BUBR1 recruits PP2A via the B56 family of targeting subunits to promote chromosome congression

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Summary

BUBR1 is a mitotic phosphoprotein essential for the maintenance of chromosome stability by promoting chromosome congression and proper kinetochore–microtubule (K-fiber) attachment, but the underlying mechanism(s) has remained elusive. Here we identify BUBR1 as a binding partner of the B56 family of Protein Phosphatase 2A regulatory subunits. The interaction between BUBR1 and the B56 family is required for chromosome congression, since point mutations in BUBR1 that block B56 binding abolish chromosome congression. The BUBR1:B56-PP2A complex opposes Aurora B kinase activity, since loss of the complex can be reverted by inhibiting Aurora B. Importantly, we show that the failure of BUBR1 to recruit B56-PP2A also contributes to the chromosome congression defects found in cells derived from patients with the Mosaic Variegated Aneuploidy (MVA) syndrome. Together, we propose that B56-PP2A is a key mediator of BUBR1's role in chromosome congression and functions by antagonizing Aurora B activity at the kinetochore for establishing stable kinetochore–microtubule attachment at the metaphase plate.

Key words: Aurora B, B56-PP2A, BUBR1, Chromosome congression

Introduction

Faithful segregation of duplicated chromosomes into two daughter cells during mitosis is essential for maintenance of genomic stability. BUBR1 is important for this process because of its essential roles in imposing the spindle assembly checkpoint (SAC) and promoting chromosome congression at the kinetochore (Ditchfield et al., 2003; Lampson and Kapoor, 2005). Strikingly, bi-allelic mutations in the *BUB1B* gene that encodes BUBR1 were identified in ~40% of families with Mosaic Variegated Aneuploidy (MVA) syndrome (Hanks et al., 2004), an autosomal recessive disorder characterized by mosaic aneuploidy, microcephaly, growth retardation, mental retardation, physical anomalies and predisposition to childhood cancers (Jacquemont et al., 2002). Consistent with the proposed chromosome congression function of BUBR1, cell lines derived from *BUB1B*-mutated MVA cases show an increased frequency of miscongressed chromosomes (Suijkerbuijk et al., 2010).

Proper chromosome congression is tightly regulated by protein phosphorylation. Aurora B kinase destabilizes erroneous kinetochore–microtubule attachments through phosphorylation of essential microtubule binding factors at the kinetochore (Kelly and Funabiki, 2009; Welburn et al., 2010). Notably, the chromosome congression defects induced by loss of BUBR1 can be partially rescued by inhibition of Aurora B kinase (Lampson and Kapoor, 2005), suggesting that BUBR1 antagonizes Aurora B at the kinetochore to promote chromosome congression. However, the underlying mechanism(s) by which BUBR1 antagonizes Aurora B has remained elusive.

PP2A is a major cellular serine–threonine phosphatase; it commonly forms a heterotrimer in which regulatory "B" subunits determine the subcellular localization and substrate specificity of the holoenzyme (Virshup and Shenolikar, 2009). Notably, the B56 subunits of PP2A (McCright and Virshup, 1995) function redundantly for chromosome congression and K-fiber formation by antagonizing Aurora B and Plk1 kinases at the kinetochore (Foley et al., 2011).

Here we show BUBR1 as a direct binding partner of all B56 family members using yeast two-hybrid screening. We have identified critical amino acid residues in BUBR1 responsible for the B56:BUBR1 interaction. Using BUBR1 depletion–reconstitution as well as rescue of MVA cell lines, we demonstrate that the B56:BUBR1 interaction is required for chromosome congression and K-fiber formation. Failure to recruit B56-PP2A to BUBR1 also contributes to the chromosome congression defects found in MVA cell lines. Notably, the mitotic defects caused by loss of the B56:BUBR1 interaction can be rescued by either chemical or siRNA-mediated inhibition of Aurora B, supporting the conclusion that a key mitotic role of BUBR1:B56-PP2A complex is antagonizing Aurora B at the kinetochore to promote chromosome congression and stable kinetochore–microtubule attachment at the metaphase plate.

Results

B56 promotes chromosome congression in a redundant manner

To confirm the importance of individual B56 members in aligning chromosome at the metaphase plate (Foley et al., 2011), we first determined the mRNA abundance of B56 family members in HeLa cells by real-time PCR (qPCR) analysis. Four of five B56 family members were expressed in asynchronously grown HeLa cells (supplementary material Fig. S1A). siRNA-mediated knockdown of individual B56s did not show any measurable effect on chromosome congression, whereas depletion of the set of four expressed B56 family members with two non-overlapping pools of siRNA dramatically increased the population of rounded-up cells arrested in mitosis (supplementary material Fig. S1A,B). The proportion of cells arrested in a prometaphase-like state with misaligned chromosomes was quantified after depleting B56 members in various combinations (supplementary material Fig. S1C). The B56 genes appear highly redundant in this assay, since at least three of the four expressed B56 members had to be depleted to measurably increase the population of mitotic arrested cells with misaligned chromosomes. Notably, depletion of the four expressed B56 family members (α, γ, δ, ε) using two non-overlapping pools of siRNAs arrested a majority of HeLa cells in mitosis with massively misaligned chromosomes (supplementary material Fig. S1D,A). This effect was not an off-target effect of siRNA-mediated depletion of B56, because re-expressing mCherry-tagged siRNA-immune B56δ efficiently restored chromosome alignment at the metaphase plate (supplementary material Fig. S1E). Furthermore, while B56β was not expressed in HeLa cells (supplementary material Fig. S1A), ectopic expression of GFP-B56β also efficiently rescued chromosome misalignment in B56-depleted cells (supplementary material Fig. S1F), confirming that all B56 genes promote chromosome congression in a redundant manner.

All B56 subunits interact with BUBR1

To determine the molecular mechanisms underlying B56 function in chromosome congression, we searched for protein partners that directly bind B56 using the yeast two-hybrid method with B56δ as bait. Screening of a HeLa cDNA library identified the known interactors PP2A Aα and Aβ and Cyclin G1 and G2, confirming the specificity of the screen (Okamoto et al., 2002). Multiple colonies containing cDNA of BUBR1 (a.a. 300–1050) were also isolated. The interaction between BUBR1 and B56δ was verified in a directed two hybrid assay (Fig. 1A). Consistent with this, Myc-tagged BUBR1 co-immunoprecipitated with HA-tagged B56δ (Fig. 1B), indicating that B56 family members are novel binding partners of BUBR1. As the B56 genes functioned redundantly in chromosome congression (supplementary material Fig. S1), we confirmed that all individual B56 isoforms interacted with BUBR1 (Fig. 1A). This interaction was specific to B56 subunits, because PR72, the B″ subunit of PP2A, failed to interact (Fig. 1A). BUBR1 is essential for chromosome congression (Lampson and Kapoor, 2005), and knockdown of BUBR1 resulted in massively misaligned chromosomes comparable to knockdown of B56 subunits (supplementary material Fig. S2A,B). Taken together, the data indicate that BUBR1 and B56 subunits may function as a complex in the chromosome congression pathway.

Isolation of BUBR1 mutants defective in B56-binding

Given that BUBR1 was isolated as a binding partner of all B56 subunits, we wished to determine if the B56:BUBR1 interaction is essential for promoting chromosome congression. First, we used the two hybrid interaction to fine-map the binding site on BUBR1. We chose B56δ for this analysis because BUBR1 was originally isolated by the yeast two-hybrid system using B56δ as bait. Notably, the BUBR1 motif (a.a. 630–720) nearly within the essential domain for chromosome congression (a.a. 484–715) (Suijkerbuijk et al., 2010) was both necessary and sufficient for binding B56δ (Fig. 1C–E). Further deletion analysis of BUBR1 revealed that two small domains within the congression regulation region of BUBR1 (a.a. 630–640 and a.a. 670–720) were required for interaction with the B56δ subunit (Fig. 1C–E). As the amino acid sequences of this second motif are evolutionally well conserved across different species (Fig. 1F) than the first motif (supplementary material Fig. S3) and also shown to be phosphorylated by mitotic kinases Cdk1, Plk1 and Mps1 in response to lack of kinetochore–microtubule attachment and tension (Elowe et al., 2007; Huang et al., 2008), we generated a series of point mutants within this second motif of BUBR1. Importantly, BUBR1 point mutants in which residue 672 was replaced with phenylalanine (I/F), residues 672 and 673 with alanine (II/AA), or residues 678–681 with alanine (EATH/AAAA) failed to bind B56δ subunit (Fig. 1G).

The B56:BUBR1 interaction is required for chromosome congression

With the point mutants of BUBR1 defective in binding B56 subunits, we determined the importance of B56:BUBR1 interaction in chromosome congression. To address this, endogenous BUBR1 in HeLa cells was depleted and replaced with GFP-S-tag (LAP)-tagged RNAi-immune BUBR1 (Fig. 2A–C). As the kinase domain of BUBR1 was dispensable for rescue of chromosome alignment (Suijkerbuijk et al., 2010), we utilized BUBR1 encompassing amino acids 1–730 (1–730-WT) in our present study. Note that endogenous BUBR1 has the same electrophoretic mobility as LAP-BUBR1(1–730-WT) (Fig. 2B,C, top panels). Cells were then treated with MG132 to arrest in metaphase, and the degree of chromosome congression defects was quantified using immunofluorescence analysis. As shown previously (Lampson and Kapoor, 2005), knockdown of BUBR1 produced severe chromosome misalignment (supplementary material Fig. S2). LAP-BUBR1(1–730-WT) restored alignment in 80% of cells, while LAP-BUBR1(1–482) lacking the chromosome congression domain was well expressed but failed to rescue (Fig. 2B). Strikingly, BUBR1 point mutants defective in binding B56 subunits in our directed two hybrid assay, while able to localize properly to kinetochores (Fig. 2B,C, lower left panels), and expressed as well as wild type (Fig. 2B,C, top panels), were unable to rescue chromosome congression defects in cells depleted of BUBR1 (Fig. 2B, lower right graphs). Importantly, these results were reproduced using a different siRNA targeting the 3′UTR of BUBR1 mRNA (Fig. 2C), excluding a possible off-target effect of siRNA. Furthermore, determined by immunoprecipitation analysis, LAP-BUBR1 point mutants defective in promoting chromosome congression showed a marked decrease in their abilities to bind HA-B56α as compared to LAP-BUBR1(1–730-WT) (Fig. 2D). Together, these results indicate that the B56:BUBR1 interaction is required for chromosome congression.

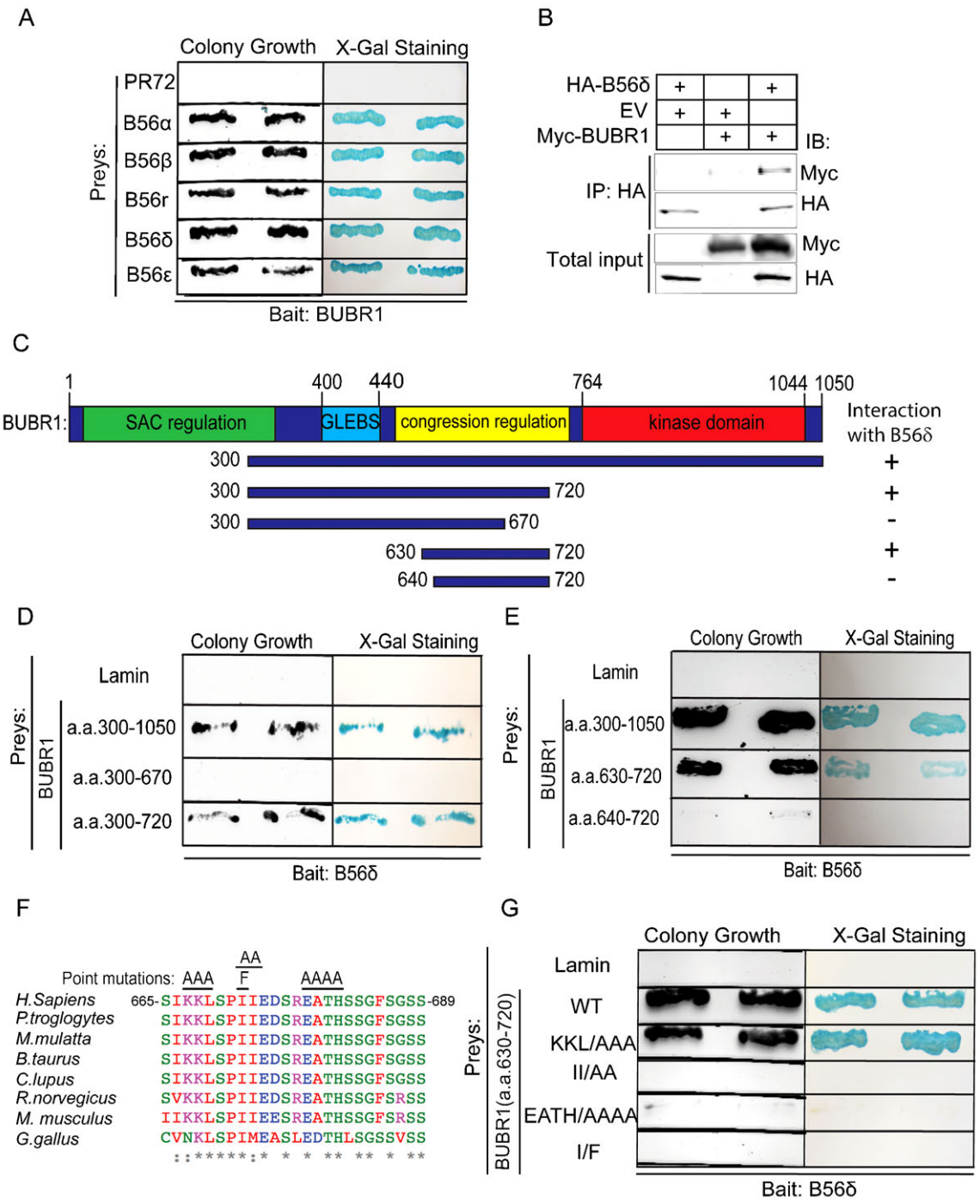


Fig. 1. Isolation and characterization of BUBR1 as a novel direct binding partner of all B56 subunits. (**A**,**D**,**E**,**G**) Yeast two-hybrid assay. The interaction between BUBR1 and B56 subunits was evaluated by colony growth as well as X-gal assay. For **C** (top panel), structural motifs of human BUBR1 with a summary of interactions between a series of deletion mutants of BUBR1 and B56δ are shown. (**B**) HeLa cell lysates transiently expressing HA-B56δ and Myc-BUBR1 were subjected to immunoprecipitation analysis with antibodies against HA-epitope. (**F**) Alignment of the putative second B56-binding sequence of BUBR1 between different species. The changes in amino acid residues to generate the indicated point mutants of BUBR1 for yeast two-hybrid assay (G) are shown.

PP2A-B56 antagonizes Aurora B for chromosome congression

Chromosome congression towards the metaphase plate is suggested as a means to maximize the efficiency of forming stable bi-oriented K-fibers at the metaphase plate. As knockdown of B56 subunits caused massive chromosome congression defects, it should also inhibit forming stable K-fibers. One characteristic of unattached K-fibers is that they depolymerize in the cold, while end-on attached K-fibers are cold-stable (Rieder, 1981). Cold-exposed HeLa cells retained K-fiber formation, while depletion of B56 subunits markedly reduced the number of K-fibers (Fig. 3A). As knockdown of B56 is likely to cause an increase in substrate phosphorylation, we tested if the loss of

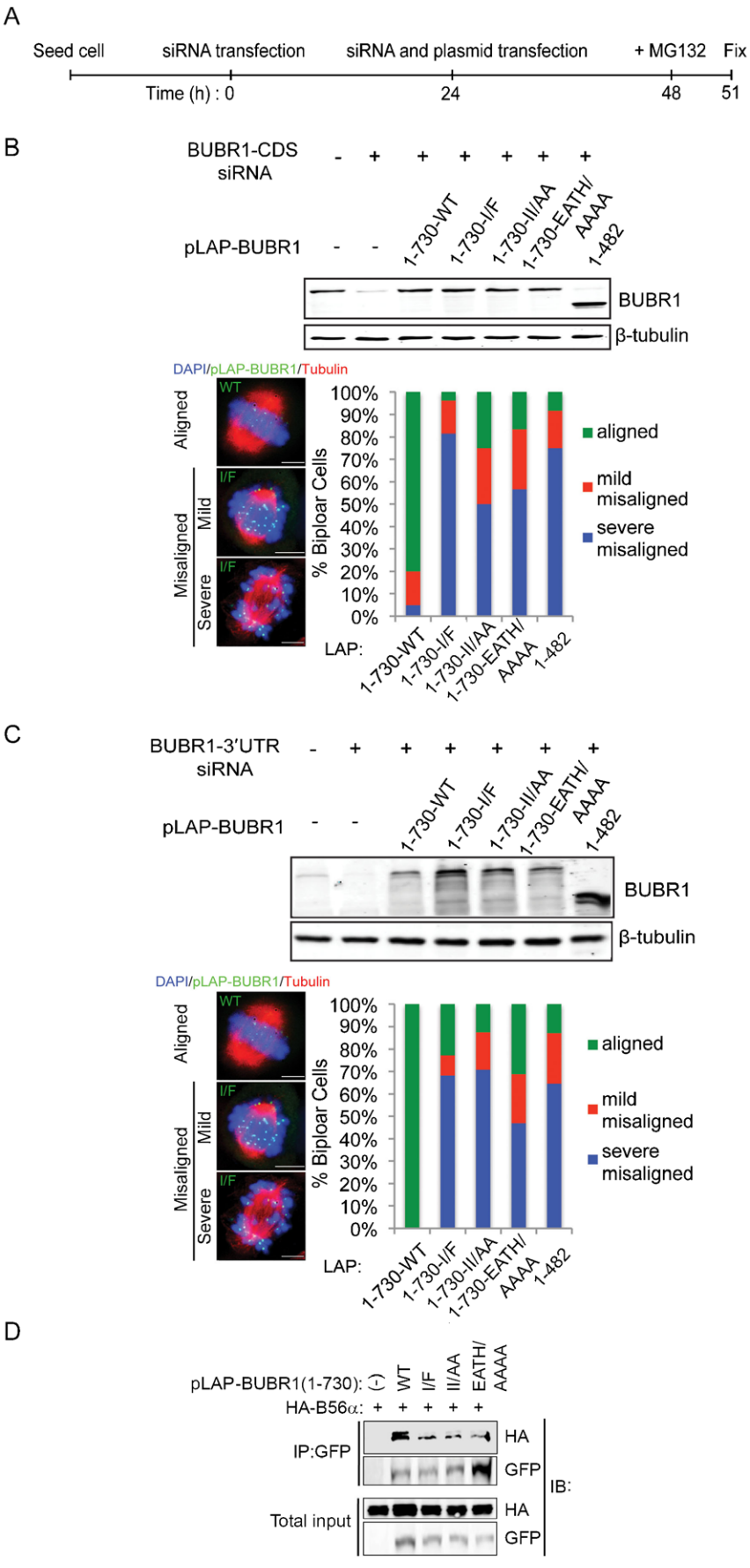


Fig. 2. The B56:BUBR1 interaction is required for chromosome congression. (**A**) Schematic experimental procedure. Asynchronously growing HeLa cells were transfected twice with a 24-hour interval using control non-silencing or two different siRNAs against BUBR1 (100 nM). During second transfection, the indicated expression vectors encoding siRNA-insensitive LAP-tagged BUBR1 were co-transfected. Forty-eight hours after initial transfection, cells were treated with MG132 for 3 hours, and subjected to immunofluorescence analysis using antibodies against α-tubulin and DAPI to visualize the mitotic spindles and chromosomes, respectively. (**B**,**C**) Quantification results of chromosome congression defects in HeLa cells (n>100 cells counted for each condition) expressing the indicated LAP-BUBR1. Immunoblot of expressed LAP-BUBR1 were shown (top panels). Scale bar=5 μm. (**D**) Lysates from HeLa cells transiently expressing HA-B56α and LAP-BUBR1 as indicated were subjected to immunoprecipitation analysis with antibodies against GFP.

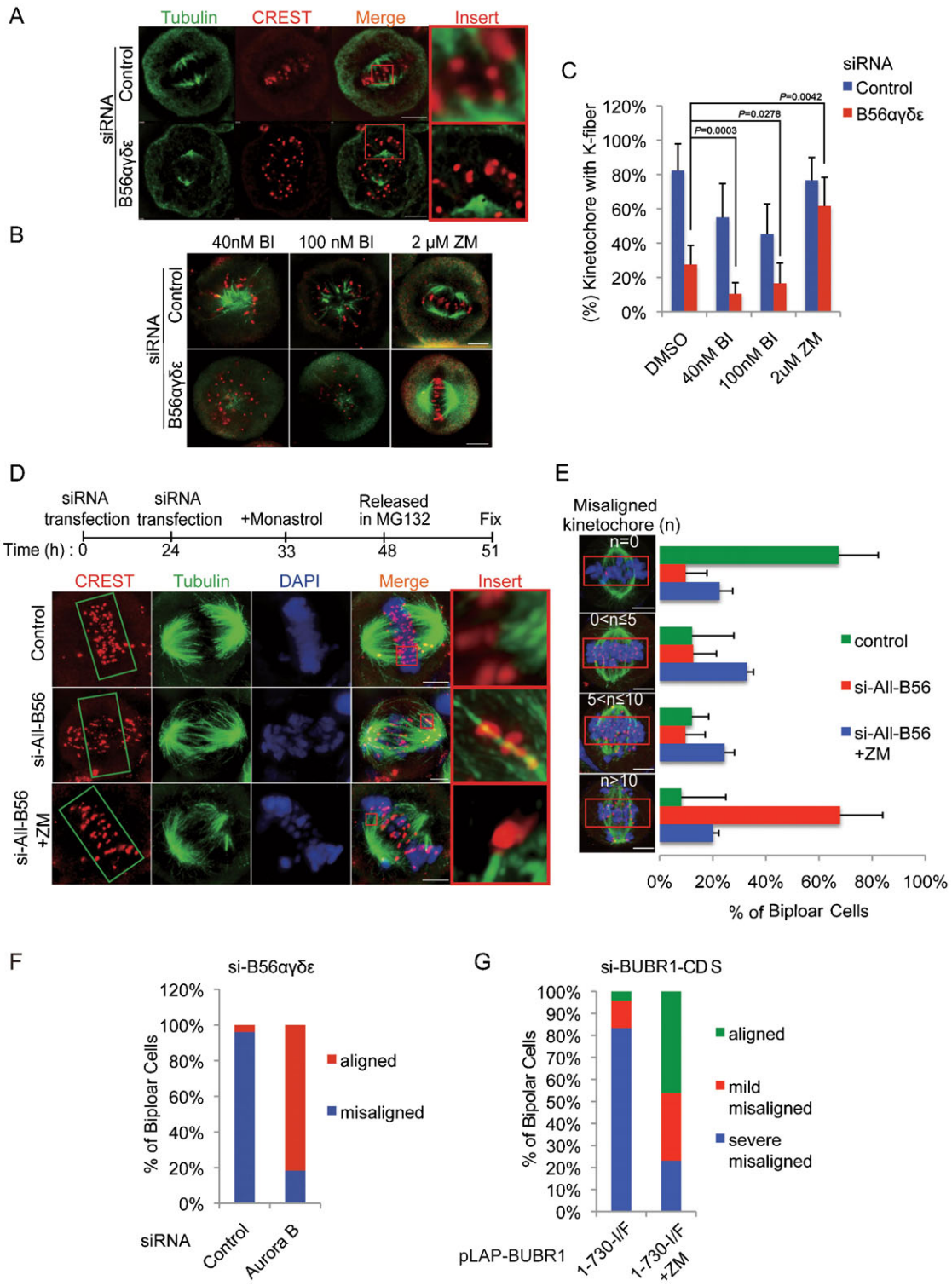


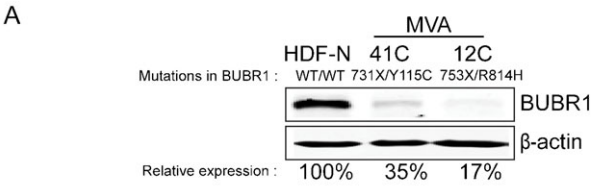
Fig. 3. PP2A-B56 antagonizes Aurora B for chromosome congression. Using the indicated siRNAs (100 nM total), asynchronously growing HeLa cells were transfected twice with a 24-hour interval. Cells were then treated with MG132 for 3 hours. (**A**,**B**) Cells were incubated on ice for 10 minutes and then subjected to immunofluorescence analysis. For B, cells were pretreated with indicated amounts of either Plk1 (BI2536) or Aurora B (ZM447439) inhibitors for 1 hour in cell culture medium containing MG132. (**C**) Quantification of cold-stable K-fiber formation on kinetochores (n>200) in HeLa cells from panels A and B. To determine the statistical significance, a Student's t-test was performed. P-values are indicated. (**D**) Immunofluorescence analysis with the schematic experimental procedure. Where indicated, ZM447439 (2 μM) was added for 1 hour into cell culture medium containing MG132 before fixation. (**E**) Quantification results of chromosome congression in HeLa cells (n>100, each) from panel D. The percentage of misaligned kinetochores falling outside the box in bipolar metaphase cells was counted. (**F**,**G**) Quantification results of chromosome congression in HeLa cells (n>100, each). For panel F, siRNA against Aurora B (20 nM) was co-transfected with a set of siRNAs (80 nM) against the indicated B56 subunits. For panel G, siRNAs and LAP-BUBR1 were transfected as described in Fig. 2A. Where indicated, ZM447439 (2 μM) was added for 1 hour in cell culture medium containing MG132 before fixation. In A,B,D,E, scale bar=5 μm.

cold-stable K-fibers could be reversed by inhibition of the appropriate kinase. Aurora B and Plk1 have been implicated in chromosome congression upon knockdown of B56 subunits (Foley et al., 2011). Both treatment of cells with the Aurora B inhibitor ZM447439 and knockdown of Aurora B by RNAi was effective in rescuing chromosome congression defects (Fig. 3D–F) and K-fiber destabilization (Fig. 3B,C). This suggests that B56-PP2A antagonizes Aurora B kinase activity for chromosome congression and K-fiber formation in a direct or indirect manner.

If BUBR1 recruits B56 to the kinetochores to antagonize Aurora B activity during chromosome congression, then inhibiting Aurora B should rescue the chromosome congression defects seen in cells expressing BUBR1 mutants defective in B56-binding (Fig. 2). Indeed, treating cells with ZM447439 rescued chromosome congression defects in cells expressing the LAP-BUBR1(1–730-I/F) mutant (Fig. 3G). This effect is specific to inhibition of Aurora B, as inhibition of Plk1 with BI2536 failed to rescue K-fiber destabilization caused by knockdown of B56 subunits (Fig. 3B,C). The lack of effect of the Plk1 inhibitor differs from a previous report (Foley et al., 2011). Plk1 is implicated in stabilizing kinetochore–microtubule attachments (Lénárt et al., 2007) and we confirmed that BI2536 treatment caused monopolar spindle formation with kinetochores detached from microtubules (Fig. 3B). Taken together, these results indicate that BUBR1 antagonizes Aurora B, rather than Plk1 activity, by recruiting B56-PP2A to the kinetochore.

The B56:BUBR1 interaction is required for rescue of chromosome congression in *BUB1B*-mutated MVA cell lines

Mosaic variegated aneuploidy (MVA) syndrome is often due to mutations in BUBR1 that result in protein loss or premature termination before the chromosome congression domain (Hanks et al., 2004). Chromosome congression defects are frequent in cell lines derived from *BUB1B*-mutated MVA cases (Suijkerbuijk et al., 2010). To test if the defect in MVA is due to failure to recruit B56-PP2A to the kinetochore, we tested if wild type or B56-interaction defective mutant of BUBR1 could rescue the MVA phenotype. We obtained fibroblast cell lines derived from MVA patients (MVA-41C, MVA-12C) as well as from a normal healthy individual (Suijkerbuijk et al., 2010). Consistent with previous reports, BUBR1 protein was markedly lower in patient as compared with normal control cell lines (Fig. 4A). To determine the degree of chromosome congression defects, these cell lines were treated with MG132 to block transition from metaphase, and fixed for quantification using immunofluorescence analysis. As reported previously, less than 35% of the cells derived from MVA patients had aligned chromosomes (Fig. 4B, right graph). Furthermore, transiently expressed LAP-BUBR1(1–730-WT) substantially increased the fraction of MVA-41C and MVA-12C cells with well aligned chromosomes (Fig. 4B). This result is consistent with the finding that MVA is a recessive disorder caused by low BUBR1 protein abundance (Suijkerbuijk et al., 2010). Strikingly, however, expressing BUBR1 mutant [LAP-BUBR1(1–730-I/F)] to the kinetochores (Fig. 4B, left panels) not only failed to rescue chromosome congression defects in both MVA cell lines, but it further increased the population of cells with markedly misaligned chromosomes (Fig. 4B, right graph), suggesting that this mutant functions in a dominant-negative fashion, possibly by competing with the remaining endogenous BUBR1. To further address whether B56-PP2A recruitment to the kinetochore by BUBR1 is specifically affected in MVA patient cell lines, we artificially targeted B56-BB2A to outer kinetochores in both MVA cell lines. For this purpose, we utilized the minimal motif of BUBR1 carrying phosphomimetic mutations for binding B56s (termed as KARD-3D), which was fused with LAP-tagged outer kinetochore protein MIS12 (LAP-MIS12-KARD-3D) (Suijkerbuijk et al., 2012). Importantly, artificially targeted PP2A-B56 to the kinetochores by transiently expressing LAP-MIS12-KARD-3D was able to



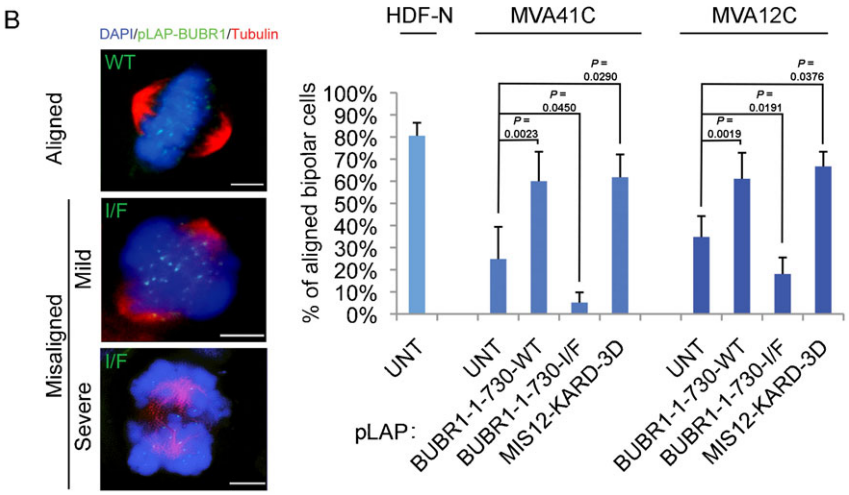


Fig. 4. The B56:BUBR1 interaction is required for chromosome congression in *BUB1B*-mutated MVA cell lines. (**A**) Immunoblot analysis of BUBR1 abundance in asynchronously growing established fibroblast cell lines from *BUB1B*-mutated MVA patients (MVA-41C, MVA-12C) in comparison with fibroblast from normal healthy individual (HDF-N). (**B**) Quantification of chromosome alignment in *BUB1B*-mutated cell lines. LAP-BUBR1 or LAP-MIS12-KARD-3D were expressed in the indicated cell lines, and 24 hours after transfection, cells were treated with MG132 for 3 hours before immunofluorescence analysis using antibodies against α-tubulin and DAPI to visualize the mitotic spindles and chromosomes, respectively. Representative images are shown in left panels. The average % aligned chromosome from three independent experiments (total $n>50$ per condition, ±s.d.) is shown. To determine the statistical significance, a Student's *t*-test was performed. *P*-values are indicated. UNT: control untransfected. Scale bar=5 µm.

efficiently restore chromosome alignment in both MVA cell lines (Fig. 4B, right graph). Together, these results show that the B56:BUBR1 interaction is crucial for chromosome congression in *BUB1B*-mutated MVA patient cell lines, and that the defect in chromosome congression is at least partly due to inefficient recruitment of B56-PP2A to the kinetochores by BUBR1.

Discussion

It has been speculated that BUBR1 antagonizes Aurora B at the kinetochore to promote chromosome alignment at the metaphase plate, but the molecular mechanism has remained unknown. Our study reveals that B56-PP2A, via interaction with BUBR1 at the kinetochore, is an essential factor antagonizing Aurora B. Importantly, we found that BUBR1 point mutants defective in binding to the B56 subunits fail to rescue chromosome congression defects both in HeLa cells depleted of BUBR1 and in cell lines derived from *BUB1B*-mutated MVA cases. In contrast, either chemical or siRNA-mediated inhibition of Aurora B rescued this mitotic defect, supporting our conclusion that a key mitotic role of BUBR1:B56-PP2A complex is antagonizing Aurora B at the kinetochore for chromosome alignment.

Our mutation analysis of BUBR1 revealed that two small motifs within the congression regulation region of BUBR1 (a.a. 630–640 and a.a. 670–720) are required for binding B56-PP2A. BUBR1 is hyperphosphorylated during mitosis, and mitotic kinases Cdk1, Plk1 and Mps1 have been shown to phosphorylate S670 (Elowe et al., 2007; Huang et al., 2008) (in response to lack of kinetochore–microtubule attachment) and S676 (Elowe et al., 2007) (in response to lack of tension) within this evolutionary conserved second motif of BUBR1. During the preparation of this manuscript, Kops and his colleague show that phosphorylation within this second motif by Plk1 promotes interaction of BUBR1 with B56α-PP2A to counter excessive Aurora B activity at the kinetochores (Suijkerbuijk et al., 2012). These findings are consistent with our conclusion that the B56:BUBR1 interaction is essential for chromosome congression. We further demonstrate that all B56 family members bind equally well to BUBR1 and promote chromosome congression in a redundant manner. However, BUBR1 mutants in which S670 was deleted or residue S676 replaced with alanine were still able to bind B56δ (data not shown), suggesting that additional phosphorylation by Plk1 within this second motif including T680 might be essential to promote this interaction (Suijkerbuijk et al., 2012). Alternatively, phosphorylation of these residues by Cdk1 and Plk1 may not be required for the basal B56:BUBR1 interaction, but it may enhance the interaction based on different degrees of kinetochore–microtubule attachment and/or tension. We note that the first motif of BUBR1 (a.a. 630–640) is also required for the B56:BUBR1 interaction (in this study), and we speculate that this first motif contributes to stability of the B56:BUBR1 complex. Thus, such fine-tuning of the B56-PP2A levels, rather than an on–off switch, may efficiently counter excessive Aurora B activity at the kinetochore for stabilization of kinetochore–microtubule attachment. Moreover, it is also important to address whether B56-PP2A directly reverses Aurora B-phosphorylation on essential microtubule binding factors at the kinetochore. In this sense, PP1 is thought to be the major phosphatase involved in directly stabilizing K-fibers, as PP1 has been shown to reverse phosphorylation on the kinetochore substrates of Aurora B (Lampson and Cheeseman, 2011; Lesage et al., 2011). Therefore, it will be of interest to investigate whether and how B56-PP2A cooperates with PP1 to establish stable kinetochore–microtubule attachment during chromosome congression and biorientation.

Chromosomal instability in MVA patients carrying *BUB1B* mutations is often due to low BUBR1 protein abundance (Suijkerbuijk et al., 2010). Affected patients either have bi-allelic BUBR1 mutations or mono-allelic BUBR1 mutations combined with allelic variants that lead to low wild-type BUBR1 protein abundance. Moreover, gene knockout study in mice supports the notion that BUBR1 protein levels tightly correlate with aneuploidy rates, cancer susceptibility, lifespan and aging-related phenotypes (Baker et al., 2004). In this study, we demonstrated that loss of the B56:BUBR1 interaction also contributes to the chromosome congression defects found in bi-allelic *BUB1B*-mutated MVA cell lines. Notably, this defect in chromosome congression caused by loss of the B56:BUBR1 interaction was not due to differences in the protein abundance between wild type and mutant BUBR1. Furthermore, artificially targeted PP2A-B56s to the kinetochores was able to rescue chromosome misalignment in MVA patient cell lines. Thus, our results indicate that misregulated B56-PP2A might also contribute to the increased frequency of chromosome instability with whole chromosome gain or loss found in MVA cases. Given that we isolated point mutants of BUBR1 defective in binding B56-PP2A, generating an *in vivo* model system (e.g. knockin mouse model) of MVA syndrome may provide an important insight into this question. Furthermore, it will be of interest to further examine whether mutations of BUBR1 occur within and surrounding BUBR1 motifs responsible for the B56:BUBR1 interaction. Since the role of the tumor suppressor PP2A in controlling tumor progression is thought to be governed by a specific set of B regulatory subunits, and several members of the B56 family have been shown to direct the tumor suppressive activity of PP2A, it is also tempting to speculate that misregulation of B56-PP2A might be also associated with tumorigenesis through increasing chromosomal instability.

Materials and Methods

Plasmids and siRNAs

B56β and B56δ open reading frame were PCR cloned into pEGFP-C1 and pCS2-mCherry respectively. pLAP-BUBR1 and pLAP-MIS12-KARD-3D plasmids were gifts from Geert J.P.L. Kops and described previously (Suijkerbuijk et al., 2010; Suijkerbuijk et al., 2012). Transfection of plasmids was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Two independent pools of siRNA targeting B56 family are the following: for pool 1, B56α (5′-CAATACAAGTGCCGAATAA-3′), B56β (5′-TCAAGTCGCTGT-CTGTCTT-3′), B56γ (5′-CAGAAGTAGTCCATATGTT-3′), B56δ (5′-CAGGA-GATTATTCTCACCAAA-3′), B56ε (5′-TTAATGAACTGGTGGACTA-3′) ; for pool 2, B56α (5′-GCTCAAAGATGCCACTTCA-3′), B56β (5′-CGCATGAT-CTCAGTGAATA-3′), B56γ (5′-GGATTTGCCTTACCACTAA-3′), B56δ (5′-GAAGTTGTTTATGGAAATGAA-3′), B56ε (5′-GCACAGCTGGCATATTGTA-3′). Other siRNAs used in this study are the following: BUBR1-CDS (5′-ACGAGAATACCTAATATGTGA-3′ (Elowe et al., 2007)), BUBR1-3′UTR (5′-GTCTCACAGATTGCTGCCT-3′ (Choi et al., 2009)), and Aurora B (Qiagen, SI02622032). The non-targeting control siRNA was purchased from Dharmacon. For siRNA transfection, Dharmafect transfection reagent 1 was used according to the manufacturer's instruction (Dharmacon).

Cell culture and drug treatment

HeLa cells are cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Thermo Scientific). MVA cells (gifts from Nazneen Rahman and Sandra Hanks) were cultured in DMEM with 10% FBS. Drugs used in this study are monastrol (100 μM; Santa Cruz Biotechnology), ZM447439 (BIOMOL international), MG132 (20 μM, Selleck Biochemicals), BI2536 (Selleck Biochemicals).

Immunofluorescence and time-lapse live-cell imaging

HeLa cells grown on coverglass-bottomed chamber slides (Lab Tek) were fixed with 4% PFA. For cold-stable microtubule assay, cells were placed on ice for 10 minutes before fixation. The fixed cells were permeabilized with 0.5% Triton X-100, and exposed to PBS containing 4% BSA for 1 hour. The following primary antibodies were diluted in PBS containing 1% BSA and 0.1% Triton X-100: CREST (Cortex Biochem; 1:500), B56α (BD Biosciences; 1:1000) BUBR1 (612503, BD Biosciences; 1:100), and α-tubulin (AA13, Sigma; 1:2000). Isotype-specific secondary antibodies (1:2000 dilution) coupled to Alexa Fluor 488, 594, or Cy5 (Molecular Probes) were used. Cells were counterstained with DAPI (Thermo Scientific). Images were acquired at RT with 3D-SIM using a Super Resolution Microscope (Nikon) equipped with an iXon^{EM}+885 EMCCD camera (Andor) mounted on a Nikon Eclipse Ti-E inverted microscope with a CFI Apo TIRF (100×/1.40 oil) objective and processed with the NIS-Elements AR software.

Immunoblotting and immunoprecipitation analysis

Protein was prepared with 4% SDS cell lysis buffer for SDS-PAGE. The following primary antibodies are used: B56δ (Forester et al., 2007), Aurora B (13E8A7, Santa Cruz), HA (Y-11, Santa Cruz), Myc(9E10, Santa Cruz), β-tubulin (Abcam), and β-actin (Sigma). For immunoprecipitation (Fig. 1B; Fig. 2D), nocodazole (200 ng/ml) arrested mitotic HeLa cells were lysed in 0.1% NP-40 cell lysis buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1% NP-40) containing 1 mM DTT, complete Mini (Roche) and phosphatase inhibitor cocktails (Sigma), and subjected to immunoprecipitation with antibodies against HA (12CA5, Santa Cruz) or GFP (sc-8334, Santa Cruz) with Protein A/G puls agarose beads (Santa Cruz) at 4℃ overnight. The beads were washed with 0.1% NP-40 cell lysis buffer and subjected to immunoblot analysis.

Yeast two hybridization

B56δ cDNA was cloned into pBTM116 vector as bait and HeLa cDNA library in pGAD vector was used as prey for screening. Interaction was evaluated by colony growth on plate dropout of Trp, Leu, and His, as well as X-gal assay for validation.

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Competing Interests

The authors have no competing interests to declare.

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