Supplemental Material

Detailed Materials & Methods

Cell Treatment

Transfections were done using the calcium phosphate method (U2OS, SV40 transformed Fibroblasts) or Effectene (QIAGEN, HeLa). Geldanamycin (1μM) was from Invivogen, Thymidine (2.5 mM), nocodazole (250 ng/ml), MG132 (5μM), STLC (10μM), Cycloheximide (10μg/ml) doxycycline and puromycin (1μg/ml HeLa, 2μg/ml U2OS) were all from Sigma.

Mutation Detection

BUB1B mutation analysis was performed by direct sequencing of genomic DNA through the full coding sequence and intron-exon boundaries of the gene. PCR primers and conditions are available on request. Amplicons were sequenced using the BigDyeTerminator Cycle Sequencing Kit and an ABI 3730 automated sequencer (Applied Biosystems). Sequence traces were analyzed using Mutation Surveyor software v3.20 (SoftGenetics) and by visual inspection.

Antibodies

Sheep anti-BUBR1 (a gift from S.Taylor) was used for immunofluoresence, rabbit anti-BUBR1 A300-386A (Bethyl Laboratories Inc.) was used for Immunoblots, both 1:1000. Other antibodies used were mouse anti-αtubulin (Sigma), ACA (Cotrex Biochem), rabbit anti-GFP (custom) and MPM-2 (Upstate). Secondary antibodies for immunoblots (alexa-680 and -800) and immunofluoresence (alexa-488 and -647) were from Molecular Probes. Secondary antibody for flow cytometry (anti-mouse Cy5) was from Jackson IR laboratories.

Immunofluoresence

Cells, plated on 12mm coverslips, were pre-extracted with 0.2% TritonX-100 in PEM (100 mM PIPES pH6.8, 1mM MgCl2 and 5mM EGTA) for 1 minute before fixation 3% PFA in PBS. Cells were cold-treated for 15 minutes and fixed while permeabilizing in 0.2% TritonX-100 in PEM supplemented with 3.7% Shandon Zinc Formal-Fixx (Thermo Scientific) for 15 min, for cold-stable microtubule staining. Coverslips were blocked with 3% BSA in PBS for 1 hour, incubated with primary antibody for 16 hours at 4°C, washed with PBS/0.1% TritonX-100 and incubated with secondary antibodies for an additional 1 hour at room temperature. Coverslips were washed and submerged in PBS containing DAPI, washed again and mounted using ProLong Antifade (Molecular Probes). Image acquisition and quantification was done as described (29), using a DeltaVision RT system (Applied Precision) with a 100X/1.40NA UPlanSApo objective (Olympus) for acquiring images and SoftWorx software for quantification.

Flow Cytometry and (Quantitative) Immunoblotting

Cells were released from a 24 hr thymidine-induced block into nocodazole for 16 hr and analyzed as described (25). As an exception, the lymphoblastoid lines were not synchronized prior to 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 fluorescent-labeled secondary antibodies.

Live Cell imaging

For live cell imaging, cells were plated in 4-well chambered glass-bottom slides (LabTek) or 24-well glass bottom plates (MatTek), transfected and imaged in a heated chamber (37°C and 5% CO2) using a 20X/0.5NA UPLFLN objective on a Olympus IX-81 microscope, controlled by Cell-M software (Olympus). 16 bits DIC (5 msec exposure) and yellow fluorescent (15 msec exposure) images were acquired every 4min (Fibroblast and HeLa studies) or 5min (U2OS studies) using a Hamamatsu ORCA-ER camera. Images of H2B-eYFP are 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. 15μg RNA was separated on a 0.8% agarose gel, transferred to a GeneScreen™ Hybridization Transfer Membrane (Perkin Elmer) and cross-linked to the membrane by UV-light irradiation. Pre-hybridization was carried out for 2-4h 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 mM Tris pH7.5, 0.1% pyrofosfaat, 1% SDS, 1M NaCl, 100 mg/ml sonicated denatured herring sperm DNA). Hybridization was performed for 4h to overnight using radiolabeled cDNA probes. A 720bp fragment of the BUBR1 coding region (bp1-720) and 650bp fragment of the αTubulin coding region (bp1-650) was used for generation of cDNA probes. The DNA fragments were labeled with Rediprime^{TM}II according to the manufacturer's protocol (Amersham). Blots were washed 5 min in 2xSSC at room temperature and at 65°C in 2xSSC/0.1% SDS and 1xSSC/0.1%SDS for 15 min each. Membranes were exposed to PhosphorImager screens for analysis and quantification with a Storm 820 scanner (Molecular Dynamics) using ImageQuant 4.2a software.

Fig. S1. All kinase-domain-localized MVA mutations affect overall BUBR1 protein abundance.

(A) Complete immunoblot of Fig. presented in 3B.

Fig. S2. The MVA-associated BUBR1 mutants that do not affect protein stability directly compromise BUBR1 function.

(A) HeLa cells transfected as in 4A and treated with MG132 for 90 minutes analyzed for Chromosome alignment. Graph indicates the percentage of cells with misaligned chromosomes (average of 3 experiments, at least 100 cells counted per experiment, ± SEM). (B) Flow cytometric analysis of MPM-2 positivity of U2OS cells transfected as in 4A and treated with nocodazole for 16 hours. Graph represents the fraction of MPM-2 positive cells of 4N population relative to the LAP-BUBR1 wild-type control (average of at least 3 experiments, ± SEM).

Fig. S3. LAP-483X-expressing cells are delayed in mitosis.

(A) Analysis of mitotic timing by live imaging of U2OS expressing H2B-EYFP and transfected as in 4A. Graph indicates the cumulative frequency of cells exiting from mitosis (1 representative experiment, at least 40 cells). (B) Flow cytometric analysis of MPM-2 positivity of U2OS cells transfected as in 4A. Graph represents the percentage of MPM-2 positive cells of the total population (1 representative experiment).

Fig. S4. Schematic representation of BUBR1.

the mapped function of domains are indicated (see text for further clarification).

Fig. S5. Molecular causes of aneuploidy in MVA patients

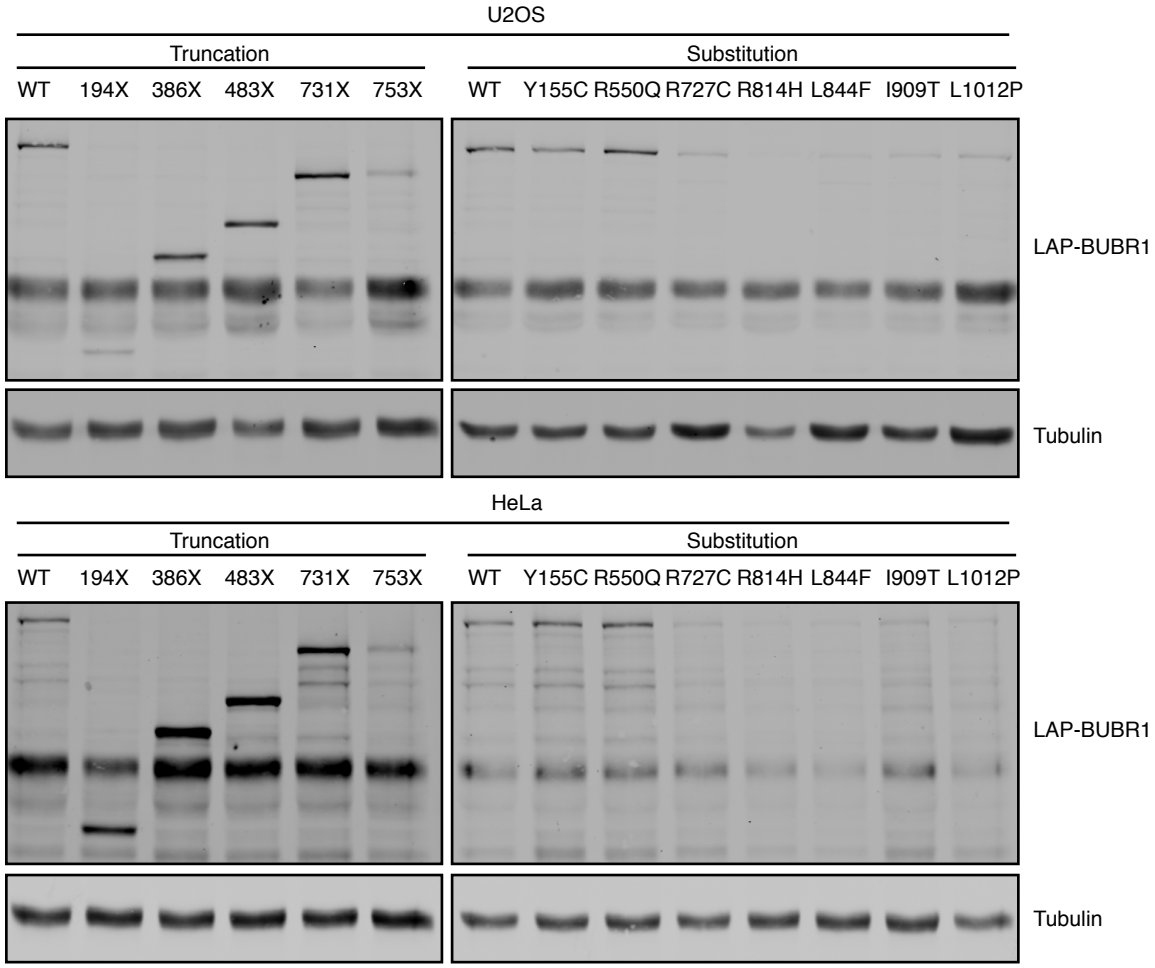
Model for the effects of MVA mutations on the function of BUBR1 in mitosis. Colors represent the combinations of truncation and substitution mutations found in patients. Asterisks indicate alleles that could not be analyzed on mRNA presence in patient lines. See text for further clarification.

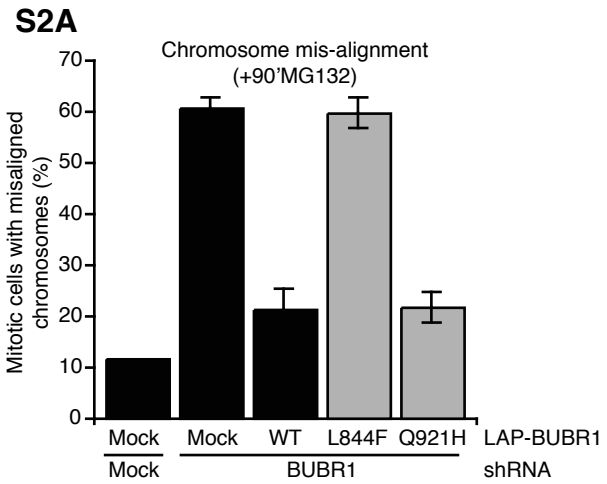
Supplemental Table 1. Overview of MVA mutations in *BUB1B*

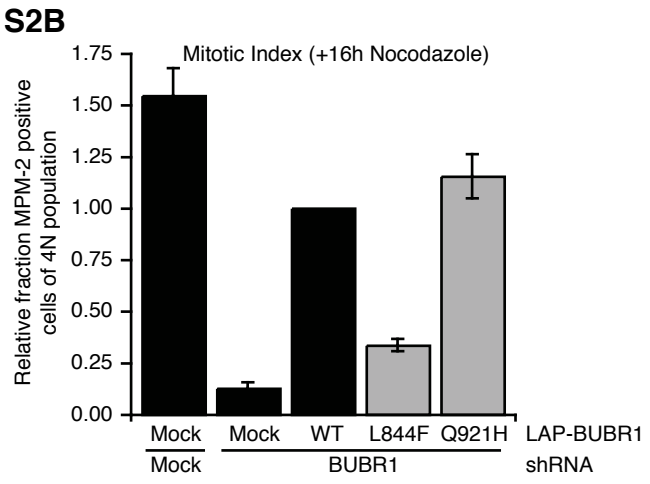
Allele 1					Allele 2	
Name	**Mutation**	**Effect**	**Amino acid sequence**		**Name**	**Patient cells?**
194X	580C>T	X194	185-PLERLQSQHX-194 185-PLERLQSQHR-195	Mutant WT	L844F & Q921H	No
386X	1156C>T*	X386	371-PGKEEGDPLQRVQSHX-386 371-PGKEEGDPLQRVQSHQ-386	Mutant WT	R727C*	Yes, Lymphoblastoid
483X	IVS10-1G>T (Δexon11)	Q467fsX483	465-GDQRNFTCGEHLAGTTSFX-483 465-GDQQEETMPTKETTKLQIA-483	Mutant WT	R550Q	No
731X	IVS16-2A>G*	S714fsX731	712-ETSGTLLSHHGVHSIADSYX-731 712-ETSENPTQSPWCSQYRRQLL-731	Mutant WT	Y155C*	Yes, Fibroblast
753X	2211-2insGTTA	S738fsX753	735-PELVKCLCRVVYRRQTNAX-753 735-PELSASAELCIEDRPMPKL-753	Mutant WT	R814H	Yes, Fibroblast
“	“	“	“	“	L1012P	No
Absent	Unknown		no transcript		I909T	Yes, Lymphoblastoid

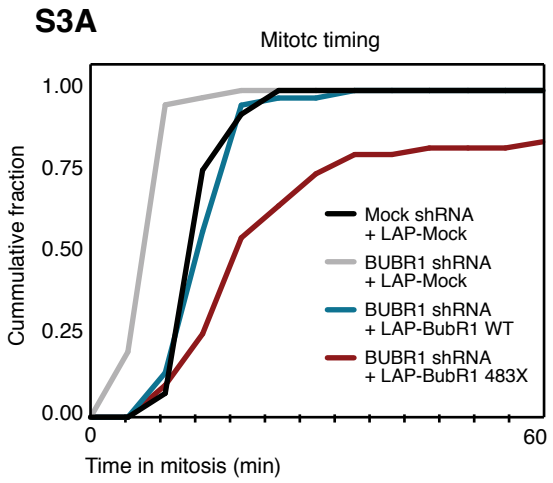
* All mutations except those marked with * have been described (Hanks et al., 2004)

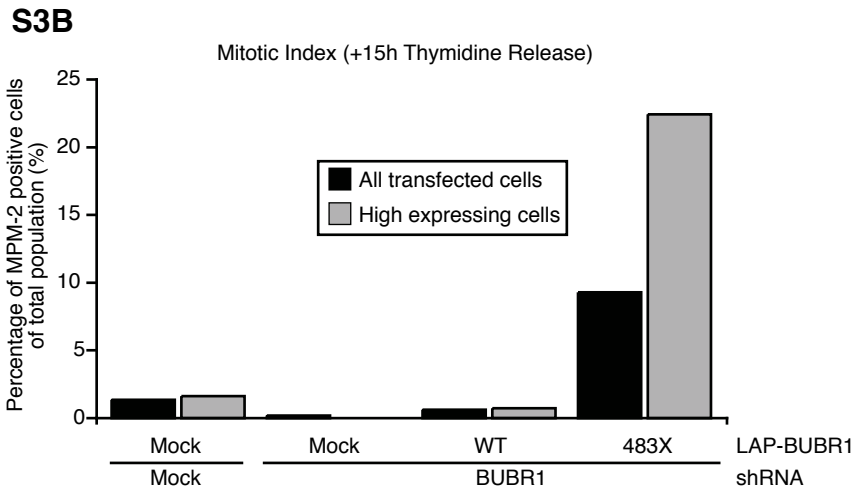
S1

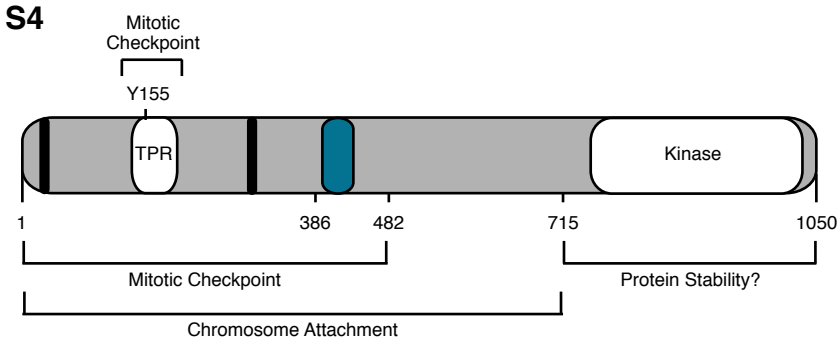


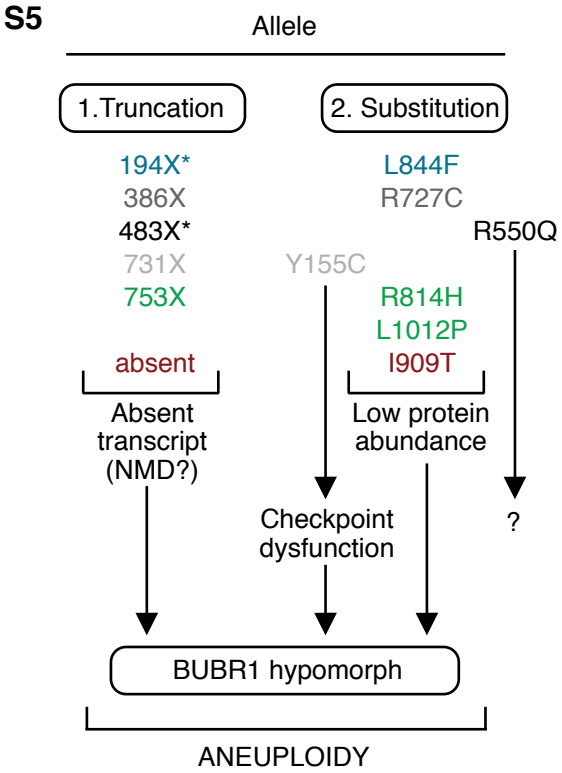












Supplementary Figures 2 Suijkerbuijk et al.