Phosphorylation and ubiquitination-dependent degradation of CABIN1 releases p53 for transactivation upon genotoxic stress

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ABSTRACT

CABIN1 acts as a negative regulator of p53 by keeping p53 in an inactive state on chromatin. Genotoxic stress causes rapid dissociation of CABIN1 and activation of p53. However, its molecular mechanism is still unknown. Here, we reveal the phosphorylation- and ubiquitination-dependent degradation of CABIN1 upon DNA damage, releasing p53 for transcriptional activation. The DNA-damage-signaling kinases, ATM and CHK2, phosphorylate CABIN1 and increase the degradation of CABIN1 protein. Knockdown or overexpression of these kinases influences the stability of CABIN1 protein showing that their activity is critical for degradation of CABIN1. Additionally, CABIN1 was found to undergo ubiquitin-dependent proteasomal degradation mediated by the CRL4DDB2 ubiquitin ligase complex. Both phosphorylation and ubiquitination of CABIN1 appear to be relevant for controlling the level of CABIN1 protein upon genotoxic stress.

INTRODUCTION

CABIN1 was initially identified as a calcineurin-binding protein acting as a negative regulator of both calcineurin and MEF2 (myocyte enhancer factor 2) (1,2). Numerous reports have thoroughly elucidated the mechanism of MEF2 repression, demonstrating that CABIN1 brings a huge complex of repressors including histone deacetylases (HDACs) and histone methyltransferase (HMT) (3–7).

We recently showed that CABIN1 plays a pivotal role in p53-dependent gene regulation by occupying the promoters of a subset of target genes with p53 as a repressive regulator in the unstressed condition (8). Our previous research provides an explanation for p53 occupancy on target promoters without activating gene expression (9–11). This study also gives rise to the necessity of CABIN1 dissociation from p53 upon genotoxic stress for activation of the target gene expression.

In response to genotoxic stress, eukaryotic cells activate conserved pathways that increase expression of many genes involved in cellular functions such as DNA repair, cell-cycle arrest and cell death (12–14). Protein kinases ATM (ataxia-telangiectasia, mutated) and ATR (ATM and Rad3-related) are emerging as potential sensors of DNA damage. Activated ATM and ATR phosphorylate downstream effector kinases including CHK1 (checkpoint kinase 1) and CHK2 (checkpoint kinase 2) for the damage-signaling cascade (15,16). ATM and ATR share consensus sites, the Ser-Gln (SQ) and Thr-Gln (TQ) motifs, and CHK1/CHK2 recognize the R–X–X–S/T motif. Moreover, CABIN1 is reported to have a putative ATM-/ATR-mediated phosphorylation site in response to UV irradiation (17). This fact prompted us to examine the possibility of CABIN1 phosphorylation upon DNA damage.

DNA-damage-binding proteins (DDB1 and DDB2) are subunits of a heteromeric complex, which is known as the primary detection device for UV-induced lesions in the genome and mediates global genome nucleotide excision

repair (GG-NER) (18–20). The CRL4DDB2 ubiquitin ligase complex participates in diverse cellular and physiological processes including DNA repair, DNA replication and chromatin remodeling. More specifically, the ligase complex facilitates NER by targeting XPC and histones H2A, H3 and H4 for ubiquitination (21–24). The complex also targets the replication licensing factor, CDT1, for degradation which in turn results in delayed cell-cycle progression, finally permitting time for DNA repair (25).

Here, we found that ATM and CHK2 mediate phosphorylation of CABIN1 and the CRL4DDB2 ubiquitin ligase complex binds and mediates CABIN1 ubiquitination, leading to proteasomal degradation upon DNA damage. These findings provide an explanation of prompt activation of bound-p53 on promoters upon DNA damage.

MATERIALS AND METHODS

Cells and reagents

HEK293 and HCT116 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 50 U/ml of streptomycin and penicillin (Invitrogen). Reagents, including puromycin, polybrene, cycloheximide, doxorubicin, etoposide, caffeine and CHK2 inhibitor II, were purchased from Sigma-Aldrich, Inc. MG132 was purchased from Calbiochem.

Plasmid constructs

Various CABIN1 expression vectors were described previously (7). Mammalian expression vectors coding for human DDB1 and CUL4A were obtained from Addgene (Cambridge, MA, USA). The expression vectors for full-length DDB2 were generated by inserting DDB2 PCR fragments from pOTB7-DDB2 (obtained from 21C Frontier Human Gene Bank, Daejeon, Republic of Korea) into pcDNA3-HA. The plasmid pcDNA4/HisMax-ubiquitin was generously provided by Prof. C.H. Chung (Seoul National University, Republic of Korea).

Lentivirus and adenovirus production

For lentiviral-mediated RNA interference, we purchased pLKO-DDB1, DDB2 and CABIN1 from Open Biosystems, pLV-ATMi and pLV-ATRi from Addgene (Cambridge, MA, USA). Lentiviruses were produced according to the manufacturer's protocol using the BLOCK-iT Lentiviral RNAi expression system (Invitrogen). Briefly, 293FT cells were transfected with the pLKO shRNA vector in combination with packaging vectors using Lipofectamine 2000 (Invitrogen). The virus containing supernatant was collected and used for target cell infection. Forty-eight hours after lentiviral infection, puromycin was added for stable cell generation. To develop the adenoviral DDB2 expression system, we used Gateway Cloning kit (Invitrogen). Briefly, DDB2 PCR fragments from pOTB7-DDB2 were subcloned into pENTR3C and recombined with pAd/CMV/V5-DEST using Gateway LR Clonase II (Invitrogen). The production and amplification of adenovirus were performed as described previously (26).

Immunoblotting and immunoprecipitation

To prepare the whole-cell extracts, cells were lysed with TETN buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% (v/v) Triton X-100, protease inhibitor cocktail (Roche) and 1 mM PMSF]. Cell lysates were incubated with the indicated antibody and protein G beads (Santa Cruz Biotechnology). Immunoprecipitates were washed three times with the same lysis buffer and boiled with sample loading buffer for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The following antibodies were used: FLAG (M2 antibody, Sigma-Aldrich), HA (16B12, Covance), MYC (9E10, Covance), β-actin (Sigma-Aldrich), p53 (DO-1, Santa Cruz Biotechnology), DDB1 (BD Transduction Laboratories), DDB2 (Santa Cruz Biotechnology), Ubiquitin (P401, Santa Cruz Biotechnology), ATM (2C1, GeneTex), CHK2 (ab8108, Abcam) and CABIN1 (polyclonal antibodies were generated by AbFrontier).

His-ubiquitin pull-down assay

Cells transfected with Myc-tagged CABIN1 and His_6-tagged Ubiquitin for 24 h were harvested and re-suspended in Buffer A (6 M guanidine–HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM imidazole at pH 8.0). The lysates were then sonicated before adding 50 μl of Ni–NTA agarose and rotating at room temperature for 4 h. The beads were washed with Buffer A, Buffer A/TI [1 vol. of Buffer A, 3 vol. of Buffer TI (25 mM Tris–Cl, 20 mM imidazole at pH 6.8)] and Buffer TI. To the washed beads, 50 μl of His-ubiquitin elution buffer [0.2 M imidazole, 5% (w/v) SDS, 0.15 M Tris–Cl at pH 6.8] was added and incubated for 20 min at room temperature. The eluted ubiquitin conjugates were boiled with SDS sample buffer and subjected to western blot analysis.

CABIN1 purification

A stable cell line expressing FLAG-tagged CABIN1 was generated and used for CABIN1 purification. Briefly, cells were lysed with TETN lysis buffer and soluble lysates were incubated with anti-FLAG-M2-agarose (Sigma-Aldrich) for 2 h. Precipitates were washed four times with lysis buffer and eluted with 50 μl elution buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1 μg/μl FLAG peptide (F4799, Sigma-Aldrich)].

Kinase assay

HEK293T cells were transiently transfected with pcDNA3-FLAG-ATM using polyethylenimine (PEI, Polysciences). Forty-eight hours after transfection, the cell lysates were prepared and immunoprecipitated with anti-FLAG antibody and protein G beads. Precipitates were washed and subjected to ATM kinase assay as

described previously (27). For CHK2 kinase assay, the purified recombinant full-length human CHK2 kinase (#7434, Cell Signaling Technology) was used following the manufacturer's protocol.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as described previously (7,8). Primers used for *GADD45* were: forward, 5′-AGCGGAAGAGATCCCTG TGA-3′ and reverse, 5′-CGGGAGGCAGGCAGATG-3′.

Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA as described previously (28). Quantitative real-time PCR was performed with the SYBR Green qPCR kit (Finnzymes, F-410L). The primers for real-time PCR were as listed previously (8). Primers used for *DDB1* RT–PCR were forward, 5′-GGCAAAGACCCCAACAC TTA-3′ and reverse, 5′-CGTGCTATTGATGCTGGC TA-3′. Primers used for *DDB2* RT–PCR were forward, 5′-TCCTCTACTCGCTGCCGCAC-3′ and reverse, 5′-CTGGGTATCGGCCCACAACA-3′.

RESULTS

CABIN1 is degraded by ubiquitin-mediated proteolysis upon DNA damage

We previously reported that CABIN1 acts as a negative regulator of p53 by co-occupying a subset of p53 target promoters in the absence of genotoxic stress (8). Under the genotoxic stress condition, CABIN1 should be dissociated from those promoters not to disturb p53 target gene expression. To determine the CABIN1 protein level in response to genotoxic stress in this study, HCT116 cells were treated with either doxorubicin ($0.4\,\mu g/ml$) or etoposide ($10\,\mu M$) and were subjected to immunoblot analysis. The protein levels of CABIN1 decreased in a time-dependent manner after the treatment of DNA damaging agents or UV irradiation (Supplementary Figure S1 and Figure 1A). To investigate whether the decrease in the CABIN1 protein level was proteasome-mediated, HCT116 cells were treated with the proteasome inhibitor MG132 prior to UV irradiation. Treatment of MG132 prevented the decrease in the CABIN1 protein level after UV irradiation (Figure 1B). These results implied that CABIN1 degradation involved CABIN1 ubiquitination. Thus, we transfected HEK293 cells with expression vectors of Myc-tagged CABIN1 along with
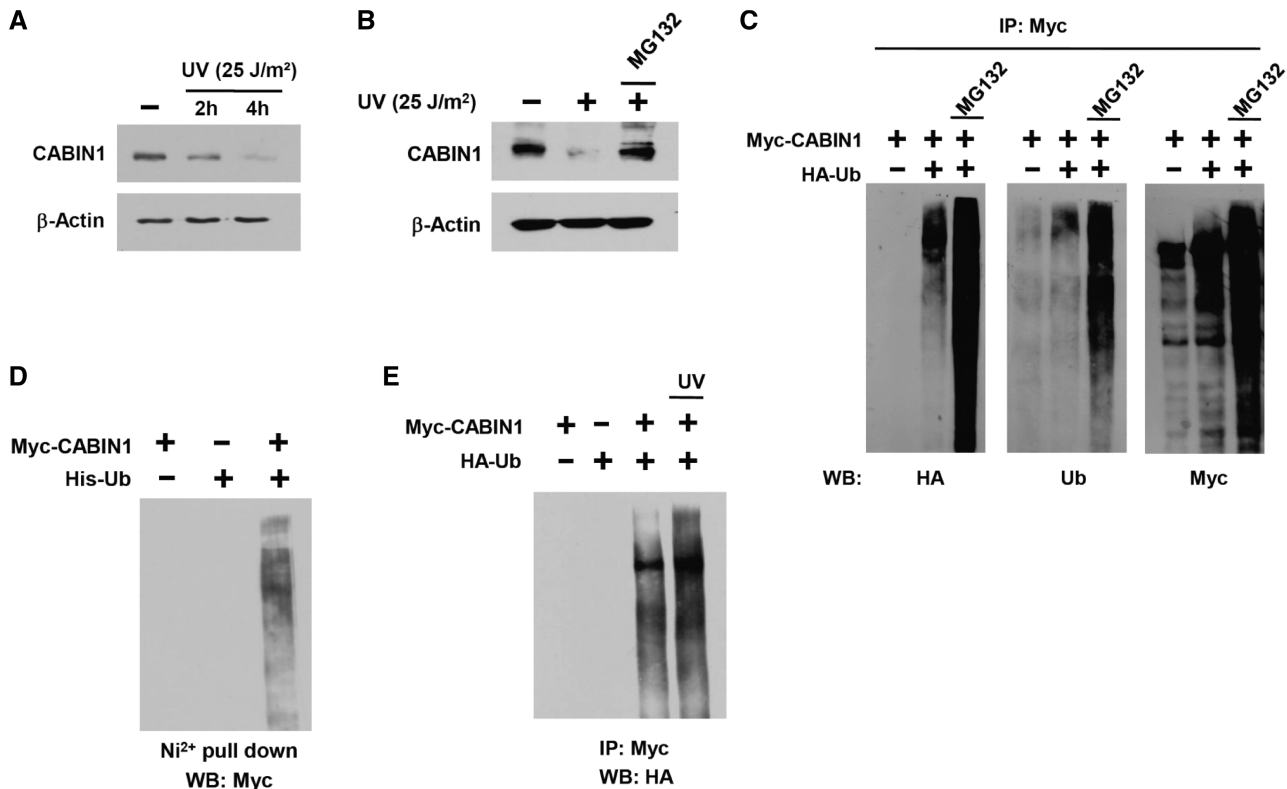

Figure 1. CABIN1 is degraded by ubiquitin-mediated proteolysis upon DNA damage. (**A**) HCT116 cells were UV-irradiated ($25\,J/m^2$) and placed back to the cell incubator. Cells were harvested after 2 or 4 h of incubation and subjected to immunoblot analysis with the indicated antibodies. β-Actin was used as a loading control. (**B**) Cells were UV-irradiated with or without MG132 ($5\,\mu M$) and incubated for 4 h. Endogenous CABIN1 protein was detected by immunoblotting. (**C**) Three sets of HEK293 cells were co-transfected with Myc-tagged CABIN1 and HA-tagged ubiquitin or the corresponding empty vector. Twenty-four hours after transfection, cells were treated with MG132 for 16 h and immunoprecipitated by anti-Myc antibody. Each set was subjected to immunoblotting with the indicated antibodies. (**D**) Cells were co-transfected with Myc-tagged CABIN1 and His₆-tagged ubiquitin or the empty vectors. His-ubiquitin pull-down assays were performed and subjected to immunoblotting with anti-Myc antibody. (**E**) The same experimental procedure with (C) was performed except for the condition of UV irradiation (lane 3).

HA-tagged ubiquitin. Cells were treated with MG132 for 16 h and then subjected to immunoprecipitation followed by immunoblotting with the indicated antibodies. The Myc-tagged CABIN1 protein exhibited smearing patterns in cells expressing exogenous ubiquitin and such protein patterns were enhanced by MG132 treatment (Figure 1C). In addition, his-ubiquitin pull-down assays were performed to confirm CABIN1 ubiquitination (Figure 1D). To investigate the role for DNA damage in stimulating CABIN1 ubiquitination, ubiquitination assay was performed with or without UV irradiation and the results showed a significant increase of CABIN1 polyubiquitination (Figure 1E).

CABIN1 is phosphorylated by DNA damage kinases

In response to UV irradiation and other genotoxic treatments, cells undergo DNA damage checkpoint response including a phosphorylation-based signal transduction cascade. To gain some insight into the mechanism leading to rapid degradation of CABIN1 protein upon DNA damage, we tested whether checkpoint kinases mediate the cue signaling of CABIN1 reduction. HEK293 cells were transfected with the FLAG-tagged ATM and the Myc-tagged CABIN1 expression vector and were subjected to immunoprecipitation assay to test the physical interaction of ATM and CABIN1. Myc-tagged CABIN1 readily co-immunoprecipitated with FLAG-tagged ATM (Figure 2A). To examine whether CABIN1 can be directly phosphorylated by ATM *in vitro*, the full-length FLAG-tagged CABIN1 protein, purified from a stable cell line expressing FLAG-tagged CABIN1, was used as a substrate in an *in vitro* kinase reaction with immunoprecipitated ATM from FLAG-ATM transfected HEK293 cells (Figure 2B). Purified CABIN1 protein was strongly phosphorylated by the immunoprecipitated FLAG-ATM wild-type (Figure 2C). We also performed binding assays with CABIN1 and other checkpoint kinases. Co-IP assays revealed that CHK2 was readily bound to CABIN1 (Figure 2D),
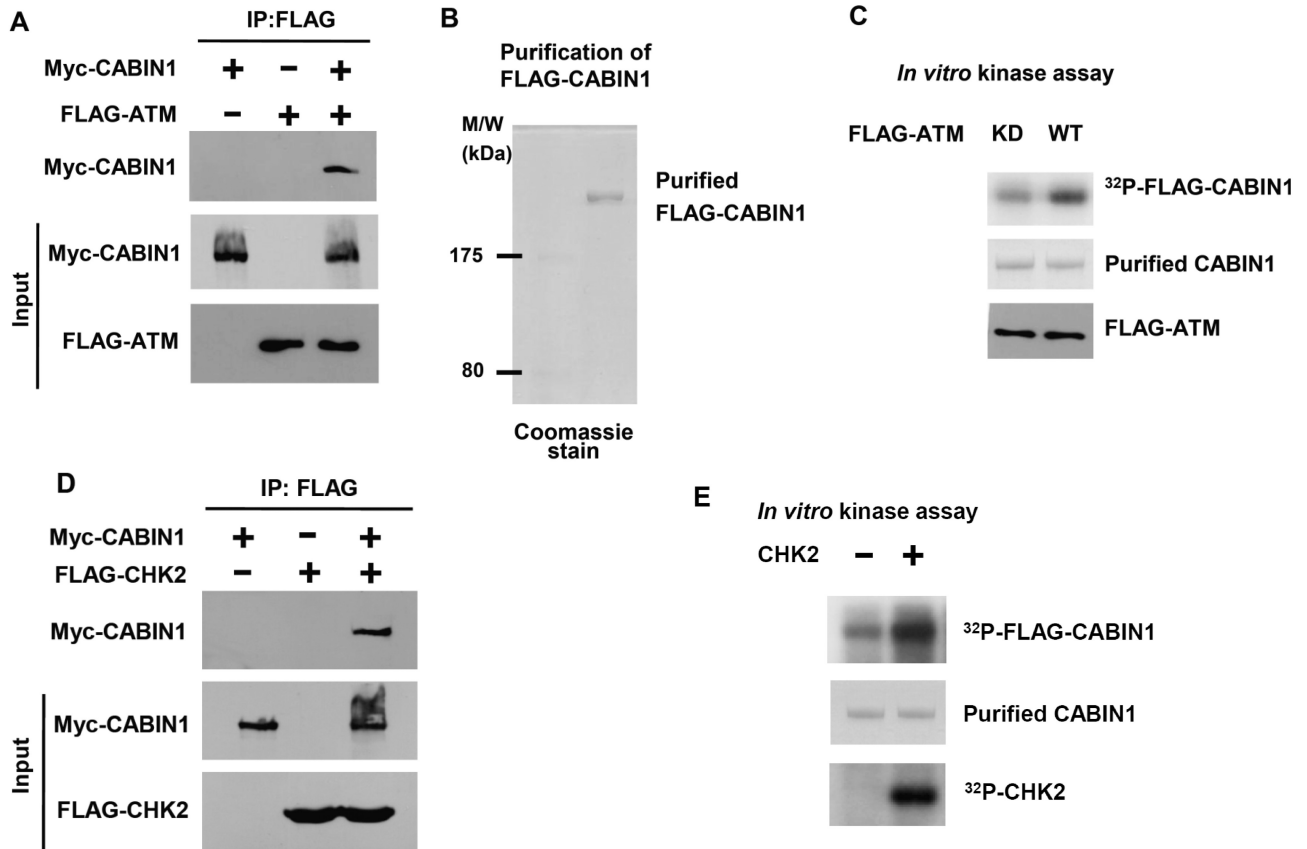
Figure 2. CABIN1 is phosphorylated by DNA damage kinase. (**A**) HEK293 cells were transfected with the indicated combination of Myc-tagged CABIN1 and FLAG-tagged ATM. To test the physical interaction, immunoprecipitation assays were performed and analyzed by immunoblotting. (**B**) A stable cell line expressing FLAG-tagged CABIN1 was used for recombinant FLAG-CABIN1 purification. Purified FLAG-tagged CABIN1 protein was resolved by SDS–PAGE and stained with Coomassie blue. (**C**) HEK293 cells were transfected with FLAG-ATM wild-type or kinase dead mutants. Using IP-ed FLAG-ATM from transiently transfected cells and purified recombinant proteins, *in vitro* kinase assay was performed and assessed by autoradiography. The middle panel shows Coomassie staining of CABIN1 and the bottom panel shows the immunoblotting results of ectopically expressed FLAG-ATM wild-type and kinase dead mutants. (**D**) Cells were transfected with the combination of Myc-tagged CABIN1 and FLAG-tagged CHK2. Lysates from the transfectants were subjected to immunoprecipitation with FLAG antibody and were analyzed by immunoblotting with the indicated antibodies. (**E**) CHK2 kinase assay was performed using purified recombinant proteins. Phosphorylated CABIN1 was visualized by autoradiography (upper panel) and phosphorylated CHK2 was also detected as a positive control (bottom panel).

whereas ATR and CHK1 did not (Supplementary Figure S2). To establish the functional relevance of CHK2/CABIN1 interaction, we tested whether CABIN1 is phosphorylated by purified CHK2. Phosphorylation of full-length CABIN1 was increased by adding of CHK2 kinase (Figure 2E). These results demonstrate that CABIN1 is a bona fide substrate of DNA damage-activated checkpoint kinases, ATM and CHK2.

Inhibition of DNA damage kinase activities blocks CABIN1 degradation upon DNA damage

To investigate the effect of these kinase activities on CABIN1 degradation, HCT116 cells were treated with an ATM/ATR inhibitor, caffeine and the protein level of CABIN1 was observed by immunoblot assay before and after UV irradiation. Caffeine blocked the reduction of the CABIN1 protein level after UV irradiation (Figure 3A). We next generated lentivirus expressing short-hairpin RNAs (shRNAs) which have specific sequences targeting ATM. Knockdown of ATM resulted in a higher level of CABIN1 protein even after UV irradiation in comparison with control (Figure 3B). We also investigated whether CHK2 contributes to the degradation of CABIN1 protein, using a specific inhibitor of CHK2 kinase activity and by shRNA-mediated CHK2 knockdown. Interestingly, both approaches markedly blocked the reduction of CABIN1 protein in response to UV irradiation (Figure 3C and D).

CABIN1 phosphorylation by DNA-damage kinases enhances ubiquitination

To verify that the activity of ATM and CHK2 affected CABIN1 ubiquitination, we transfected HEK293 cells with the Myc-CABIN1 expression vector in combination with HA-ubiquitin and FLAG-ATM or the FLAG-CHK2 expression vectors. Overexpression of ATM or CHK2 increased the ubiquitination of CABIN1 protein (Figure 4A and B). We also observed that the ubiquitination of CABIN1 in response to UV irradiation was attenuated in the presence of caffeine (Figure 4C). Thus, these results indicate that the DNA-damage kinase-mediated phosphorylation of CABIN1 is prone to ubiquitination upon DNA damage.

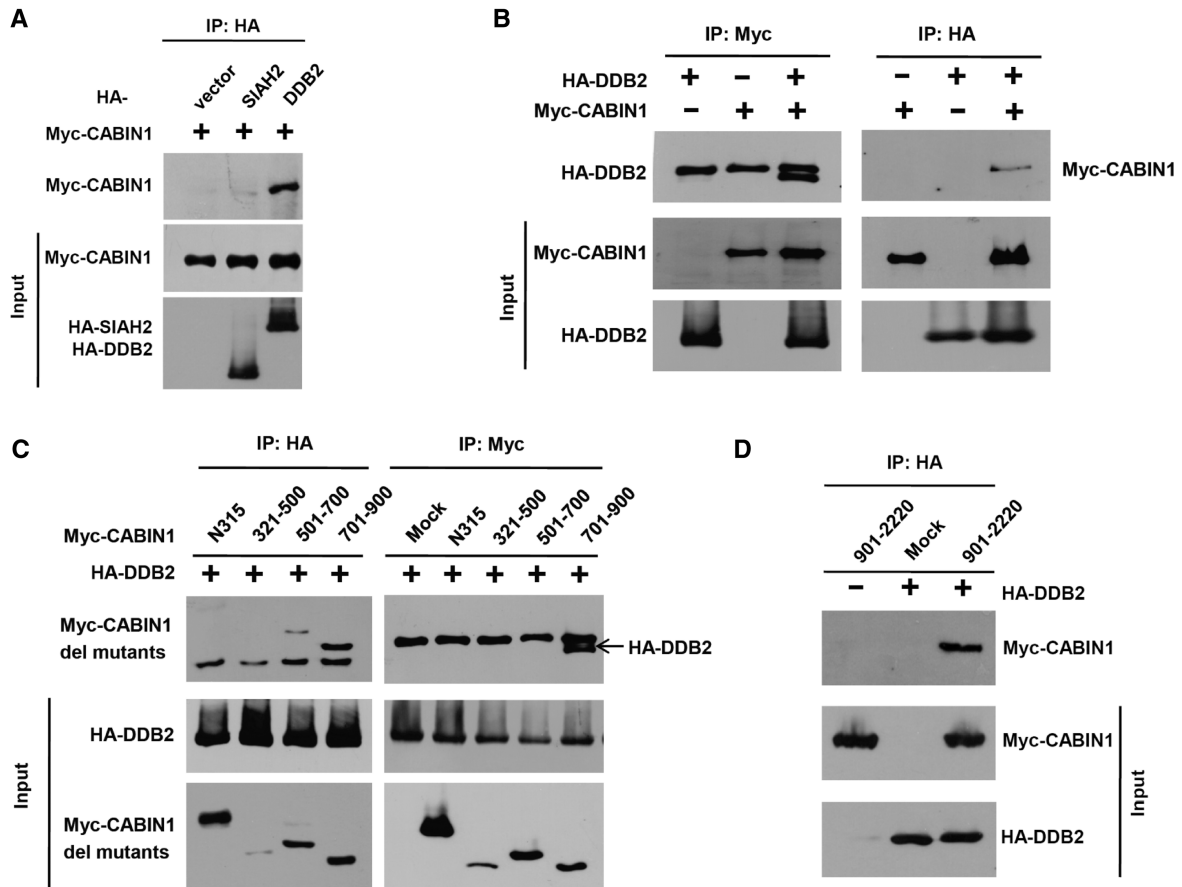### CABIN1 associates with CRL4DDB2 ubiquitin ligase

CRL4DDB2 ubiquitin ligase participates in degradation of several target proteins upon DNA damage (21–24). We thus postulated that CRL4DDB2 may be involved in CABIN1 degradation upon DNA damage. To address this, the binding of DDB2 to CABIN1 was first tested. Indeed, HA-tagged DDB2 readily co-immunoprecipitated with Myc-tagged CABIN1 and vice versa (Figure 5A and B). To identify the region of CABIN1 protein involvement in interaction with DDB2, Co-IP assays were performed with HA-tagged DDB2 and Myc-tagged CABIN1 deletion mutants. Myc-tagged CABIN1 fragments carrying 701–900 and 901–2220aa (amino acid) regions showed
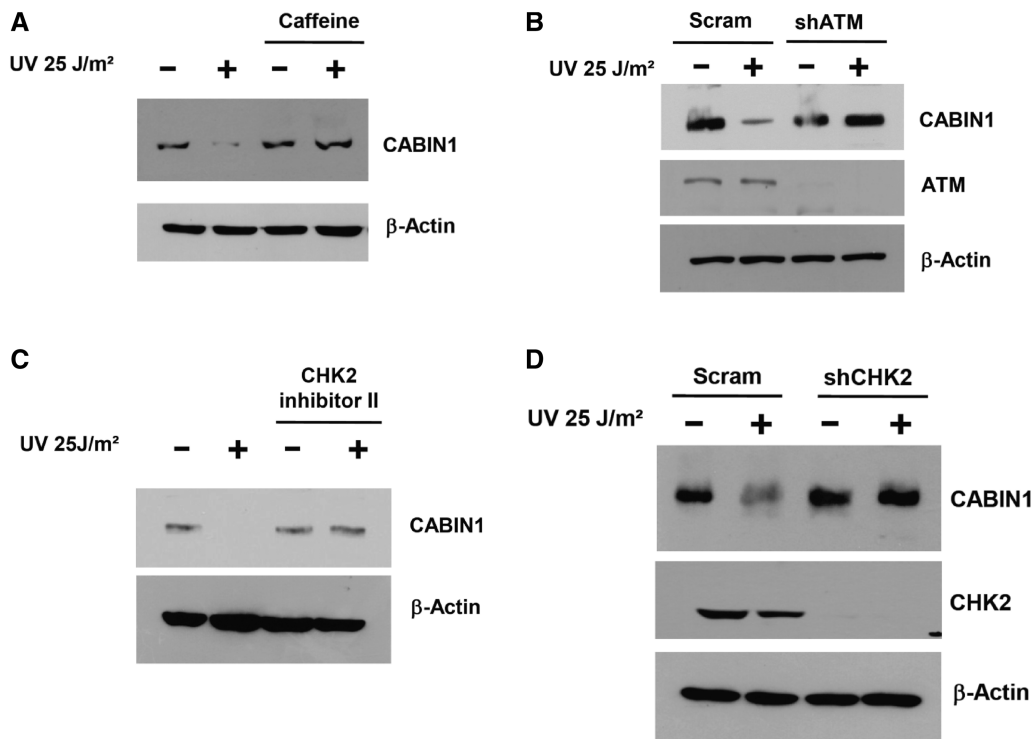
Figure 3. Inhibition of DNA damage kinase activities blocks CABIN1 degradation upon DNA damage. (**A**) HCT116 cells were UV-irradiated with or without caffeine and harvested 4 h later. The CABIN1 protein levels were checked by immunoblotting. (**B** and **D**) Using the lentiviral shRNA knockdown system, ATM or CHK2 was reduced and the indicated protein levels were checked under UV irradiation condition. (**C**) Cells were UV-irradiated with or without CHK2 inhibitor II and then, the CABIN1 protein levels were determined by immunoblotting.
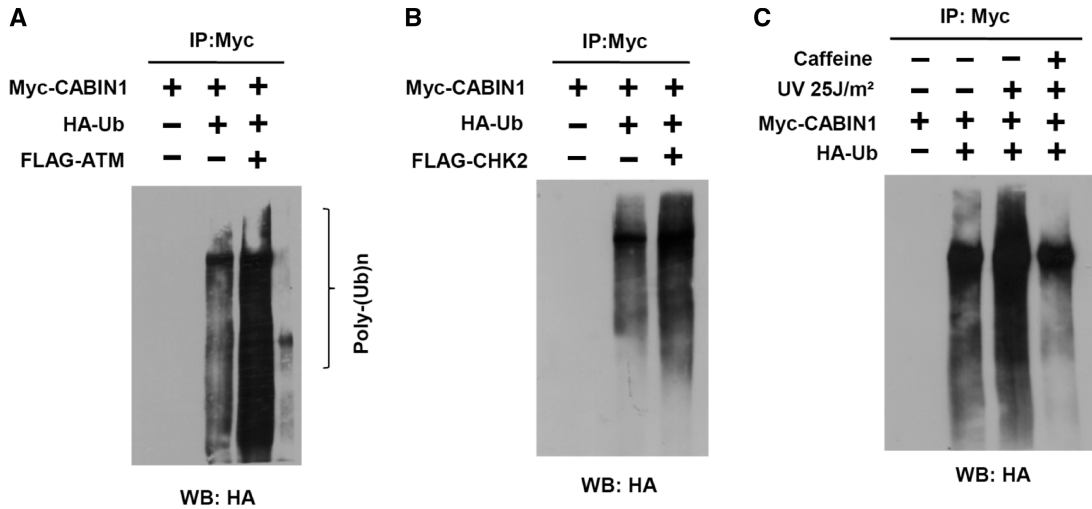
Figure 4. CABIN1 phosphorylation by DNA-damage kinases enhances ubiquitination. (**A** and **B**) HEK293 cells were transfected with the indicated expression vectors or the corresponding empty vectors. All cells were treated with MG132 (5 μM) for 16 h prior to lysis. Total lysates were immunoprecipitated using anti-Myc antibody and subjected to immunoblot analysis. (**C**) HEK293 cells were transfected with the indicated plasmids. Twenty-four hours after transfection, cells were pre-treated with caffeine (2 mM, lane 4) and UV-irradiated (lane 3 and 4). Cells were then harvested and prepared for immunoprecipitation and immunoblotting.

Figure 5. CABIN1 associates with CRL4DDB2 ubiquitin ligase. (**A**) HEK293 cells were co-transfected with Myc-tagged CABIN1 and HA-tagged SIAH2 or HA-tagged DDB2. To test the binding ability with Myc-tagged CABIN1, cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-Myc antibody. (**B**) Co-IP assays were performed using cells expressing Myc-tagged CABIN1 and HA-tagged DDB2. The immunoprecipitates and input lysates were analyzed by immunoblotting. (**C** and **D**) Co-IP assays were performed with HA-tagged DDB2 and Myc-tagged CABIN1 deletion mutants carrying 1–315, 321–500, 501–700, 701–900 and 901–2220aa regions. (**E**) HEK293 cells were transfected with HA-tagged DDB1 along with Myc-tagged full-length CABIN1 or CABIN1 deletion mutants. Co-IP assays and immunoblotting with the indicated antibodies were performed. (**G**) HA-tagged CUL4A and Myc-tagged CABIN1 expression constructs were transfected with the indicated combination, and the transfected cells were subjected to Co-IP, followed by immunoblotting. (**F**) A schematic illustration of CABIN1 deletion mutants and the results of their interaction with DDB1 or DDB2.
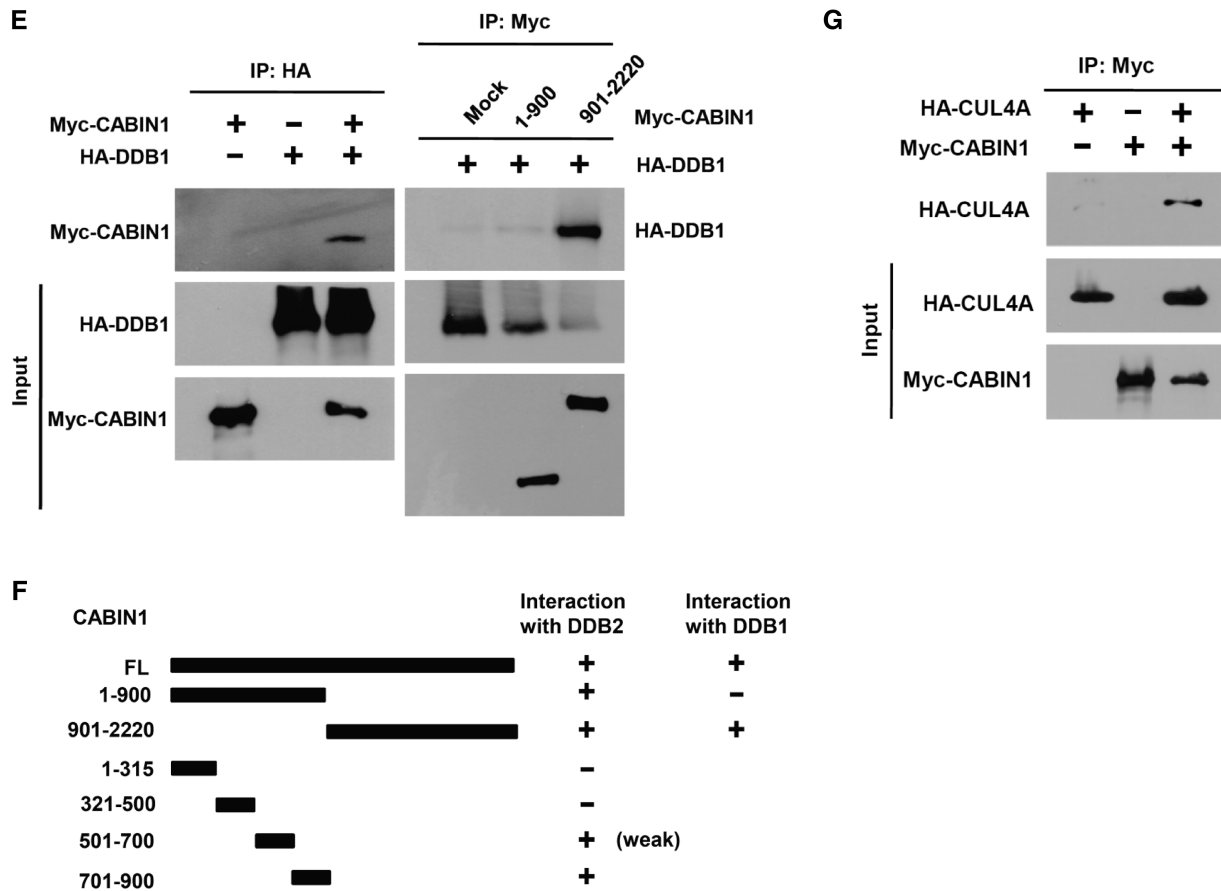
Figure 5. Continued.

strong binding ability with DDB2 and rather weaker binding affinity in 501–700 aa regions (Figure 5C and D). We next performed binding assays with DDB1 and CUL4A which acted as an E3 ligase complex with DDB2. The results showed a strong interaction with CABIN1 (Figure 5E and F). As an adaptor protein of CUL4 ligase, DDB1 associates with numerous substrate receptors including DDB2 which brings the specific substrates to the E3 ligase complex. To determine whether DDB1 and DDB2 share the binding region of CABIN1, Co-IP assay was carried out using CABIN1 deletion mutants and DDB1. DDB1 was bound to CABIN1 fragments carrying 901–2220 aa regions that partially overlapped with the binding sites of DDB2. In addition, we confirmed that Myc-CABIN1 associated with HA-CUL4A by Co-IP assay (Figure 5G).
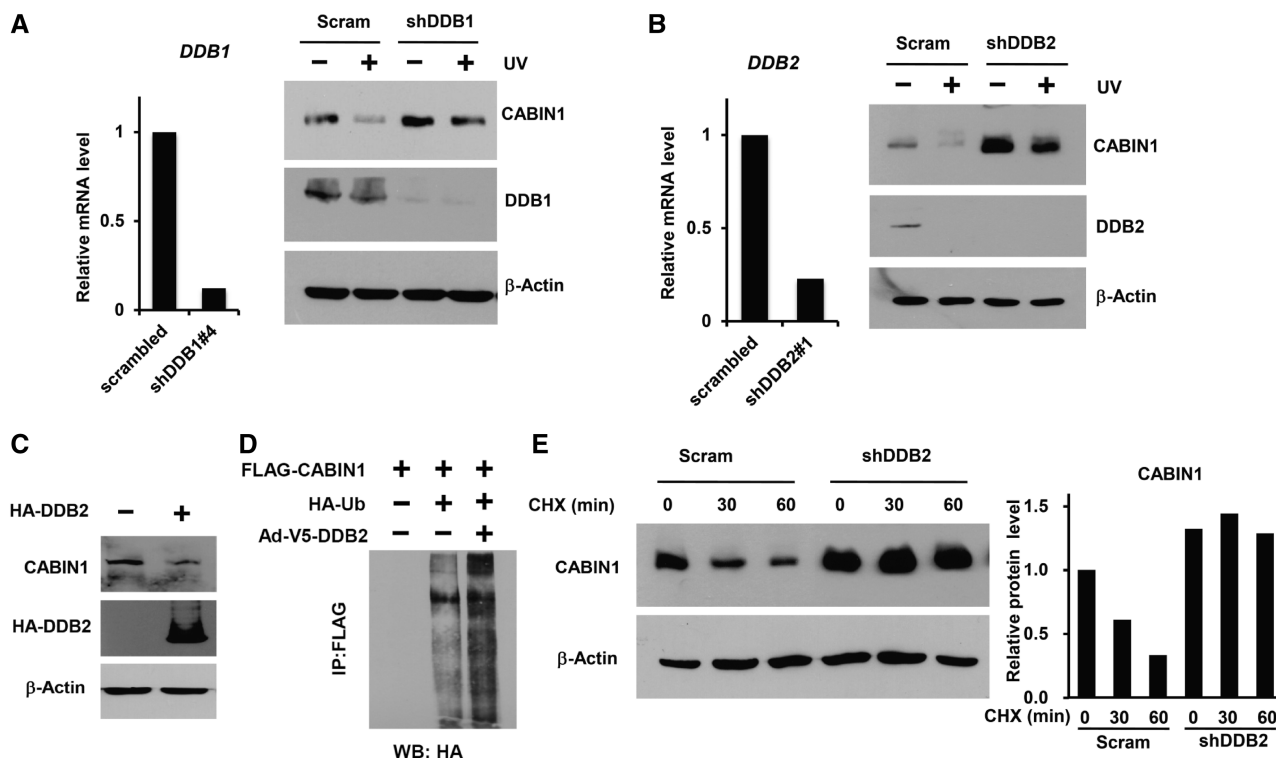
UV-DDB controls the CABIN1 protein stability upon DNA damage

To address the possibility that the CRL4DDB2 ubiquitin ligase may be involved in the degradation of CABIN1 under DNA damage condition, we next examined the knockdown effect of DDB1 or DDB2 on the CABIN1 protein level upon UV irradiation. Using the lentivirus-mediated shRNA silencing system, DDB1 and DDB2 were efficiently knocked down in HCT116 cells as evaluated by real-time qPCR and immunoblot analysis

(Figure 6A and B). A notable observation was made in the UV-DDB knockdown cells which showed a significant increase in the amount of CABIN1, suggesting that degradation of CABIN1 might be mediated by CRL4DDB2. Furthermore, overexpression of HA-tagged or V5-tagged DDB2 caused reduction of endogenous CABIN1 protein and increased the ubiquitination of CABIN1 (Figure 6C and D). HCT116 cells were infected with scrambled shRNA or DDB2 shRNA lentivirus and CABIN1 stability was subsequently determined using the cycloheximide chase. As expected, knockdown of DDB2 expression delayed CABIN1 protein decay.

UV-DDB regulates p53 activity through degradation of CABIN1

We have previously shown that CABIN1 affects p53 target gene expression by co-occupying their promoters in the absence of genotoxic stress. Therefore, the changes in protein levels of CABIN1 influence the mRNA expression of p53 target genes. To test whether UV-DDB could regulate p53 activity through degradation of CABIN1, we assessed the mRNA levels of *GADD45* and *p21* in sh-DDB-treated cells. Two different combinations of effective shRNAs were used to address off target effects (Supplementary Figure S6). Transient depletion of UV-DDB reduced UV-induced expression of *GADD45* and *p21* due to the high levels of CABIN1 even after

Figure 6. UV-DDB controls the CABIN1 protein stability upon DNA damage. (**A** and **B**) Using the lentivirus-mediated shRNA silencing system, DDB1 or DDB2 was knocked down specifically. The mRNA level of *DDB1* or *DDB2* was measured by real-time qPCR, and the protein levels were determined by immunoblotting. (**C**) HCT116 cells were transfected with the HA-tagged DDB2 expression vector or the corresponding empty vector. Whole-cell lysates were prepared and subjected to immunoblotting with the indicated antibodies. (**D**) HEK293 cells were co-transfected with FLAG-tagged CABIN1 and HA-tagged ubiquitin or the corresponding empty vector. On the next day, cells were infected either with the adenovirus encoding V5-tagged DDB2 or the control LacZ adenovirus. All cells were treated with MG132 (5 μM) for 16 h and then subjected for the ubiquitination assay. (**E**) DDB2 knockdown increased CABIN1 protein stability and half-life. HCT116 cells infected with scrambled shRNA or shDDB2 lentivirus was treated with cycloheximide (CHX; 5 μg/ml) for the indicated times. Whole-cell lysates were subjected to western blot with CABIN1 antibodies. The protein levels of CABIN1 were quantified using ImageJ software.

DNA damage (Figure 7A). However, double RNA interference of UV-DDB and CABIN1 restored the mRNA levels to those of controls. We also observed an increased expression of *GADD45* in cells expressing exogenous DDB1 and DDB2, which coincided with the reduction of CABIN1. Moreover, the increase of *GADD45* expression was not observed in p53-deficient cells (Figure 7B). To investigate the effect of UV-DDB knockdown on promoter co-occupancy, ChIP assays were performed. The results showed that knockdown of UV-DDB blocked CABIN1 dissociation from *GADD45* promoter upon genotoxic stress (Figure 7C).

Based on the current results, we propose a working model for CABIN1 degradation upon DNA damage (Figure 8). CABIN1 acts as a repressor of p53 in the absence of genotoxic stress. When DNA damage is inflicted on the whole cells, however, CABIN1 is rapidly phosphorylated, ubiquitinated, and degraded in order to activate the expression of p53 target genes.

DISCUSSION

We demonstrated that the CABIN1 protein is phosphorylated and ubiquitinated in response to DNA damage and that this post-translational modifications cause the rapid degradation of the CABIN1 protein. In this study, we defined a mechanism of CABIN1 degradation including the factors responsible for these modifications. Phosphorylation of CABIN1 is mediated by ATM and CHK2 kinases and occurs on multiple residues of CABIN1 (Supplementary Figure S3A and B). Depletion of ATM or CHK2 increases CABIN1 protein stability upon DNA damage, and overexpression of either one causes an increase in the ubiquitination of CABIN1.

In addition, the CRL4DDB2 ligase complex was found to target CABIN1 for ubiquitination and degradation. No reports have suggested that UV-DDB recognizes and binds to a specific phosphorylated motif, but it is still possible that phosphorylation of CABIN1 at multiple sites could alter the binding affinity between CABIN1 and the CRL4DDB2 complex. To test this hypothesis, we performed reciprocal co-IP assays of CABIN1 and DDB2 in various conditions. Our results show that UV irradiation increased the binding affinity between them, but pre-treatment of caffeine or CHK2 inhibitor II blocked that effect (Supplementary Figure S5). These results suggest that activities of DNA-damage kinases affect the binding affinity of CABIN1 and DDB2. Moreover, ATM- and CHK2-dependent phosphorylation of MDMX is reported to be important for its degradation
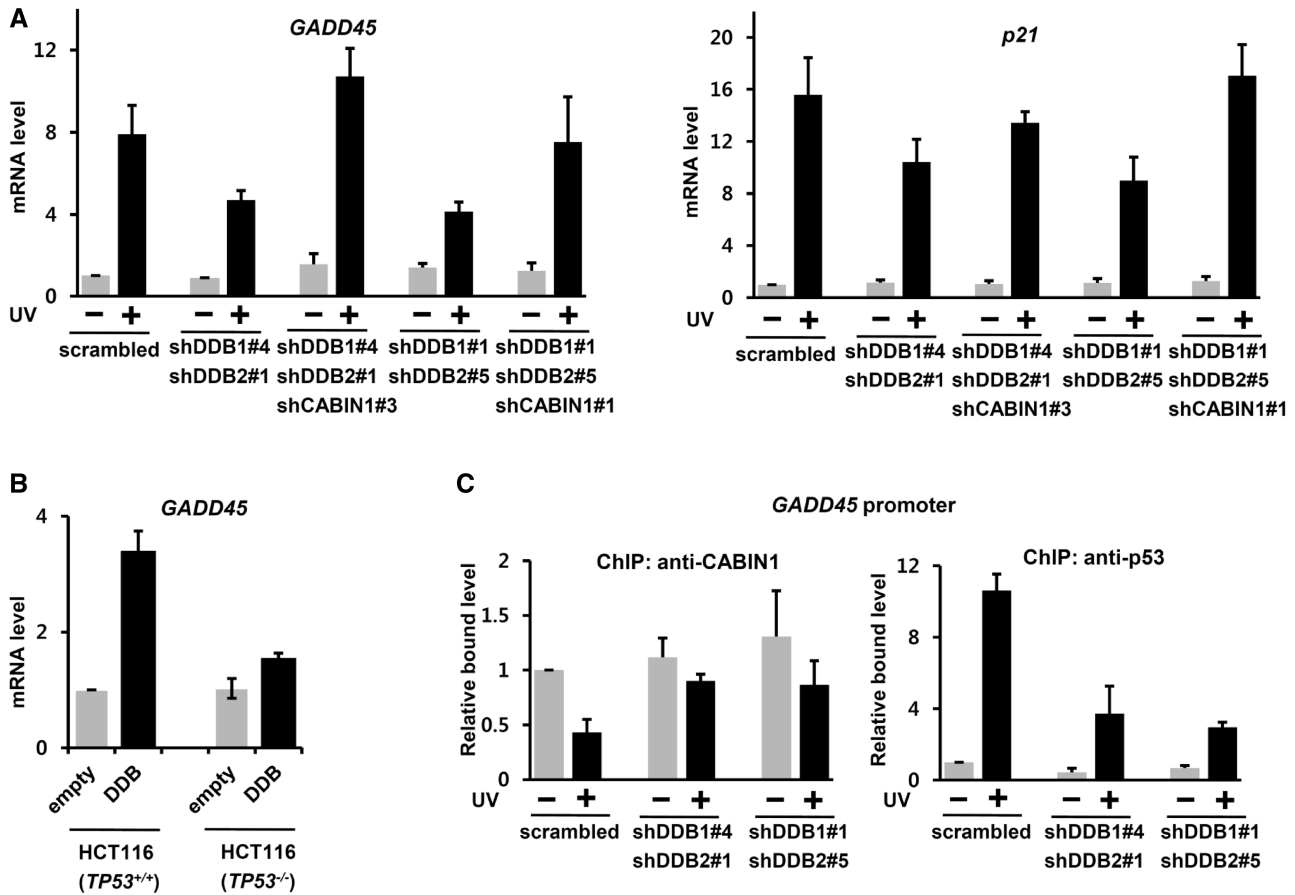
Figure 7. UV-DDB regulates p53 activity through degradation of CABIN1. (**A**) HCT116 cells were treated with scrambled shRNA or two sets of shDDB1/2 or shDDB1/2 and shCABIN1 together. After 48 h of infection, cells were selected with puromycin (2 µg/ml) for 2 days and split into two dishes. On the next day, control and UV-irradiated cells were harvested and the mRNA levels of *GADD45* and *p21* were measured by qRT–PCR. The values represent mean relative change ± SD ($n \geq 3$). (**B**) HCT116 (*TP53*$^{+/+}$) and HCT116 (*TP53*$^{-/-}$) cells were transfected with HA-tagged DDB1/2 or the empty vector. Cells were harvested 24 h after transfection and mRNA levels of *GADD45* were measured by qRT–PCR. (**C**) HCT116 cells were infected with scrambled shRNA or two sets of shDDB1/2 lentivirus and selected for 2 days with puromycin. After UV-irradiation ChIP assays were performed using anti-CABIN1 and anti-p53 antibodies, respectively. ChIPed DNA was analyzed by qRT–PCR using *GADD45* ChIP primers.
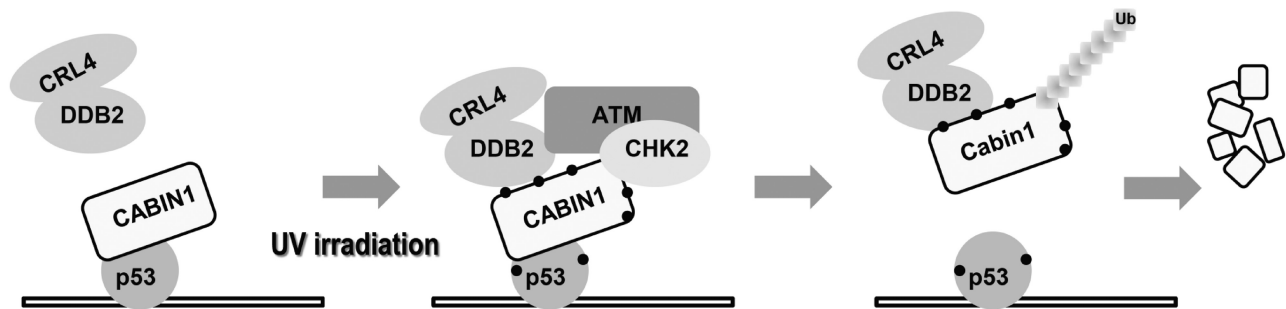
Figure 8. Schematic model for CABIN1 degradation mechanism upon DNA damage. CABIN1 acts as a repressive regulator of p53 by co-occupying a subset of p53 target promoters in the unstressed condition. Under the genotoxic stress condition, CABIN1 is phosphorylated by ATM and CHK2 and undergoes ubiquitin-dependent proteasomal degradation mediated by the CRL4DDB2 ubiquitin ligase complex.

and p53 activation which is a similar consequence to CABIN1 phosphorylation (29–31). At present, the precise mechanism of the functional interplay between damage kinases and CRL4DDB2 ubiquitin ligase remains to be elucidated, and further studies are required to determine the exact phosphorylation and

ubiquitination sites that are critical for CABIN1 protein degradation.

In fact, we found several phosphorylation sites on CABIN1, but a single residue mutation did not cause a significant difference in CABIN1 protein stability, suggesting that phosphorylation of multiple residues are

needed for its degradation. We recently found a mutant construct which seemed to be more stable than wild-type protein under DNA damage; however, it is yet to be tested thoroughly (Supplementary Figure S3C). We also tested whether deletion mutants of CABIN1 are subjected to ubiquitination. Both Myc-tagged CABIN1 fragments carrying 1–900 and 901–2220 aa regions were highly ubiquitinated, implying that ubiquitination also occurs on multiple residues (Supplementary Figure S4).

Our previous studies demonstrated that CABIN1 brings a repressive complex consisting of histone modifying enzymes including HDACs and HMT to the binding partner for repressive regulation (8). It has been shown that CABIN1 restrains p53 activity by regulating chromatin structure on the p53 target promoters. This explains how p53 can occupy the target promoters without activating the gene expression and also suggests a need for their rapid dissociation mechanism after the DNA-damage signal. In this study, we showed that CABIN1 is a target of the CRL4DDB2 ubiquitin ligase complex, thus the protein level of CABIN1 is influenced by overexpression or knockdown of UV-DDB.

To confirm the correlation between CABIN1 and p53 target gene expression, the mRNA level of *GADD45* was checked in cells expressing either a high level of UV-DDB or depleted UV-DDB. As expected, the protein level of CABIN1 fluctuated with UV-DDB expression and therefore, the mRNA level of p53 target genes was, in turn, influenced by them.

It is noteworthy that DDB2 is also known as a p53 target gene (32–34). Genotoxic stress causes the transcriptional activation of the p53 target genes including *DDB2*, which mediates the ubiquitination of CABIN1 and finally boosts p53 activity by eliminating the repressor. We assume that this is a very efficient mechanism for positive regulation of p53 activity upon DNA damage.

Previous studies have also shown that p53 is required for efficient NER of UV-induced DNA lesions (35). It has been proposed that p53 acts not only as a transcriptional activator of DDB2 and XPC, the global genome repair (GGR)-specific damage recognition genes, but also as a chromatin accessibility factor required for chromatin relaxation in order to make DNA lesions accessible for NER (36,37). In the context of the relationship between p53 and CABIN1, there is a high possibility that CABIN1 may influence the NER process.

In this work, we demonstrated a novel mechanism of CABIN1 degradation mediated by the cooperation of the damage kinases and the CRL4DDB2 ubiquitin ligase. Our findings broaden the functional understanding of CABIN1 in gene repression and reveal a possible interplay between damage kinases and the CRL4DDB2 complex.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–6.

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