. This assay allows for the identification of gene products that might
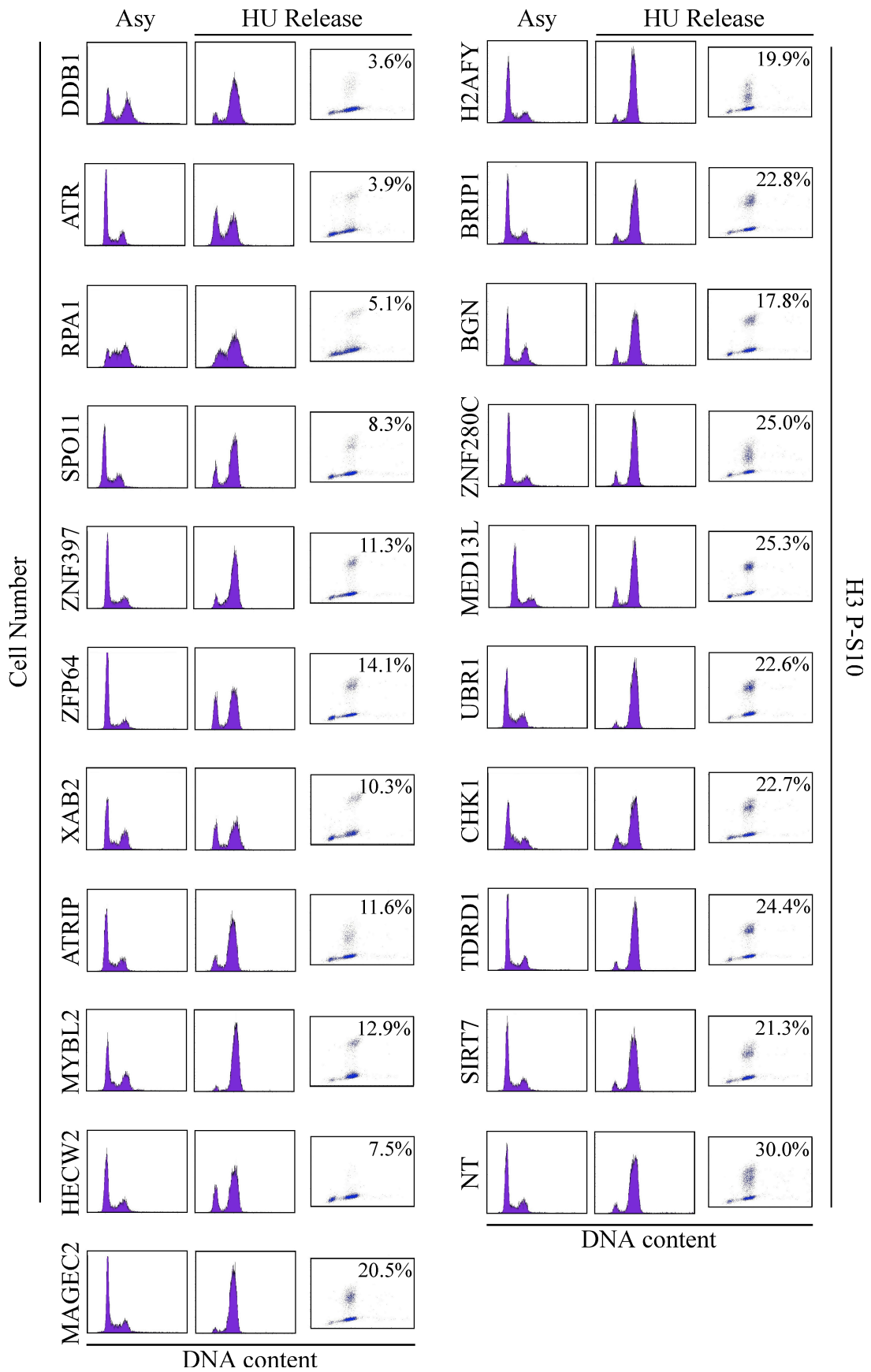
specifically be required to stabilize stalled replication forks or promote the recovery of stalled forks—genome maintenance functions associated with ATR-mediated pathways [228]. Following RNAi silencing, replication forks were stalled by a 16 hour treatment with a high dose of HU. The HU was removed and the ability of the cells to complete DNA synthesis was monitored by flow cytometric analysis of DNA content, BrdU incorporation, and entry into mitosis (histone H3 phospho-S10 staining).

Cells transfected with a non-targeting siRNA show no defect in the maintenance or recovery from stalled replication forks, as a significant proportion of cells progress into mitosis following release from the HU arrest (where they were trapped with the microtubule depolymerization agent nocodazole; Figure 4.7A). Most cells possess a 4n DNA content, and 30% have entered mitosis based on the positive staining for phosphorylated histone H3. Additionally, there is minimal incorporation of the thymidine analog BrdU, indicating that these cells have completed DNA synthesis at the time-point examined.

In contrast, *ATR*-silenced cells released from the HU arrest show an inability to efficiently resume DNA synthesis (Figure 4.7A). Many cells continue to exhibit 2n DNA content, and there is a low level of BrdU incorporation in a significant percentage of cells. The majority of cells that progressed to 4n DNA content have not entered mitosis based on the dramatically reduced percentage of cells staining positive for phosphorylated histone H3 (4%). This is consistent with the presence of an active G2 checkpoint, and is likely due to activation of an ATM-dependent checkpoint response resulting from DNA damage that accumulated during the aberrant attempt at replication in the absence of ATR. The failure to complete DNA replication is consistent with an
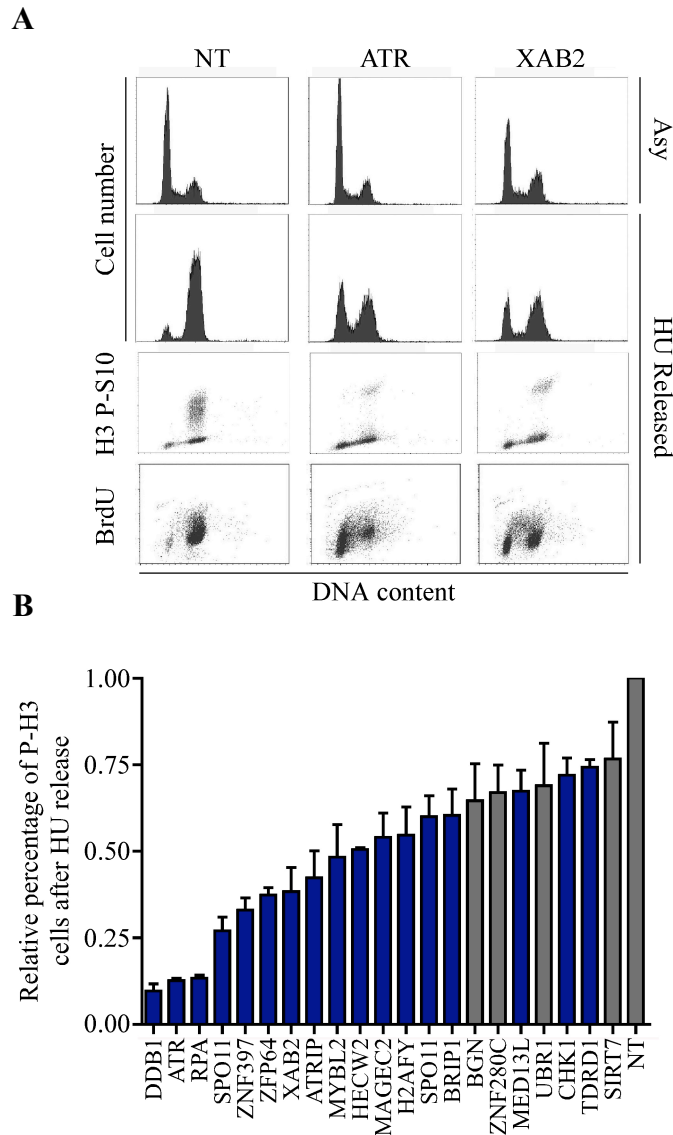
Figure 4.7. Replication-dependent DNA damage prevents mitotic entry after HU release. **(A and B)** Cell cycle recovery from a transient HU replication block was examined in RNAi transfected U2OS cells. After the HU block, cells were released into fresh growth media containing the mitotic spindle inhibitor nocodazole for 16hrs, and recovery was assessed by flow cytometric analysis of DNA content, the percentage of mitotic cells (histone H3 S10 phosphorylation), and DNA synthesis (based on BrdU incorporation during the last 2hrs of release). **(A)** Representative data for the non-targeting and *ATR* controls are displayed, along with *XAB2* gene silencing. The DNA content of untreated (asynchronous) cells was also measured for each RNAi transfection. **(B)** HU recovery was examined for all gene targets displaying HU sensitivity. Gene silencing siRNAs showing evidence of a defect in HU recovery in the initial experiment were assayed in three independent transfections and are graphed. Those demonstrating significant reductions in phospho-H3 staining, relative to the non-targeting control, are shaded blue ($p \leq 0.05$).

Asy HU Release Asy HU Release

DDB1 — 3.6%   H2AFY — 19.9%
ATR — 3.9%   BRIP1 — 22.8%
RPA1 — 5.1%   BGN — 17.8%
SPO11 — 8.3% ZNF280C — 25.0%
ZNF397 — 11.3% MED13L — 25.3%
ZFP64 — 14.1% UBR1 — 22.6%
XAB2 — 10.3% CHK1 — 22.7%
ATRIP — 11.6% TDRD1 — 24.4%
MYBL2 — 12.9% SIRT7 — 21.3%
HECW2 — 7.5% NT — 30.0%
MAGEC2 — 20.5%

Cell Number

DNA content

H3 P-S10

DNA content

97

Figure 4.8 (previous page). Identification of genome maintenance genes with potential roles in replication fork maintenance and recovery. Cell cycle recovery from a transient HU replication block was examined in RNAi transfected U2OS cells. Cells were then released into fresh growth media containing the mitotic spindle inhibitor nocodazole for 16hrs, and recovery was assessed by flow cytometric analysis of DNA content (propidium iodide staining) and mitotic cells (histone H3 S10 phosphorylation staining). HU recovery was examined for all gene targets displaying HU sensitivity. Those demonstrating a reduction in phospho-H3 staining, relative to the non-targeting control, are shown. The average percent of phospho-H3 cells from three independent experiments is indicated. The DNA content of untreated (asynchronous) cells was also measured for each RNAi transfection.

inability to maintain stalled DNA replication forks in the presence of HU or efficiently resume DNA synthesis following removal of this replication inhibitor.

The analysis of genes required for the maintenance and recovery from stalled replication forks was extended to all genes that displayed reduced viability after HU treatment of siRNA-silenced cells. Gene-silencing siRNAs that reduced phospho-histone H3 staining relative to the non-targeting control were then evaluated in triplicate.

The silencing of 10 genome maintenance genes caused a reduction in phospho-histone H3 staining after HU release relative to cells transfected with a non-targeting siRNA (Figure 4.7B and Appendix A, Table 5). Two distinct phenotypes are observed within this group of genes. RNAi silencing of six genes does not appear to significantly inhibit the ability of cells to resume DNA synthesis, since the majority of the population reaches 4n DNA content (*BRIP1, CHK1, DDB1, H2AFY, MAGEC2,* and *MED13L*; Figure 4.8). However, the lack of phospho-histone H3 staining in these cells with 4n DNA content is consistent with the presence of an active G2 checkpoint. This suggests that despite the resumption of DNA synthesis, the silencing of these genes likely caused replication stress that resulted in the formation of DNA damage and thus inhibition of

entry into mitosis. Consistent with this hypothesis, in Chapter III I demonstrated that silencing of *DDB1* resulted in replication-dependent DNA damage, due in part to the misregulation of replication origin firing. Silencing of *DDB1* would therefore not prevent the resumption of DNA synthesis after HU removal, but would induce replication-dependent DNA damage and a G2 arrest, consistent with the flow cytometry data here. The gene depletions exhibiting this phenotype may or may not have a role in the maintenance and recovery of stalled replication forks, but likely do possess replication-dependent genome maintenance activities.

The second phenotype observed is reminiscent of *ATR* silencing, with cell populations demonstrating an inability to efficiently resume DNA replication after release from the HU challenge. This phenotype was observed with gene-silencing siRNAs targeting four of the genome maintenance genes (*HECW2*, *RPA1*, *SPO11*, and *XAB2;* Figure 4.8). Silencing of *RPA1* is expected to yield a phenotype similar to silencing of *ATR* given its critical functions in the ATR signaling pathway [36]. Two independent siRNAs targeting both *SPO11* and *XAB2* produce a phenotype that is similar to that observed in *ATR*-silenced cells (Figures 4.7A, 4.8, and data not shown). *SPO11*- and *XAB2*-silenced cells display an inability to recover from the HU arrest with the persistence of cells with less than 4n DNA content, continued BrdU incorporation, and reduced phospho-histone H3 staining. The similarity in phenotypes between *SPO11*-, *XAB2*-, and *ATR*-silenced cells in terms of sensitivity to replication stress, and specifically with regards to an inability to recover DNA synthesis after replication fork arrest, suggests that SPO11 and XAB2 may have important roles in the maintenance or recovery of stalled DNA replication forks.

Silencing of XAB2 promotes genome instability by decreasing DDR protein expression

The striking similarity in phenotypes between *XAB2*- and *ATR*-silenced cells suggests that XAB2 may function in ATR-mediated replication stress responses, and thus silencing of *XAB2* could disrupt the ATR signaling pathway. The recruitment of ATR-ATRIP to sites of DNA damage is important for ATR activation because it concentrates the kinase with its activating protein, TopBP1 [66]. Silencing of *XAB2* had no adverse effect on the HU-induced foci formation of ATR-ATRIP, suggesting that the localization of this complex to sites of DNA damage and replication stress is not dependent on XAB2 (Figure 4.9).

I next examined whether silencing of *XAB2* disrupted ATR-mediated phosphorylation of target proteins in response to DNA damage. Most DNA damaging agents can activate both ATR and ATM, and these apical DDR kinases phosphorylate an overlapping set of target proteins. However, the phosphorylation of CHK1 on S317 is exclusively an ATR-mediated event [229]. Examination of CHK1 S317 phosphorylation in *XAB2*-silenced cells revealed a significant defect in this ATR-mediated phosphorylation event after treatment with a variety of DNA damaging agents (Figure 4.10).

This inhibition of DDR signaling is not limited to ATR-dependent pathways, as silencing of XAB2 also inhibits phosphorylation of the ATM-specific substrate CHK2 in response to DNA damaging agents (Figure 4.10). However, these phosphorylation defects appear to be the result of a reduction in protein levels for both CHK1 and CHK2. This suggests that silencing of *XAB2* may not directly affect the ability of these kinases to phosphorylate their respective substrates, but instead compromises DDR signaling
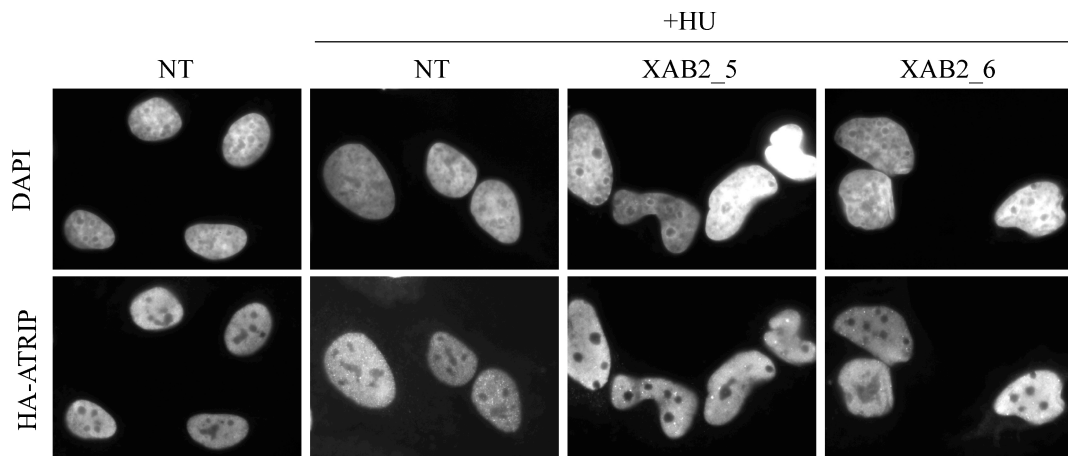
Figure 4.9. XAB2 is not required for the localization of ATR-ATRIP to sites of damage. U2OS cells stably expressing HA-epitope tagged ATRIP were transfected with non-targeting or XAB2 siRNAs. Three days after transfection cells were treated with 2mM HU for 5hrs and recruitment of ATR-ATRIP to sites of damage was monitored by immunofluorescence staining using HA. Cell nuclei are distinguished by DAPI.
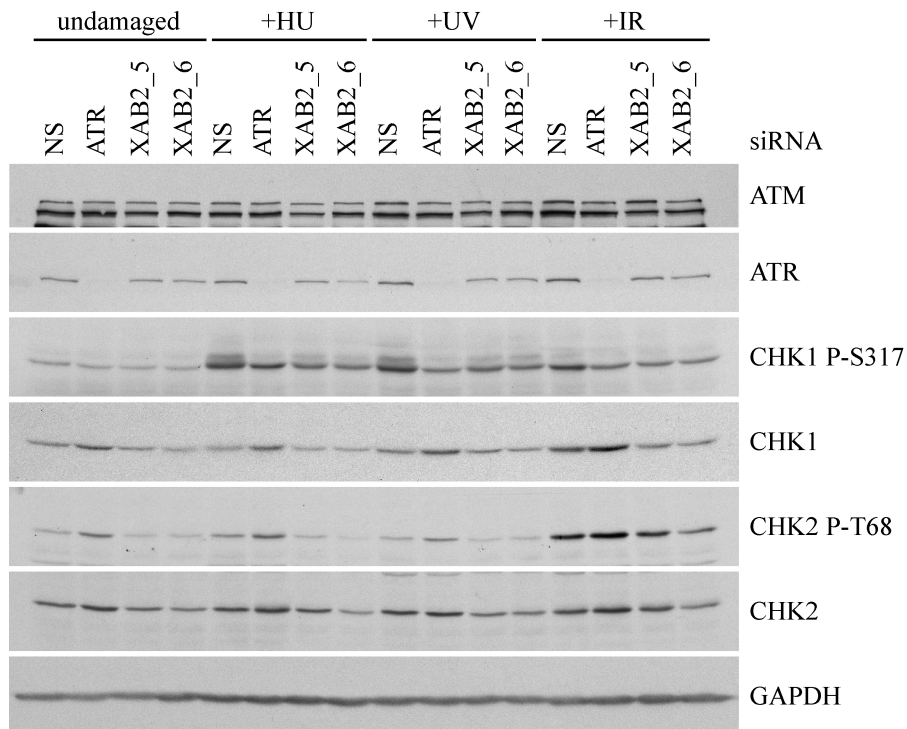
**Figure 4.10. Silencing of XAB2 disrupts ATR signaling**. U2OS cells were transfected with non-targeting (NT), ATR, or XAB2 siRNAs. Three days after transfection cells were left untreated, or treated with 2mM HU for 5hrs, 30 J/m^2 UV for 2 hours, or 5Gy IR for 2 hours. Checkpoint activation was monitored by immunoblotting total cell lysates with the indicated phospho-peptide-specific and total protein antibodies.

indirectly by reducing the amount of substrate available for phosphorylation. ATR protein levels are also reduced in *XAB2*-silenced cells, but this effect does not extend to all DDR proteins since ATM is unaffected (Figures 4.10). XAB2 is unlikely to regulate CHK1 protein levels through a post-translational mechanism since CHK1 protein turnover is not significantly altered by silencing of XAB2 (Figure 4.11). These results suggest that the inability of *XAB2*-silenced cells to resume DNA synthesis after HU treatment is the result of compromised ATR signaling through reduced CHK1 protein levels. Further experiments will be necessary to determine the mechanism by which XAB2 regulates the abundance of CHK1, CHK2, and ATR, as well as the extent of the proteins whose expression is dependent on XAB2.

Discussion

I developed a functional genomic screen for the identification of genome maintenance activities, and identified 74 genes that reproducibly demonstrate genome maintenance defects following RNAi silencing based on the activation of DDR pathways. Further analyses demonstrated that 35 of these genes display sensitivity to replication stress agents after RNAi silencing, suggesting potential replication-dependent genome maintenance activities for these gene products. The identification of gene function defects that induce genome instability and DDR activation, particularly through replication-dependent processes, can begin to provide necessary insight into the mechanisms that promote tumorigenesis in pre-cancerous lesions [209, 210, 218].
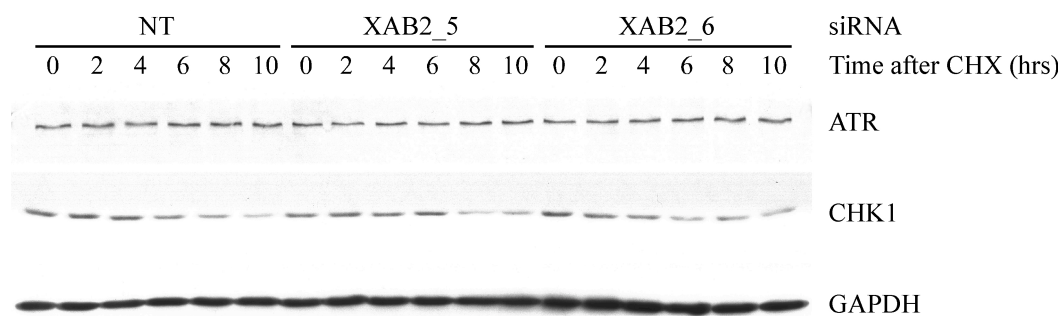
Figure 4.11. CHK1 protein stability is not affected by silencing of *XAB2*. U2OS cells were transfected with non-targeting (NT) and XAB2 siRNAs. Two days after transfection cells were treated with 100uM cycloheximide (CHX) to inhibit protein synthesis. Cells were harvested at the indicated times after CHX addition, and protein turnover was monitored by immunoblotting total cell lysates with the indicated antibodies.

Genome maintenance genes with tumor suppressor activity

Our RNAi screen identified 74 genes with genome maintenance activities using a functional and biologically relevant assay that monitors genome integrity challenges by activation of DDR pathways after RNAi-mediated gene silencing. DDR activation is observed in pre-cancerous lesions as a result of gene function defects [210, 217-220]. The ability of these gene disruptions to induce DDR activation suggests they may have important roles in early lesions to promote the genome instability that can facilitate tumorigenesis.

Recent sequencing efforts identified hundreds of genes that are mutated in breast and colorectal cancer genomes, but the mechanisms by which these mutations may contribute to the process of tumorigenesis remains unclear [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. Our RNAi screen can begin to provide functional information about the mutations observed in 6 genes from these cancer genome sequencing efforts (*ANAPC2*, *CENTB1, CNTN4, ERCC6, OTOF*, and *TBX22*), and suggests that mutations inhibiting the functions of these gene products may facilitate tumorigenesis by promoting genome instability.

Additional genome maintenance genes identified in our RNAi screen are clearly relevant for the process of tumorigenesis because they display tumor suppressor phenotypes (Appendix A, Table 3). Overall, 17 of the 74 genome maintenance genes have previously been suggested to function as tumor suppressors. While many of the

putative tumor suppressors displaying DDR activation after RNAi silencing have established connections to DNA replication, DNA repair, and DDR signaling, the tumor suppressors *TDRD6* and *MGC33407* have no identified functions. Our results suggest that loss of these gene functions may promote tumorigenesis by causing genomic instability, thus characterizing them as caretaker tumor suppressors.

*Cellular processes that are important for genome maintenance*

As important as the identification of individual genes that function to maintain genome integrity is the identification of cellular 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.

An extensive bioinformatics search using PubMed, BOND, IntAct, BioGRID, and STRING databases revealed potentially interesting connections that may provide further insight into the genome maintenance functions of these gene products, and also highlights cellular pathways that are critical for the maintenance of genome integrity. The largest interacting group of proteins is centered on the DDR kinases ATM and ATR, and includes gene products involved in DNA replication, repair, and checkpoint activities (Figure 4.12A). Several gene products (*FANCL, BRIP1, USP1, USP11,* and *BRE*) interact with the double strand break repair proteins RAD51, BRCA2, and FANCD2, suggesting genome maintenance activities associated with homologous recombination (HR). ATM/ATR substrates are encoded by 18 of the gene products displaying DDR

activation after RNAi silencing [96, 231]. In a few of these cases, such as *CHK1* and *CHK2*, their function is well defined. However, in several cases, including *PLEKHQ2*, *SCFD1*, *MED13L*, *COPZ1*, *RBBP5*, and *PPP1R12C*, it is unclear how these gene products execute a genome maintenance activity. The regulation by ATM/ATR suggests they may have important roles in checkpoint-mediated processes.

In addition to repair, replication, and checkpoint pathways, proper regulation of mitosis is also important for the maintenance of genome integrity, as evidenced by the numerous mitotic genes identified in this screen (Figure 4.12B). Genes involved in mitotic entry (*CHK1*, *CHK2*, *WEE1*), mitotic spindle assembly (*CDCA8*), and the mitotic checkpoint (*ANAPC2*, *ANAPC5*) were identified. All of these genome maintenance genes are directly or indirectly involved in the control of cell division cycle 2 (CDC2) activity or the execution of its cellular functions, emphasizing the importance of CDC2 regulation in genome maintenance.

Genes involved in chromatin regulation were also prominent among the genome maintenance genes identified (Figure 4.12C). Silencing of two histones, *H2AFY* and HIST1H2AA, causes DDR activation. The proper regulation of histone post-translational modifications is also critical for genome maintenance. A recent report demonstrates that deletion of the histone deacetylase HDAC3 causes replication-dependent double strand breaks and defects in double strand break repair [232]. Histone modifying activities identified in our screen include those involved in the regulation of histone methylation (*RBBP5*, *SETDB2*) and histone ubiquitination (*BMI1*, *DDB1*). In addition to post-translational modifications that can regulate chromatin structure, two gene products with
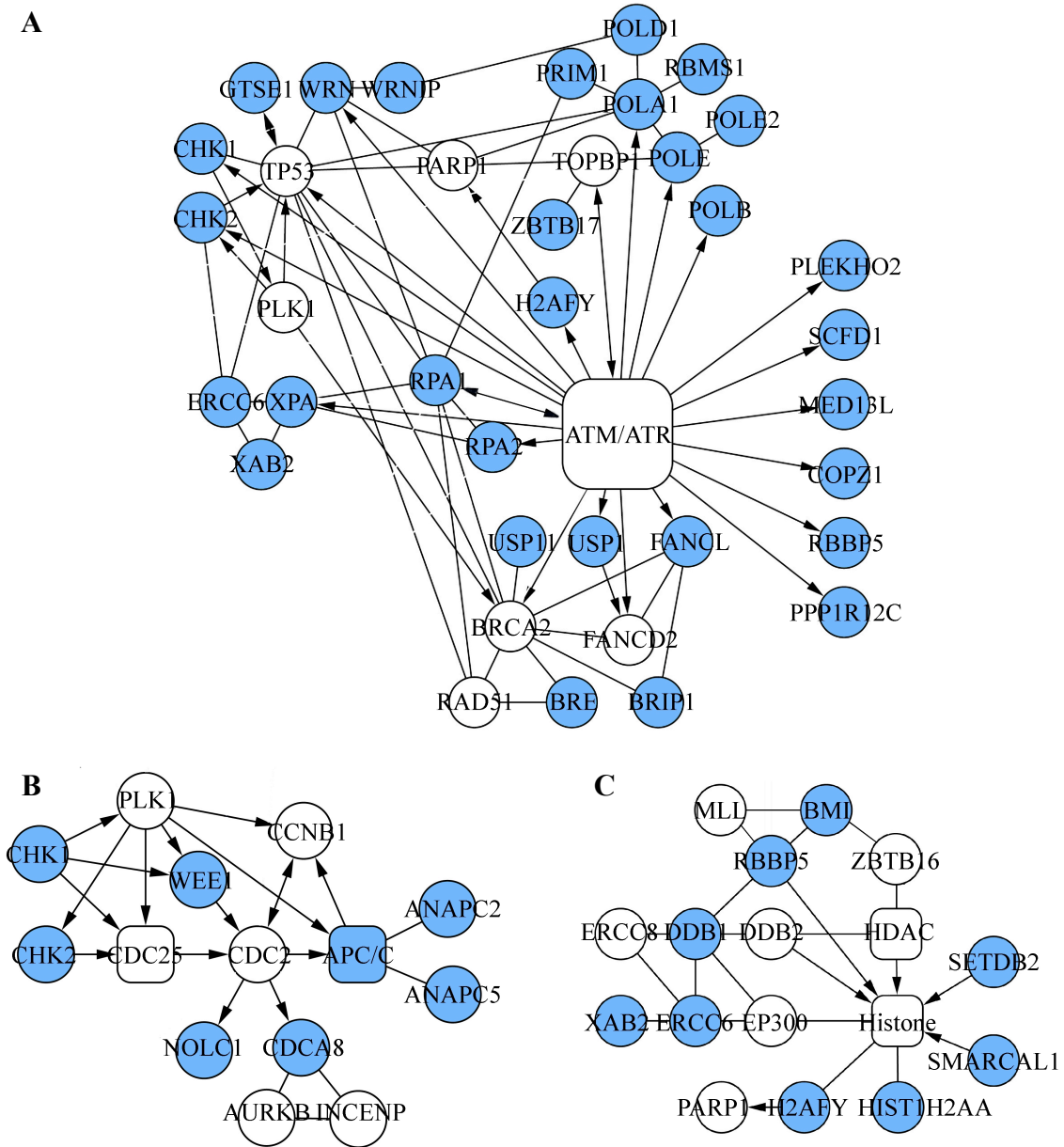
Figure 4.12. Network modeling of genome maintenance genes. (A) A connectivity map centered on the DDR kinases ATM and ATR highlights the importance of replication, repair, and checkpoint genes in the maintenance of genome integrity. Genome maintenance genes with connections to gene products that function in mitotic control (B) and chromatin regulation (C) are also highlighted. In each network, the genome maintenance genes identified in the screen are colored in blue. A solid line between two gene products indicates a direct protein-protein interaction; an arrow indicates that one gene product acts on the gene product to which the arrow is drawn (for example, a kinase-substrate relationship). Cellular activities containing more than one gene product, such as the anaphase promoting complex (APC/C), are diagramed as rounded rectangles. Transcriptional relationships are not diagrammed. The connectivity maps were generated by David Cortez.

ATPase and nucleosome remodeling activity were also identified as genome maintenance genes (*ERCC6*, *SMARCAL1*).
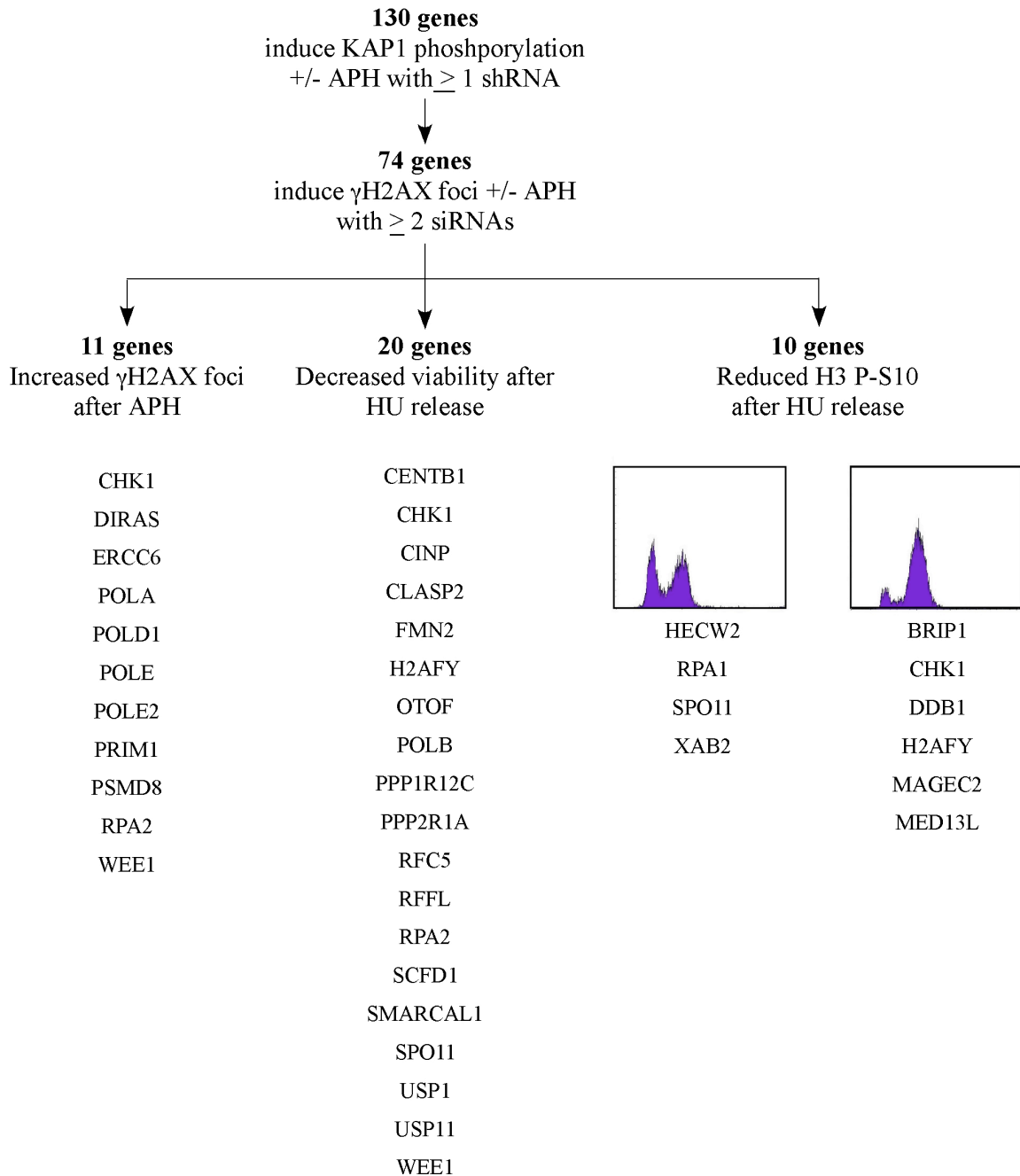
Identification of replication-dependent genome maintenance activities

Replication stress is a key contributor to the genome instability and DDR activation observed in pre-cancerous lesions. To distinguish gene products with potential replication-dependent genome maintenance activities, I performed a series of assays to identify the gene disruptions that cause sensitivity to replication stress. The disruption of DNA replication and replication stress response pathways can sensitize cells to further replication interference, thus facilitating identification of replication-dependent genome maintenance activities.

We first examined cellular sensitivity to replication stress by identifying genetic disruptions that cause a synergistic increase in DDR activation when combined with a low level of replication stress. Silencing of 11 genes caused an increase in γH2AX foci formation after aphidicolin treatment, compared to the gene disruptions in untreated cells, with at least two independent siRNA oligonucleotides (Figures 4.4 and 4.13).

Seven of these genes have established roles in DNA replication and replication stress responses, likely accounting for the observed sensitivity. Polymerases alpha (*POLA*), delta (*POLD*), and epsilon (*POLE*), as well as primase (*PRIM1*) and replication protein A (*RPA*) are all components of the replisome. The local unwinding at an initiated origin of replication results in the recruitment of the heterotrimeric protein RPA to bind and stabilize the ssDNA. After loading of RPA, polymerase alpha/DNA primase is recruited and synthesizes a short RNA/DNA primer. This primer is extended by the

130 genes
induce KAP1 phoshporylation
+/- APH with ≥ 1 shRNA

74 genes
induce γH2AX foci +/- APH
with ≥ 2 siRNAs

11 genes Increased γH2AX foci after APH	**20 genes** Decreased viability after HU release	**10 genes** Reduced H3 P-S10 after HU release	
CHK1	CENTB1		
DIRAS	CHK1		
ERCC6	CINP		
POLA	CLASP2		
POLD1	FMN2	HECW2	BRIP1
POLE	H2AFY	RPA1	CHK1
POLE2	OTOF	SPO11	DDB1
PRIM1	POLB	XAB2	H2AFY
PSMD8	PPP1R12C		MAGEC2
RPA2	PPP2R1A		MED13L
WEE1	RFC5		
	RFFL		
	RPA2		
	SCFD1		
	SMARCAL1		
	SPO11		
	USP1		
	USP11		
	WEE1		

Figure 4.13. Flow chart depicting progression of the RNAi screening assays. The primary screen in HeLa cells identified 130 genes that caused DDR activation after shRNA-mediated gene silencing. The genome maintenance activity of these 130 genes was confirmed by examining γH2AX in U2OS cells after siRNA-mediated silencing, and identified 74 genes that reproducibly cause DDR activation. Potential replication-dependent genome maintenance activities were assessed by examining the synergistic induction of γH2AX foci by siRNA and replication stress, reduced cell viability after replication stress, and the ability of cell populations to resume and complete DNA synthesis after release from replication arrest. Genes that were positive in these replication-dependent assays are listed. APH = aphidicolin, HU = hydroxyurea.

leading and lagging strand polymerases epsilon and delta, respectively, as the replisome commences DNA replication [6]. CHK1 activities during S phase include the regulation of replication origin firing, as well as a critical role in the maintenance and recovery of stalled replication forks [228, 233]. Silencing of *PSMD8* also increased DDR activation in the presence of replication stress; however, this gene product is a component of the proteosome and could indirectly affect replication-dependent processes by disrupting the degradation of key regulatory proteins. The remaining genes (*DIRAS3*, *ERCC6*, and *WEE1*) are notable for their increased DDR activation in siRNA-silenced cells following aphidicolin treatment because they lack recognized roles in replication-dependent processes.

GTP-binding RAS-like 3 (*DIRAS3*) encodes a GTPase with homology to the RAS superfamily and displays tumor suppressor activity [234-237]. DIRAS3 inhibits growth, motility, and invasion; maps to a chromosomal region that exhibits loss of heterozygosity in ovarian, breast, and oligodendroglial tumors; and is downregulated in 60% of breast and ovarian cancers [234-237]. *ERCC6* encodes a protein containing DNA-dependent ATPase and DNA binding activities, as well as nucleosome remodeling activity [238]. ERCC6 is also known as Cockayne's syndrome B (CSB), and is required for transcription-coupled nucleotide excision repair (TCR) [239]. Cockayne's syndrome is a disease characterized partially by sun sensitivity, which is the hallmark of Xeroderma Pigmentosum patients with defects in nucleotide excision repair (NER) proteins. However, Cockayne's syndrome patients also have developmental and growth defects, suggesting additional functions of ERCC6 that are distinct from its established role in NER [240].

Neither DIRAS3 nor ERCC6 have reported roles in DNA replication. The observation that two independent siRNAs targeting *DIRAS3* and *ERCC6* display enhanced DDR activation in response to aphidicolin treatment suggests that this phenotype is not an off-target effect, and that these gene products may have novel roles in regulating DNA replication. Furthermore, the inability of NER defects to account for the complexity of the Cockayne's syndrome phenotype suggests there are NER-independent functions of ERCC6. Interestingly, ERCC6 recruits NER proteins to a stalled RNA polymerase II enzyme [241]. Given the sensitivity of *ERCC6*-silenced cells to a replication stress agent that stalls DNA polymerase alpha, an intriguing possibility is that ERCC6 may be recruited to and promote recovery from stalled polymerases in general, rather than responding to a stalled RNA polymerase specifically.

WEE1 encodes a tyrosine kinase that catalyzes the inhibitory phosphorylation of CDC2/cyclin B kinase, thus functioning as a critical regulator of the entry into mitosis [103]. The sensitivity of *WEE1*-silenced cells to replication inhibitors is thus unclear since a role for WEE1 in DNA replication has not been described in mammalian cells. One possibility may be that silencing of *WEE1* compromised G2 checkpoint integrity and allowed cells containing DNA damage to progress through mitosis. Chromosome gaps or breaks that were not efficiently repaired in the previous cell cycle could then disrupt replication and activate a DDR is the subsequent cell cycle. However, silencing of *WEE1* with two distinct siRNAs caused DDR activation in 24% and 31% of cells in the absence of any exogenous genotoxic agent. This significant amount of DDR activation suggests that the DNA damage was not passively created by lack of a G2 checkpoint, but was actively induced by the loss of *WEE1*. Interestingly, WEE1 may have an uncharacterized

role independently of regulating the G2/M transition. The yeast homolog of WEE1, Swe1, is proposed to have a role in cell cycle re-entry from a G1 arrest that is independent of Cdc28 phosphorylation [242].

H2AFY and SPO11 exhibit sensitivity to replication stress in multiple assays

The remaining two assays examined cellular viability and replication recovery after cell cycle arrest and release from HU treatment. Silencing of 20 genome maintenance genes demonstrated sensitivity to replication stress with two independent siRNAs by reduced viability after HU treatment, and 10 genes displayed defects in the resumption of DNA synthesis after replication arrest (Figures 4.7 and 4.13, and Appendix A, Tables 4 and 5). Three genes were common to both assays: *CHK1*, *H2AFY*, and *SPO11*. The presence of *CHK1* in every replication sensitivity assay is not surprising considering the essential function role this gene product has in DNA replication, including a role in the maintenance and recovery of stalled replication forks [228].

H2AFY is a macrodomain containing histone that may have an important role in the establishment or maintenance of heterochromatin, as well as transcriptional repression [243-248]. H2AFY is an ATM/ATR substrate, and interacts with PARP1 through its macrodomain, resulting in the inhibition of PARP1 auto-ADP-ribosylation activity [231, 249]. This suggests that H2AFY may have genome maintenance functions in cell cycle checkpoint or DNA repair pathways that contribute to the genome instability and sensitivity to replication stress agents observed after RNAi silencing.

The phenotype of *SPO11* silencing is somewhat unexpected, since this gene product is thought to function primarily in meiotic cells to create the double strand breaks

required for initiation of meiotic crossover events [250, 251]. The reduced viability and inability to resume DNA synthesis after HU release is unlikely to be an off-target effect since two siRNAs targeting *SPO11* yield similar results in both assays. An interesting possibility is that this endonuclease has a previously unrecognized activity in processing stalled replication forks.

Gene products with potential roles in the maintenance and recovery of stalled replication forks

In addition to *SPO11*, silencing of three other genes (*RPA1*, *HECW2*, and *XAB2*) produced a phenotype similar to *ATR* silencing by displaying an inability to recover from an HU arrest with the persistence of cells with less than 4n DNA content, continued BrdU incorporation, and reduced phospho-histone H3 staining (Figures 4.7, 4.8, and 4.13). Silencing of *RPA1* is expected to yield a phenotype similar to *ATR* given its critical functions in the ATR signaling pathway [36]. The similarity in phenotypes between *XAB2-* and *ATR*-silenced cells, as well as the unstudied ubiquitin ligase *HECW2*, suggest that these gene products may also have important roles in the maintenance or recovery of stalled DNA replication forks.

The remaining 6 genes that displayed reduced phospho-histone H3 staining after release from an HU arrest were able to resume DNA synthesis since the populations have largely progressed to 4n DNA content (*BRIP1*, *CHK1*, *DDB1*, *H2AFY*, *MAGEC2*, *MED13L;* Figure 4.8). However, the reduced phospho-histone H3 staining is consistent with the arrest of these cells containing 4n DNA content in G2 phase of the cell cycle. Thus, silencing of these gene products may not have affected the stability of stalled replication forks, but likely did cause aberrant replication that generated DNA damage

and induced activation of a checkpoint response. The replication-dependent genome maintenance activities of *BRIP1*, *CHK1*, and *DDB1* are at least partially understood [221, 228, 252]. Confirmation of this phenotype and further characterization of *H2AFY*, *MAGEC2*, and *MED13L* will be necessary to identify their potential replication-dependent genome maintenance functions.

The genome maintenance functions of XAB2 may be attributable to regulation of DDR protein expression

The inability of *XAB2*-silenced cells to resume DNA synthesis after replication fork arrest was observed with two independent siRNA oligonucleotides, suggesting that this phenotype is unlikely to be an off-target effect. The striking similarity of *XAB2*-silenced cells to *ATR*-silenced cells prompted us to examine whether XAB2 functions in the ATR damage response pathway. The localization of ATR-ATRIP to sites of DNA damage was unperturbed by silencing of *XAB2*, however, phosphorylation of both the ATR-and ATM-specific substrates CHK1 and CHK2 was compromised in response to multiple DNA damaging agents. This inhibition of DDR signaling results from reduced expression of these target proteins in *XAB2*-silenced cells. The mechanism of regulation is unlikely to be post-translational since the protein turnover rate of CHK1 is unaltered by silencing of *XAB2*.

Recent reports indicate XAB2 may have functions independent of its role in transcription-coupled nucleotide excision repair. One report demonstrated that injection of antibodies against XAB2 into fibroblasts inhibited RNA synthesis, while a second report isolated XAB2 as part of a multiprotein complex containing factors involved in pre-mRNA splicing [253, 254]. This suggests that XAB2 may regulate CHK1 and CHK2

by promoting the transcription or splicing of these gene products. Immunoblots of *XAB2*-silenced cells suggest that the expression of additional DDR proteins may also be regulated by XAB2. The expanse of gene products affected by silencing of *XAB2* will require a global examination of mRNA expression before and after silencing.

The sensitivity to replication stress caused by silencing of *XAB2* is thus likely explained by its apparent role in the regulation of DDR protein expression, as opposed to a direct role for XAB2 in replication or replication stress responses. The reduction in CHK1 protein levels can readily account for the HU sensitivity of *XAB2*-silenced cells in these assays. Furthermore, the PARP sensitivity produced by loss of *XAB2* may also be explained by the effect on CHK1 protein levels [255].

Our functional RNAi screen identified 74 genes with genome maintenance activity. Seventeen of these genes have already been shown to possess tumor suppressor activity, and the genome instability observed here after silencing of these genes allows us to further characterize these products as caretaker tumor suppressors. The identification of gene products with replication-dependent genome maintenance activities is important to facilitate our understanding of the potential mechanisms through which replication stress is generated and how it promotes genome instability and DDR activation in precancerous lesions. In addition to providing insight into the genetic basis of cancer, these gene products may reveal novel biomarkers for the identification of cancer at its earliest stages, or perhaps suggest treatments that may be particularly useful based on the genetic and functional profiling of the tumor. Further research into both is necessary to more efficiently diagnose and treat this disease.