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Molecular Causes for BUBR1 Dysfunction in the Human Cancer Predisposition Syndrome Mosaic Variegated Aneuploidy

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Abstract

Genetic mutations in the mitotic regulatory kinase BUBR1 are associated with the cancer-susceptible disorder mosaic variegated aneuploidy (MVA). In patients with biallelic mutations, a missense mutation pairs with a truncating mutation. Here, we show that cell lines derived from MVA patients with biallelic mutations have an impaired mitotic checkpoint, chromosome alignment defects, and low overall BUBR1 abundance. Ectopic expression of BUBR1 restored mitotic checkpoint activity, proving that BUBR1 dysfunction causes chromosome segregation errors in the patients. Combined analysis of patient cells and functional protein replacement shows that all MVA mutations fall in two distinct classes: those that impose specific defects in checkpoint activity or microtubule attachment and those that lower BUBR1 protein abundance. Low protein abundance is the direct result of the absence of transcripts from truncating mutants combined with high protein turnover of missense mutants. In this group of missense mutants, the amino acid change consistently occurs in or near the BUBR1 kinase domain. Our findings provide a molecular explanation for chromosomal instability in patients with biallelic genetic mutations in BUBR1. Cancer Res; 70(12): 4891–900. ©2010 AACR.

Introduction

Mosaic variegated aneuploidy (MVA; OMIM: 257300), also referred to as premature chromatid separation syndrome, is an autosomal recessive syndrome characterized by constitutional aneuploidy and very early onset cancer predisposition. Typically, individuals with MVA display microcephaly and growth and mental retardation, as well as other, milder, physical anomalies. In addition, 37% of patients develop cancers including rhabdomyosarcoma, Wilms’ tumor, and leukemia, mostly within the first 3 years of life and not seldom even in utero (see Table 1 of ref. 1 and refs. 2, 3). The MVA syndrome has been linked to monoallelic or biallelic mutations in the BUB1B locus, encoding the predicted serine/threonine protein kinase BUBR1 (BUBR1 is the accepted alias name for the BUB1B protein, and therefore used throughout this study; refs. 2, 3). As indicated by the name, mosaic aneuploidies are found in cells of various tissues from MVA patients, suggesting underlying defects in the fidelity of chromosome segregation during development. Consistent with this, BUBR1 is critical for several processes that govern chromosome segregation during cell divisions. Error-free chromosome segregation requires that each sister of a duplicated chromosome is attached via their kinetochores to spindle microtubules from two opposing spindle poles (4). Onset of cell division before each and every kinetochore is attached to the mitotic spindle is normally prevented by the mitotic checkpoint (5). One of the essential components of this checkpoint is BUBR1 (6–9). BUBR1 directly inhibits the E3 ubiquitin ligase anaphase-promoting complex/cyclosome, which promotes chromosome segregation by targeting essential cell cycle regulators such as CYCLIN B and SECUKRIN/PTTG for destruction (10, 11). This inhibitory property of BUBR1 resides in the highly conserved NH2-terminal 450 amino acids and does not absolutely require the COOH-terminal kinase domain (12–14). Consistent with the presence of BUBR1 mutations as a cause for aneuploidy in MVA patients, cells from a Japanese patient had an impaired ability to respond to the microtubule poison colcemid (3, 15). In addition to a role in the mitotic checkpoint, BUBR1 is required for the establishment of stable interactions between kinetochores and spindle microtubules (16, 17).

The high incidence of tumors in MVA patients suggests a causal link between aneuploidy and tumor formation. In sporadic cancers, chromosomal instability, the frequent misregulation of whole chromosomes, has also been proposed to be a contributing force in carcinogenesis (18, 19). The causes of chromosomal instability in human tumors are unknown but likely involve dysfunction of some machineries...
that normally promote error-free chromosome segregation. Defects in attachment error-correction mechanisms, centrosome duplication, cytokinesis, or the mitotic checkpoint have all been postulated to promote chromosomal instability in tumors (16, 19–21). In rare cases, mutations in regulators of chromosome segregation in sporadic human tumors have been reported, including in BUB1 and the homologous kinase BUB1 (18), but no clear functional link between these mutations and chromosome segregation errors in such tumors has been established.

The mutations in BUBR1 associated with MVA fall in two classes: missense or frameshift mutations that result in truncation of the protein products (hereafter referred to as "truncation") and missense mutations that cause single amino acid substitutions (hereafter referred to as "substitution"). In four families with individuals that carried biallelic mutations, a truncating mutation in one allele was combined with one amino acid substitution, often in the kinase domain, in the other (2). In eight other families, one predominantly truncating mutation was found (3). We set out to examine the molecular causes of chromosome segregation errors in MVA patients. Our results present a rationale for why specific combinations of biallelic mutations cause aneuploidy in MVA patients.

Materials and Methods

Plasmids and shRNA-based protein replacement

The pSuper-based shRNA plasmids used in this study were Mock (22) and BUBR1 (23). LAP-BUBR1-WT has been created by cloning the RNAi-resistant allele from pcDNA3-myc-BUBR1ΔshRNA (23) to pIC58 (24). Mutants were obtained by site-directed mutagenesis and the LAP-mock control was created by mutating the second codon after the LAP tag of LAP-BUBR1-WT to a stop codon.

Cells were cotransfected with a marker plasmid along with pSuper-BUBR1 or pSuper-mock and shRNA-insensitive LAP-BUBR1-WT or mutants in a 1:8:5 ratio (U2OS) or 1:10:5 (HeLa). This ratio was based on the functional rescue by wild-type in relation to the shRNA. Marker plasmids were pSpectrin-GFP for flow cytometry and pEYFP-H2B for live cell imaging and immunofluorescence. pBabe-puro was used for expression level studies in U2OS and HeLa, and transfected cells were selected by treatment with puromycin.

Cell culture

HeLa and U2OS cells were grown in DMEM supplemented with 8% fetal bovine serum (FBS) and pen/strep (50 μg/mL). EBV-transformed lymphoblastoid cell lines were obtained from cell repositories (European Collection of Cell Cultures, Salisbury, United Kingdom, or Coriell Institute for Medical Research, Camden, NJ). Fibroblasts were obtained as primary cell lines and were subsequently SV40 transformed using standard procedures. All samples were obtained with informed consent from the family and under multicenter ethics approval (MREC05/02/17).

SV40-transformed fibroblasts and EBV-transformed lymphoblastoids were grown in DMEM and RPMI, respectively, supplemented with 10% FBS and pen/strep (50 μg/mL). HTR34 cells were created by infection of HeLa cells stably expressing Tet repressor (a gift of M. Timmers, Physiological Chemistry, University Medical Center Utrecht, Utrecht, Netherlands) with retrovirus carrying pSuperior-tet-PURO-BUBRI and grown on medium with TetSystem-approved FBS (Clontech).

Immunofluorescence

Cells, plated on 12-mm coverslips, were pre-extracted with 0.2% Triton X-100 in PEM [100 mmol/L PIPES (pH 6.8), 1 mmol/L MgCl2, and 5 mmol/L EGTA] for 1 minute before fixation within 3% paraformaldehyde in PBS. Cells were cold-treated for 15 minutes and fixed while permeabilizing in 0.2% Triton X-100 in PEM supplemented with 3.7% Shandon Zinc Formal-Fixx (Thermo Scientific) for 15 minutes, for cold-stable microtubule staining. Coverslips were blocked with 3% bovine serum albumin (BSA) in PBS for 1 hour, incubated with primary antibody for 16 hours at 4°C, washed with PBS/0.1% Triton X-100, and incubated with secondary antibodies for an additional 1 hour at room temperature. Coverslips were washed and submerged in PBS containing 4',6-diamidino-2-phenylindole, then washed again and mounted using ProLong antifade (Molecular Probes). Image acquisition and quantification were done as described (29), using a DeltaVision RT system (Applied Precision) with a 100×/1.40-numerical aperture (NA) UPlanSapo objective (Olympus) for acquiring images and SoftWorx software for quantification.

Flow cytometry and (quantitative) immunoblotting

Cells were released from a 24-hour thymidine-induced block into nocodazole for 16 hours and analyzed as described (25). An exception, the lymphoblastoid lines were not synchronized before the nocodazole block. Flow cytometric analysis of transfected cells was based on Spectrin-GFP expression. Immunoblotting was done using standard protocols; the signal was visualized and analyzed on an Odyssey scanner (LI-COR Biosciences) using fluorescently labeled secondary antibodies.

Live cell imaging

For live cell imaging, cells were plated in four-well chambered glass-bottomed slides (LabTek) or 24-well glass-bottomed plates (MatTek), transfected, and imaged in a heated chamber (37°C and 5% CO2) using a 20×/0.5-NA UPLFLN objective on a Olympus IX-81 microscope, controlled by Cell-M software (Olympus). Sixteen-bit DIC (5-ms exposure) and yellow fluorescent (15-ms exposure) images were acquired every 4 minutes (fibroblast and HeLa studies) or 5 minutes (U2OS studies) using a Hamamatsu ORCA-ER camera. Images of H2B-eYFP were maximum intensity projections of all Z-planes and were processed using Cell-M software.

Northern blotting

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN) from asynchronous cells. RNA (15 μg) was separated on a 0.8% agarose gel, transferred onto a GeneScreen hybridization
transfer membrane (Perkin-Elmer), and cross-linked to the membrane by UV-light irradiation. Prehybridization was carried out for 2 to 4 hours at 45°C in 10 ml of hybridization mix [25% deionized formamide, 10% dextran sulfate, 0.2% BSA, 0.2% polyvinyl pyrrolidone, 0.2% Ficoll, 50 mmol/L Tris (pH 7.5), 0.1% pyrophosphate, 1% SDS, 1 mol/L NaCl, 100 mg/ml sonicated denatured herring sperm DNA].

Hybridization was performed for 4 hours to overnight using radiolabeled cDNA probes. A 720-bp fragment of the BUBR1 coding region (basepairs 1–720) and a 650-bp fragment of the α-tubulin coding region (basepairs 1–650) was used for generation of cDNA probes. The DNA fragments were labeled with Rediprime II according to the manufacturer’s protocol (Amersham). Blots were washed for 5 minutes in 2× SSC at room temperature and at 65°C in 2× SSC/0.1% SDS for 15 minutes each.

Results and Discussion

MVA patient cell lines are checkpoint deficient and have chromosome alignment defects

Monoallelic and biallelic mutations in BUB1B were identified in British and Japanese MVA patients (2, 3). Previous studies had shown that centromere cohesion was lost in colcemid-treated MVA fibroblasts (3, 25), and that colcemid addition to the media of fibroblasts from Japanese MVA patients did not cause an accumulation in mitosis and increased the amount of cells with micronuclei (3). To examine the fidelity of chromosome segregation in MVA patients, we established lymphoblastoid and fibroblast cell lines of MVA patients as well as of their respective parents or unrelated healthy individuals (an overview is presented in Supplementary Table S1). Flow cytometric analysis combined with time-lapse imaging revealed that the MVA patient cells failed to accumulate in mitosis in a checkpoint-dependent manner after treatment with the spindle-depolymerizing drug nocodazole (Fig. 1A) or the EG5 inhibitor S-trityl-l-cysteine (STLC; refs. 26, 27; Fig. 1B). Whereas control STLC-treated cells were delayed in mitosis for the duration of the experiment, 50% of the population of MVA patient cell lines exited within 68 minutes (753X/R727C) and 114 minutes (731X/Y155C). Furthermore, MVA patient cells, treated with the proteasome inhibitor MG132 to prevent mitotic exit, showed a 2- to 3-fold increase in the amount of chromosome misalignment compared with control lines (Fig. 1C).

Strikingly, restoration of high levels of BUBR1 by transient expression of localization and affinity purification (LAP)-tagged BUB1B cDNA (23) induced a 2-fold increase in the response to nocodazole in patient cells (Fig. 1D). The amount of mitotic cells was, however, unchanged in LAP-BUBR1–expressing control cells (Fig. 1D). Importantly, comparison of the relative activity of the mitotic checkpoint of cells from one of the MVA patients (731X/Y155C) to that of cells of the relevant parental control (731X/WT) showed that mitotic checkpoint activity was almost fully restored to parental levels on reexpression of BUBR1 in the patient cells (Fig. 1D). Together, these findings show that the two mitotic processes in which BUBR1 participates, mitotic checkpoint signaling and chromosome alignment, are severely impaired in cells from MVA patients.

All kinase domain–localized mutations in BUBR1 reduce overall BUBR1 protein abundance

Most BUBR1 substitution mutations identified in British MVA patients are located in or in close proximity to the region encoding the kinase domain of BUBR1. This has inspired the hypothesis that BUBR1 kinase activity and/or substrate recognition is important for chromosome segregation and is affected by the MVA mutations. Although an intriguing hypothesis, analysis of BUBR1 protein expression showed that abundance of full-length substitution mutant BUBR1 protein in all patient lines (absent/I909T, 386X/R727C, 731X/R814H, and 731X/Y155C) was decreased 2- to 6-fold compared with wild-type alleles (Fig. 2A). Interestingly, BUBR1 protein abundance was most severely decreased when mutations occurred in or near the kinase domain, but less by a mutation in the NH2-terminal TPR domain (Fig. 2A). Importantly, levels of full-length BUBR1 in the parent cell lines, carrying one mutant allele, were consistently in between those of patients and unrelated healthy controls (Fig. 2A). Low overall protein abundance translated to low levels of BUBR1 on unattached kinetochores (Fig. 2B). Enhancing contrast settings, however, showed that the residual protein could be recruited to kinetochores, indicative of at least partial functionality of mutant BUBR1 (Fig. 2B, lower insets). Similar results were obtained by expression of wild-type and mutant LAP-BUBR1 in human tissue culture cells. This approach allowed analysis of all MVA mutations including those of which no patient-derived cell lines were available (a schematic representation of the mutants is shown in Fig. 2C). Please note that fusing the LAP tag to the NH2 terminus of BUBR1 did not affect its function or subcellular localization (see below). All mutant proteins with substitution mutations in or near the kinase domain showed a 5- to 10-fold decrease in levels compared with wild-type BUBR1 in HeLa and U2OS cells (Fig. 2D; Supplementary Fig. S1). These data show that a wide range of mutations in or near the kinase domain can destabilize BUBR1. Importantly, the two substitution mutations located away from the kinase domain, Y155C and R550Q, did not affect protein levels when compared with wild-type BUBR1 (Fig. 2D).

Substitution mutations affect BUBR1 protein stability

To investigate the cause of low BUBR1 protein abundance, mRNA levels and protein turnover were measured in patient cells for which relevant parental control cells were available to allow analysis of specific single gene products. For instance, comparison of mRNA from 386X/R727C cells to that from 386X/WT cells provides information on the level of R727C transcript relative to wild-type. As shown by Northern blotting, mRNA expression of three substitution alleles, I909T, R727C, and Y155C, was unaffected (Fig. 3A). We next addressed whether the substitution mutations, rather than
affecting mRNA levels, promote protein turnover. Treatment of the lymphoblastoid cell lines with the translation inhibitor cycloheximide for 5 hours showed that the protein turnover of the substitution mutant proteins I909T and R727C was increased ~2-fold compared with wild-type protein (Fig. 3B). Additionally, U2OS cells stably expressing LAP-tagged wild-type I909T or L1012P mutant BUBR1 were treated with inhibitors to three major pathways that control protein abundance [protein translation (cycloheximide), folding (geldanamycin), and degradation (MG132)]. As expected from data of the patient cells, treatment with cycloheximide reduced mutant levels more severely than wild-type (Fig. 3C). Furthermore, whereas levels of wild-type BUBR1 showed only minor changes, levels of both mutant proteins were severely decreased after 5-hour treatments with the heat-shock protein 90 (HSP90) inhibitor (Fig. 3C, GA). Importantly, combined...
treatments of cycloheximide and geldanamycin removed virtually all mutant BUBR1 protein (Fig. 3C). This shows that the stability of these substitution mutants but not that of wild-type relied on heat-shock proteins and suggested that the mutations cause protein misfolding. In support of this, inhibition of proteasomal degradation with MG132 (Fig. 3C, MG) prevented the enhanced protein turnover caused by HSP90 inhibition (Fig. 3C, MG + GA). Thus, HSP90 activity is needed for folding of BUBR1 substitution mutants and for preventing their clearance via proteasomal degradation. Thus, low abundance of substitution mutants is, at least in part, caused by decreased protein stability.

None of the truncated proteins, 386X, 731X, and 753X, could be detected in lysates of MVA patient cell lines or in parental controls (Fig. 2A). However, most truncations, except LAP-194X and LAP-753X, showed expression levels comparable to or higher than wild-type BUBR1 in U2OS and HeLa cells (Fig. 2D; Supplementary Fig. S1). This indicates that...
truncations also affected BUBR1 protein levels, although not necessarily by decreased protein stability. In patient cells, mRNA from the 386X allele could not be detected and although the relevant parental controls are missing, the 2-fold reduction in mRNA levels of 731X/WT relative to healthy controls suggested that transcripts from the 731X allele were also absent (Fig. 3A). These findings suggested that the premature STOP codons decreased transcript stability and thereby contributed to reducing BUBR1 levels. This was in agreement with a previous suggestion that expression of truncated BUBR1 proteins may be prevented by nonsense-mediated mRNA decay (3, 28), and supported by the finding that both 386X and 731X are expressed when nonsense-mediated mRNA decay is circumvented by plasmid-driven expression in HeLa or U2OS cells. It is, however, unclear what the status of 194X, 483X, or 753X is in patient cells. Plasmid-driven expression in cancer cells of 194X and 753X shows levels that are nonetheless lower than wild-type BUBR1, suggesting that the truncations may also affect protein stability; however, BUBR1 abundance of truncation mutants is mainly affected at the mRNA level.

MVA-associated mutations in the kinase domain of BUBR1 cause chromosome segregation defects primarily by lowering protein abundance

The previous analyses showed that the MVA-associated substitution and truncation mutations in BUBR1 could be divided in two categories: those that affect its protein abundance and those that do not. To examine if mutants from the "low abundance" category compromise BUBR1 function, various aspects of chromosome segregation were examined in cells in which endogenous BUBR1 was transiently replaced with exogenous epitope-tagged mutant BUBR1 (23). As a consequence of checkpoint inactivation, cells depleted of BUBR1 were unable to delay mitosis in the presence of nocodazole (Fig. 4A), and more than 80% of the cells showed massive chromosome missegregations as determined by time-lapse microscopy (Fig. 4B). Checkpoint function was restored by expression of wild-type, shRNA-insensitive LAP-BUBR1, but the truncated proteins LAP-194X and LAP-753X as well as the five kinase domain–localized substitution mutants were unable to sustain a mitotic delay in response to spindle depolymerization and caused severe chromosome missegregations (Fig. 4B). In addition, these mutants also affected the ability of chromosomes to form stable microtubule attachments: While wild-type LAP-BUBR1 restored efficient chromosome alignment in BUBR1-depleted cells, neither of the unstable truncations nor the five substitution mutants could restore chromosome alignment in BUBR1-depleted cells (Fig. 4C).

The inability of the low-abundance mutants to restore BUBR1 functionality on our assays raised the possibility that these disease-associated mutations affected chromosome segregation primarily by lowering BUBR1 protein abundance without affecting BUBR1 function directly. Three lines of evidence strongly support this. First, forced overexpression

Figure 3. All kinase domain–localized MVA mutations affect overall BUBR1 protein abundance.
A, Northern blot of mRNA isolated from immortalized MVA lymphoblastoid (left) and fibroblast (right) cell lines. Band intensity of BUBR1/tubulin relative to healthy control cells is shown (representative of two experiments). B, quantitative immunoblot of lysates of immortalized MVA lymphoblastoid cell lines. Band intensity of BUBR1/tubulin of cells treated with cycloheximide for 5 h relative to untreated cells is shown (average of three experiments). C, quantitative immunoblot of lysates of U2OS cells stably expressing wild-type or mutant LAP-BUBR1 together with BUBR1 shRNA, treated with DMSO (−), cycloheximide (CHX), geldanamycin (GA), MG132 (MG), or their combinations for 5 h (representative experiment is shown).
Figure 4. Low BUBR1 levels is the primary cause of BUBR1 dysfunction by MVA mutations in the kinase domain. A, flow cytometric analysis of MPM-2 positivity of U2OS cells transfected with control (Mock) or BUBR1 shRNA in combination with control or RNAi-insensitive LAP-BUBR1 cDNA, and treated with nocodazole for 16 h. Boxes indicate percentage of cells positive for MPM-2. Graph represents the fraction of MPM-2-positive cells of 4N population relative to the LAP-BUBR1 WT control (average of at least three experiments, ± SEM). B, analysis of chromosome segregation by live imaging of U2OS cells expressing H2B-EYFP and transfected as in A. Graph indicates the fraction of cells with chromosome missegregations relative to BUBR1-depleted cells (average of three experiments, ± SEM). C, HeLa cells, transfected as in A, were treated with MG132 for 90 min and analyzed for chromosome alignment. Graph indicates the fraction of cells with misaligned chromosomes relative to BUBR1-depleted cells (average of three experiments, ± SEM). D, top, flow cytometric analysis of MPM-2 positivity of U2OS cells transfected with control (Mock) or BUBR1 shRNA in combination with control or RNAi-insensitive LAP-BUBR1 cDNA; substitution mutants were overexpressed to levels comparable to wild-type and treated with nocodazole for 16 h. Graph represents the percentage of MPM-2-positive cells of 4N population. Immunoblot shows the expression of wild-type and mutant LAP-BUBR1 in lysates from the same experiment (representative experiment). D, middle, analysis of chromosome segregation by live imaging of U2OS cells expressing H2B-EYFP and transfected as in A. Graph indicates the percentage of cells with chromosome missegregations (average of three experiments, ± SEM). Immunoblot shows the expression of wild-type and mutant LAP-BUBR1 in lysates from a representative experiment. D, bottom, analysis of chromosome segregation by live imaging of HeLa cells expressing inducible BUBR1 shRNA and H2B-EYFP, treated with 0, 1, or 1,000 ng/mL doxycycline. Graph indicates the percentage of cells with chromosome missegregations (average of three experiments, ± SEM). Quantitative immunoblot shows the expression of BUBR1 and tubulin in lysates from a representative experiment; band intensity of BUBR1/tubulin relative to untreated control is indicated (average of three experiments).
of the poorly expressed substitution mutants I909T and L1012P to levels comparable to wild-type BUBR1 fully restored the response to nocodazole (Fig. 4D, top graph). This showed that these mutations do not impose significant constraints on BUBR1 function other than affecting overall BUBR1 protein abundance. Second, of the two substitution mutations that were reported to occur within one allele (L844F and Q921H; ref. 2), L844F was poorly expressed and unable to restore any of the defects in BUBR1-depleted cells (Fig. 4D, middle graph; Supplementary Fig. S2A and B). In striking contrast, the Q921H mutation was indistinguishable from wild-type LAP-BUBR1, both in protein level and functionality. This suggests that the L844F substitution is the likely cause of mitotic defects in the patient carrying the doubly mutated allele. Third, the amount of segregation errors is highly sensitive to the amount of BUBR1 present in the cell. BUBR1 abundance was reduced to different levels by titration of doxycyline to a HeLa cell line stably carrying inducible expression of an shRNA to BUBR1. Whereas saturating amounts of doxycyline reduced BUBR1 protein to ~6% and caused chromosome missegregation in the majority of cells, low amounts of doxycyline, while still significantly reducing BUBR1 levels to ~13%, had virtually no effect on the fidelity of chromosome segregation (Fig. 4D, bottom graph). These findings support the hypothesis that mitotic checkpoint deficiency in MVA patient cells carrying mutations in the BUBR1 kinase domain is caused by low BUBR1 levels. Interestingly, of the two newly identified substitution mutations reported here (Y155C and R727C), only the mutation located near the kinase domain, R727C, confers a similar level of protein instability. As there is no information on structural properties of BUBR1, it is at present unclear why these specific mutations affect protein abundance. Interestingly, however, mapping of the orthologous residues in the recently published crystal structure of the highly similar BUB1 kinase (29) suggests that these residues are needed for the overall integrity of the BUB1 kinase domain. From these comparisons, it is to be expected that substitution of these amino acids for their respective disease-causing counterparts will disturb kinase domain folding and stability of the protein. In support of this, we have observed a 2-fold increase in the turnover of BUBR1 as a result of the substitution mutations. Furthermore, inhibition of HSP90, a chaperone that aids protein folding, further decreased protein levels of substitution mutants. This finding indicates a strong tendency of kinase domain–located substitution mutants to misfolding, which raises the interesting possibility that this region of BUBR1 is highly sensitive to amino acid substitutions. Detailed insights into BUBR1 structure will be crucial to further investigate this.

Stable MVA mutants cause direct functional BUBR1 defects and reveal novel aspects of the roles of BUBR1 in mitosis

We next examined functionality of the MVA mutations in BUBR1 that have no significant effect on BUBR1 protein levels. Again, all mutants were assayed on the capability to rescue mitotic checkpoint defects and chromosome misalignment in cells depleted of endogenous BUBR1 (as in Fig. 4A–C). Analysis of the two stable substitution mutants revealed that expression of LAP-R550Q rescued both functions of BUBR1 (Fig. 5A–C). Interestingly, however, the Y155C substitution mutation that is located in the NH2-terminal TPR domain was a separation-of-function allele: LAP-Y155C restored chromosome alignment to BUBR1-depleted cells (Fig. 5C) but failed to reconstitute mitotic checkpoint activity (Fig. 5A and B). The TPR domain of BUBR1 is essential for BUBR1 functionality in checkpoint signaling via interaction with KNL-1/Blinkin (30, 31). Similar to previously reported BUBR1 TPR mutants, LAP-Y155C properly localized to unattached kinetochores (Fig. 5D).

Expression of LAP-386X, a protein that lacks the kinase domain, the BUB3-interaction domain, and a CDC20-binding region, could not restore checkpoint activity nor chromosome alignment (Fig. 5A–C). As expected from the absence of the BUB3 interaction domain, LAP-386X could not localize to unattached kinetochores (Fig. 5D). In contrast, the slightly longer protein LAP-483X that has the BUB3-binding region included could be recruited to unattached kinetochores (Fig. 5D) and was fully functional in restoring mitotic checkpoint activity (Fig. 5A and B). This indicates that the extended NH2 terminus of BUBR1 is sufficient for sustained mitotic checkpoint activity. LAP-483X, however, could not rescue chromosome misalignments in BUBR1-depleted cells, and as a result, LAP-483X–reconstituted cells had a significantly extended mitosis (Supplementary Fig. S3A and B), indicative of a sustained mitotic checkpoint response to unattached chromosomes. Thus, amino acids 483–1,050 of human BUBR1 were indispensable for attachment of chromosomes to the mitotic spindle (Fig. 5C). This is in agreement with studies in Saccharomyces cerevisiae and mice describing two conserved KEN motives, both of which are intact in 483X, which are essential for the budding yeast and murine checkpoints (12, 14). However, unlike murine BUB1, which can act as a functional checkpoint component without localizing to unattached kinetochores (14), amino acids 1–386 of human BUBR1 lacking the BUB3-binding domain, essential for kinetochore localization, are not sufficient.

Surprisingly, the kinase domain did not contribute to chromosome alignment, as LAP-731X has no kinase domain yet is fully capable of supporting the microtubule attachment function of BUBR1 (Fig. 5C). Furthermore, LAP-731X restored the response to nocodazole as well as proper chromosome segregation in unperturbed mitosis (Fig. 5A and B), suggesting that, at least in our assays and in these cells, the kinase domain of human BUBR1 is not required for the mitotic checkpoint nor for stable microtubule attachments. Combined, these findings have two important implications: First, neither the kinase activity nor the 265-amino-acid kinase domain is required for establishing mitotic checkpoint activity or stable microtubule attachments. Second, stable kinetochore-microtubule attachments rely on the central part of human BUBR1 [484–715 (note that 731X contains only 715 amino acids of BUBR1

*S.J.E. Suijkerbuijk and G.J.P.L. Kops, unpublished observation.
sequence). If 731X restores all functions of BUBR1, what is then the role, if any, of the kinase domain? Although careful live cell analyses of 731X did not reveal subtle defects in our experiments, it cannot be excluded that such defects would become apparent under certain conditions. However, our demonstration that BUBR1 is destabilized by all kinase domain–localized MVA mutations offers an alternative explanation for the role of the BUBR1 kinase domain. Our data show that the integrity of the kinase domain needs to be preserved for chromosome segregation to be free of errors. This opens the possibility that the kinase domain is involved in regulating BUBR1 protein stability in a noncatalytic fashion, perhaps through ATP binding or cofactor association (Supplementary Fig. S4).

A model for aneuploidy by biallelic mutations in BUBR1

The appearance of aneuploidy in MVA patients carrying the specific combination of biallelic mutations is explained by a combination of a reduction in the efficiency of chromosome attachment with an impaired mitotic checkpoint response. Our molecular analyses provide a rationale for how the specific combinations of mutations in each patient can cause these defects. In one group of patients, both mutations in a biallelic combination reduce BUBR1 protein levels to such an extent that both chromosome attachment and the mitotic checkpoint are significantly impaired, whereas in other patients the same outcome is reached by a reduction in protein combined with direct functional impairment (Supplementary Fig. S5). In conclusion, we propose that chromosomal instability in MVA patients carrying BUB1B mutations is a result of low BUBR1 protein abundance. It will be of interest to examine whether genetic or epigenetic alterations of BUBR1 protein expression may also underlie other pathologies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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