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SHORT REPORT

Chk1 protects against chromatin bridges by constitutively phosphorylating BLM serine 502 to inhibit BLM degradation

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ABSTRACT

Chromatin bridges represent incompletely segregated chromosomal DNA connecting the anaphase poles and can result in chromosome breakage. The Bloom's syndrome protein helicase (BLM, also known as BLMH) suppresses formation of chromatin bridges. Here, we show that cells deficient in checkpoint kinase 1 (Chk1, also known as CHEK1) exhibit higher frequency of chromatin bridges and reduced BLM protein levels compared to controls. Chk1 inhibition leads to BLM ubiquitylation and proteasomal degradation during interphase. Furthermore, Chk1 constitutively phosphorylates human BLM at serine 502 (S502) and phosphorylated BLM localises to chromatin bridges. Mutation of S502 to a non-phosphorylatable alanine residue (BLM-S502A) reduces the stability of BLM, whereas expression of a phospho-mimicking BLM-S502D, in which S502 is mutated to aspartic acid, stabilises BLM and prevents chromatin bridges in Chk1-deficient cells. In addition, wild-type but not BLM-S502D associates with cullin 3, and cullin 3 depletion rescues BLM accumulation and localisation to chromatin bridges after Chk1 inhibition. We propose that Chk1 phosphorylates BLM-S502 to inhibit cullin-3-mediated BLM degradation during interphase. These results suggest that Chk1 prevents deleterious anaphase bridges by stabilising BLM.

KEY WORDS: Chk1, BLM, Mitosis, Cullin 3, Chromatin bridge

INTRODUCTION

Failure to eliminate replication or recombination intermediates can lead to chromatin bridges in anaphase (i.e. strings of chromatin connecting the two segregating masses of chromosomes) (Chan et al., 2007; Wyatt et al., 2013; Germann et al., 2014). If unresolved, chromatin bridges can be fragmented during furrow ingression in cytokinesis and contribute to genetic instability in cancer cells (Hoffelder et al., 2004).

The Bloom's syndrome protein helicase (BLM, also known as BLMH) unwinds double-stranded DNA (Chu and Hickson, 2009). BLM suppresses homologous recombination (Wang et al., 2000) and, in a complex with topoisomerase III, promotes the dissolution of double Holliday junctions to produce non-crossover homologous recombination products (Karow et al., 2000; Wu and Hickson, 2003; Raynard et al., 2006). It has been proposed that BLM suppresses chromatin bridges by inhibiting homologous recombination initiated from hemicatenated or unreplicated DNA and also by localising, together with the helicase PICH (also known as ERCC6L), to chromatin bridges to unravel chromatin and promote bridge resolution (Chan et al., 2007; Ke et al., 2011; Germann et al., 2014).

Checkpoint kinase 1 (Chk1, also known as CHEK1), a well-established effector of the DNA damage and DNA replication responses (Zhang and Hunter, 2014), is also required for faithful chromosome segregation in vertebrate cells (Zachos et al., 2007; Peddibhotla et al., 2009). Chk1 phosphorylates Aurora B S331 to induce Aurora B catalytic activity and promote correction of mis-attached kinetochores (Petsalaki et al., 2011; Petsalaki and Zachos, 2013). Chk1 also phosphorylates BLM S646 to increase BLM localisation to promyelocytic leukaemia protein (PML) nuclear bodies (Kaur et al., 2010) and regulates BLM accumulation during replication stress through an undescribed mechanism (Sengupta et al., 2004). In the present study, we show that Chk1 protects against chromatin bridges by constitutively phosphorylating BLM at the newly identified phosphorylation site S502 to prevent its cullin-3-mediated degradation.

RESULTS AND DISCUSSION

Chk1 prevents chromatin bridges

Human colon carcinoma BE cells transfected with Chk1 siRNA (siChk1) or treated with the selective Chk1 inhibitor UCN-01 exhibited increased the proportion of anaphases with chromatin bridges (11.8% or 13.7%, respectively) compared to controls transfected with negative siRNA (2.7%) (Fig. 1A,B). Transfection of BLM siRNA (siBLM) also increased the frequency of anaphases with chromatin bridges (13.6%) compared to controls (Fig. 1B; supplementary material Fig. S1A).

BLM localised across the length or near the midpoint of chromatin bridges in control cells (Fig. 1C, supplementary material Fig. S1B). In contrast, Chk1-depleted cells exhibited impaired BLM staining on chromatin bridges compared to controls (Fig. 1C). PICH is required for BLM recruitment to anaphase bridges (Ke et al., 2011); however, in control and Chk1-deficient cells, PICH was localised to chromatin bridges (supplementary material Fig. S1C). These results show that Chk1 prevents chromatin bridges and is required for BLM localisation to anaphase bridges.

Chk1 promotes BLM protein stability

Chk1-deficient cells had reduced BLM protein levels (by 80–90%) in the absence of drugs (untreated), and after treatment with nocodazole (to enrich for mitotic cells) or hydroxyurea (HU; to enrich for S phase cells) compared to controls (Fig. 1D). Treatment of cells with the proteasome inhibitor MG132 rescued BLM protein levels after Chk1 inhibition by UCN-01, and induced accumulation of poly-ubiquitylated BLM (Fig. 1E,F). These

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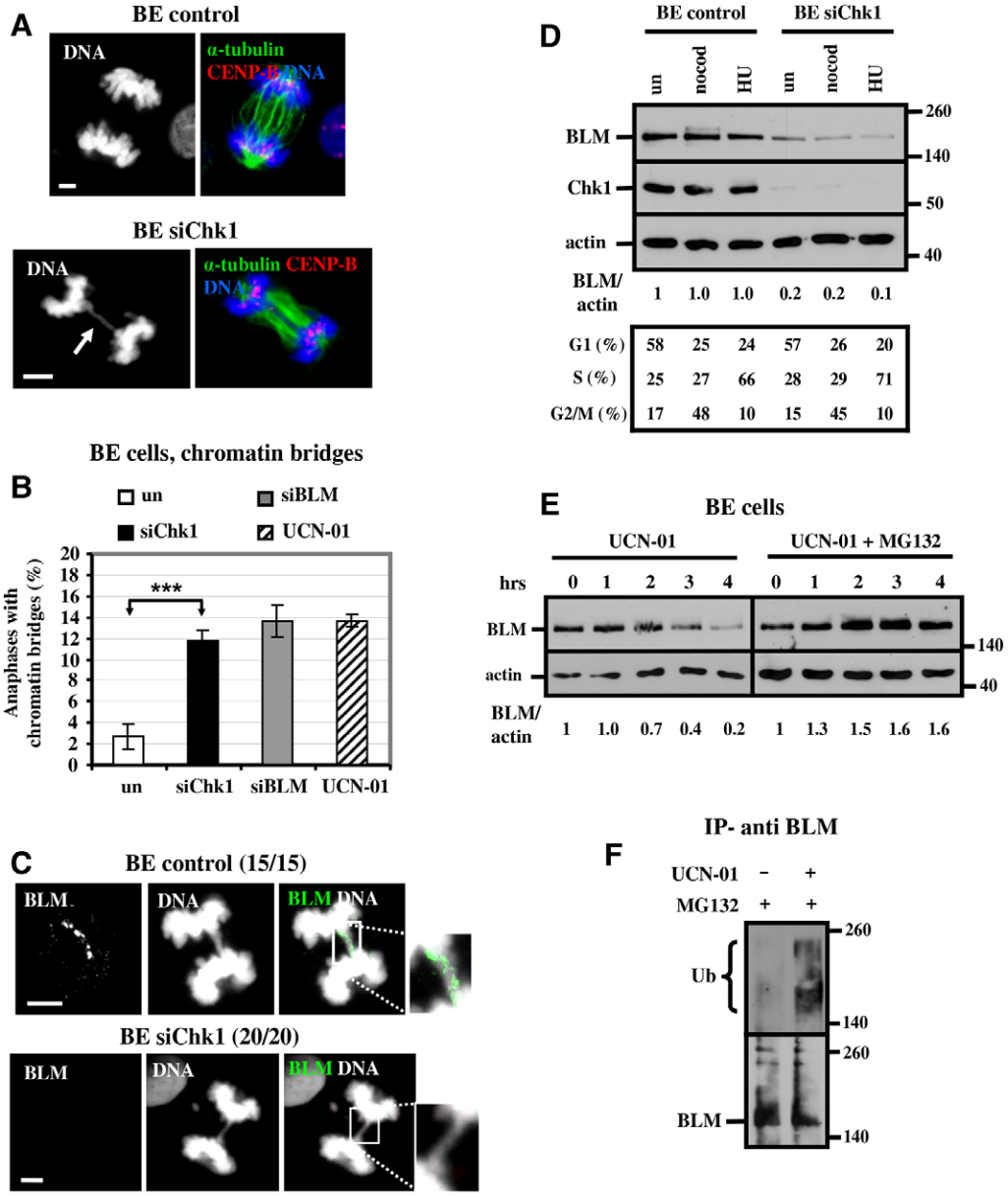

Fig. 1. Chk1 prevents chromatin bridges. (A) BE cells were transfected with negative siRNA (control) or Chk1 siRNA (siChk1). A chromatin bridge is indicated by arrow. (B) Frequencies of anaphases with chromatin bridges. Cells were untreated (un), transfected with Chk1 or BLM siRNA (siBLM), or treated with UCN-01 for 8 h. Results are mean±s.d. from three independent experiments. A minimum of 100 anaphases were analysed per experiment. ***$P<0.001$. (C) BLM localisation to chromatin bridges in cells transfected as in A. The frequency of cells exhibiting the respective phenotype is shown. Insets show magnified chromatin bridges. Scale bars: 5 µm. (D) Top: western blot analysis of total BLM, Chk1 or actin in cells transfected as in A and treated with nocodazole (nocod) or hydroxyurea (HU) for 16 h, or left untreated (un). Numbers below the blot show the relative level of BLM, normalized to actin, compared to that in the control (set as 1). Bottom: percentage of cells in G1, S or G2/M as determined by flow cytometry. (E) Total BLM or actin in cells treated with UCN-01 in the absence or presence of MG132. Numbers below the blot show the relative level of BLM, normalized to actin, compared to that at 0 h (set as 1). (F) Western blot analysis of immunoprecipitated (IP) BLM or associated ubiquitin (Ub) in cells expressing Myc–ubiquitin and treated with MG132 plus or minus UCN-01 for 8 h.

results indicate that Chk1 protects BLM from proteasomal degradation in different phases of the cell cycle.

Phosphorylation at S502 stabilises BLM

To investigate whether Chk1 stabilises BLM through phosphorylation, GST–BLM fusion proteins spanning amino acids 9–474, 469–679 or 674–1417 of human BLM were used as substrates in Chk1 *in vitro* kinase assays. GST–BLM-(469–679) was radiolabeled at levels ~20-fold higher compared to the other two GST–BLM substrates (Fig. 2A–C; supplementary material Fig. S1D). Phosphorylated GST–BLM-(469–679) was analysed by liquid chromatography-mass spectrometry and BLM-S502, -S499, -S517 and -S646 were identified as major Chk1-phosphorylation sites based on the number of times the phosphopeptides were selected for mass spectrometry versus the dephosphorylated peptides (supplementary material Fig. S1E–G). In the absence of the Chk1 inhibitor UCN-01, cells expressing full-length BLM fused to GFP harboring the non-phosphorylatable mutation S502A (GFP–BLM-S502A) had reduced GFP–BLM levels compared to those expressing wild-type (WT), S499A, S517A or S646A GFP-tagged proteins (Fig. 2D), suggesting that S502 phosphorylation is required for BLM accumulation. In addition, UCN-01-treatment did not further reduce GFP–BLM-S502A levels compared to cells without UCN-01 (Fig. 2D), indicating that S502 phosphorylation mediates BLM regulation by Chk1. In addition, the S502A mutation reduced phosphorylation of GST–BLM-(469–679) by Chk1 *in vitro* by ~25% compared to the wild-type fragment (supplementary material Fig. S1H).

GFP–BLM-S502A exhibited reduced protein stability in the presence of cycloheximide (CHX, an inhibitor of protein synthesis) compared to wild-type or the phospho-mimicking GFP–BLM-S502D mutant (Fig. 2E; supplementary material Fig. S2A). Furthermore, GFP–BLM-S502D accumulated to similar levels in the absence or presence of Chk1 inhibitor (Fig. 2F). These results show that Chk1 phosphorylates BLM-S502 to stabilise BLM protein.

Endogenous Chk1 associated with BLM in untreated and nocodazole-treated cells (Fig. 2G; supplementary material Fig.

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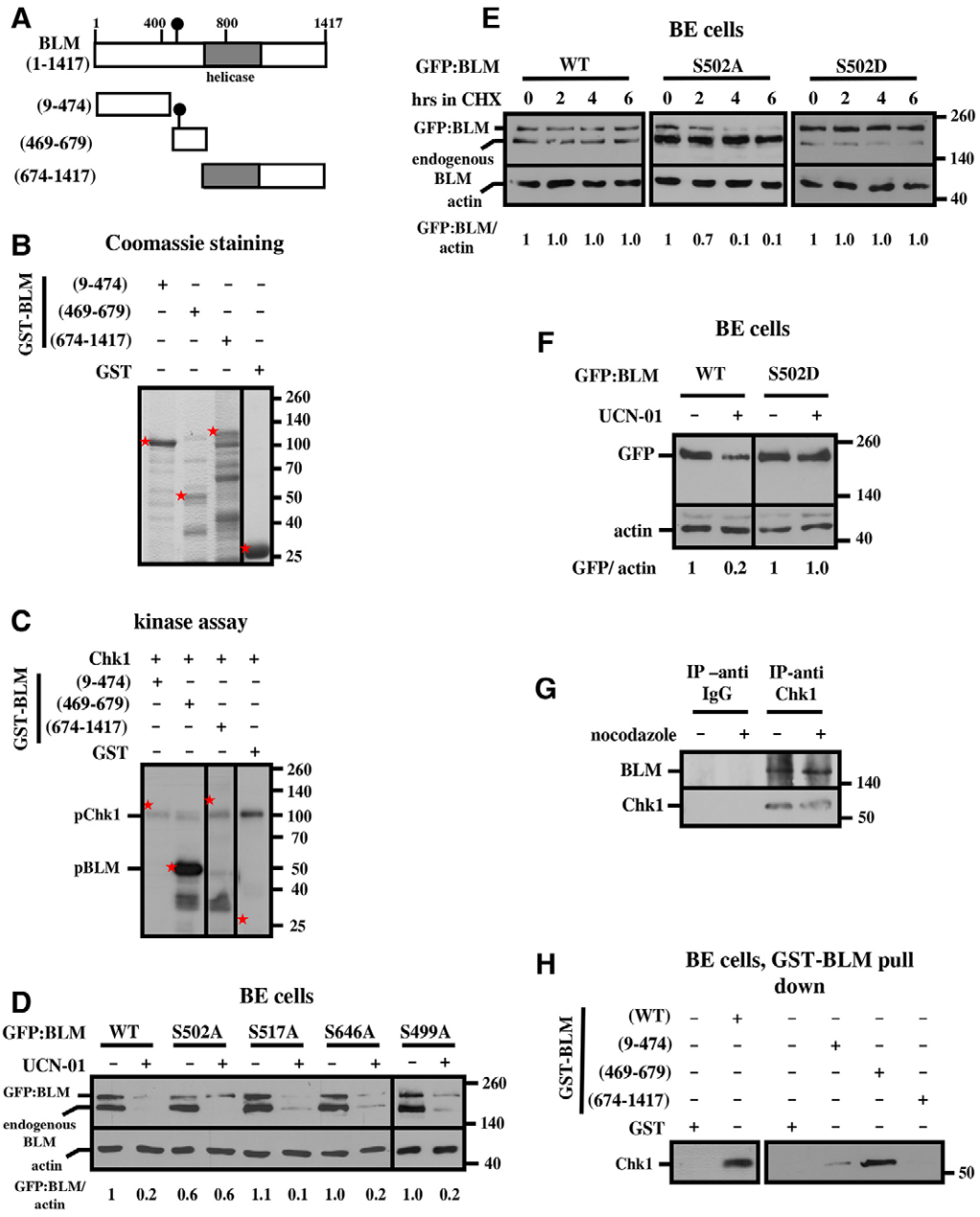

Fig. 2. Phosphorylation at S502 stabilises BLM. (A) A schematic of BLM fragments used. S502 is indicated by the lollipop. (B) Levels of glutathione–agarose-bound GST or GST–BLM proteins as detected by Coomassie Blue staining. 10 µg of protein was loaded in each lane. (C) Chk1 *in vitro* kinase assay. Autoradiography analysis of phosphorylated GST–BLM (pBLM) or GST. 10 µg substrate was used per reaction. Predicted molecular mass are indicated by asterisks. pChk1, autophosphorylated Chk1. (D) Western blot analysis of total BLM and actin in cells expressing WT, S502A, S499A, S517A or S646A GFP–BLM (GFP:BLM) in the absence or presence of UCN-01 for 8 h. Numbers below the blot show the relative level of GFP–BLM, normalized to actin, compared to that in the control (set as 1). (E) Total BLM and actin in cells expressing WT, S502A, or S502D GFP–BLM in the presence of cycloheximide (CHX). Numbers below the blot show the relative level of GFP–BLM, normalized to actin, compared to that at 0 h (set as 1). (F) Total GFP and actin in cells expressing WT or S502D GFP:BLM and treated as in (D). Numbers below the blot show the relative level of GFP–BLM, normalized to actin, compared to that in the control (absence of UCN-01, set as 1). (G) Western blot analysis of immunoprecipitated (IP) BLM and Chk1 in the absence or presence of nocodazole for 16 h. (H) Cell lysates were incubated with 10 µg of glutathione–agarose-bound GST–BLM or GST. Associated Chk1 was detected by western blotting.

S2B), indicating that Chk1–BLM interaction was not a consequence of mitotic accumulation. In addition, endogenous Chk1 predominantly associated with GST–BLM-(469–679) in GST pulldown assays (Fig. 2H).

BLM-S502 is conserved in mammals (Fig. 3A) and is an atypical Chk1 phosphorylation site; however, phosphorylation of atypical sites by Chk1 has been reported previously (Shieh et al., 2000; Gonzalez et al., 2003).

Phosphorylated BLM-S502 localises to chromatin bridges

To further investigate BLM-S502 phosphorylation, an anti-phospho-BLM-S502 (anti-pS502) antiserum was raised against the human protein sequence. This antiserum detected phosphorylated BLM-S502 at chromatin bridges in control cells (Fig. 3B,C; supplementary material Fig. S2C) and immunoreactivity was abolished after BLM depletion or incubation of the antiserum with the phosphorylated synthetic peptide (supplementary material Fig. S2C). Phosphorylated BLM-S502 colocalised with GFP–BLM at chromatin bridges in control cells, and this localisation was impaired after Chk1 inhibition (Fig. 3B,C).

The anti-pS502 antiserum immunoprecipitated WT, but not S502A, GFP–BLM in immunoprecipitation experiments followed by western blotting against GFP (Fig. 3D), verifying that this reagent is specific for the phosphorylation. In addition, similar levels of WT or S646A GFP–BLM associated with pS502 suggesting that BLM-S502 phosphorylation is not dependent on BLM-S646 phosphorylation (Fig. 3D; Kaur et al., 2010). In addition, untreated cells, cells treated with nocodazole, to enrich for mitosis, cells treated with hydroxyurea, to enrich for S phase, and cells treated with the DNA damage agent etoposide showed similar levels of phosphorylated BLM-S502 (Fig. 3E,F), indicating that BLM-S502 is a constitutive phosphorylation.

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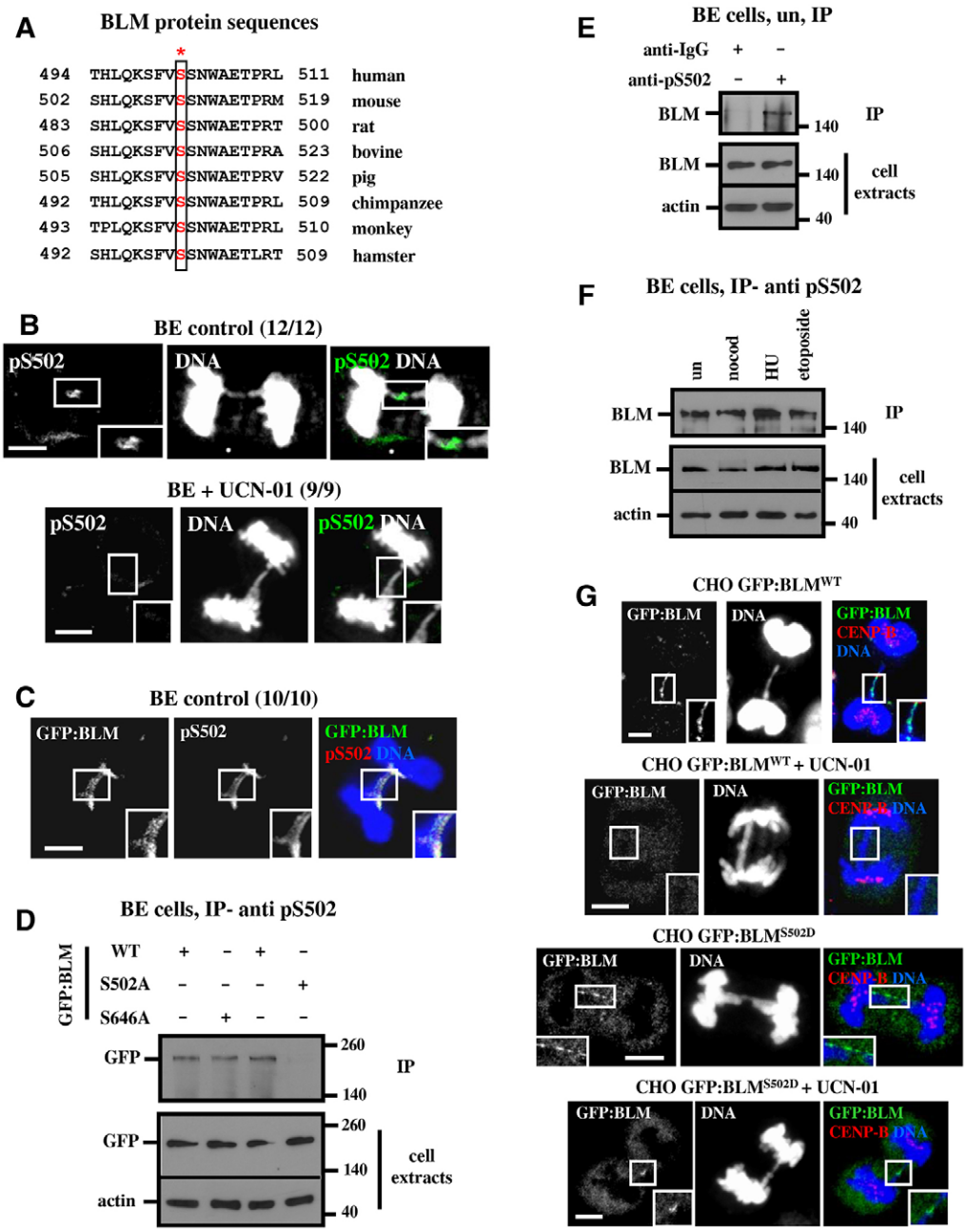

Fig. 3. S502D mutation rescues BLM localisation to chromatin bridges in Chk1-deficient cells. (A) Alignment of BLM protein sequences. S502 is marked by an asterisk. (B,C) Localisation of phosphorylated BLM-S502 (pS502) in the absence (control) or presence of UCN-01 for 8 h. The frequency of cells exhibiting the respective phenotype is shown. (D) *I*mmunoprecipitation (IP) assay. Top: western blot analysis of pS502-associated GFP–BLM (GFP). Bottom: western blot analysis of total GFP and actin. (E,F) Top: western blot analysis of pS502-associated BLM. Cells were treated with nocodazole (nocod), hydroxyurea (HU) or etoposide for 16 h, or left untreated (un). Bottom: western blot analysis of total BLM and actin. (G) Localisation of GFP–BLM (GFP:BLM). CHO cells expressing WT or S502D GFP–BLM were induced with tetracycline for 16 h and treated with UCN-01 in the continuous presence of tetracycline for a further 8 h. Insets show magnified chromatin bridges. Scale bars: 5 μm.

BLM-S502 phosphorylation protects against chromatin bridges

To investigate the significance of BLM-S502 phosphorylation for protection against anaphase bridges, Chinese hamster ovary (CHO) cells expressing WT or S502D GFP–BLM under control of a tetracycline-induced promoter were generated (supplementary material Fig. S2D,E). This is a cell system we have previously used for conditional expression of proteins (Petsalaki et al., 2011; Petsalaki and Zachos, 2013). S502D, but not WT, GFP–BLM localised to chromatin bridges in Chk1-deficient cells and accumulated to similar levels in the absence or presence of the Chk1 inhibitor UCN-01 (Fig. 3G; supplementary material Fig. S2F). Furthermore, expression of GFP–BLM-S502D suppressed the number of anaphases with chromatin bridges compared to WT GFP–BLM after Chk1 inhibition (Fig. 4A). These results show that BLM-S502 phosphorylation is required for protection against chromatin bridges in anaphase.

Depletion of cullin 3 prevents BLM degradation after Chk1 inhibition

Cullin complexes are E3 ubiquitin ligases (Bosu and Kipreos, 2008). To determine the type of cullins that associate with full-length WT GST–BLM, Myc-tagged human cullins were expressed in CHO cells and affinity purified proteins were analysed by western blotting against Myc. We found that cullin 3, but not cullin 1, 2, 4A, 4B or 5, specifically associated with WT GST–BLM (Fig. 4B; supplementary material S3A–F). Remarkably, GST–BLM-S502D exhibited diminished binding to Myc–cullin-3 compared to WT (Fig. 4B; supplementary material Fig. S3A). Furthermore, cullin 3 depletion (siCul3)

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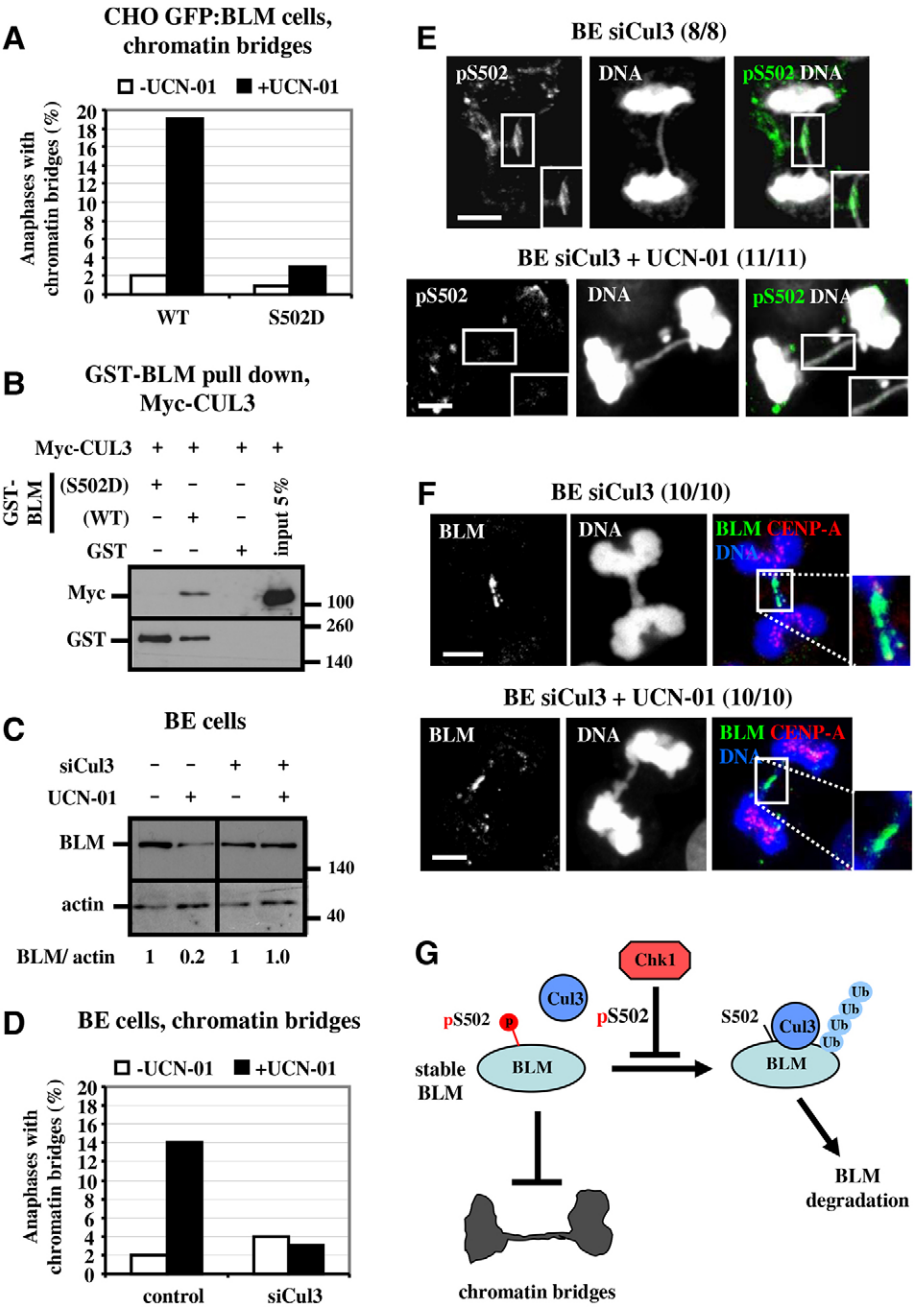

Fig. 4. Cullin 3 depletion prevents chromatin bridge formation after Chk1 inhibition. (A) Frequencies of anaphases with chromatin bridges. CHO cells expressing WT or S502D GFP–BLM (GFP:BLM) were induced with tetracycline for 16 h and treated with UCN-01 in the continuous presence of tetracycline for a further 8 h. A minimum of 100 anaphases were analysed. (B) Lysates from CHO cells expressing Myc–cullin-3 (Myc-CUL3) were incubated with 10 µg of glutathione–agarose-bound GST–BLM or GST. Associated Myc or GST was detected by western blotting. (C) Western blot analysis of total BLM and actin. Cells were transfected with Cullin 3 siRNA (siCul3) in the absence or presence of UCN-01 for 8 h. Numbers below the blot show the relative level of BLM, normalized to actin, compared to that in the control (absence of UCN-01, set as 1). (D) Frequencies of anaphases with chromatin bridges in cells treated as in C. A minimum of 100 anaphases were analysed. (E,F) Localisation of phospho-S502 (pS502, E) or total BLM (F) in cells treated as in C. The frequency of cells exhibiting the respective phenotype is shown. Insets show magnified chromatin bridges. Scale bars: 5 µm. (G) The proposed mechanism by which Chk1 prevents chromatin bridges. Cul3, Cullin 3; p, phosphorylation; Ub, ubiquitylation.

inhibited BLM degradation and prevented anaphases with chromatin bridges in cells treated with the Chk1 inhibitor UCN-01 compared to controls (Fig. 4C,D; supplementary material Fig. S3G). These results show that S502 phosphorylation stabilises BLM by inhibiting binding of cullin 3 to BLM.

After cullin 3 depletion, localisation of phosphorylated BLM-S502 to anaphase bridges was diminished in cells treated with UCN-01 compared to controls (Fig. 4E). However, Chk1-deficient or control cells exhibited similar total BLM staining at chromatin bridges (Fig. 4F). These results show that S502 phosphorylation is dispensable for BLM localisation to chromatin bridges when BLM degradation is inhibited.

On the basis of the above findings, we propose the following model (Fig. 4G): Chk1 phosphorylates BLM at S502 and this phosphorylation stabilises BLM by inhibiting its association with cullin 3, and the subsequent BLM poly-ubiquitylation and proteosomal degradation, during interphase. Stable BLM can suppress chromatin bridges in anaphase.

TopBP1, a Chk1 regulator during replication stress, has recently been proposed to prevent anaphase bridges through stimulation of kinase ATR (Germann et al., 2014). It is possible that TopBP1 and ATR activate Chk1 to stabilise BLM and suppress recombination events giving rise to chromatin bridges in anaphase. In addition, because BLM-S502 phosphorylation by Chk1 is a constitutive phosphorylation, it will likely impact on several aspects of BLM function (Karow et al., 2000; Wu and Hickson, 2003).

MATERIALS AND METHODS

Antibodies, plasmids and siRNAs

Anti-pS502 antiserum was generated in rabbits by immunisation against the phosphorylated peptide [FV(pS)SNWAETPRLGKC] of human BLM (GenScript).

Monoclonal antibodies against Chk1 (G-4), GST (B-14), Myc (9E10), BLM (BFL103; supplementary material Fig. S1D) and polyclonal antibodies against CENP-B and GFP (FL) were from Santa Cruz Biotechnology. Monoclonal antibodies against α-tubulin (DM1A) and actin (AC-40) were from Sigma, anti-CENP-A (3-19) was from GeneTex, anti-PICH (142-26-3) from Millipore and anti-Ubiquitin (P4D1) from Cell Signaling. Polyclonal antibody against BLM (ab2179) was from Abcam. Recombinant Chk1 was from Millipore.

Plasmids pcDNA3-myc3-CUL2 (#19892) and pcDNA3-myc-CUL5 (#19895) were from Addgene (Ohta et al., 1999). pCMV-Myc-Cullin 1 was from Maria Masucci (Gastaldello et al., 2012), pCS2/Cullin 3-myc from Jeffrey Singer (Wimuttisuk and Singer, 2007), pcDNA/myc-His-Cul4B from Yaoqin Gong (Zou et al., 2009), pcDNA3/myc-Ubiquitin from Emi Nakayama (Maegawa et al., 2010), pCS2/GFP-BLM from Hongtao Yu (Ke et al., 2011) and pGEX/BLM (1-1417) from Sagar Sengupta (Kaur et al., 2010).

Negative siRNA and human Chk1 or BLM siRNAs were from Thermo Scientific. Cullin 3 siRNA was from Santa Cruz Biotechnology.

Mutagenesis, cloning and generation of cell lines

Point mutations were generated using the QuikChange site-directed mutagenesis kit (Agilent Technologies). To produce inducible CHO cell lines, wild-type or mutant GFP–BLM cDNAs were subcloned into the pcDNA5/FRT/TO plasmid (Invitrogen). These plasmids were transfected into CHO cells (T-REx; Invitrogen) stably expressing the tetracycline repressor together with the pTK-Hyg selection vector (Agilent Technologies).

Cell culture and treatments

Human colon carcinoma BE cells and Chinese hamster ovary CHO cells were grown as described previously (Petsalaki et al., 2011). Cells were treated with 3.32 µM nocodazole (AppliChem), 10 µg/ml MG132 (Millipore), 300 nM UCN-01 (Sigma), 100 µg/ml cycloheximide (AppliChem), 2 mM hydroxyurea (Sigma) or 2 µM etoposide (Sigma). To induce expression of GFP–BLM transgenes, CHO GFP–BLM WT or S502D cells were treated, respectively, with 300 ng/ml or 1 µg/ml tetracycline (Sigma) for 16 h.

Indirect immunofluorescence microscopy

For total BLM staining, cells were fixed in ice-cold methanol for 5 min at −20℃, washed twice with PBS at room temperature and immunostained. pS502 staining was performed using the Phem-paraformaldehyde-microcystin protocol as described previously (Petsalaki and Zachos, 2014). For all other fluorescence microscopy applications, cells were fixed in 4% paraformaldehyde in cytoskeleton buffer (Petsalaki et al., 2011).

Immunoprecipitations and kinase assays

Cells were sonicated in ice-cold immunoprecipitation buffer (Petsalaki et al., 2011). 1 mg cell lysate was incubated with 0.5 µg antibody or 10 µg agarose-bound GST proteins. For Chk1 *in vitro* kinase assays, 0.5 µg recombinant Chk1 was incubated with 10 µg protein substrate on agarose beads as described previously (Petsalaki et al., 2011).

Mass spectrometry

GST–BLM bands were excised from the gel, digested with trypsin and the extracted tryptic peptides analysed by liquid chromatography-mass spectrometry as described previously (Roget et al., 2012).

DNA content

Propidium iodide staining was as described previously (Petsalaki et al., 2011).

Western blotting and densitometry

Cells were lysed in ice-cold whole-cell extract buffer (Petsalaki et al., 2011). Densitometric analysis was performed using ImageJ (NIH).

Statistical analysis

The *P*-values were calculated using the Student's *t*-test.

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Competing interests

The authors declare no competing interests.

Author contributions

E.P., M.D. and G.Z. performed experiments and analysed the results. N.M. did the mass-spectrometry. G.Z. designed the study and wrote the paper.

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Supplementary material

Supplementary material available online at
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