CHAPTER III

DDB1 MAINTAINS GENOME INTEGRITY THROUGH REGULATION OF REPLICATION ORIGIN LICENSING[1]

Introduction

Damaged DNA binding protein 1 (DDB1) was identified as part of a heterodimer that tightly associates with DNA following UV damage [143]. This heterodimer of DDB1 and damaged DNA binding protein 2 (DDB2) was subsequently discovered to have a role in nucleotide excision repair (NER). There are two subpathways of NER: global genomic repair (GGR) removes lesions throughout the genome, while transcription-coupled repair (TCR) removes lesions more readily from the transcribed strand of active genes. Defects in the chromatin binding property of the DDB1-DDB2 complex reduce the GGR capacity of cells by approximately 50% [144, 145]. Genetic defects in NER give rise to the autosomal recessive disorder Xeroderma Pigmentosum (XP), which is characterized by extreme sun-sensitivity, premature aging, and an increased incidence of skin cancer. Eight complementation groups have been defined for XP, with seven resulting from unique defects in proteins required for NER [146, 147]. Mutations in *DDB2* are classified as XP complementation group E (XP-E) [148, 149].

Further studies revealed that it is not simply a heterodimer of DDB1-DDB2 that

[1]The majority of this chapter has previously been published in reference 221. Lovejoy CA, Lock K, Yenamandra A, & Cortez D (2006) DDB1 maintains genome integrity through regulation of CDT1. *Mol Cell Biol* 26, 7977-7990.

binds DNA and stimulates GGR following UV damage, but a larger complex of proteins that possess an active ubiquitin ligase activity. In addition to DDB1 and DDB2, the soluble complex contains the E3 protein Cullin 4A (CUL4A), the Roc1 RING subunit that is required for CUL4A activity, and a cullin regulatory complex termed the COP9 signalosome (CSN) [150]. Following chromatin association, this active ubiquitin ligase targets XPC, a damage sensor for the GGR pathway. Ubiquitination of XPC does not result in proteosomal degradation, but instead enhances the ability of XPC to remain associated with chromatin [151]. Another target of this chromatin-bound complex is the DDB2 protein itself. DDB2 is rapidly degraded after UV damage, and is ubiquitinated *in vitro* by DDB1-CUL4A [152-154]. The ubiquitination of DDB2 reduces the DNA binding ability of DDB1-CUL4A and is necessary for efficient repair of UV-induced lesions [151]. Recently, the monoubiquitination of histone H2A at sites of UV-induced damage was also shown to require DDB1-CUL4 [155]. This modification may have an important role in facilitating GGR as well. Interestingly, an identical complex to that of DDB1-DDB2-CUL4A-Roc1-CSN was identified in which DDB2 is substituted by CSA, a protein that is defective in patients with Cockayne Syndrome (CS). Unlike the DDB2 complex, the CSA complex has a role in TCR [150].

DDB1 has important roles outside of NER as an adaptor molecule for the CUL4A ubiquitin ligase complex. Studies from *S. pombe* revealed that DDB1 and CUL4 promote the ubiquitin-mediated degradation of an inhibitor of ribonucleotide reductase (RNR) [156-158]. This inhibitor prevents the association of the two RNR subunits, an event that is necessary for the catalytic activity that converts NTPs to dNTPs for DNA synthesis and repair [158, 159]. Additionally, the DDB1-CUL4A complex promotes CDT1

degradation in human cells after IR and UV damage, and also has a role in the replication-dependent destruction of CDT1 [160-165]. CDT1 is a component of the pre-replication complex, which assembles in an ordered fashion during G1 to license origins of replication for initiation of DNA synthesis. Regulation of CDT1 is a critical means by which human cells prevent re-replication. Re-replication occurs when a replication origin fires more than once in a single cell division cycle [166, 167]. This protein is targeted for proteasome-mediated degradation by two ubiquitin ligase complexes, SCF^{Skp2} and DDB1-CUL4A, and is also functionally inhibited by the binding of Geminin [160-163, 168-171]. These multiple levels of CDT1 regulation emphasize the importance of restraining re-replication, and highlight the need to understand how these distinct processes cooperate to maintain the integrity of the genome.

In addition to DDB1-DDB2, several other protein complexes are recruited to and activated by UV-radiation-induced lesions. Principal among these UV-response proteins is ATR. ATM- and Rad3-related kinase (ATR) is a member of the phosphatidylinositol 3-kinase related kinase (PIKK) family, which also includes the damage response protein ataxia-telangiectasia mutated (ATM). These proteins are situated at the apex of the DNA damage response pathways, and function to protect the stability of the genome by initiating signal transduction cascades that result in the inhibition of cell cycle progression and the coordination of DNA repair [172, 173]. While ATM responds primarily to the formation of DNA double strand breaks, ATR is activated by a wide variety of lesions and stresses, including UV radiation, DNA alkylation and replication inhibitors, as well as double strand breaks. Downstream targets of these active kinases that are important in mediating cell cycle arrest and facilitating DNA repair include the

checkpoint kinases CHK1 and CHK2, p53, and the histone variant H2AX [76, 174-180]. Since ATR is responsible for initiating the checkpoint response to UV radiation, and both DDB1 and ATR are recruited to UV lesions, we examined whether DDB1 functions in the ATR-mediated DNA damage response to this genotoxic stress. While silencing of *DDB1* did not impair ATR-dependent responses to UV, surprisingly, we found that silencing of *DDB1* caused DNA double strand breaks and activation of checkpoint responses. This phenotype is due, in part, to the misregulation of DNA replication.

Results

Silencing of DDB1 activates cell cycle checkpoints

Both DDB1 and ATR are activated by UV radiation and are recruited to sites of UV-induced DNA damage. In *S. pombe* these proteins are both required for the proteolysis of the replication inhibitor Spd1. The damage-inducibility of DDB1, along with its functional relationship to the ATR homologue Mec1 in yeast, led us to test whether DDB1 has a role in the UV response initiated by ATR in human cells. We did not observe any defects in ATR activation in response to DNA damage in *DDB1*-silenced cells (data not shown). However, during the course of testing this hypothesis we examined the cell cycle distribution of *DDB1*-silenced cells to ensure that there was no adverse effect on cell cycle progression. Surprisingly, we found a striking change in the cell cycle distribution of undamaged, *DDB1*-silenced cells (Figure 3.1A). This population shows a considerable increase in cells with 4n DNA content compared to that observed following treatment with a non-targeting, control siRNA. To determine if this

accumulation is occurring in G2 or mitosis, cells were stained with an antibody to phosphorylated histone H3 on serine 10. This phosphorylation event occurs during chromosome condensation and is routinely used as a marker of mitotic cells [181]. Silencing of *DDB1* does not increase the percentage of mitotic cells relative to that observed in control cells (Figure 3.1A). In fact, there was a significant decrease in the percentage of mitotic cells in the *DDB1*-silenced population. Similar results were obtained with two independent *DDB1* siRNA oligonucleotides in both U2OS and HeLa cell lines (data not shown). This observation suggests that silencing of *DDB1* causes a cell cycle arrest in G2.

An arrest in G2 may indicate that cell cycle progression is halted due to the activation of a cell cycle checkpoint. Treatment with caffeine abrogates the G2/M checkpoint [182, 183]; therefore, if the G2 arrest in *DDB1*-silenced cells results from checkpoint activation, the addition of caffeine should overcome this accumulation. Treatment with caffeine for 24 hours does reverse the G2 accumulation of *DDB1*-silenced cells, but also induces apoptosis (as measured by cells with < 2n DNA content, Figure 3.1B). Since caffeine has numerous cellular effects, we also directly inhibited two of the checkpoint proteins that initiate the DNA damage response signaling cascade, ATR and ATM. Co-silencing of *DDB1* with *ATR* also suppresses the G2 accumulation that is normally observed following silencing of *DDB1* (Figure 3.1B). Interestingly, co-silencing of *DDB1* with *ATM* cannot prevent the accumulation of cells in G2 (Figure 3.1B). The sensitivity of the 4n accumulation to treatment with caffeine and silencing of *ATR* provides further evidence that the arrest is in G2 and that it is dependent on the activation of cell cycle checkpoints.

To determine which checkpoint pathways are induced after silencing of *DDB1*, we immunoblotted with phosphopeptide-specific antibodies that detect activated checkpoint proteins. Immunoblotting of HeLa extracts revealed that ATM S1981, CHK2 T68, and CHK1 S345 are all phosphorylated after silencing of *DDB1* (Figure 3.2A). A similar observation was made in U2OS cells, where p53 S15 is phosphorylated and p21 is induced specifically after silencing of *DDB1* (Figure 3.2B). To confirm that these results are not limited to transformed cells, the experiment was repeated in an untransformed, telomerase-immortalized epithelial cell line (RPE-hTERT). The results again show p53 S15 phosphorylation and p21 induction specifically after silencing of *DDB1* (Figure 3.2C). These data indicate that silencing of *DDB1* causes activation of both ATM- and ATR-dependent cell cycle checkpoints, since CHK2 T68 is predominantly an ATM phosphorylation site and CHK1 S345 is an ATR phosphorylation site [76, 175, 184, 185].

Even though both ATM and ATR signaling pathways are activated in *DDB1*-silenced cells, siRNA targeting *ATR* but not *ATM* relieved the G2 checkpoint, despite efficient silencing of both proteins (Figure 3.1C). The dependency of the G2 arrest on ATR, but not ATM, may be explained by the role each of these proteins play in the G2/M checkpoint. While both proteins are important for initiation of the G2/M checkpoint response, maintenance of the cell cycle arrest largely relies on the activity of ATR [186].

To confirm that activation of cell cycle checkpoints is due specifically to the silencing of *DDB1*, we created a DDB1 construct using site directed mutagenesis of wobble base pairs that is resistant to RNA inhibition by one of the siRNA oligonucleotides. U2OS cell lines were generated that stably express an empty vector or

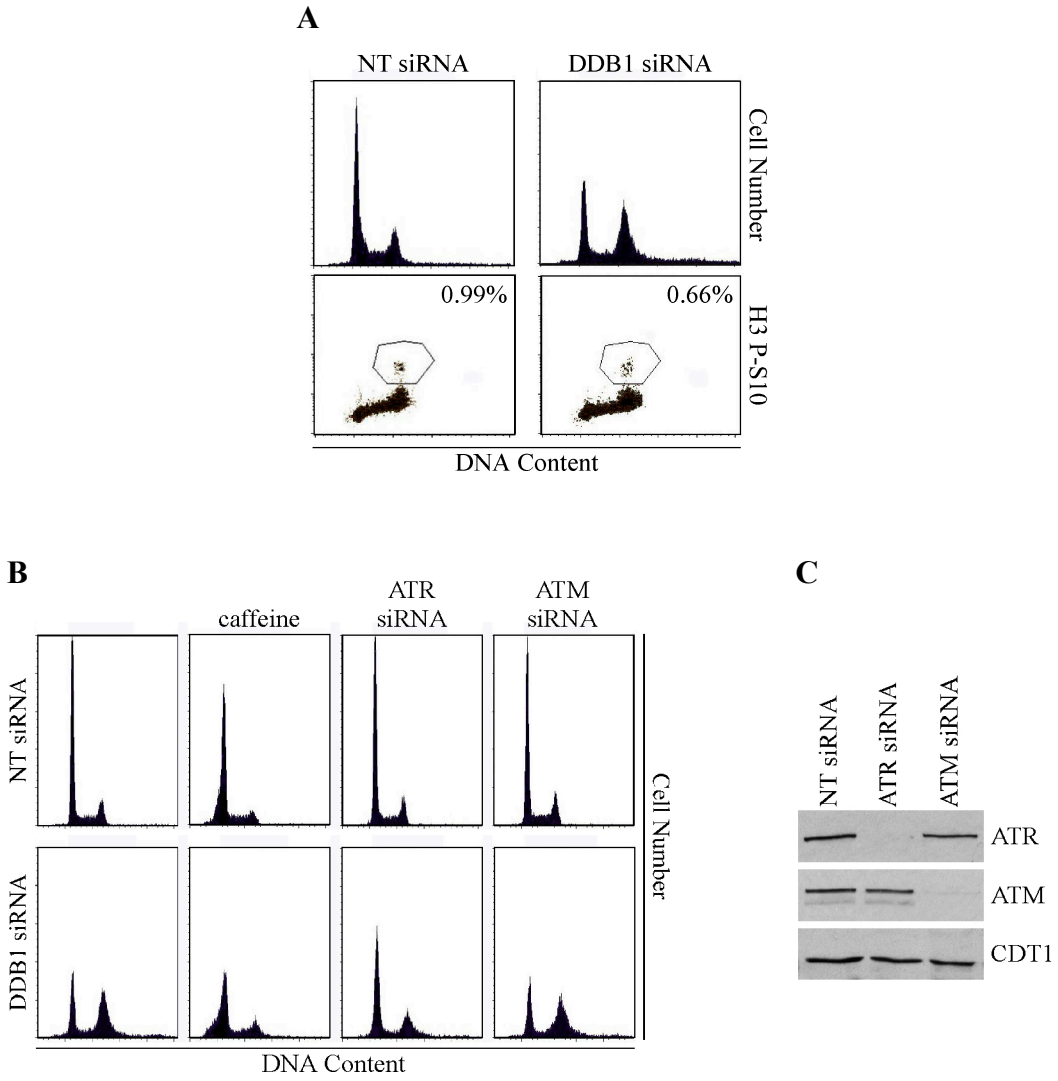


Figure 3.1. Silencing of DDB1 arrests cells in G2 phase of the cell cycle. (A) The cell cycle distribution of HeLa cells transfected with non-targeting (NT) or DDB1 siRNAs was analyzed by flow cytometry detection of DNA content following propidium iodide (PI) staining three days after transfection. The percentage of mitotic cells in each population was determined by immunostaining with a phospho-peptide-specific antibody to histone H3 S10. **(B)** HeLa cells transfected with non-targeting (NT) or DDB1 siRNAs were processed for flow cytometry four days after transfection. Where indicated, the cells were co-transfected with ATM- or ATR-specific siRNA, or treated with 8mM caffeine for 24hrs prior to analysis. **(C)** The efficiency of silencing by the ATR and ATM siRNAs was monitored by immunoblot analysis.

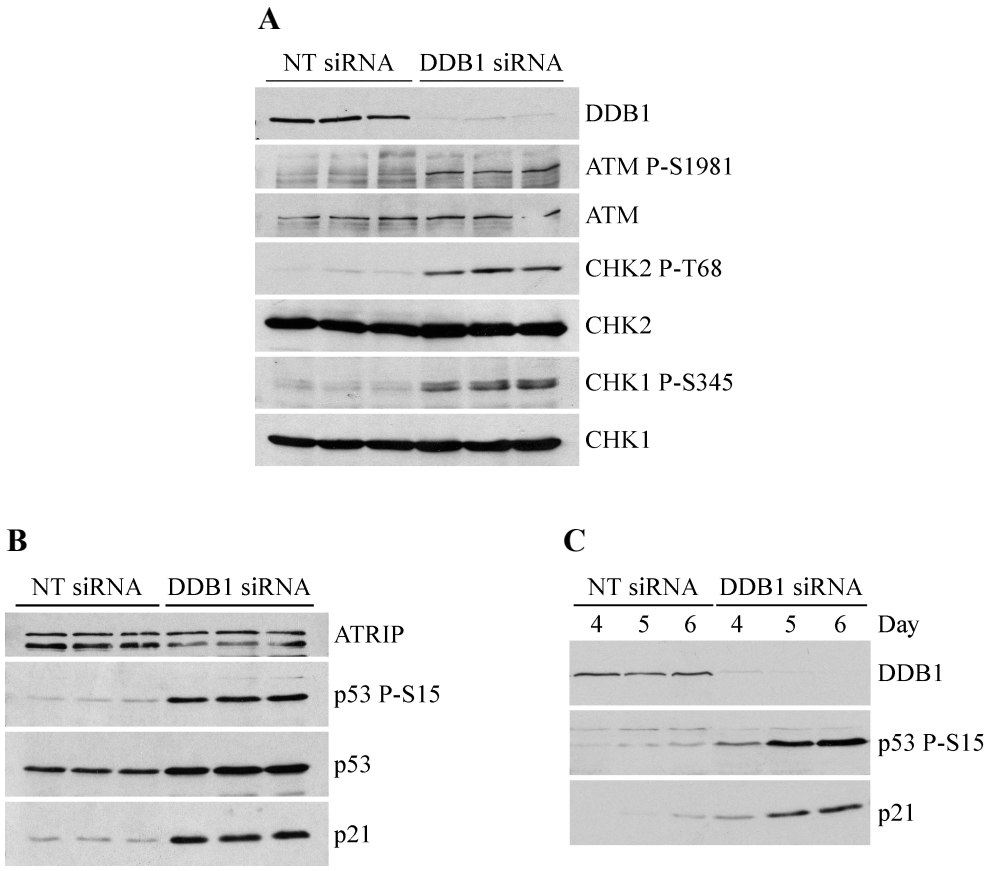


Figure 3.2. Silencing of DDB1 activates ATM- and ATR-mediated DDR pathways. DDR activation was examined in HeLa **(A)**, U2OS **(B)**, and RPE-hTERT **(C)** cells three days after transfection with non-targeting (NT) or DDB1 siRNAs. Cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

a Flag-tagged siRNA-immune DDB1 mRNA (DDB1*). Silencing of *DDB1* from the cell line stably expressing an empty vector results in phosphorylation of CHK2 T68 and p53 S15, as well as p21 induction (Figure 3.3A). However, silencing of *DDB1* from U2OS cells expressing the siRNA-resistant DDB1 mRNA produces significantly less checkpoint protein phosphorylation and p21 induction (Figure 3.3A). The slight amount of residual checkpoint signaling observed in some experiments is likely due to variability in the expression of the DDB1* cDNA in the polyclonal population. Analysis of cell cycle profiles by flow cytometry also revealed that expression of the siRNA-resistant DDB1 mRNA can suppress the G2 accumulation that is observed in *DDB1*-silenced cells (Figure 3.3B). The findings that checkpoint activation and cell cycle arrest can be complemented by expression of a siRNA-resistant DDB1 construct confirm that the observed phenotype is due specifically to silencing of *DDB1*.

Silencing of DDB1 results in γH2AX, MRE11, and RPA foci formation

The phosphorylation of CHK2 T68 and CHK1 S345 indicate that both the ATM and ATR damage response pathways are activated. In principle, activation of these kinases could be due to the elimination of a negative regulatory mechanism or the presence of genotoxic stress. To differentiate between these hypotheses, we examined whether phosphorylation of H2AX by checkpoint kinases is restricted to foci or distributed diffusely throughout the genome. If the silencing of *DDB1* relieves an inhibitory mechanism restraining the activity of ATM or ATR, then these kinases will be generally active without the presence of DNA damage and H2AX phosphorylation is expected to occur throughout the chromatin. A similar phenomenon is observed when

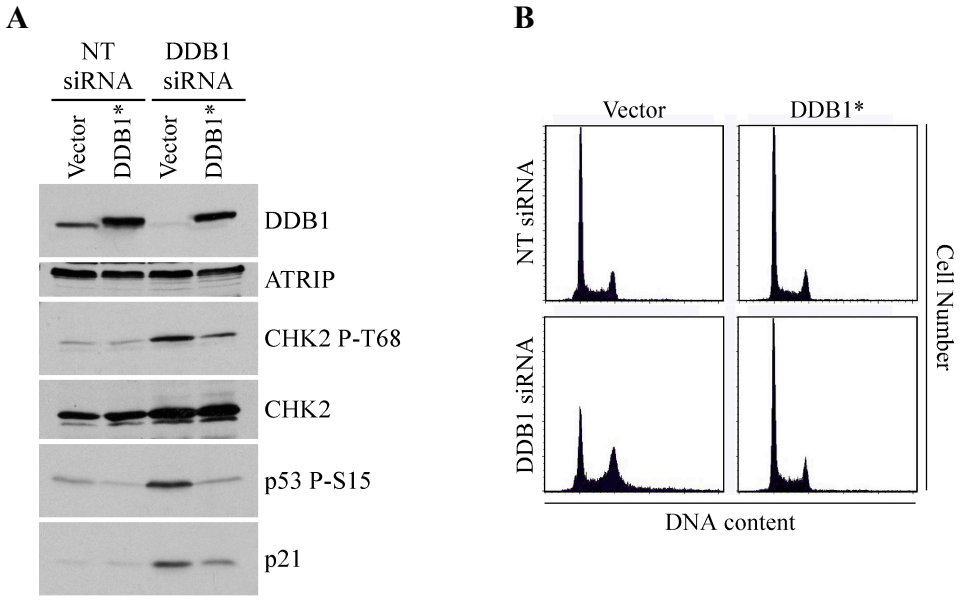


Figure 3.3. DDR activation and cell cycle arrest are complemented by a siRNA-resistant DDB1 construct. U2OS cells were infected with retroviruses encoding the empty vector or a siRNA-immune Flag-DDB1 cDNA (DDB1*). Following selection, the cells were transfected with non-targeting (NT) or DDB1 siRNAs. DDR activation was examined three days after transfection by **(A)** immunoblotting with the indicated antibodies and **(B)** monitoring DNA content by flow cytometry analysis of propidium iodide-stained cells.

the ATR-activating fragment of TopBP1 is overexpressed in cells [60]. Under these circumstances ATR is activated and H2AX is phosphorylated, but the phosphorylation is pan-nuclear rather than occurring in distinct foci because there is no actual DNA damage created by overexpression of the TopBP1 fragment (D.C. unpublished data). In contrast, if silencing of *DDB1* causes the accumulation of DNA damage, then phosphorylated H2AX should be restricted to discrete intranuclear foci [187].

We monitored the phosphorylation of H2AX (γH2AX) in U2OS cells by indirect immunofluorescence using a phosphopeptide-specific antibody to S139. Cells treated with a non-targeting siRNA show little γH2AX foci formation (Figures 3.4A and B). Approximately 5% of the undamaged control cells contain γH2AX foci. Irradiation of control cells with 8Gy IR, to induce the formation of DNA double strand breaks, results in a corresponding increase in cells with γH2AX foci to 75% (Figures 3.4A and B). Silencing of *DDB1* also induces γH2AX foci formation, with 67% of *DDB1*-silenced cells displaying intranuclear foci (Figures 3.4A and B). Irradiation of DDB1-silenced cells further enhances the percentage with γH2AX foci to 84% (Figure 3.4B). The formation of γH2AX foci in the majority of cells following silencing of *DDB1* suggests that the loss of this protein generates DNA damage.

To further support the finding that silencing of *DDB1* results in DNA damage, U2OS cells transfected with non-targeting or *DDB1* siRNA were also examined for MRE11 foci formation. MRE11 is one component of the MRN complex (MRE11-RAD50-NBS1), which localizes to sites of double strand breaks and facilitates repair of the damaged DNA [188]. Immunostaining with γH2AX and MRE11 reveals that the γH2AX foci co-localize with MRE11 foci in the irradiated cells, as well as in the *DDB1*-

silenced cells (Figure 3.4A). This suggests that the γH2AX foci do indeed represent sites of DNA damage, as proteins necessary for the repair of double strand breaks are also recruited to these sites within the cell.

The extent of DNA damage was characterized by examining the number of γH2AX foci generated per cell in control, irradiated, and *DDB1*-silenced cells. While 80% of cells transfected with a non-targeting siRNA contained no γH2AX foci, 90% of *DDB1*-silenced cells displayed γH2AX foci ranging in numbers from 3-86, with an average of 22 γH2AX foci per cell (Figure 3.4C). In comparison, control cells display an average of one foci per cell, while those irradiated with the lethal dose of 8Gy IR display an average of 66 γH2AX foci per cell (Figure 3.4C).

In addition to monitoring γH2AX and MRE11 foci formation as markers of DNA damage, we also examined RPA foci formation. Replication protein A (RPA) is a three-subunit protein complex that binds single-stranded DNA in eukaryotic cells, and like γH2AX and MRE11, forms intranuclear foci after DNA damage [189]. Immunostaining for the RPA34 subunit of this heterotrimeric protein complex indicates that similar to irradiated cells, *DDB1*-silenced cells display numerous RPA34 foci (Figure 3.5). These results provide additional evidence that depletion of DDB1 results in DNA damage.

DDB1 maintains genome integrity as part of a CUL4A ubiquitin ligase complex

All currently known roles of DDB1 involve it functioning as part of a multi-protein complex containing CUL4A that targets substrates for ubiquitination [151, 155, 160, 163, 190-193]. To determine if defective ubiquitination contributes to the

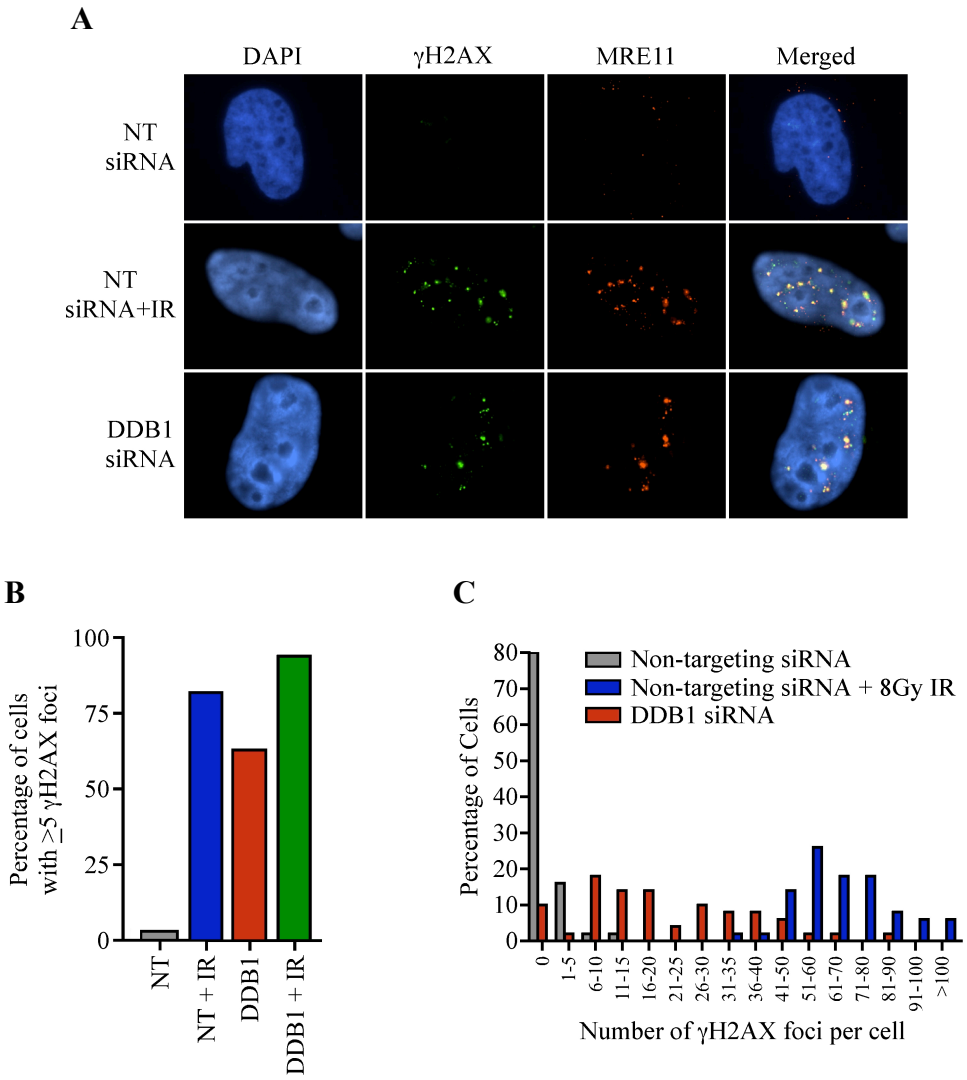


Figure 3.4. Silencing of DDB1 causes γH2AX foci formation. U2OS cells were transfected with non-targeting (NT) or DDB1 siRNAs. Cells transfected with NT siRNA were also irradiated with 8Gy IR two hours prior to analysis where indicated. **(A)** Three days after siRNA transfection the cells were fixed and stained with antibodies to H2AX phospho-S139 (γH2AX) and MRE11, followed by the appropriate FITC and Rhodamine Red-X secondary antibodies. The nucleus was visualized with DAPI. **(B)** The percentage of U2OS cells displaying greater than or equal to 5 intranuclear γH2AX foci three days after transfection with NT or DDB1 siRNAs was quantified. **(C)** The number of γH2AX foci per cell was quantitated after transfection with NT and DDB1 siRNAs.

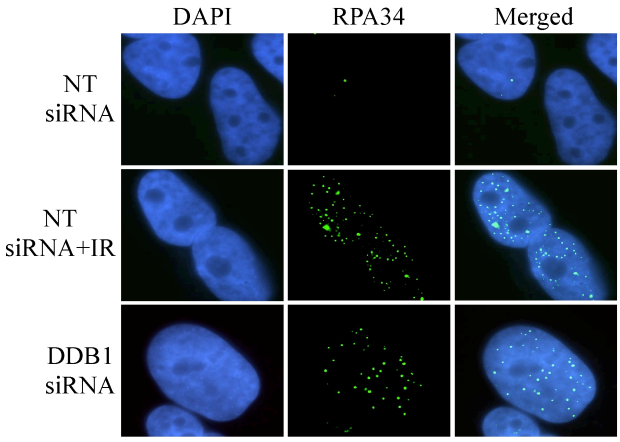


Figure 3.5. DDB1-silenced cells display RPA foci. U2OS cells were transfected with non-targeting (NT) or DDB1 siRNAs, and NT cells were also irradiated with 8Gy IR two hours prior to analysis. Three days after siRNA transfection the cells were fixed and stained with antibodies to RPA34. DAPI staining was used to visualize the nucleus.

phenotype observed after silencing of *DDB1*, we used siRNA to silence *CUL4A*. Silencing of *CUL4A* from HeLa cells results in the phosphorylation of CHK2 T68, analogous to what is observed following silencing of *DDB1* (Figure 3.6A). Similar phenotypes are also observed in the untransformed RPE-hTERT cells. Checkpoint activation is absent from undamaged RPE-hTERT cells treated with a non-targeting siRNA and is induced following irradiation, as indicated by p53 S15 phosphorylation (Figure 3.6B). Silencing of *DDB1* and *CUL4A* results in p53 S15 phosphorylation and p21 induction in the absence of exogenous DNA damage, and further increases in p53 phosphorylation are observed after ionizing radiation (Figure 3.6B). Additionally, silencing of *CUL4A* produces a modest increase in cells with 4n DNA content (Figure 3.6C). This phenotype is reminiscent of that observed following DDB1 depletion and correlates with the phosphorylation of checkpoint proteins; however, the cell cycle arrest following silencing of *CUL4A* is less striking than the arrest typically observed following silencing of *DDB1*. This may be due to differences in the silencing efficiency of the siRNA oligonucleotides, or may indicate that DDB1 is limiting in the DDB1-CUL4A ubiquitin ligase complex. The activation of checkpoint pathways following depletion of CUL4A is consistent with the hypothesis that the DNA damage observed following silencing of DDB1 results from defective ubiquitination of a DDB1-CUL4A substrate(s).

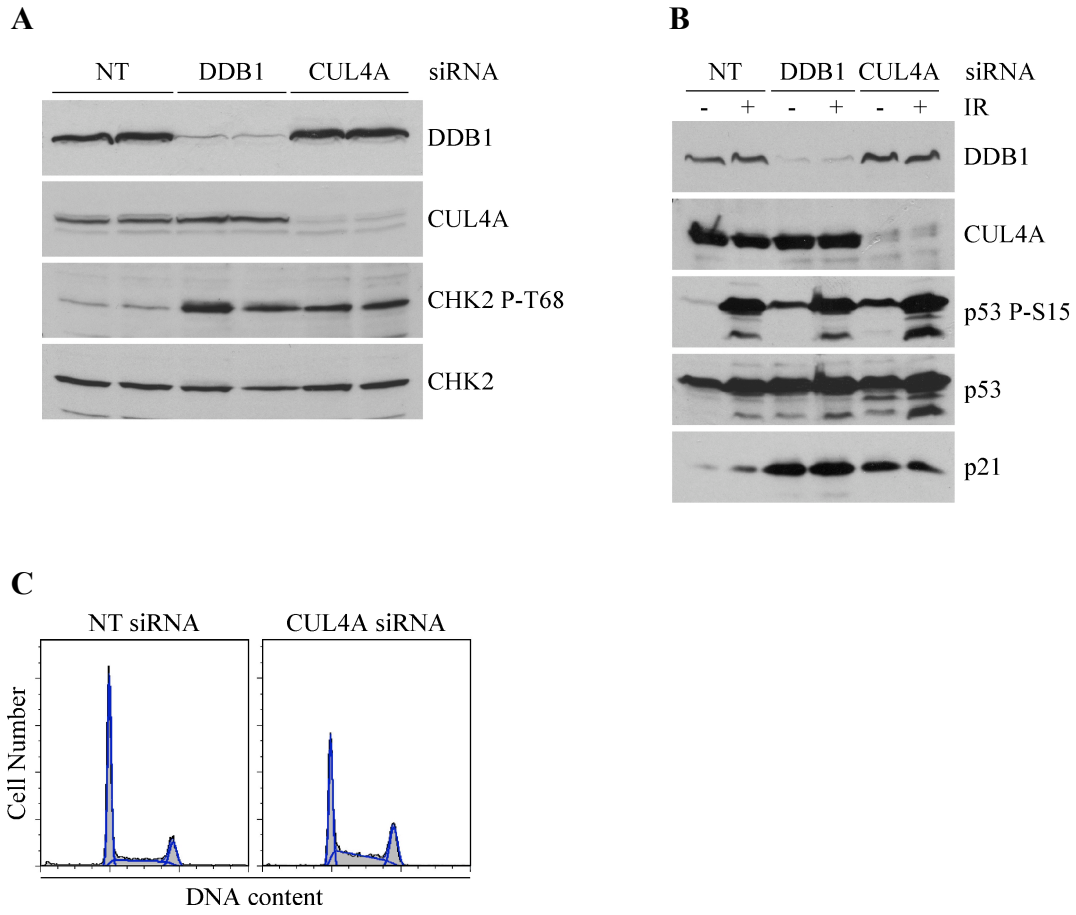


Figure 3.6. Silencing of CUL4A causes DDR activation and G2 accumulation. (A) HeLa cells were transfected with non-targeting (NT), DDB1, or CUL4A siRNAs. Three days after transfection cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. **(B)** RPE-hTERT cells were transfected with NT, DDB1, or CUL4A siRNAs. Three days after transfection the indicated samples were irradiated with 8Gy IR and incubated two hours prior to analysis. DDR activation was monitored by immunoblotting with the indicated antibodies. **(C)** Cells transfected with either NT or CUL4A siRNA were analyzed for DNA content by flow cytometry.

Defective NER is not responsible for the DNA damage observed after silencing of DDB1

One way in which DDB1 may exert its genome protective function is through its role in nucleotide excision repair (NER) [144, 145, 151, 155]. Since the chromatin binding activity of the DDB1-DDB2-CUL4A ubiquitin ligase complex is important for the global genomic repair (GGR) subpathway of NER, we determined whether a defect in this repair process was the cause of the phenotype observed following silencing of *DDB1* [149, 150]. To examine this possibility, we disrupted GGR by silencing *DDB2*. Silencing of *DDB2* using siRNA oligonucleotides does not resemble the phenotype observed following silencing of *DDB1* (Figure 3.7A). ATM S1981 and CHK2 T68 phosphorylation, as well as increased p21 levels, are observed after silencing of *DDB1* but are minimal after silencing of *DDB2*. Also, silencing of *DDB2* does not cause a G2 accumulation (Figure 3.8).

We further examined the potential role that impaired GGR activity may have in contributing to the damage phenotype by silencing *XPC*, a damage sensor protein required for the GGR subpathway of NER [194-196]. Loss of XPC function is more detrimental to GGR than the loss of DDB1-DDB2-CUL4A ubiquitin ligase activity [197]. However, despite an effective reduction in protein levels (Figure 3.7D), silencing of *XPC* again does not produce the phenotypic characteristics of *DDB1*-silenced cells. ATM S1981 and CHK2 T68 phosphorylation are not observed following silencing of *XPC* from cells, nor is there a significant increase in p21 levels (Figure 3.7B). Silencing of *XPC* also does not result in a G2 accumulation of cells (Figure 3.8). These results suggest that defects in GGR are unlikely to be the cause of the DNA damage observed in *DDB1*-silenced cells.

In addition to functioning in the GGR subpathway of NER, DDB1 also has a role in transcription-coupled repair (TCR) [150]. The DDB1-CUL4A ubiquitin ligase complex functions independently of DDB2 in this capacity. To determine whether defective TCR contributes to the phenotype observed after DDB1 depletion, we silenced *XPA* using siRNA oligonucleotides. Like DDB1, XPA is required for both GGR and TCR, with the loss of this protein reducing the repair capacity of cells to approximately 10% [195-197]. Silencing of XPA from cells does not produce a phenotype reminiscent of *DDB1*-deficient cells. There is no phosphorylation of checkpoint proteins, no increase in p21 levels, and no aberration in the cell cycle distribution of these cells (Figures 3.7C and 3.8). Immunoblots for XPA demonstrate that the siRNA oligonucleotides effectively reduce XPA protein levels in these experiments (Figure 3.7D). This data indicates that the phenotype observed after silencing of *DDB1* is not due to defective GGR or TCR.

Misregulation of CDT1 contributes to the DNA damage observed following silencing of DDB1

Aside from its role in NER, another function ascribed to DDB1 is the regulation of CDT1 by DDB1-CUL4A. The DDB1-CUL4A ubiquitin ligase complex targets this replication licensing factor for degradation following UV and IR damage [160, 161]. It also regulates CDT1 degradation during normal cell cycle progression in combination with a SCF ubiquitin ligase complex [162-165]. Regulation of CDT1 is critical, as overexpression of this protein leads to re-replication of genomic DNA. During the course of characterizing the phenotype resulting from silencing of *DDB1*, it was apparent in the DNA content profiles generated by flow cytometry that silencing of *DDB1* increases the percentage of cells with more than 4n DNA content (Figure 3.9A). While only 4.5% of

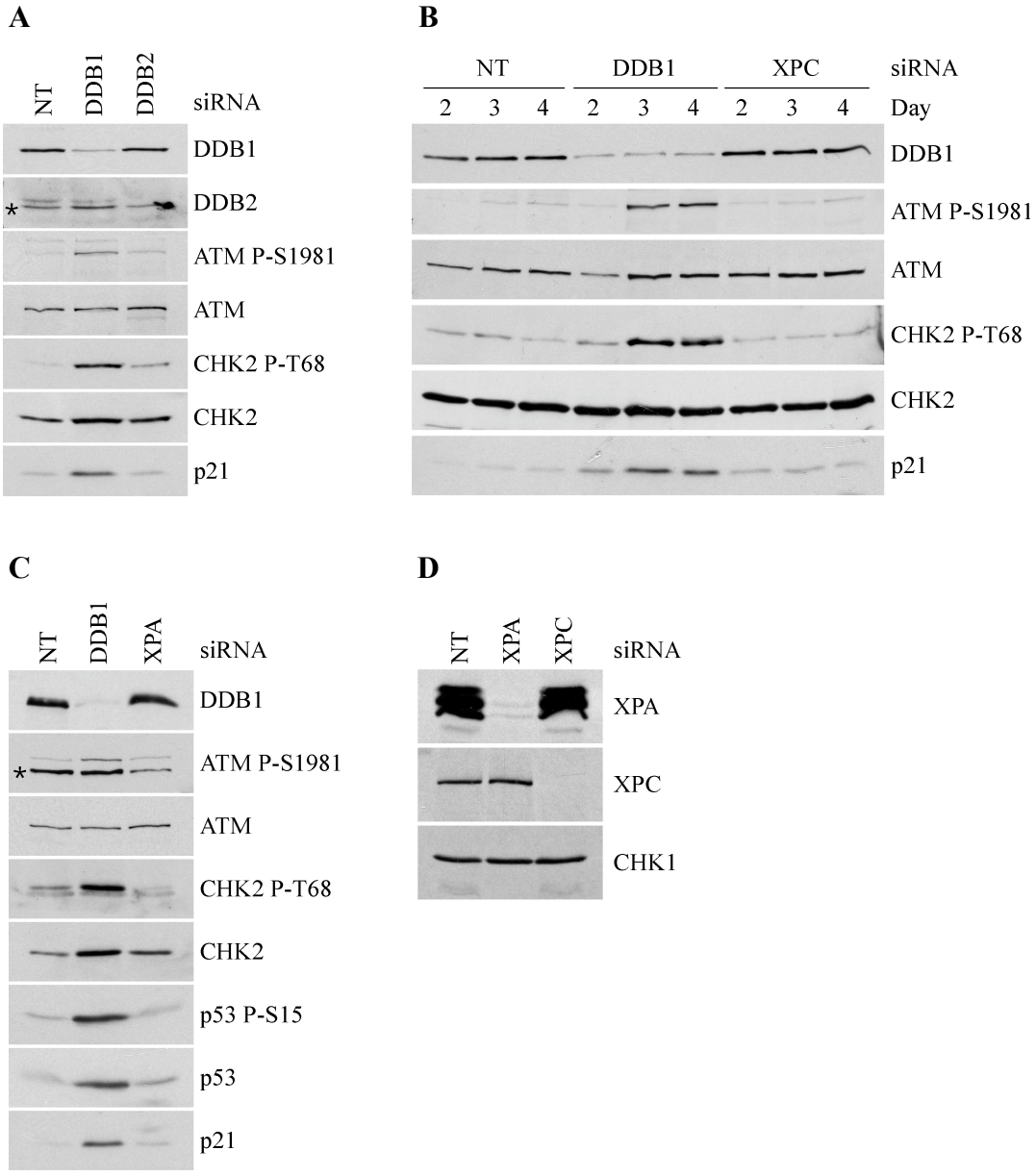


Figure 3.7. Defective NER does not cause DDR activation. (A) U2OS Cells were transfected with non-targeting (NT), DDB1, or DDB2 siRNA oligonucleotides. Three days after transfection cell lysates were harvested, separated by SDS-PAGE, and immunoblotted with the indicated antibodies. **(B)** DDR activation was monitored on days two, three, and four after transfection with NT, DDB1, and XPC siRNAs by immunoblotting with the indicated antibodies. **(C)** Cells were transfected with NT, DDB1, or XPA siRNAs. Three days after transfection, DDR activation was examined by immunoblotting with the indicated antibodies. **(D)** The efficiency of silencing by the XPA and XPC siRNA oligonucleotides was monitored by immunoblot analysis. The asterisks (*) denote nonspecific, cross-reacting proteins.

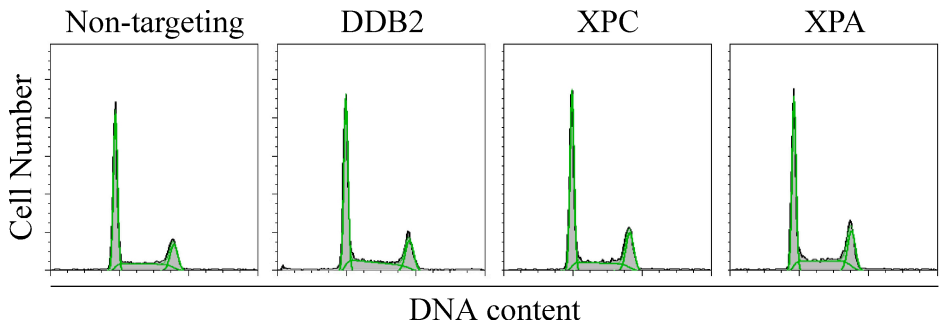


Figure 3.8. Defective NER does not cause a G2 accumulation of cells. U2OS cells transfected with non-targeting, DDB2, XPC, or XPA siRNAs were analyzed for DNA content by flow cytometry analysis of propidium iodide-stained cells three days after transfection.

cells transfected with a non-targeting siRNA reside in this population, 18.1% of *DDB1*-silenced cells display a DNA content greater than 4n. The existing link between DDB1-CUL4A and CDT1 degradation suggests that the increase in the percentage of cells with more than 4n DNA content after silencing of *DDB1* may result from re-replication due to the misregulation of CDT1.

To more definitively determine whether silencing of *DDB1* causes re-replication, we monitored incorporation of the thymidine analogue bromodeoxyuridine (BrdU) by flow cytometry. Silencing of *DDB1* from U2OS cells again enhanced the percentage of cells with greater than 4n DNA content (Figure 3.9B). Furthermore, nearly 36% of *DDB1*-silenced cells with greater than 4n DNA content are actively undergoing DNA synthesis and incorporating BrdU (6% of the total *DDB1*-silenced population). DNA synthesis in cells with greater than 4n DNA content suggests that this population does arise from re-replication. The presence of active cell cycle checkpoints has likely limited the extent of BrdU incorporation in the *DDB1*-silenced cells displaying greater than 4n DNA content, as well as in some S phase cells, as evidenced by the diminished BrdU incorporation in cells possessing a DNA content between 2n and 4n. The cells with greater than 4n DNA content after silencing of *DDB1* are unlikely to result from defects in cytokinesis since we found no enhancement in the number of multinucleated cells in the *DDB1*-silenced population (Figure 3.9C).

The presence of re-replication in *DDB1*-silenced cells suggests that regulation of the replication licensing factor CDT1, a known substrate of the DDB1-CUL4A ubiquitin ligase complex, may be disrupted. Consistent with this interpretation, we observed an

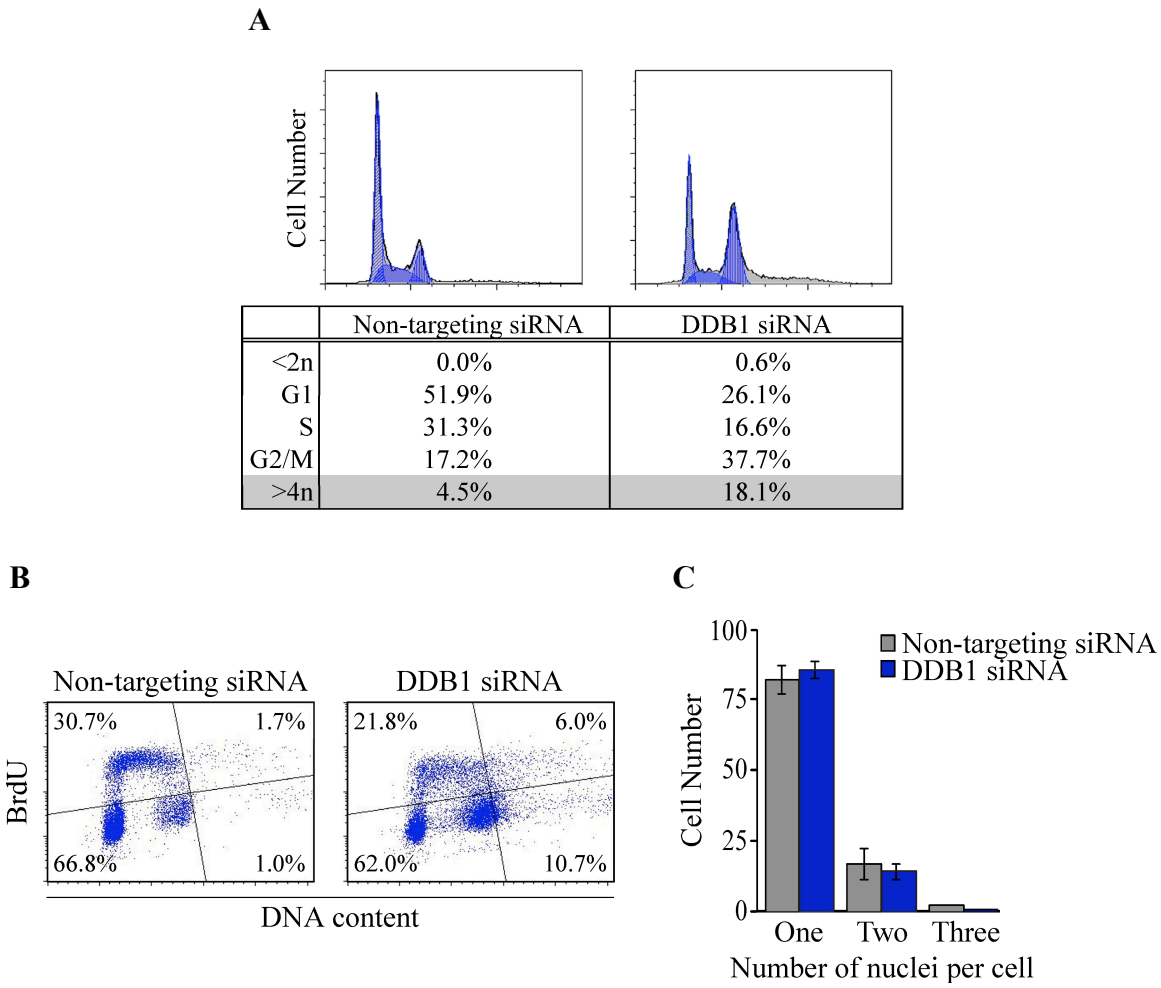


Figure 3.9. Silencing of DDB1 causes re-replication. (A) The DNA content of HeLa cells was analyzed by flow cytometry following propidium iodide staining four days after transfection with non-targeting (NT) or DDB1 siRNAs. Cell cycle distributions were quantified using FlowJo FACS analysis software. **(B)** U2OS cells were transfected with NT or DDB1 siRNAs. Four days after transfection the cells were labeled with bromodeoxyuridine (BrdU). After fixation, the cells were immunostained with an anti-BrdU antibody and DNA content was monitored by flow cytometry using propidium iodide staining. **(C)** Cells were transfected with NT or DDB1 siRNA. Three days after transfection the cells were fixed, permeabilized and stained with rhodamine-phalloidin. The number of nuclei per cell were counted. Error bars represent the standard error from three samples.

increase in the abundance of CDT1 protein after silencing of *DDB1* (Figure 3.10A). Furthermore, the CDT1 protein is more stable in the *DDB1*-silenced cells compared to the non-targeting control (approximate half-life is 120 minutes and 65 minutes, respectively; Figure 3.10B). Thus, CDT1 turnover is misregulated in *DDB1*-silenced cells.

The increased stability of CDT1 and the re-replication observed following silencing of *DDB1* suggest that misregulation of CDT1 may be contributing to the phenotype observed in *DDB1*-silenced cells. To examine this possibility we used siRNA to co-silence *CDT1* and *DDB1* from U2OS cells. If deregulation of CDT1 is promoting re-replication in *DDB1*-silenced cells, then silencing of *CDT1* should reduce the percentage of cells with greater than 4n DNA content. The amount of *CDT1* silencing that was achieved in combination with a non-targeting siRNA did not alter the cell cycle profile, the percentage of replicating cells, or the percentage of cells with more than 4n DNA content (Figures 3.11A and B). In contrast, co-silencing of *CDT1* with *DDB1* reduces the percentage of cells with more than 4n DNA content from 14% to 5%; a level similar to that observed in control cells (Figures 3.11A and B). This confirms that the population of cells with greater than 4n DNA content after silencing of *DDB1* does represent cells in which re-replication has occurred due to CDT1 misregulation.

Since co-silencing of *CDT1* with *DDB1* can reduce the percentage of re-replicating cells, we next asked whether the co-silencing of *CDT1* with *DDB1* can alleviate the DNA damage and checkpoint activation caused by silencing of *DDB1*. As observed previously, silencing of *DDB1* significantly enhances the percentage of cells displaying γH2AX foci. The co-silencing of *DDB1* with *CDT1* considerably reduces the

amount of DNA damage generated by the loss of DDB1, as the percentage of cells with γH2AX foci was reduced by nearly 50% (Figure 3.11C). A similar alleviation in the DNA damage phenotype was observed by immunoblot analysis. As noted previously, silencing of *DDB1* results in phosphorylation of p53 S15 and CHK2 T68 (Figure 3.10A). These events are not observed in control or *CDT1*-silenced cells. When *DDB1* is silenced in combination with *CDT1*, the levels of phosphorylated p53 S15 and CHK2 T68 are significantly reduced (Figure 3.10A). Collectively, these results indicate that misregulation of CDT1, and the subsequent re-replication, contribute to the formation of DNA damage and the activation of checkpoint responses after silencing of *DDB1*. It should also be noted that reducing the level of CDT1 below that of control cells only partially alleviates the DNA damage associated with silencing of *DDB1*. Thus, misregulation of CDT1 may be only one of the factors that cause DNA damage in *DDB1*-silenced cells.

Disruption of SCF^{SKP2}-mediated CDT1 regulation does not cause DNA damage

Multiple mechanisms of CDT1 regulation exist, and our results suggest that disruption of a single mechanism is sufficient to cause deregulation of CDT1, re-replication, and DNA damage. To more precisely examine the ubiquitin-dependent mechanisms of CDT1 regulation and their potential to generate DNA damage when disrupted, we transiently expressed wild type CDT1 or CDT1 mutants where one or both of the ubiquitin-mediated degradation pathways were abrogated. Disruption of the DDB1-CUL4A-dependent ubiquitination was accomplished by mutating residues of CDT1 that are critical for its interaction with the DNA polymerase processivity factor,

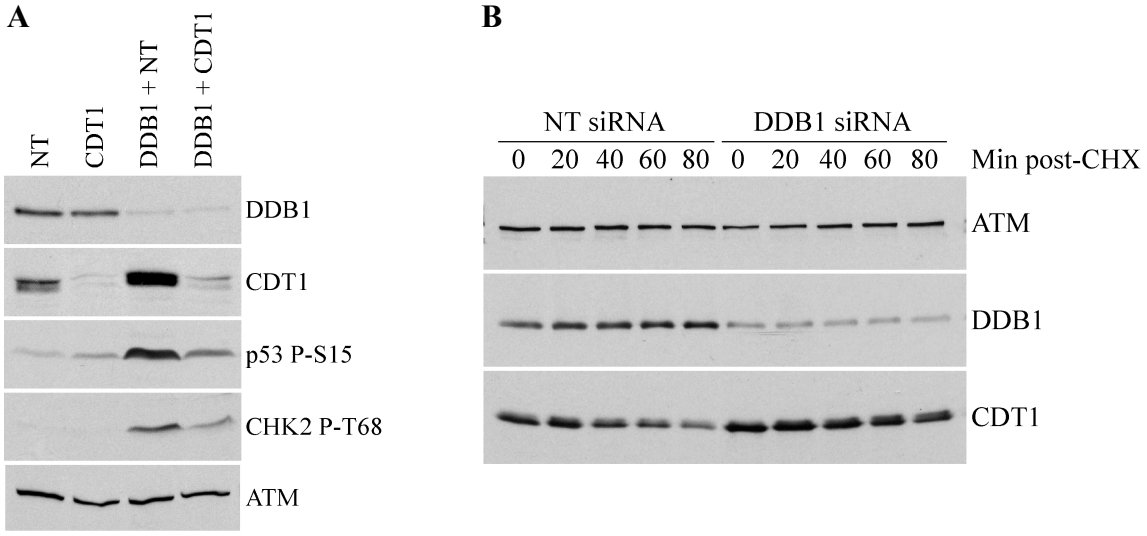


Figure 3.10. CDT1 protein turnover is delayed in DDB1-silenced cells. (A) U2OS cells were transfected with non-targeting (NT), CDT1, and DDB1 siRNAs alone or in combination, as indicated. CDT1 abundance and DDR activation was examined four days after transfection by immunoblotting with the indicated antibodies. (B) Cells stably expressing a HA-tagged CDT1 protein were transfected with NT or DDB1 siRNAs. Two days after transfection cells were treated with 100μM cycloheximide (CHX) and harvested at the times indicated. Silencing of *DDB1* and the stability of CDT1 were monitored by immunoblot analyses.

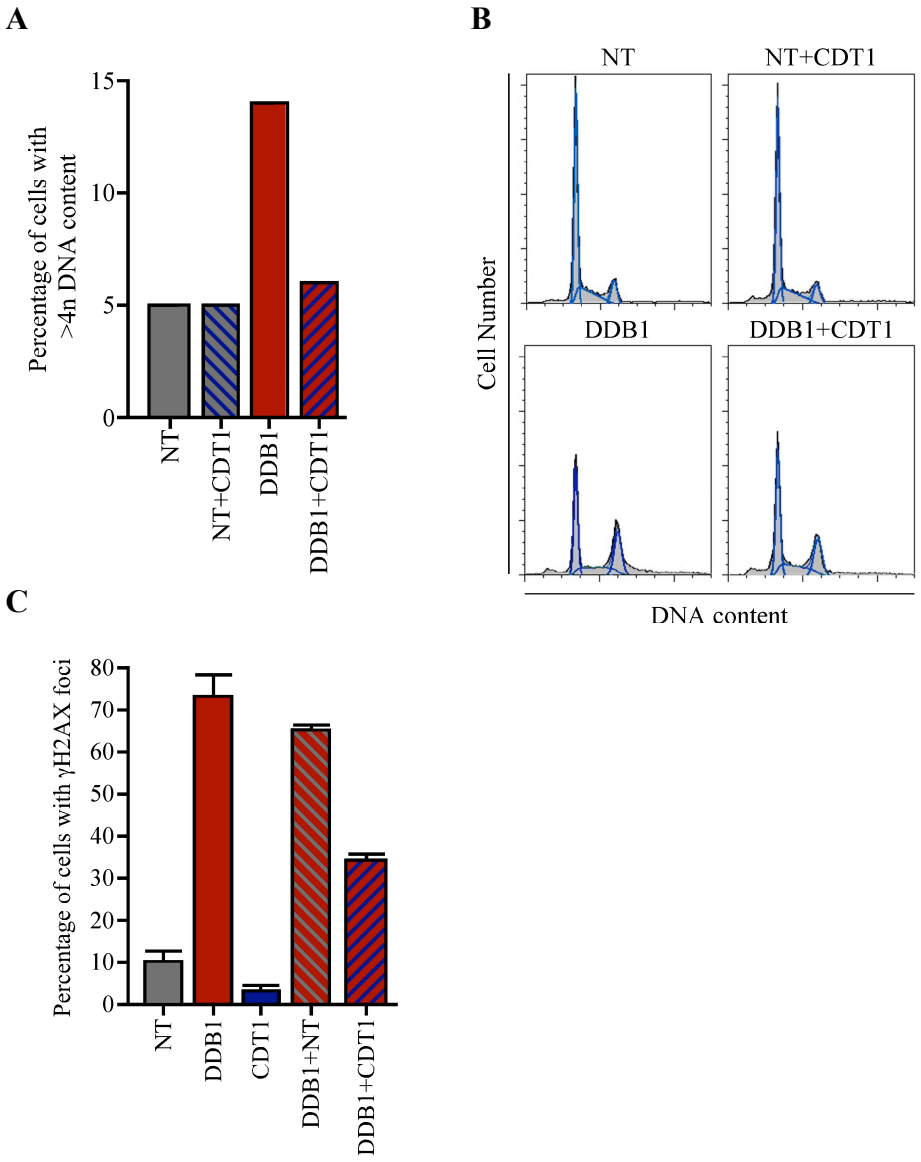


Figure 3.11. Deregulation of CDT1 accounts for re-replication and contributes to DNA damage in DDB1-silenced cells. U2OS cells were transfected with non-targeting (NT), CDT1, and DDB1 siRNAs alone or in combination, as indicated. The extent of re-replication **(A)** and G2 accumulation **(B)** was monitored by flow cytometric analysis of DNA content. Quantitiation was performed using the FlowJo software analysis program. **(C)** Intranuclear γH2AX foci formation was examined by indirect immunofluorescence. The graph depicts the percentage of cells in each population displaying γH2AX foci.

PCNA (Q3A, V6A, F9A, F10A=ΔPIP). Binding of CDT1 to PCNA is required for degradation of CDT1 by DDB1-CUL4A [162-165]. We also disrupted the SCF^{SKP2} ubiquitination pathway for CDT1 by removing a cyclin-dependent kinase (CDK) phosphorylation site in CDT1 at threonine 29 (T29A). The binding and degradation of CDT1 by SCF^{SKP2} requires the prior phosphorylation of CDT1 on T29 by CDKs [170, 171, 198, 199].

Introduction of an empty vector into HeLa cells does not cause DNA damage; however, overexpression of a wild type CDT1 protein induces DNA damage signaling (Figure 3.12A), as previously reported [200-203]. A similar level of p53 S15 phosphorylation is observed upon disruption of the SCF^{SKP2} degradation pathway (T29A), indicating that the loss of this regulatory mechanism does not cause any further damage than overexpressing wild-type CDT1. Notably, disruption of DDB1-CUL4A-dependent CDT1-degradation (ΔPIP) resulted in greater p53 S15 phosphorylation than overexpression of either wild type CDT1 or the T29A mutant. This suggests that the two ubiquitin-dependent mechanisms of CDT1 degradation are not redundant, but that disruption specifically of the DDB1-CUL4A-mediated CDT1 degradation pathway has a greater potential to generate DNA damage.

To further examine the ability of these ubiquitin ligase complexes to maintain genome stability, we assessed checkpoint activation following silencing of *DDB1* or *SKP2* by siRNA. While silencing of *DDB1* results in the phosphorylation of CHK1 S345, CHK2 T68, and p53 S15, these markers of DNA damage signaling are not observed after silencing of *SKP2* (Figure 3.12B). Interestingly, silencing of *DDB1* elevates CDT1 levels to a greater extent than that observed following silencing of *SKP2*,

suggesting that disruption of the DDB1-CUL4A ubiquitin ligase complex is more detrimental to the proper regulation of CDT1 protein levels than loss of the SCF^{SKP2} ubiquitin ligase complex. Together, these results support our conclusion that misregulation of CDT1 generates DNA damage, and suggests that disruption of DDB1-CUL4A-mediated CDT1 degradation is more detrimental to genome stability than disruption of the SCF^{SKP2}-mediated degradation pathway.

Accumulation of DNA damage following silencing of DDB1 requires cell cycle progression

To further test the model that re-replication resulting from misregulation of CDT1 is contributing to the phenotype observed after silencing of *DDB1*, we examined the cell cycle dependency of the phenotype. If our model is correct, then the accumulation of DNA damage should require progression into S phase. RPE-hTERT cells were transfected with non-targeting or *DDB1* siRNA and grown to confluency as a means of causing contact inhibition and a G_0 arrest. Cell cycle analysis by propidium iodide staining shows a significant accumulation of cells with a 2n DNA content following the arrest by contact inhibition (Figure 3.13A). Approximately 93% of control cells and 88% of *DDB1*-silenced cells arrested by this method display a DNA content of 2n. G_0-arrested cells were also released into the cell cycle and harvested at a point in time when cells were advancing into S phase. This population allows us to examine the extent of DNA damage that occurs during progression through a single S phase. It should be noted, however, that *DDB1*-silenced cells take more time to release from a G_0 arrest and do not release as synchronously as control cells (Figure 3.13A). Finally, control and *DDB1*-silenced cells were allowed to cycle unperturbed through the course of the

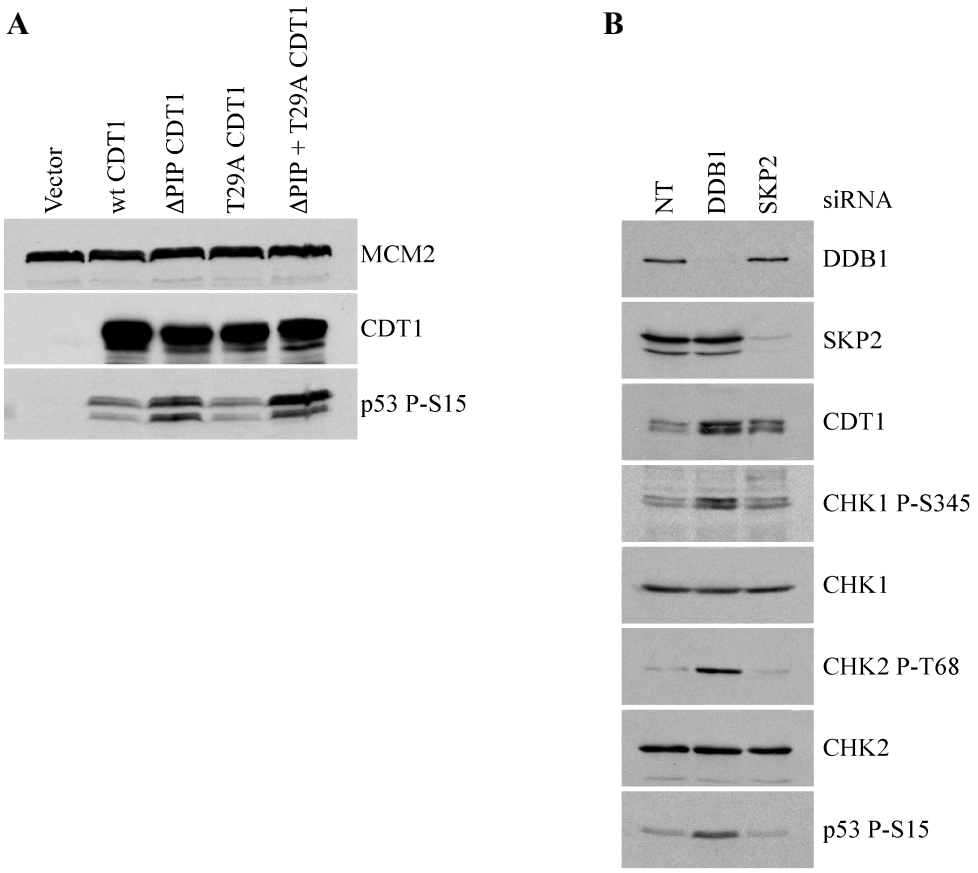


Figure 3.12. Disruption of DDB1-CUL4A-mediated CDT1 degradation has greater potential to generate DNA damage. (A) HeLa cells were transfected with an empty vector or vectors encoding wild type CDT1, CDT1 Q3A,V6A,F9A, F10A (ΔPIP), or CdDT1 T29A. DDR activation was examined in these cells two days after transfection by immunoblotting. (B) HeLa cells were transfected with NT, DDB1, or SKP2 siRNAs. Three days after transfection cell lysates were harvested, separated by SDS-PAGE, and DDR activation was monitored by immunoblotting with the indicated antibodies.

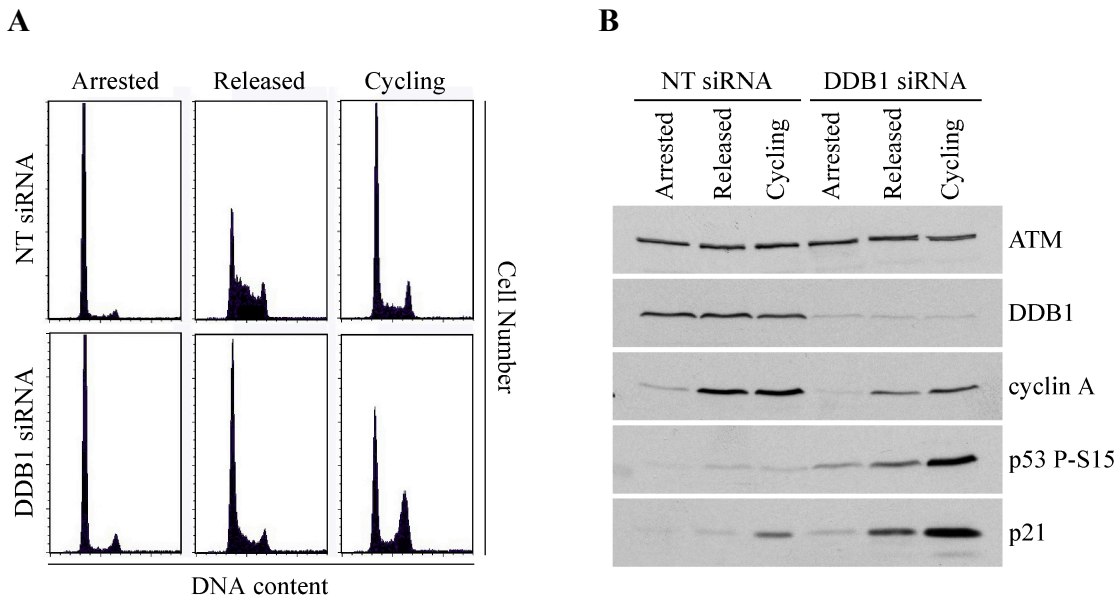


Figure 3.13. Cell cycle progression is required for accumulation of DNA damage in DDB1-silenced cells. RPE-hTERT cells were transfected with non-targeting (NT) or DDB1 siRNAs. Cells were arrested by growth to confluency, arrested and released back into the cell cycle by splitting into sub-confluent densities, or maintained at sub-confluent densities during the course of the experiment. All samples were harvested on the fourth day after siRNA transfection, and **(A)** DNA content was measured by flow cytometry analysis of propidium iodide-stained cells; or **(B)** cell lysates were prepared, resolved by SDS-PAGE, and DDR activation was monitored by immunoblotting.

experiment. The extent of DNA damage signaling in these samples was then examined by immunoblotting. *DDB1*-silenced cells that are arrested by contact inhibition show very little p53 S15 phosphorylation or p21 induction (Figure 3.13B). The entrance of *DDB1*-silenced cells into S phase corresponds with an increase in the levels of p53 S15 phosphorylation and p21 induction, whereas the analogous control cells showed no increase in these markers of DNA damage. While it is unclear why *DDB1*-silenced cells release slowly from G_0, these data indicate that the damage observed after silencing of *DDB1* can occur during a single cell cycle, and that the damage requires progression into S phase. *DDB1*-silenced cells that are allowed to cycle during the course of the experiment show further increases in p53 S15 phosphorylation and p21 induction, suggesting that the damage is accumulating and further activating checkpoint responses over time. Cyclin A immunoblotting of these samples was used as a marker of S and G2 phases of the cell cycle, and corresponds with the flow cytometry data (Figure 3.13B).

Analysis of the location of chromosomal breaks in DDB1-silenced cells

Our data indicate that lack of DDB1 function causes DNA damage, partially though deregulation of CDT1 that allows re-replication to occur. Since the DNA damage is associated with replication, we hypothesized that the double strand breaks may preferentially occur at chromosomal fragile sites. These chromosome regions are particularly vulnerable to breakage, especially when cells experience replication stress such as that caused by low doses of aphidicolin [30, 138]. Therefore, we examined whether the DNA double strand breaks that form following silencing of *DDB1* occur preferentially at fragile sites. Metaphase spreads from U2OS cells transfected with non-

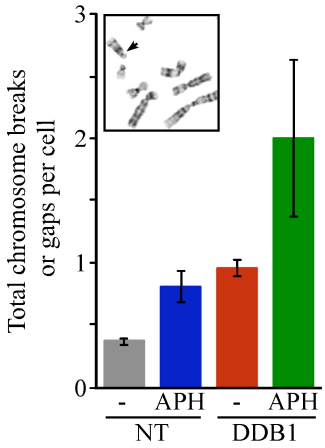
targeting or *DDB1* siRNAs were analyzed for chromosomal gaps and breaks by trypsin-Giemsa banding. Silencing of *DDB1* from U2OS cells enhances the occurrence of chromosomal gaps and breaks in mitotic cells more than two-fold over the number of events observed in control cells (Figure 3.14A). This increase is similar to what we observed upon treating cells with aphidicolin. Interestingly, treatment of *DDB1*-silenced cells with aphidicolin merely caused an additive effect.

Further characterization of the location of these chromosomal breaks reveals that while the addition of aphidicolin enhances breakage events at common fragile sites, such as FRA3B, silencing of *DDB1* alone causes breakage events to occur at a wide variety of loci throughout the genome, with no enhancement in the number of breaks at any known fragile site (Figure 3.14B). For example, over 10% of breaks in aphidicolin-treated cells occur at a single fragile site (FRA3B), but only 0.7% of breaks caused by silencing of *DDB1* were at this site. These results were replicated in the untransformed RPE-hTERT cells, where silencing of *DDB1* again enhanced the number of chromosome breakage events observed relative to control cells (data not shown). This data indicates that the cause of the double strand breaks in DDB1-silenced cells is distinct from the replication problems created by aphidicolin, and suggests that re-replication does not cause DNA damage due to the stalling of replication forks at fragile sites.

Discussion

Our data indicate that DDB1 is required for maintaining genome stability in human cells. Silencing of *DDB1* expression results in an accumulation of DNA damage

A



B

	NT	NT+ APH	DDB1	DDB1+ APH
Fra3B (% of breaks)	2 (3.6)	13 (10.8)	1 (0.7)	12 (4.0)
Fra2G	0	5	1	3
Fra16D	0	2	0	2
Total Breaks per 150 cells	56	120	142	299

Figure 3.14. Silencing of DDB1 enhances chromosome breaks. (A) U2OS cells were transfected with non-targeting (NT) or DDB1 siRNAs and processed for metaphase spread analysis three days after transfection. Fragile sites were induced by the addition of 0.1μM aphidicolin (APH) 24 hours prior to harvest. The identification of chromosome gaps and breaks was facilitated by trypsin-Giemsa banding. The graph depicts the average number of chromosome gaps or breaks per cell. **(B)** The chromosome location of the gaps and breaks was recorded based on banding patterns. FRA3B, FRA2G, and FRA16D breakages were scored as described [138]. The number and location of chromosome gaps and breaks in *DDB1*-silenced cells was determined by Kimberly Lock of Genetics Associates, Inc.

and activation of cell cycle checkpoints. DDB1 protects the integrity of the genome as part of an E3 ubiquitin ligase complex containing CUL4A. One target of this complex that must be degraded to preserve genome integrity is the replication licensing factor CDT1. Multiple levels of CDT1 regulation exist in human cells. These mechanisms possess unique and necessary roles in the regulation of CDT1, to properly restrain this protein and prevent the adverse cellular consequences associated with its misregulation.

A role for DDB1 in maintaining genome stability has previously been noted in other organisms. Shimanouchi and colleagues found that silencing of *DDB1* in *Drosophila* promoted the loss of heterozygosity in somatic cells [204]. Holmberg et al. (2005) also found that deletion of *DDB1* in *S. pombe* enhances the mutation rate more than 20-fold [157]. This data correlates well with our data from human cells. Silencing or deletion of *DDB1* results in genome instability, as assessed by the generation of DNA double strand breaks in human cells (this study) or an enhanced mutation rate in yeast, and in neither organism is this instability the result of defective NER.

In *S. pombe*, the genetic instability associated with deletion of *DDB1* could be partially suppressed by removing *Spd1*, an inhibitor of ribonucleotide reductase (RNR) [157]. DDB1 and CUL4 promote the ubiquitin-mediated degradation of Spd1 to allow association of the RNR subunits and production of dNTPs for DNA synthesis and repair [156-159]. A human homologue of Spd1 has not been identified; therefore, it is unclear whether this function of DDB1 is conserved in higher eukaryotes. However, removal of *Spd1* could not completely alleviate the genetic instability created by the loss of *DDB1* in *S. pombe*. This suggests that DDB1 is required for other aspects of mutation avoidance in addition to regulating DNA replication through the degradation of Spd1. Likewise, we

suggest that human DDB1 mediates genome stability, in part by controlling DNA replication through the degradation of CDT1. However, the inability of *CDT1* silencing to completely alleviate the phenotype observed in *DDB1*-silenced cells indicates that additional functions of DDB1 are critical for its role in the maintenance of genome integrity.

The DDB1-CUL4A ubiquitin ligase complex regulates DNA replication in multicellular eukaryotes by mediating the degradation of CDT1. Exposure to exogenous DNA damaging agents induces the destruction of CDT1 [160, 161]. Our data indicate that DDB1 also has an important role in the regulation of CDT1 in the absence of exogenous DNA damage. In agreement with our proposal, recent reports have implicated DDB1-CUL4A in the replication-dependent degradation of CDT1, and identified PCNA as a critical mediator of the DDB1-CUL4A-dependent CDT1 ubiquitination [162-165]. The destruction of CDT1 by the DDB1-CUL4A ubiquitin ligase complex is one of three mechanisms known in human cells to regulate CDT1. Additionally, the replication-dependent degradation of this protein can be accomplished by an SCF^{SKP2} ubiquitin ligase complex, which is targeted to CDT1 by a CDK-mediated phosphorylation event [170, 171, 198, 199]. CDT1 is also functionally inhibited by the binding of Geminin [168, 169]. These multiple levels of CDT1 regulation suggest that the proper restraint of this protein activity is crucial.

Disruption of CDT1 regulation is detrimental to genome stability and cell viability [157, 202, 204]. Highly elevated levels of CDT1 expression lead to significant amounts of re-replication in human cells, *Drosophila*, and *Xenopus* [200, 201, 205, 206]. Re-replication induces activation of DNA damage response pathways in humans,

Xenopus, and yeast [200-203, 207]. The phenotype observed after *DDB1* silencing is consistent with the phenotype observed after re-replication. We found a significant increase in DNA damage and activation of both ATR- and ATM-mediated damage response pathways (Figures 3.1 and 3.2). Notably, we see this enhancement of genome instability with only moderate increases in CDT1 levels (Figure 3.10A), in contrast to the significantly greater levels of CDT1 overexpression that were used in previous studies.

We also observe direct evidence that re-replication is occurring in *DDB1*-silenced cells with the incorporation of BrdU by cells with greater than 4n DNA content (Figure 3.9). The re-replication observed after silencing of *DDB1* is not extensive, perhaps due in part to the restraints imposed by the checkpoint response [201]. The elevated levels of CDT1 protein, re-replication, and DNA damage suggest that misregulation of CDT1 is contributing to the phenotype observed after silencing of *DDB1*. In support of this hypothesis, we found that there was indeed deregulation of CDT1, as the degradation of CDT1 proteins was significantly delayed following silencing of *DDB1* (Figure 3.10B). Additionally, reducing CDT1 protein levels in *DDB1*-silenced cells could prevent re-replication and eliminate approximately half of the DNA damage and checkpoint activation (Figure 3.11). Importantly, the level of CDT1 reduction achieved in these experiments did not interfere with normal DNA replication. Therefore, the reduction in DNA damage by co-silencing *CDT1* with *DDB1* is unlikely to be an indirect consequence of slowing the cell cycle. Our results clearly demonstrate that silencing of *DDB1* results in the misregulation of CDT1, which stabilizes and elevates the cellular levels of this protein. The consequence of this misregulation is re-replication, which contributes to the DNA damage and checkpoint activation observed after silencing of *DDB1*.

Our data indicates that re-replication occurs in *DDB1*-silenced cells despite the presence of other mechanisms that operate to suppress the re-firing of replication origins. Previous data from human systems has suggested that the DDB1-CUL4 and SCF^{SKP2} mechanisms of CDT1 destruction are redundant in the absence of exogenous DNA damage [162, 164]. However, the disruption of a single mode of CDT1 regulation in other organisms was shown to be sufficient to have adverse effects. In *Xenopus*, disruption of the DDB1-CUL4 degradation pathway stabilizes CDT1 and induces significant levels of re-replication [163]. Depletion of CUL4 from *C. elegans* also stabilizes CDT1 and results in massive re-replication, with cells exhibiting up to 100n DNA content [208]. The stabilization of CDT1 and the presence of re-replication after silencing of *DDB1* argue against redundant roles for DDB1-CUL4A and SCF^{SKP2} in the destruction of CDT1 in human cells. Additionally, this suggestion is supported by our observation that expression of a CDT1 mutant insensitive to DDB1-CUL4A degradation results in greater DNA damage than expression of either wild-type CDT1 or a mutant that is insensitive to the SCF^{SKP2} destruction pathway (Figure 3.12).

We propose that the DDB1-dependent degradation of CDT1 is particularly important because the loss of DDB1 creates a situation in which one other mechanism of CDT1 regulation is also inactivated. Silencing of *DDB1* generates DNA damage that activates cell cycle checkpoints, which in turn function to inactivate CDK complexes [173]. Since the SCF^{SKP2}-mediated destruction of CDT1 requires a CDK-mediated phosphorylation event, this pathway is inhibited by the presence of active checkpoints. Therefore, disruption of *DDB1* eliminates both ubiquitin-dependent mechanisms of CDT1 regulation, which will likely result in further re-replication, greater DNA damage,

and amplification of genome instability (Figure 3.15). It should be noted that cells generate DNA damage intrinsically as a consequence of respiration and DNA metabolism. Replication forks encounter DNA lesions and experience difficulty in replicating through specific regions of the genome during every round of DNA synthesis. The act of growing cells in culture can also increase cellular stresses. Thus, the distinction between DNA damage-dependent CDT1 degradation from replication-dependent degradation is largely one of degree.

DDB1-dependent regulation of CDT1 is not sufficient to explain all of the genome instability that arises from silencing of *DDB1*. Co-silencing of *CDT1* with *DDB1* eliminates re-replication; however, it does not completely prevent DNA damage or checkpoint activation. Additional genome maintenance functions of DDB1 likely have cell cycle-dependency, since the loss of DDB1 from G_0 arrested cells does not cause DNA damage (Figure 3.13). Identifying other substrates of DDB1-CUL4A whose degradation is important in preventing genetic instability will be important.

Another question that remains unanswered is the mechanism by which re-replication activates checkpoint pathways. Our analyses suggest that re-replication causes DNA double- strand breaks in a manner that is distinct from those arising at chromosome fragile sites during replication stress. It is unclear whether these breaks are at random locations or whether they may cluster near specific genomic regions that are prone to re-replication. The breaks do not appear to cluster near centromeres. This suggests that attachment of the mitotic spindle to a re-replicated centromere on a single chromatid, and the subsequent breakage of the chromosome during anaphase, is not a major mechanism contributing to these breaks. One possibility is that re-replication

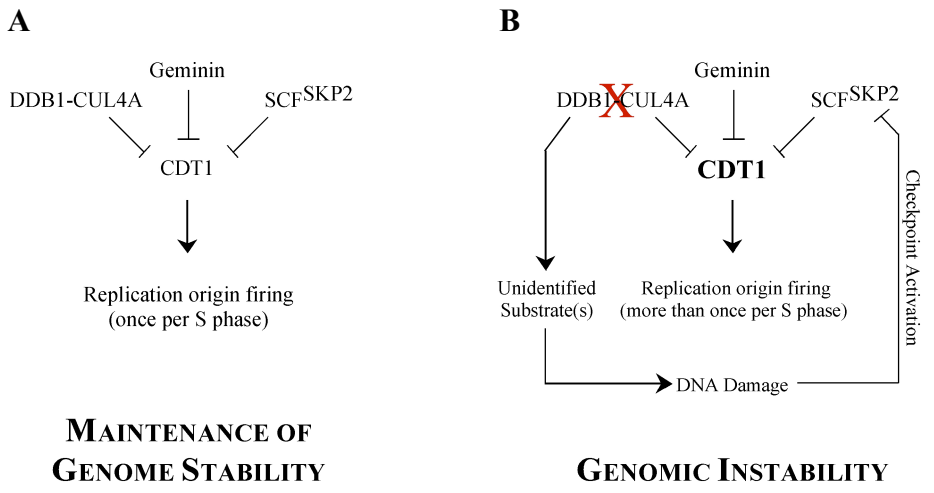


Figure 3.15. Model highlighting the importance of DDB1 in CDT1 regulation. (A) Geminin, DDB1-CUL4A, and SCF^{SKP2} cooperate to restrain the activity and levels of CDT1, ensuring that origins of replication fire only once per S phase. **(B)** Silencing of *DDB1* disrupts the DDB1-CUL4A-dependent regulation of CDT1 and causes DNA damage due to re-replication and impaired regulation of an additional unidentified substrate. DNA damage activates cell cycle checkpoints, which in turn function to inactivate CDK complexes. SCF^{SKP2}-mediated degradation of CDT1 requires a prior phosphorylation event by CDK; therefore, this mechanism of CDT1 regulation is disrupted by the presence of active checkpoints. The deregulation results in increased CDT1 levels that promote re-replication, leading to greater DNA damage, and amplification of genomic instability.

causes double strand breaks as the second fork originating from a re-fired origin encounters the Okazaki fragments generated by the original replication fork. A second possibility is that the forks initated at a re-fired origin are defective, stall, and eventually collapse, thus generating DNA damage.

Recent studies have highlighted a role for replication stress as an early event in the initiation of cancer [209-211]. Aberrant DNA replication in pre-cancerous lesions produces DNA damage and activates checkpoint pathways. Inactivation of these damage response pathways is associated with tumor progression due to genetic instability. Disruption of DDB1-dependent functions also has the ability to create replication stress and genetic instability, perhaps providing an avenue through which cancer progression can be facilitated. Changes in the activity of the DDB1-CUL4A ubiquitin ligase complex, or in mechanisms regulating replication origin firing, may therefore play important roles in the process of tumorigenesis.