Candidate tumor suppressor BTG3 maintains genomic stability by promoting Lys63-linked ubiquitination and activation of the checkpoint kinase CHK1

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***B-cell translocation gene 3* (*BTG3*) is a member of the antiproliferative *BTG/ Transducer of ErbB2* gene family and is induced by genotoxic stress in a p53- and Checkpoint kinase 1 (CHK1)-dependent manner. Down-regulation of BTG3 has been observed in human cancers, suggesting that it plays an important role in tumor suppression, although the underlying mechanisms are unclear. Here, we report that BTG3 interacts with CHK1, a key effector kinase in the cell cycle checkpoint response, and regulates its phosphorylation and activation. Upon interaction, BTG3 mediates K63-linked ubiquitination of CHK1 at Lys132 through the cullin-RING ligase 4^{Cdt2} E3 complex, thus facilitating CHK1 chromatin association. We show that BTG3-depleted cells phenocopy those CHK1-deficient cells, exhibiting increased cell death after replication block and impaired chromosome alignment and segregation. These defects could be corrected by wild-type BTG3 but not by a mutant impaired in CHK1 interaction. We propose that BTG3-dependent CHK1 ubiquitination contributes to its chromatin localization and activation and that a defect in this regulation may increase genome instability and promote tumorigenesis.**

The protein encoded by the *B cell translocation gene* (*BTG*) *3* is a member of the anti-proliferative BTG/ Transducer of ErbB2 (Tob) family which also includes *BTG1*, *BTG2*, *BTG4*, *TOB*, and *TOB2*. Members of the BTG family are very similar in their N-terminal domains but appear to diverge in their C-terminal domains (1).

Expression of mouse Btg3 peaks at the end of the G_1 phase in peripheral blood cells after stimulation with phytohemagglutinin (2). Induction of BTG3 by genotoxic stress depends on the tumor suppressor protein p53 and the kinase Checkpoint kinase 1 (CHK1) (3). Once induced, BTG3 associates with and inhibits the transcription factor E2F1 and is involved in the regulation of S phase entry and maintenance of G_2 arrest after DNA damage (3). Btg3-deficient (*Btg3*$^{-/-}$) mice display a higher incidence of lung tumors, which correlates with reduced expression in clinical specimens from lung cancer (4). Down-regulation of BTG3 has been observed in renal, breast, and prostate cancers and occurs mainly because of promoter methylation (5–7). These results suggest that BTG3 plays an important role in tumor suppression, although the underlying mechanisms are unknown.

The DNA damage response (DDR) is critical for cell survival and maintenance of genome stability. Ataxia telangiectasia and Rad3-related (ATR)-CHK1 and ataxia telangiectasia mutated (ATM)-CHK2 are two main signaling axes in the DDR network (8). The ATR-CHK1 pathway responds principally to single-strand DNA generated at the stalled replication fork or in the double-strand break–repair process (9, 10). Upon UV exposure or replication stress, CHK1 is activated through phosphorylation at Ser317 and Ser345 by the ATR kinase (11). CHK1 is a key effector kinase in the DDR; CHK1 phosphorylates an array of substrates and is required for cell cycle checkpoint maintenance and cell survival after certain types of DNA damage (12, 13). For example, CHK1-depleted cells undergo massive cell death after treatment with hydroxyurea, a ribonucleotide reductase inhibitor, or camptothecin (CPT), a DNA topoisomerase I inhibitor (14). In addition, CHK1 functions in the spindle checkpoint to maintain genome stability. CHK1 phosphorylates Aurora B and promotes its activity to enhance (Budding uninhibited by benomyl)-related 1 (BubR1) kinetochore localization after paclitaxel treatment (15, 16). Defects in ATR and CHK1 have been found in human cancers (17–19). Similarly, CHK1 with frameshift mutations was identified in human colon and endometrial cancer samples (20).

In addition to ATR-mediated CHK1 phosphorylation, other modifications that modulate the stability, activity, and cellular localization of CHK1 have also been described. AKT phosphorylates CHK1 at Ser280, which promotes its monoubiquitination and cytosolic localization (21, 22). CHK1 is also polyubiquitinated and degraded after CPT treatment, and such ubiquitination is Cullin (CUL)1 and CUL4 dependent (23). The F box protein (Fbx) 6 was identified as the CHK1 E3 enzyme that ubiquitinates CHK1 and promotes its proteasome-mediated degradation after CPT treatment (24).

In this study, we demonstrate that BTG3 promotes Lys (K) 63-linked ubiquitin chain modification of CHK1 through the cullin-RING ligase (CRL)^{Cdt2} E3 complex, thus increasing CHK1 chromatin localization and activation. We also show that, like CHK1, BTG3 plays an important role in maintaining cell survival after replication block and, importantly, in safeguarding the spindle assembly checkpoint.

Results

BTG3 Is Required for CHK1 Phosphorylation and Activation After Genotoxic Stress. We showed that the G_2/M checkpoint was impaired in BTG3 down-regulated cells (3). To understand the underlying mechanism, we investigated whether the checkpoint kinase CHK1, a key player in the G_2/M checkpoint, is affected by BTG3 ablation. CHK1 activation after UV irradiation was diminished after down-regulation of BTG3 by either of the two different targeting siRNAs in HCT116 cells, as evidenced by decreased CHK1 Ser345 phosphorylation after UV (Fig. 1*A*). The effect was confirmed in a different cell line U2OS (Fig. S1*A*) and by using a different DNA damage agent CPT (Fig. S1*C*). By contrast, overexpression of BTG3 significantly increased CHK1 Ser345 phosphorylation after UV treatment (Fig. 1*B* and Fig. S1*B*). Comparison of the cell cycle profiles indicated that BTG3 down-regulation in HCT116 did not grossly alter the progression of cell cycle before genotoxic stress (Fig. S2), thus ruling out an indirect effect through altering cell cycle distribution. Finally, using in vitro kinase assay with immunoprecipitated CHK1, we confirmed that damage-induced CHK1 activity was markedly reduced in BTG3-depleted cells (Fig. S1*D*). These results point to

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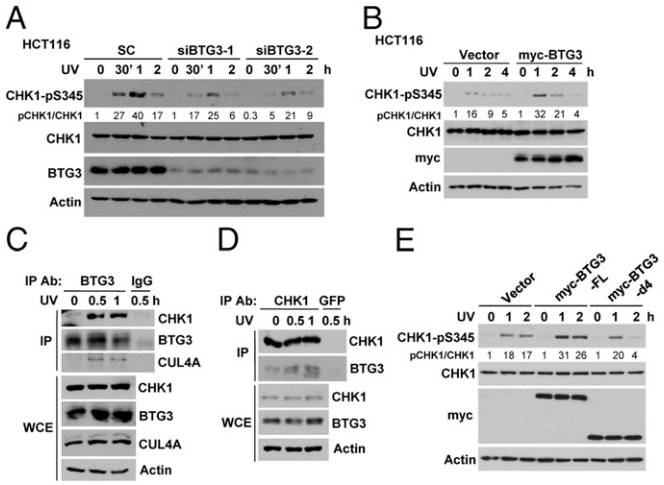

Fig. 1. BTG3 interacts with CHK1 and promotes CHK1 phosphorylation and activation after DNA damage. (*A*) UV-induced CHK1 phosphorylation was diminished in BTG3-down-regulated cells. HCT116 was transfected with control (SC) or indicated BTG3 siRNA, and the levels of CHK1 Ser345 phosphorylation (CHK1-pS345) and CHK1 after UV damage (15 J/m^2) were examined by immunoblotting. (*B*) UV-induced CHK1-pS345 was enhanced by overexpression of BTG3 in HCT116. (*C* and *D*) BTG3 interacts with CHK1 in vivo. HCT116 cells were irradiated or not with UV (15 J/m^2), and soluble extracts were prepared at the time points indicated. The interaction was analyzed by coimmunoprecipitation (co-IP) using anti-BTG3 antibody (*C*) or anti-CHK1 antibody (*D*). Normal IgG or anti-GFP was used as a negative control. (*E*) The interaction-defective d4 mutant could not promote CHK1 phosphorylation in HCT116.

a possibility that BTG3 is directly involved in regulating CHK1 activation after DNA damage.

To determine whether the observed effect of BTG3 on CHK1 was mediated through direct protein interaction, GST pull-down assays were performed. The results showed that the N-terminal domain of CHK1 (amino acids 1–276) interacted mainly with the C-terminal domain of BTG3 (amino acids 147–252) (Fig. S3 *A* and *B*). This interaction did not require the kinase activity of CHK1 because BTG3 also pulled down kinase-defective (KD) CHK1 (D130A) (Fig. S3*C*). In vivo, the interaction between endogenous BTG3 and CHK1 was observed after UV in HCT116 cells by using coimmunoprecipitation with either anti-BTG3 antibody (Fig. 1*C*) or anti-CHK1 antibody (Fig. 1*D*). Furthermore, the BTG3 mutant with the defective interaction domain (d4, amino acids 147–252 deleted) failed to promote CHK1 Ser345 phosphorylation after UV (Fig. 1*E*), supporting the idea that direct protein–protein interaction is required for BTG3 to affect CHK1 phosphorylation and activation.

BTG3 Promotes K63-Linked CHK1 Ubiquitination. During our investigations, we observed multiple slower-migrating forms and a smearing pattern of the full-length CHK1 or the CHK1 N-terminal domain when they were coexpressed with the full-length BTG3 but not with the d4 mutant (Fig. 2*A*). Because CHK1 ubiquitination has been noted (21–23), we speculated that these slower-migrating forms are ubiquitinated CHK1. This possibility was later confirmed by coexpressing CHK1 with BTG3 and His-tagged ubiquitin followed by a nickel-nitrilotriacetic acid (Ni-NTA) bead pull-down assay (Fig. 2*B*). Interestingly, the amount of ubiquitinated CHK1 did not increase further in the presence of the proteasome inhibitor MG132, suggesting that the ubiquitin chains are not K48 linked (Fig. 2*B*). In agreement, substitution of wild-type (WT) ubiquitin with the K48R mutant did not affect the extent of CHK1 ubiquitination in this assay, whereas substitution with the K63R ubiquitin nearly obliterated the ubiquitination (Fig. 2*C*). Ubiquitination was observed only when CHK1 was coexpressed with the full-length BTG3 but not with the interaction-defective d4 mutant (Fig. 2*D*). Taken together, these results demonstrate that interaction is required for BTG3-mediated K63-linked CHK1 ubiquitination.

To verify the existence of K63-linked CHK1 ubiquitination in cells, endogenous CHK1 was immunoprecipitated from cells treated with or without UV, and ubiquitination was determined by immunoblot using linkage-specific ubiquitin antibody. The result shown in Fig. 2*E* demonstrates that UV induces K63-linked ubiquitination of CHK1. Importantly, this modification of CHK1 was abrogated in BTG3-knockdown cells. Notably, we also found that the chromatin-associated CHK1 was proportionally more ubiquitinated than the CHK1 in soluble fractions (evidenced by the K63-Ub/CHK1 ratio) (Fig. 2*E*), suggesting a possible role of the specific modification in the regulation of CHK1 cellular localization after UV.

BTG3-Mediated CHK1 Ubiquitination Depends on the CRL4^{Cdt2} E3 Complex. Ubiquitination and degradation of CHK1 requires CUL1 or CUL4, and CHK1 interacts with CUL1 and CUL4 after DNA damage-inducing treatment (23–25). Interestingly, BTG3-promoted CHK1 ubiquitination was markedly reduced upon siRNA-mediated down-regulation of CUL4A (Fig. 3*A*) and, to

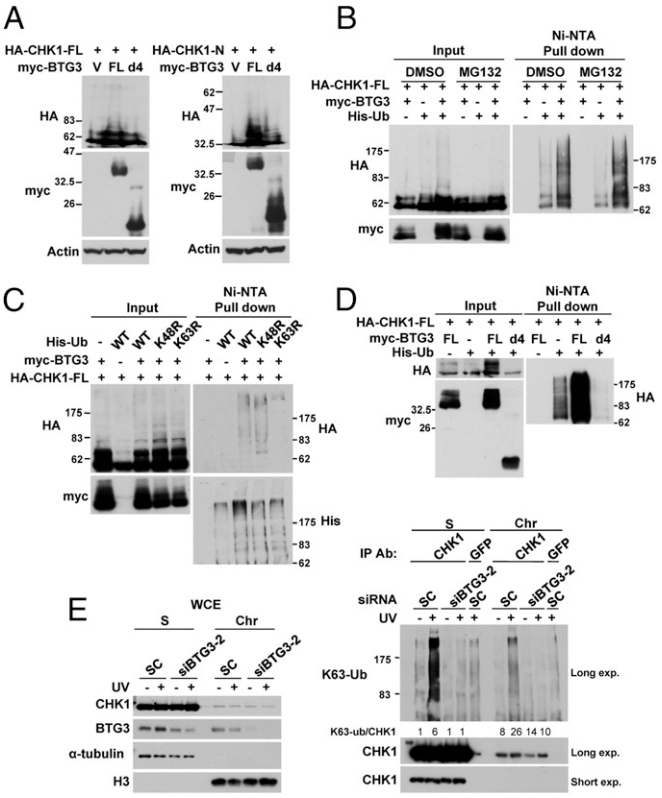

Fig. 2. BTG3 promotes K63-linked polyubiquitination of CHK1. (*A*) Coexpression of BTG3-FL but not BTG3-d4, results in the appearance of multiple slower-migrating forms of CHK1 in 293T cells. (*B*) BTG3-mediated CHK1 ubiquitination is not targeted for proteasome degradation. HA-CHK1, myc-BTG3, and His-ubiquitin (His-Ub) were coexpressed in 293T cells. Cells were treated with or without 25 μM MG132 for 3 h before collection. A Ni-NTA bead pull-down assay was used to detect CHK1 ubiquitination. (*C*) BTG3-mediated CHK1 ubiquitination is mainly K63-linked. The in vivo ubiquitination assay was performed as described above by using WT, K48R, or K63R ubiquitin. (*D*) BTG3-FL but not BTG3-d4 promotes CHK1 ubiquitination. (*E*) UV-induced K63-linked ubiquitination of CHK1 requires BTG3. HCT116 cells were first transfected with scramble control (SC) or BTG3-2 siRNA and the next day with His-Ub for 24 h. Cells were treated with 15 J/m^2 UV 1 h before collection. CHK1 was immunoprecipitated from the soluble (S) or chromatin (Chr) fractions, and ubiquitinated CHK1 was detected with the K63-linked-specific ubiquitin antibody.

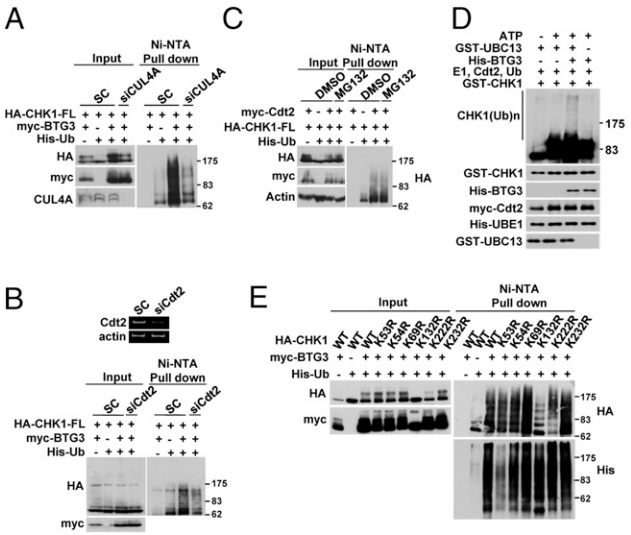

Fig. 3. BTG3-mediated CHK1 ubiquitination depends on the $CRL4^{Cdt2}$ E3 complex. (*A* and *B*) CUL4A and Cdt2 are required for BTG3-mediated CHK1 ubiquitination. The in vivo ubiquitination assay was performed in 293T cells transfected with siRNA targeting CUL4A (*A*) or Cdt2 (*B*). (*C*) CHK1 ubiquitination was increased by overexpressed Cdt2 in 293T cells. (*D*) CHK1 ubiquitination assembled in vitro. Cdt2 immunoprecipitated from transfected 293T cells mediates UBC13-dependent CHK1 ubiquitination in vitro. (*E*) Mutation of K132 to R markedly reduced BTG3-promoted CHK1 ubiquitination. The 293T cells were transfected with various CHK1 KR mutants, and in vivo ubiquitination was assessed as described above.

a lesser extent, of CUL4B (Fig. S4*A*), suggesting that BTG3-mediated CHK1 ubiquitination also depends on CUL4, especially CUL4A. Of note, depletion of CUL1 did not appear to affect BTG3-promoted CHK1 ubiquitination (Fig. S4*B*).

DNA damage-binding protein 2 (DDB2) and chromatin licensing and DNA replication factor 2 (Cdt2) are two known substrate receptors that associate with CRL4 in DDR, DNA replication, and DNA repair (26). Significantly, knockdown of Cdt2 but not DDB2 resulted in reduced BTG3-mediated CHK1 ubiquitination (Fig. 3*B* and Fig. S4*C*). Conversely, overexpression of Cdt2 increased CHK1 ubiquitination, which was not increased further by the proteasome inhibitor MG132 (Fig. 3*C*). In vitro with purified components, immunoprecipitated Cdt2 could ubiquitinate GST-CHK1 in a ubiquitin-conjugating enzyme (UBC) 13- and BTG3-dependent manner, indicating that the $CRL4^{Cdt2}$ complex directly mediated the ubiquitination (Fig. 3*D*). In support, the GST-fused BTG3 C-terminal domain pulled down CUL4A and Cdt2 in cell lysates (Fig. S5 *A* and *B*), suggesting that BTG3 could interact directly with the $CRL4^{Cdt2}$ complex through the C-terminal domain. In vivo, UV treatment enhanced the interaction between endogenous BTG3 and CUL4A (Fig. 1*C*) and between coexpressed BTG3 and Cdt2 (Fig. S5*C*). Furthermore, CHK1 interacted directly with Cdt2 in vitro (Fig. S5*D*), and their interaction in cells was transiently enhanced after UV (Fig. S5*E*). Importantly, GST-UBC13, the E2 involved in the synthesis of K63-linked ubiquitin chain, could pull down CUL4A in cell lysates, indicating a possible direct association of UBC13 with the CRL4 complex (Fig. S5*F*). Taken together, these results physically link the involvement of the $CRL4^{Cdt2}$ complex in BTG3-mediated, K63-linked CHK1 ubiquitination.

Next, we sought to identify the ubiquitinated residues in CHK1. Of the six lysine (K) residues within the N-terminal domain of CHK1 investigated, only the mutation of K132 to arginine (R) reproducibly affected CHK1 ubiquitination (Fig. 3*E*), suggesting that K132 is the major ubiquitination site promoted by BTG3.

Nuclear Localization of CHK1 After UV Irradiation Is Impaired in BTG3-Depleted Cells. Unlike the K48-linked ubiquitin chain that mediates protein degradation, the K63-linked ubiquitin chain functions to dictate the cellular localization, stability, and modification of proteins (27). Because CHK1 activation by DNA damage was regulated by BTG3 (Fig. 1), and BTG3-dependent ubiquitination was also detected in CHK1 resided on chromatin (Fig. 2*E*), we next investigated the functional link between these observations.

As shown in Fig. 4*A*, endogenous CHK1 localized mainly in the cytoplasm in control untreated U2OS cells, and its nuclear distribution increased after UV irradiation. Similarly, this change of distribution was observed with ectopically expressed HA-tagged CHK1 in HeLa cells (Fig. S6*A*). Of note, *Schizosaccharomyces pombe* Chk1 has also been shown to accumulate in the nucleus after CPT treatment (28). Significantly, this nuclear shuttling was diminished in BTG3 knockdown cells (Fig. 4 *A* and *B*). Conversely, induction of BTG3 expression in stable Tet-On U2OS cells caused enhanced nuclear retention of CHK1 after UV irradiation (Fig. 4 *D* and *E*), which was not observed in control vector-transfected stable cells (Fig. S6 *B* and *C*). Of note, pretreating cells with leptomycin B, the CHK1 nuclear export inhibitor, although enhanced the nuclear presence of CHK1, did not abolish the effect of either BTG3 down-regulation (Fig. 4*C*) or overexpression (Fig. 4*F*), suggesting BTG3 did not act on preventing CHK1 nuclear export. In addition, cell fractionation using BTG3 and CHK1 cotransfected 293T cells revealed increased chromatin association of CHK1 upon coexpression of BTG3 (Fig. 4*G*). Interestingly, most of the ubiquitinated form of CHK1 was found in the chromatin fraction. Taken together, these data strongly support a role of BTG3 in regulating CHK1 cellular distribution after DNA damage.

To determine whether the effect of BTG3 on CHK1 was mediated through ubiquitination, the cellular distribution of CHK1 was compared between WT CHK1 and the K132R mutant deficient in BTG3-dependent ubiquitination. Unlike WT CHK1, the K132R mutant, which by itself bound chromatin less efficiently, exhibited no significant increase in chromatin association when coexpressed with BTG3 (Fig. 4*G*). This absence of enhancement was not because the K to R mutation disrupts the interaction with BTG3, because the K132R mutant could be coimmunoprecipitated with BTG3 (Fig. S7*A*). Because the K132R mutant was inherently kinase-inactive (*Discussion* and Fig. S7*B*), we also determined whether the reduced chromatin association was due to the loss of the kinase activity. This possibility was subsequently eliminated because the D130A KD mutant could be ubiquitinated (Fig. S7*C*), and its chromatin association could be increased by BTG3 (Fig. 4*G*). In line with its reduced chromatin association, UV-induced Ser345 phosphorylation was impaired in the K132R CHK1 mutant compared with WT CHK1 (Fig. 4*H*).

To determine whether phosphorylation at Ser345 or Ser317 would, in turn, regulate K63-linked ubiquitination and chromatin association, the double A mutant (AA) in which both residues were mutated to alanine were coexpressed with BTG3. Our results showed that ubiquitination and chromatin association of the AA mutant was similarly promoted by BTG3 (Fig. S7 *D*–*F*), suggesting that phosphorylation at Ser317 or Ser345 is not involved in these events.

Taken together, these results support the idea that BTG3-mediated K132 ubiquitination promotes CHK1 chromatin association and activation.

BTG3 Is Required for Maintaining Cell Survival After Prolonged Replication Block. Based on our understanding of the role of CHK1 in replication stress, we next examined the survival of BTG3-knockdown cells after replication block. Similar to CHK1-depleted cells, which displayed massive cell death, BTG3–down-regulated cells showed an increase in the sub-G_1 population after prolonged low-dose CPT treatment (Fig. 5*A*). Cell death could be rescued by reexpression of siRNA-resistant full-length BTG3 but not by the d4 mutant that cannot bind and promote CHK1 ubiquitination (Fig. 5*B*). These results provide a functional link

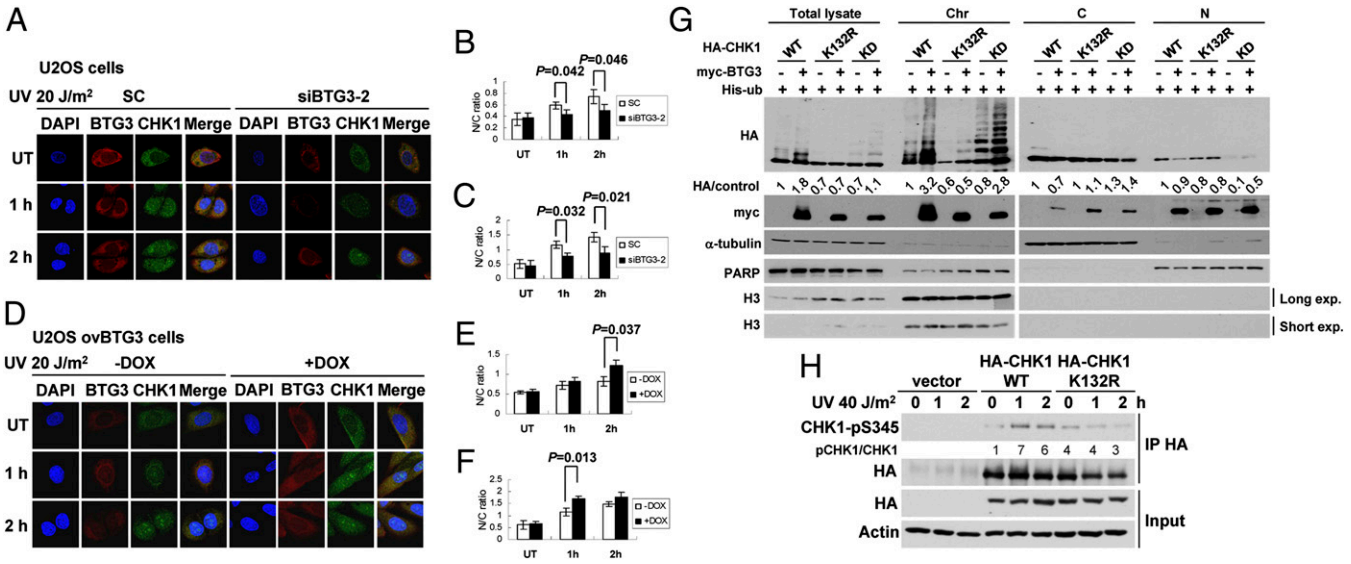

Fig. 4. BTG3 promotes CHK1–chromatin association. (*A–C*) CHK1 nuclear localization after UV damage was diminished in BTG3-depleted cells. BTG3-2 or control siRNA (sc)-transfected U2OS cells were pretreated or not with 5 ng/mL leptomycin B (LMB) for 3 h before UV irradiation. CHK1 cellular localization was analyzed by immunofluorescence microscopy after staining with anti-CHK1 and anti-BTG3 antibodies (*A*). The CHK1 intensity in the nucleus (N) and the cytosol (C) was quantified, and the N/C ratio is shown in *B* without LMB treatment or in *C* with LMB treatment. Images in *A* are without LMB pretreatment. (*D–F*) Overexpression of BTG3 promotes CHK1 nuclear localization after UV damage. Stable Tet-On U2OS cells were induced to express BTG3 with doxycycline (Dox) for 24 h, pretreated or not with 5 ng/mL LMB for 3 h, and then irradiated with UV. CHK1 localization was analyzed as in *A*. The N/C ratio of CHK1 is shown in *E* without LMB treatment or in *F* with LMB treatment. Images in *D* are without LMB treatment. (*G*) BTG3 promotes chromatin association of WT CHK1 and KD CHK1 (D130A) but not CHK1-K132R. Cell fractionation was performed with 293T cells transfected with myc-BTG3, His-Ub, and indicated HA-CHK1 constructs. PARP1, α-tubulin, and histone H3 were used as nuclear, cytosolic, and chromatin markers, respectively. Values underneath the HA image represent quantified HA-CHK1 signals normalized to α-tubulin (for total lysates and cytosolic fractions), Poly (ADP-ribose) polymerase (PARP) 1 (nuclear soluble fractions), or histone H3 (for chromatin fractions). (*H*) UV-induced CHK1-pS345 was diminished in CHK1-K132R. HA-CHK1 was immunoprecipitated from transfected 293T cells, and CHK1-pS345 was detected by Western blotting.

between BTG3 and CHK1 in maintaining cell survival after replication block. Note that rescue with either WT CHK1 or CHK1 K132R in CHK1 knockdown cells was not feasible because the K132R mutant is catalytically inactive (Fig. S7*B*).

BTG3 and Cdt2 Act in the Same Pathway as CHK1 in Safeguarding Mitotic Progression. CHK1 has been implicated in the mitotic checkpoint by ensuring proper kinetochore attachment of the mitotic spindle through regulation of Aurora B and BubRI (16). Misaligned and lagging chromosomes, cytokinetic regression, or even fragmented nuclei have been reported in CHK1-depleted cells (15, 29, 30). To provide biological evidence that BTG3 regulates the activity of CHK1 in mitotic checkpoint, we first determined whether CHK1 is modified by K63-linked ubiquitin chain in mitosis-arrested cells. As shown in Fig. 6*A*, K63-linked CHK1 ubiquitination was induced by paclitaxel and was detected in both soluble and chromatin fractions, although by the K63-Ub/CHK1 ratio, preferentially associated with the latter. Significantly, such enhancement was abrogated upon depletion of BTG3 (Fig. 6*A*). Consistently, the interaction between BTG3 and CHK1 in cells was increased upon paclitaxel treatment (Fig. 6*B*). We then explored the role of BTG3 in safeguarding mitotic progression. After paclitaxel treatment, down-regulation of BTG3 phenocopied CHK1 depletion in inducing mitotic slippage or early mitotic exit and cytokinesis defect (Fig. 6*C*). This abnormality was accompanied by reduced levels of cyclin B1 and phospho-H3 (Fig. 6*D*). As a result, the multinucleated population also increased significantly in BTG3- or CHK1-depleted cells, indicating impaired mitotic checkpoint in these cells (Fig. 6*E*).

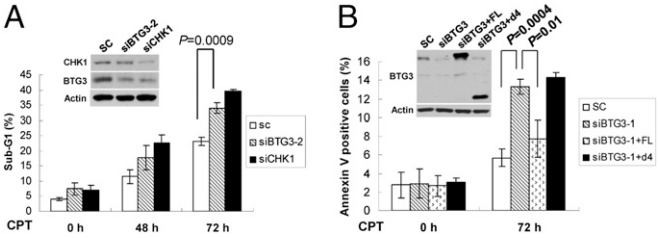

Fig. 5. BTG3 is required for maintaining cell survival after replication block. (*A*) BTG3 or CHK1 depletion increases cell death after prolonged CPT treatment. HCT116 cells transfected with indicated siRNA were treated or not with 20 nM CPT for the indicated time and analyzed by flow cytometry. Quantification of results from three independent experiments (mean ± SD) is shown. *Inset* is an immunoblot showing the extent of BTG3 and CHK1 down-regulation. (*B*) Full-length (FL) BTG3 but not the CHK1-binding defective d4 mutant reverses the apoptosis phenotype induced upon BTG3 depletion. Rescue experiments were performed with siRNA-resistant full-length BTG3 or the d4 construct. Cells were treated with CPT for 72 h and then stained with Annexin V-PE to detect apoptotic cells.

Next, we examined whether kinetochore attachment was intact in these cells. Cells were first treated with monastrol, an inhibitor of the kinesin Eg5 that blocks spindle bipolarity. Upon removal of the drug, a bipolar spindle was assembled with properly aligned chromosomes in control siRNA-transfected cells, whereas misaligned and lagging chromosomes were observed in BTG3- or CHK1-depleted cells (Fig. 7 *A* and *B* and Fig. S8*A*). These results provide evidence that, like CHK1, BTG3 contributes to the proper maintenance of the spindle checkpoint.

If BTG3 regulates CHK1 in the spindle checkpoint, we would expect that CHK1 downstream effector such as BubRI would be dysfunctional in BTG3 knockdown cells because of failure in the CHK1-mediated Aurora B activation (16). Consistently, BubRI kinetochore localization (Fig. S9 *A* and *B*) and its phosphorylation (Fig. S9*C*) were impaired in BTG3-depleted, mitosis-arrested cells, thus providing a mechanistic explanation for the observation.

In addition, we conducted rescue experiments with either siRNA-resistant WT BTG3 or the d4 mutant that is impaired in binding CHK1 and is unable to promote CHK1 ubiquitination. As a result, only the full-length BTG3, and not the d4 mutant,

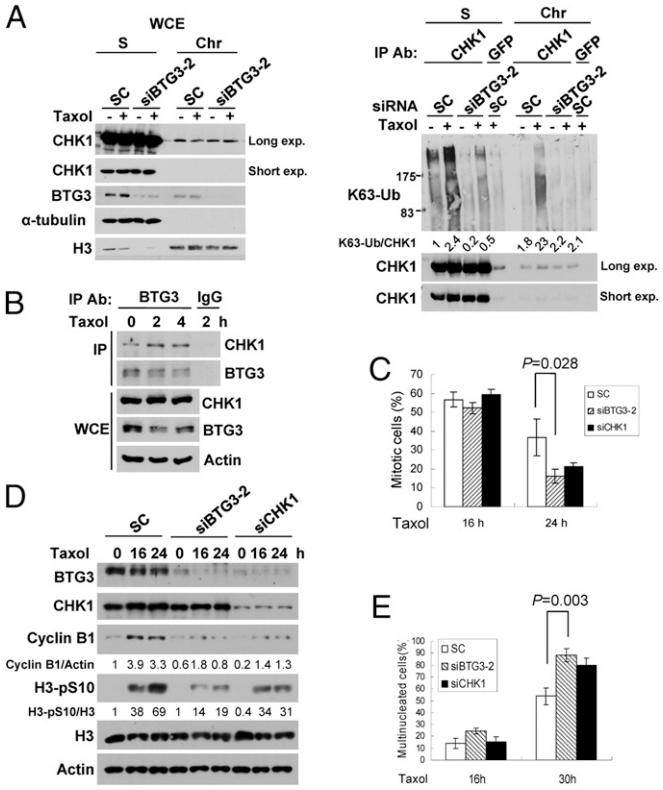

Fig. 6. Impaired mitotic checkpoint in BTG3-depleted cells. (*A*) Paclitaxel-induced K63-linked ubiquitination of CHK1 requires BTG3. HCT116 cells were first transfected with scramble control (SC) or BTG3-2 siRNA and the next day with His-Ub for 24 h. Cells were treated with 100 nM paclitaxel for 2 h before harvest. CHK1 was immunoprecipitated from the soluble (S) or chromatin (Chr) fractions, and ubiquitinated CHK1 was detected with the K63-linked–specific ubiquitin antibody. (*B*) Paclitaxel enhances the interaction between endogenous CHK1 and BTG3. (*C*–*E*) Down-regulation of BTG3 or CHK1 induces mitotic slippage or early mitotic exit. HCT116 cells transfected with BTG3 or CHK1 siRNA were treated with 10 μM paclitaxel and collected at the time points indicated. Cells were stained with DAPI, and percent cells with condensed chromosomes (mitotic cells) are shown in *C*. The lysates were analyzed by immunoblotting and are shown in *D*. Multinucleated cells (indicated by arrows), revealed by staining with DAPI and fluorescence microscopy, were counted, and the result is shown in *E*.

could rescue the mitotic defect (Fig. 7*C* and Fig. S8*B*), thus lending further support to the functional link between BTG3 and CHK1.

As CHK1 knockdown also caused down-regulation of BTG3 possibly through reduced p53 activity (Figs. 5*A* and 6*D*; ref. 3), a question arose regarding whether the defect observed in CHK1-depleted cells was due to reduced BTG3. This possibility was ruled out because ectopically expressed BTG3 could not correct the mitotic defect observed in CHK1–down-regulated cells (Fig. S10), suggesting BTG3 acts upstream of CHK1 in the mitotic checkpoint.

If the mitotic function of BTG3 is mediated through promoting $CRL4^{Cdt2}$-dependent CHK1 ubiquitination, one would expect that down-regulation of Cdt2 should result in a similar mitotic defect. Indeed, we observed a similar increase in chromosome misalignment and segregation defect in Cdt2-depleted mitotic cells (Fig. 7*D* and Fig. S8 *C* and *D*), further implicating a role of Cdt2 and possibly the $CRL4^{Cdt2}$ complex in the functional interplay between CHK1 and BTG3.

Discussion

The involvement of BTG3 in CHK1 ubiquitination is both interesting and unexpected. Apart from the N-terminal domain encompassing box A and box B that are conserved across the BTG family, no significant homology suggestive of its connection with ubiquitination was found. However, we demonstrated the functional interplay among BTG3, CHK1, and $CRL4^{Cdt2}$. As suggested in our model (Fig. 7*E*) and supported by our data, it appears that genotoxic stress promotes the interaction and K63-linked ubiquitination of CHK1. The ubiquitination enhances CHK1 association with chromatin (Figs. 2*E*, 4*G*, and 6*A*), which would in theory promote its phosphorylation and activation by ATR (upon DNA damage) or other upstream kinase (upon spindle disruption). At present, we do not know whether ubiquitination occurs before CHK1 localization to chromatin to promote its chromatin association or after its localization to stabilize its association with chromatin. Our observation that BTG3, CHK1, and Cdt2 can be coimmunoprecipitated from the soluble fractions (Fig. 1 *C* and *D* and Fig. S5 *C* and *E*) seems to support the former although does not exclude the latter possibility.

In this study, we also observed that DNA damage induces transient movement of endogenous CHK1 from the cytoplasm into the nucleus in U2OS cells (Fig. 4*A*) and, in addition, in HeLa cells (Fig. S6*A*). This observation is in conflict with the observations made by other groups, for example, Wang et al. (31), with ectopically expressed CHK1 that showed persistent nuclear localization. We speculate that the discrepancy may result from the

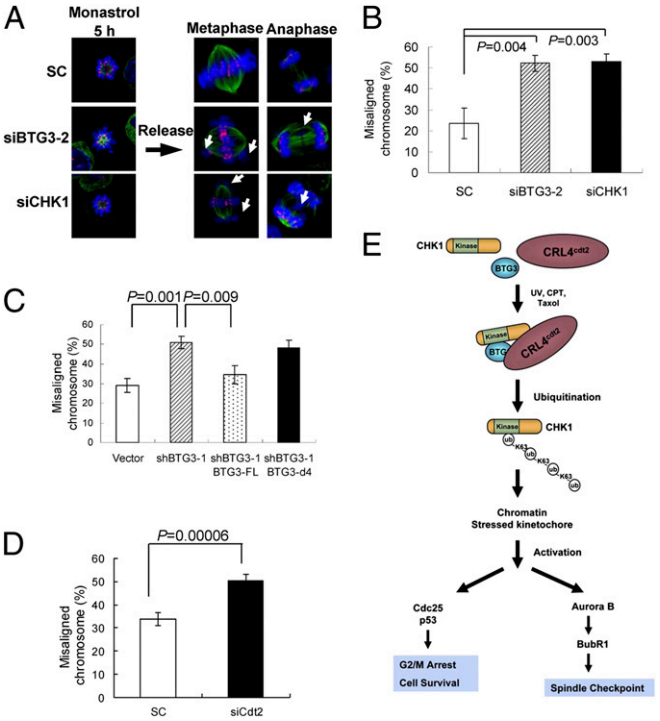

Fig. 7. BTG3 and Cdt2 are essential for proper chromosome alignment and segregation. (*A* and *B*) Knockdown of BTG3 or CHK1 promotes chromosome misalignment and missegregation. HCT116 cells transfected with control, BTG3-2, or CHK1 siRNA were treated with 68 μM monastrol for 5 h and then released. The populations with misaligned chromosomes at metaphase or lagging chromosomes at anaphase (indicated by white arrows) were analyzed by DAPI staining and immunofluorescence by using anti–α-tubulin (green) and anti-CENP-B (red) antibodies (*A*). Quantitative results from three independent experiments are shown in *B* and Fig. S8*A*. (*C*) BTG3-FL but not BTG3-d4 corrects the chromosome alignment defect observed in BTG3-depleted HCT116 cells. Quantitative results showing misaligned chromosomes are displayed ($n = 3$). Results on lagging chromosomes are shown in Fig. S8*B*. (*D*) Depletion of Cdt2 promotes chromosome misalignment and missegregation in HCT116 cells. Additional results on lagging chromosomes are displayed in Fig. S8 *C* and *D*. (*E*) Model depicting the role of the BTG3-mediated, K63-linked ubiquitination in CHK1 activation.

difference between endogenous CHK1 and ectopically expressed protein and also in the level of expression for the latter, as we also observed in HA-CHK1–transfected cells that cells expressing higher levels of CHK1 tend to retain the protein in the nucleus. Of note, Li et al. (32) also observed cytoplasmic localization of CHK1 in RPE1 cells before serum stimulation.

How CHK1 is activated in M phase remains an enigma, although phosphorylation was shown to be involved (16). The mitotic defects we saw in BTG3-depleted cells bear striking resemblance to those observed in CHK1-deficient cells (Figs. 6 and 7 and Fig. S8). Because these defects could be rescued by WT BTG3 but not by the d4 mutant impaired in CHK1 interaction, we reason that BTG3 is also required for proper CHK1 function upon spindle disruption. It is possible that binding of BTG3 and, consequently, CHK1 ubiquitination upon spindle disruption may promote the activation of CHK1 by its upstream activator. The K63-linked ubiquitin chain on CHK1 may serve as a recognition module or protein assembly platform. Of note, survivin, a component of the chromosome passenger complex, is modified by K63-linked ubiquitination, and such modification is essential for its kinetochore localization and function in chromosome alignment and segregation (33). One cannot but be intrigued by a potential general role of the K63-linked ubiquitin chain in the assembly of a functional kinetochore checkpoint complex.

Our study also raises an issue regarding the activity of CHK1 modified with an ubiquitin chain at K132. The crystal structure solved by Chen et al. (34) suggests that the side chains of D130, K132, and N135 are essential for the kinase active site. Therefore, one would predict that a substitution (such as the K132R mutant) or a bulky ubiquitin chain at K132 will disrupt the CHK1 active site and render the kinase inactive. It is likely that once the chromatin-associated K132-ubiquitinated CHK1 is phosphorylated, it would need to be deubiquitinated at K132 to be active. It would be interesting to know whether a specific deubiquitinase is involved to fully activate CHK1.

Nevertheless, the physiological consequence of the loss of BTG3 expression is evident. The failure in G_2/M arrest (3) leads to abnormal cell division; the defective spindle checkpoint characterized here causes polyploidy and possibly aneuploidy, which are often associated with the development of cancer (35). Although these defects can be functionally linked to impaired CHK1 activation, our study does not exclude the possibility that events involving additional BTG3 targets may together contribute to the observed abnormalities. Identification of these targets would certainly provide a more complete understanding as to how BTG3 functions as a tumor suppressor.

Materials and Methods

Detailed protocols regarding cell treatment, immunoblotting, immunoprecipitation, GST pull-down, cell fractionation, in vitro and in vivo ubiquitination, flow cytometry, immunofluorescence, and information regarding expression vectors, and antibodies can be found in *SI Materials and Methods*. Sequences targeted by siRNAs are listed in Table S1. Dosage and concentration of drugs and treatment are indicated in respective figure legends.

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