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Supporting Information for

The Arabidopsis BUB1/MAD3 family protein BMF3 requires BUB3.3 to recruit CDC20 to kinetochores in spindle assembly checkpoint signaling

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SI MATERIALS AND METHODS

Plant Materials and Growth Conditions

All T-DNA insertional lines of *A. thaliana* were obtained from the Arabidopsis Biological Research Center (ABRC) at Ohio State University. They were *bub3.3* (SALK_022904 at the AT1g69400 locus), *bmf1* (SALK_122554 at AT2g20635), *bmf2* (SAIL_303_E05 at AT2g33560), *bmf3* (SALK_032111 at AT5g05510), *mad1* (SALK_073889 at AT5g49880), *cdc20.1* (GABI_438H03 at AT4g33270) and *mps1* (GABI_663H07 at AT1g77720) and their insertion positions were shown in Supplemental Figure S1. All plants were grown in a controlled environment with 16-h-light and 8-h-dark cycle at 22°C. Seedlings for live-cell imaging and immunolocalization were produced on solid medium supplied with 1/2 Murashige Skoog (MS) (ICN, 092623022) and 0.8% phytagel (Sigma, P8169).

Oryzalin, as a highly specific and potent anti-plant microtubule agent (1), was used at 100 nM to cause weak microtubule depolymerization coupled with SAC activation but did not obviously inhibit root growth in the control plants. For oryzalin sensitivity experiments, seeds were germinated on 1/2 MS solid medium supplied with 100 nM oryzalin in DMSO and equal volumes of DMSO served as the control. Ten-day-old seedlings grown on plates with or without oryzalin were photographed and root length was measured by ImageJ. For live-cell imaging, seedlings were germinated on 1/2 MS solid medium for 4 days, then transferred to plates containing either 100 nM oryzalin or DMSO for 12h.

Plasmid Construction

To produce GFP-BUB3.3 or FLAG-BUB3.3 constructs, a 2541-bp *BUB3.3* genomic fragment containing a 736-bp promoter region was amplified from genomic DNA and cloned into the Gateway pENTR/D-TOPO vector (Thermo Fisher). To create GFP-BUB3.3 construct, the Entry vector containing genomic BUB3.3 was linearized by inverse PCR, then an enhanced green fluorescence protein (GFP) version of VisGreen fragment (2) or FLAG fragment followed a GAGA (Gly-Ala- Gly-Ala) linker coding sequence was inserted in front of the start codon via Gibson Assembly method (New England Biolabs). The resulting entry clone were recombined with pGWB1 (3) by LR clonase. The entry vectors containing CDS of MAD1, MAD2, BMF2 or BMF3, Pro_{MAD1}:GFP:MAD1 and Pro_{BMF3}:BMF3:GFP were described as previously (4). Primer pairs for plasmid construction are listed in SI Table S1.

Genomic fragments of *BUB1* (4126-bp) and *CDC20* (3115-bp), containing 1154 bp and 1071 bp hypothesized promoter regions, respectively, were amplified from genomic DNA and cloned into pDONR221 by BP reactions, followed by LR recombination reactions with pGWB650 (5) to yield a C-terminus GFP fusion. The entry vectors containing CDS of SAC components, binary vectors of BMF3-GFP, GFP-MAD1, histone H1.2-TagRFP

and GFP-TUB6 were described previously (4). Primers used in the amplification of these genes are listed in Supplemental Table S1.

Yeast Two-Hybrid Assay

For yeast two-hybrid assay, all cDNAs to be tested were amplified from a cDNA library (CD4-30, ABRC) and cloned into pDONR221. The subcloned cDNAs were recombined into the pGADT7-GW (AD) (Addgene #61702) or pGBKT7-GW (BD) (Addgene, plasmid #61703) through LR recombination reactions. The resulting constructs were transformed into the yeast strain AH109 and were spotted on SD plates without Leu and Trp (-L/-W; control media) or without Leu, Trp, His and Ade (-L/-W/-H/-A; selection media) by following the instruction described in the article reporting the two yeast two-hybrid vectors (6). Yeast colonies were photographed after the plates were incubation at 30°C for 2 days.

Transient Expression Assay in Tobacco Leaves and Stable Transformation in *A. thaliana*

Transient expression experiments were carried out by using 5-week-old tobacco (*Nicotiana benthamiana*) plants grown at 26°C under a 16 h light/8 h dark cycle. Leaves were infiltrated with *Agrobacterium tumefaciens* strain GV3101 carrying the plasmids of interest. *A. tumefaciens* cultures were grown overnight at 28°C, pelleted, washed twice with 1/2 strength liquid MS medium, and resuspended to OD600 = 0.8 before infiltration.

To carry out the BiFC experiments, the entry clones carrying the coding sequences of tested SAC components were recombined into the pGTQL1211YN (Addgene, plasmid #61704) or pGTQL1221YC (Addgene, plasmid #61705) vectors via LR clonase to generate N- or C-terminal YFP fusions. *A. tumefaciens* strains carrying nYFP and cYFP constructs were mixed 1:1 and co-infiltrated into young *N. benthamiana leaves*. After 48 hours, YFP fluorescence was examined by confocal microscopy using 488 nm excitation and 500-550 nm emission. *A. tumefaciens* carrying the GUS gene fused to nYFP and cYFP were infiltrated as negative controls.

Stable agrobacterium-mediated transformation experiments were carried out by using ~1.5-month-old *A. thaliana* plants and following a floral dip protocol (7). Seeds produced by the plants were screened according to resistance against hygromycin for pGWB4-derived plasmids or glufosinate for pGWB650-derived ones. More than 10 transformants were selected for each transformation experiments. After obtaining consistent results in two or three transgenic lines, we used one of the lines to carry out imaging experiments or compare growth to reference plants.

In vitro Protein Expression and Pull-Down Assay

To produce recombinant proteins, entry clones containing coding sequences of BUB3.3, BMF3 and CDC20.1 were recombined with pDEST565 (Addgene, plasmid #11520) or pDEST566 (Addgene, plasmid #11517) by Gateway cloning through LR clonase. The

resulting plasmids rendered the expression of GST- or MBP-tagged fusion proteins in the *E. coli* BL21 host cells. The fusion proteins were purified by using Glutathione HiCap matrix (Qiagen, catalog #30900) or MBPSep Dextrin Agarose Resins (Yeasen Biotechnology, catalog # 20515ES) according to the manufacturer's instructions.

For co-sedimentation/pull-down assays, MBP-BUB3.3 and MBP proteins were incubated with equal amounts of GST-tagged BMF3 or truncated BMF3 proteins immobilized on the Glutathione resin. Binding reactions were performed in the pull-down buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.2% Triton X-100) at 4°C for 2 hours with rotation. After washing 5 times with wash buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Triton X-100), the beads were collected by centrifugation. Bound proteins were eluted by boiling in 50 µL 1X SDS sample buffer for 10 minutes at 100°C and examined by SDS-PAGE and immunoblotting using anti-MBP or anti-GST antibodies.

Immunolocalization and Fluorescence Microscopy

Root meristematic cells from 5-day-old seedlings were prepared for immunofluorescence staining as described previously (8). Primary antibodies used in this study included GFP recombinant rabbit monoclonal antibody (ThermoFisher, Product # G10362), DM1A mouse anti-α-tubulin monoclonal antibody (Abcam, Product # ab7291) and 9A3 mouse anti-FLAG monoclonal antibody (Cell Signaling Technology, Catalog # 8146). Secondary antibodies were Alexa fluor 488-conjugated goat anti-rabbit IgG and Alexa fluor 555-conjugated goat anti-mouse IgG (ThermoFisher, Product # A32731 and A32727). Stained cells were observed under an Eclipse 600 microscope equipped with 60X Plan-Apo and 100X Plan-Fluor objectives (Nikon). Images were acquired by an OptiMOS camera (Q Imaging) controlled by the µManager software package (9).

For live-cell observation, meristem cells of 5-day-old seedlings were observed under an LSM710 laser scanning confocal module (Carl Zeiss) with a 40X C-Plan (water) objective. Images were acquired using the ZEN software package (Carl Zeiss) and processed in ImageJ/Fiji (https://fiji.sc/).

Micrographs and plant images were assembled into figure plates by using Photoshop software (Adobe).

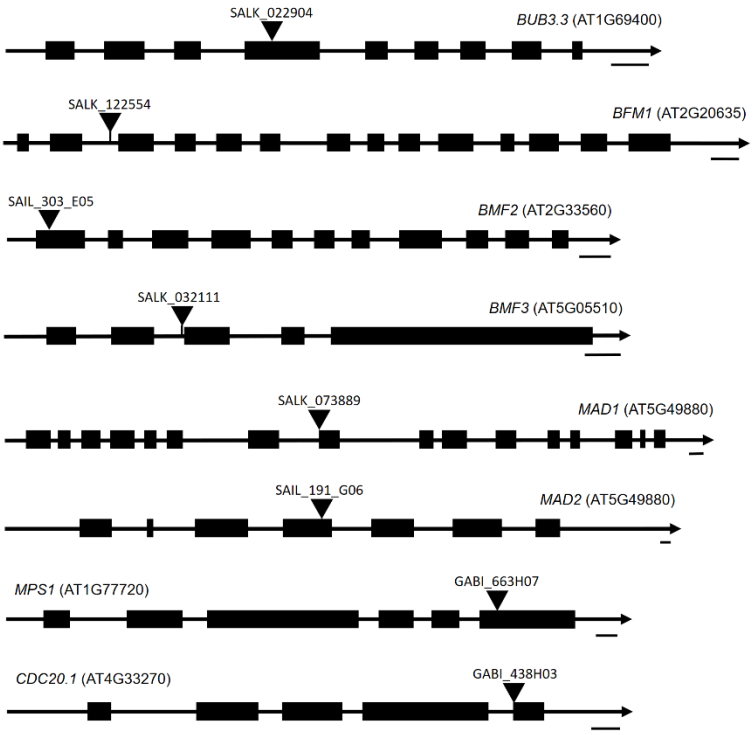

Fig. S1. Gene structures and T-DNA insertions in mutants included in this study. Exons are represented by black boxes and introns by lines. Insertions in the *BUB3.3*, *BMF1*, *BMF2*, *BMF3*, *MAD1*, *MAD2*, *MPS1*, and *CDC20.1* genes, accompanied by their corresponding locus numbers in TAIR, are highlighted by inversed black triangles associated with the insertion numbers identified at SIGnAL (http://signal.salk.edu/).

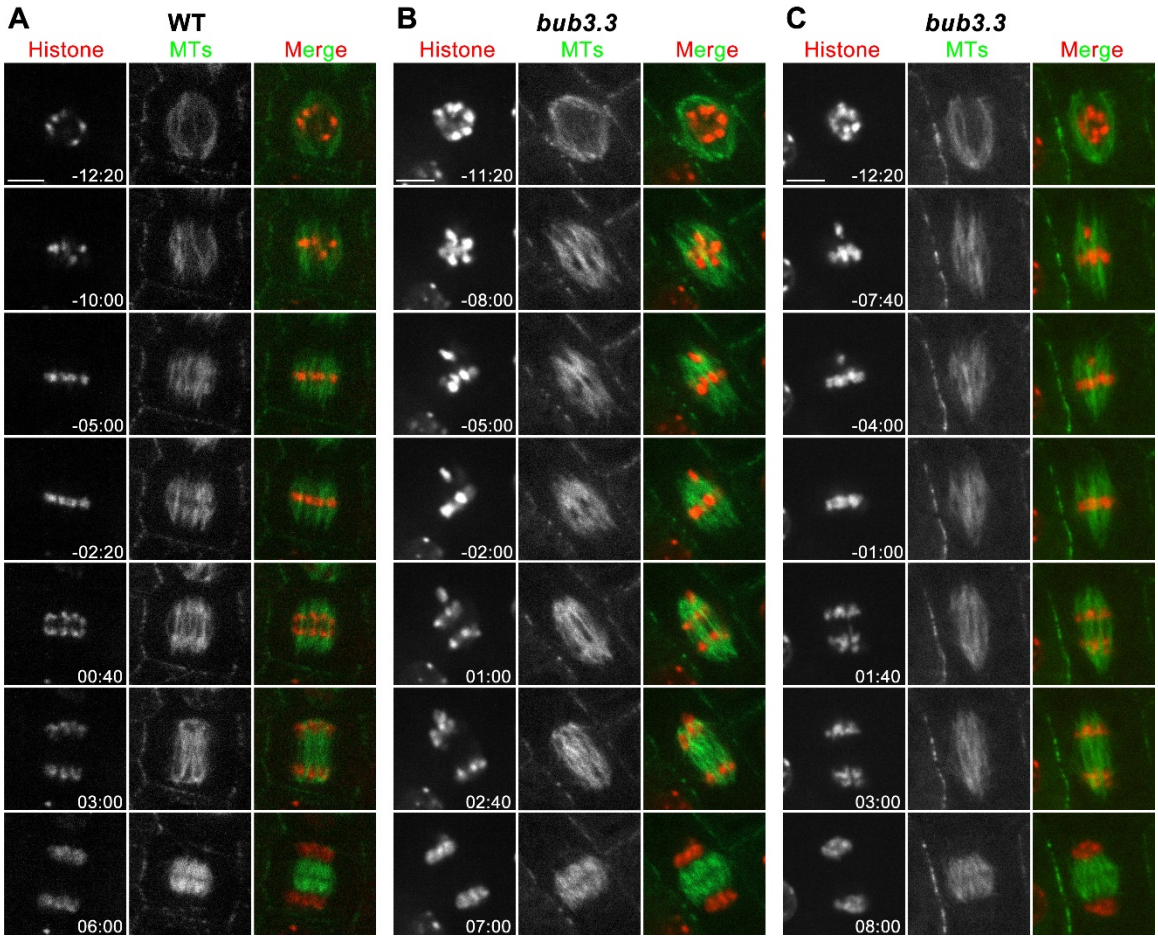

Fig. S2. Snapshots of mitotic wild-type and *bub3.3* cells undergoing mitotic cell division from Supplemental Movies S4, S5, and S6. (A) A wild-type cell has all chromosomes aligned at metaphase plate before anaphase onset. (B) A *bub3.3* mutant cell enters anaphase when a polar or misaligned chromosome remains outside the metaphase plate. (C) A *bub3.3* cell has an uncongressed chromosome at prometaphase which joins the metaphase plate, followed by chromosome segregation. Time stamps, min:sec with anaphase onset set at 0:0; scale bars, 5 μm.

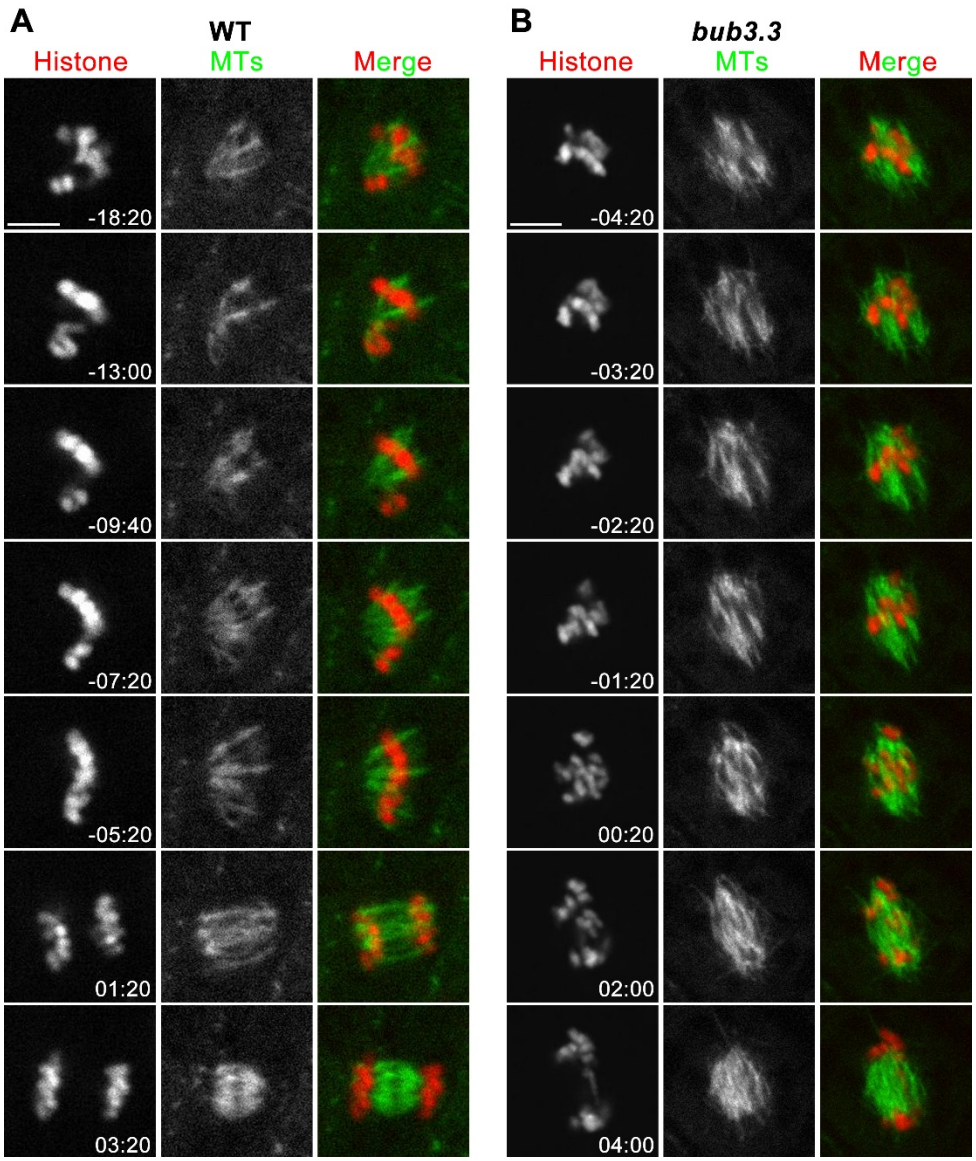

Fig. S3. Wild-type and *bub3.3* cells show polar chromosomes after oryzalin treatment. Snapshots are taken from Supplemental Movies S7 and S8. (**A**) The wild-type cell has the misaligned chromosome engaged in the metaphase plate prior to anaphase onset. (**B**) The *bub3.3* mutant cell enters anaphase when the polar chromosome remains outside the metaphase plate, and has chromosomes lagged behind in the middle of the cell. Time stamps, min:sec with anaphase onset set at 0:0; scale bars, 5 μm.

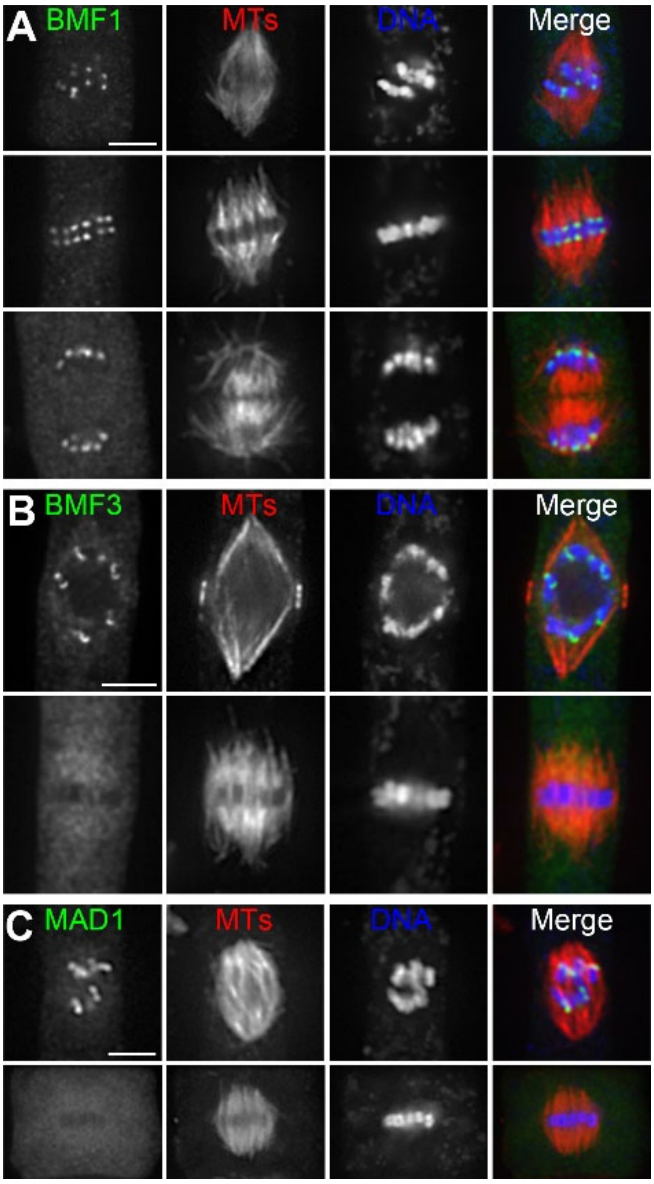

Fig. S4. Localization of BMF1, BMF3, and MAD1 during mitosis. **(A)** BMF1-GFP continuously decorated kinetochores from prometaphase (top) to metaphase (middle) and telophase/cytokinesis (bottom) when expressed in the *bmf1* mutant cells. **(B, C)** The BMF3-GFP and MAD1-GFP fusion proteins decorated kinetochores at late prophase or prometaphase (top) but disappear from there when chromosomes are aligned at metaphase plate (bottom) when expressed in the *bmf3* and *mad1* mutant cells. scale bars, 5 µm.

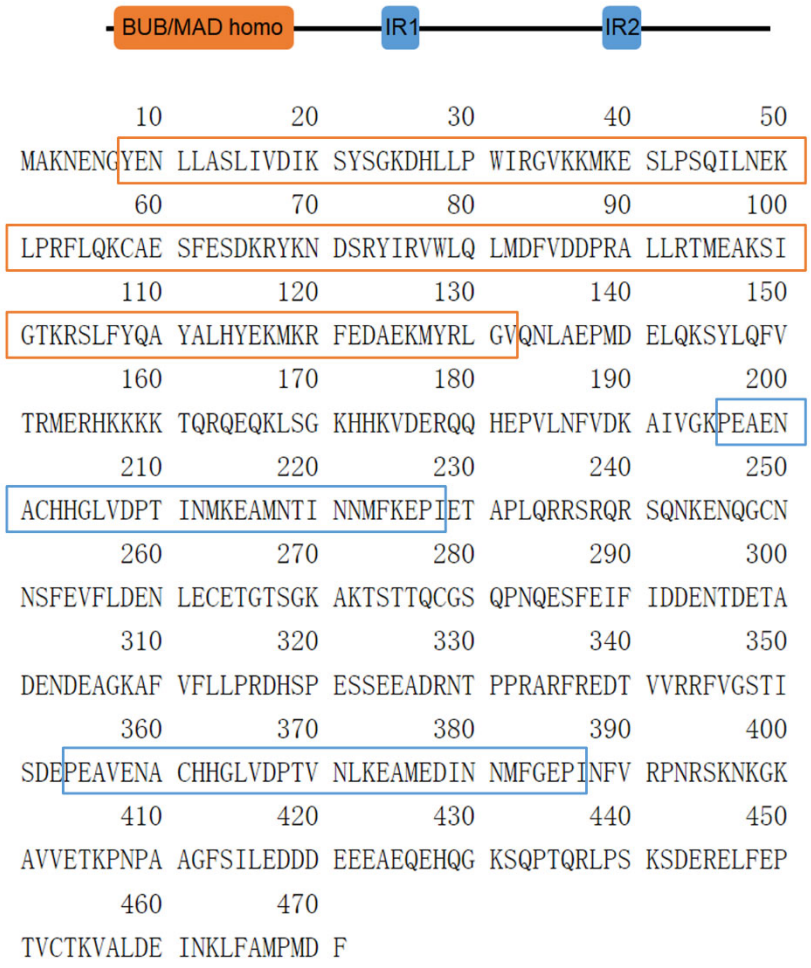

Fig. S5. Schematic representations of the functional domains and corresponding sequences of the BMF3 protein in *Arabidopsis thaliana*. The BUB/MAD homology domain is highlighted by orange boxes and internal repeats (IR) are highlighted by blue boxes.

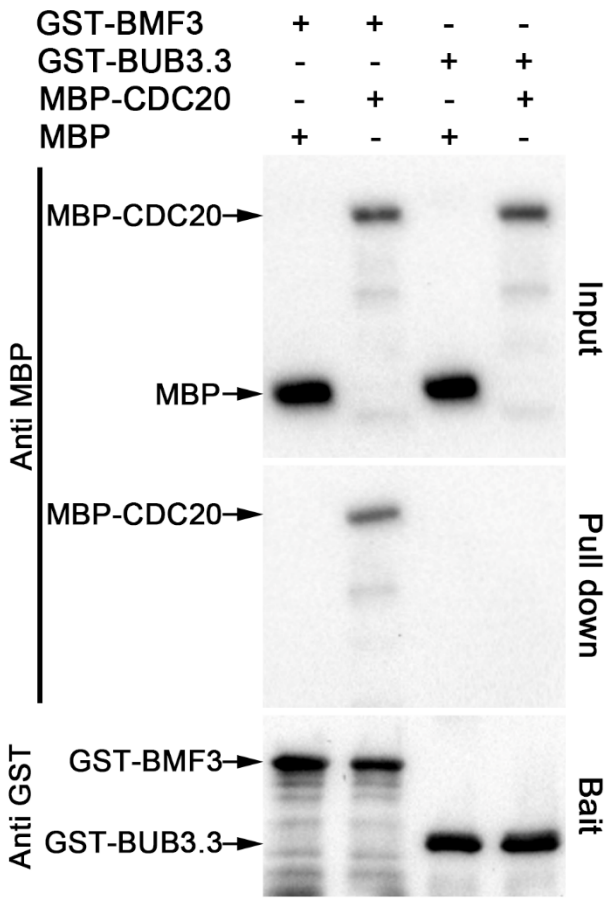

Fig. S6. Interaction of BMF3 and CDC20 by in vitro co-sedimentation/pull-down assay. Bacterially expressed GST-BMF3, GST-BUB3.3, MBP-CDC20, and MBP are used in the assay and detected by either anti-GST or anti-MBP antibodies. Only GST-BMF3 and MBP-CDC20 interact in the assay.

Table S1. Primers, their sequences, and information

Primer name	Sequence	Information
T-1-23	5'-AAG TGG TTT TGT TGT GTC TCT CTG-3'	SALK_022904 LP
T-1-24	5'-ACT TAA TCT CAC TGG AGC ATG ATG-3'	SALK_022904 RP
T-1-25	5'-AAG AAA GAA ACT CAG TGG ACA AGC-3'	SALK_122554 LP
T-1-26	5'-TCC CAC TCT CTC TCT CTC TCT CTC-3'	SALK_122554 RP
T-1-27	5'-TCA CTT GCC ATA TAA ACC AAT GAC-3'	SAIL_303_E05 LP
T-1-28	5'-TTT CTC GTT GTT TTC ATT ATC TGC-3'	SAIL_303_E05 RP
T-1-29	5'-AGC GTT TTA CAA TTC CTG TCA ATA C-3'	SALK_032111 LP
T-1-30	5'-GCA ATC AAA TTA TGG AAA CAG TAC C-3'	SALK_032111 RP
T-1-31	5'-ATA GAC TCA TGT TTG GAT ACG AAG G-3'	SALK_073889 LP
T-1-32	5'-GGT GCT ACA GAA GTA TGT CAA CAT G-3'	SALK_073889 RP
T-1-33	5'-TAG TCC TCT CCA TAT ACC TCG TCT G-3'	GABI_663H07 LP
T-1-34	5'-TGG TAC TTG ACG ATA CCT TCT TTT C-3'	GABI_663H07 RP
T-1-67	5'-AGG AAT GGA TGG ACT GAT CAT C-3'	GABI_438H03 LP
T-1-68	5'-TCC AAA ATC CCT AAT CGA ACA C-3'	GABI_438H03 RP
LBb1.3	5'-ATT TTG CCG ATT TCG GAA C-3'	BP for SALK lines
LB3	5'-TAG CAT CTG AAT TTC ATA ACC AAT CTC GAT ACA C-3'	BP for SAIL lines
8474	5'-ATA TTG ACC ATC ATA CTC ATT GC-3'	BP for GABI lines
gBMF1-attB1-F	5'-GAC AAG TTT GTA CAA AAA AGC AGG CTA TGA CAA TCG GTT ACA GAG ACG C-3'	genomic BMF1 primers cloned into pDONR221 via BP reaction
gBMF1-attB2-R	5'-GAC CAC TTT GTA CAA GAA AGC TGG GTA CGA AGA GCA TAA CGA GAT GCG TTG-3'	genomic BMF1 primers cloned into pDONR221 via BP reaction BMF1 CDS F primer for BP reaction
BMF1-CDS-attB1	5'-GAC AAG TTT GTA CAA AAA AGC AGG CTT GAT GAC AAT CGG TTA CAG AGA CGC-3'	
gBUB3;3-attB1-F	5'-GAC AAG TTT GTA CAA AAA AGC AGG CTG GCA CAG TCT TAG ATT TAT TTG CT-3'	genomic BUB3;3 primers cloned into pDONR221 via BP reaction
gBUB3;3-attB2-R	5'-GAC CAC TTT GTA CAA GAA AGC TGG GTA AAG CCT GTG TAT GAA CAC TTG AGG-3'	genomic BUB3;3 primers cloned into pDONR221 via BP reaction BUB3;3 CDS F primer for BP reaction
BUB3;3-CDS-attB1	5'-GAC AAG TTT GTA CAA AAA AGC AGG CTT GAT GAG CGG AGA TAG ACT TGA AT-3'	
L-BUB3;3-F	5'-AGC GGA GAT AGA CTT GAA TTT GA-3'	Primers for linearizing pEN-gBUB3;3
L-BUB3;3-F	5'-ATT TCA AAC CCA GAG AGA CAC AA-3'	

GFP-gBUB3;3-F	5’-TGT CTC TCT GGG TTT GAA ATA TGG TGA GCA AGG GCG AGG AGC TGT T-3’	Primers for linearizing pEN-gBUB3;3 Primers for GFP gibson into pEN-gBUB3;3
GFP-gBUB3;3-R	5’-TCA AGT CTA TCT CCG CTA GCT CCT GCA CCC TTG TAC AGC TCG TCC ATG CCG TGA-3’	Primers for GFP gibson into pEN-gBUB3;3
gCDC20.1-attB1-F	5’-GAC AAG TTT GTA CAA AAA AGC AGG CTT ACA GAC AAT CCC ATA CAA ATG GTG-3’	genomic CDC20.1 primers cloned into pDONR221 via BP reaction
gCDC20.1-attB2-R	5’-GAC CAC TTT GTA CAA GAA AGC TGG GTA ACG AAT ACG ATT CAC GTG AGA AAA-3’	genomic CDC20.1 primers cloned into pDONR221 via BP reaction
CDC20.1-CDS-attB1	5’-GAC AAG TTT GTA CAA AAA AGC AGG CTT GAT GGA TGC AGG TTT GAA TCG GT-3’	CDC20.1 CDS F primer for BP reaction
gNDC80-attB1-F	5’-GAC AAG TTT GTA CAA AAA AGC AGG CTT ACT ACT GCT GAA GCA TTG AAA GT-3’	genomic NDC80 primers cloned into pDONR221 via BP reaction
gNDC80-attB2-R	5’-GAC CAC TTT GTA CAA GAA AGC TGG GTA GAT ACC AAG GTT CTT CTT GAA GTT-3’	genomic NDC80 primers cloned into pDONR221 via BP reaction
G-1-3	5’-AAG CTG GGT AGT GCT TTC CAG ACA GTT TTT GT-3’	Primers to get BMF3 (1-172aa) CDS
G-1-4	5’-TGG AAA GCA CTA CCC AGC TTT CTT GTA CAA A-3’	Primers to get BMF3 (1-172aa) CDS Primers to get BMF3 (173-471aa) CDS
G-1-7	5’-AGG CTT GAT GCA CAA GGT TGA TGA GAG GCA AC-3’	
G-1-8	5’-CAA CCT TGT GCA TCA AGC CTG CTT TTT TGT AC-3’	Primers to get BMF3 (173-471aa) CDS Primers to get BMF3 (1-450aa) CDS
G-1-27	5’-ATT TGA ACC ATA CCC AGC TTT CTT GTA CAA AG-3’	
G-1-28	5’-AAG CTG GGT ATG GTT CAA ATA GTT CGC GCT CAT-3’	Primers to get BMF3 (1-450aa) CDS Primers to get BMF3 (1-400aa) CDS
G-1-29	5’-CAA AGG AAA ATA CCC AGC TTT CTT GTA CAA AG-3’	
G-1-30	5’-AAG CTG GGT ATT TTC CTT TGT TTT TAG AGC GGT-3’	Primers to get BMF3 (1-400aa) CDS Primers to get BMF3 (1-350aa) CDS
G-1-31	5’-TTC AAC AAT CTA CCC AGC TTT CTT GTA CAA AG-3’	

G-1-32	5’-AAG CTG GGT AGA TTG TTG AAC CAA CAA ATC TAC-3’	Primers to get BMF3 (1-350aa) CDS
G-2-25	5’-AGG GAA GCC TAA AGA GCC TAT AGA AAC TGC TCC A-3’	Primers to get $BMF3^{-IR1}$
G-2-26	5’-TAG GCT CTT TAG GCT TCC CTA CAA TGG CTT TAT-3’	Primers to get $BMF3^{-IR1}$
G-1-63	5’-CCT GAA GCA GAA CCA ATC AAT TTT GTT AGA CC-3’	Primers to get $BMF3^{-IR2}$
G-1-64	5’-GAT TGG TTC TGC TTC AGG CTC ATC GGA GAT TG-3’	Primers to get $BMF3^{-IR2}$

Movie S1 (separate file). Live-cell imaging of GFP-BUB3.3 and mCherry-labelled microtubules in the *bub3.3* background. Images have intervals of 15 s.

Movie S2 (separate file). Live-cell imaging of WT cells expressing NDC80-TagRFP following the treatment with 100 nM oryzalin. Images have intervals of 20 s.

Movie S3 (separate file). Live-cell imaging of *bub3.3* cells expressing NDC80-TagRFP following the treatment with 100 nM oryzalin. Images have intervals of 20 s.

Movie S4 (separate file). Live-cell imaging of VisGreen-TUB6 and histone H1-TagRFP in control cells without oryzalin treatment.

Movie S5 & S6 (separate file). Live-cell imaging of VisGreen-TUB6 and histone H1-TagRFP in *bub3.3* cells without oryzalin treatment.

Movie S7 (separate file). Live-cell imaging of VisGreen-TUB6 and histone H1-TagRFP in control cells treated with 100 nM oryzalin.

Movie S8 (separate file). Live-cell imaging of VisGreen-TUB6 and histone H1-TagRFP in *bub3.3* cells treated with 100 nM oryzalin.

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