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Supplemental Material to:

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Dma/RNF8 proteins are evolutionarily conserved E3 ubiquitin ligases that target septins

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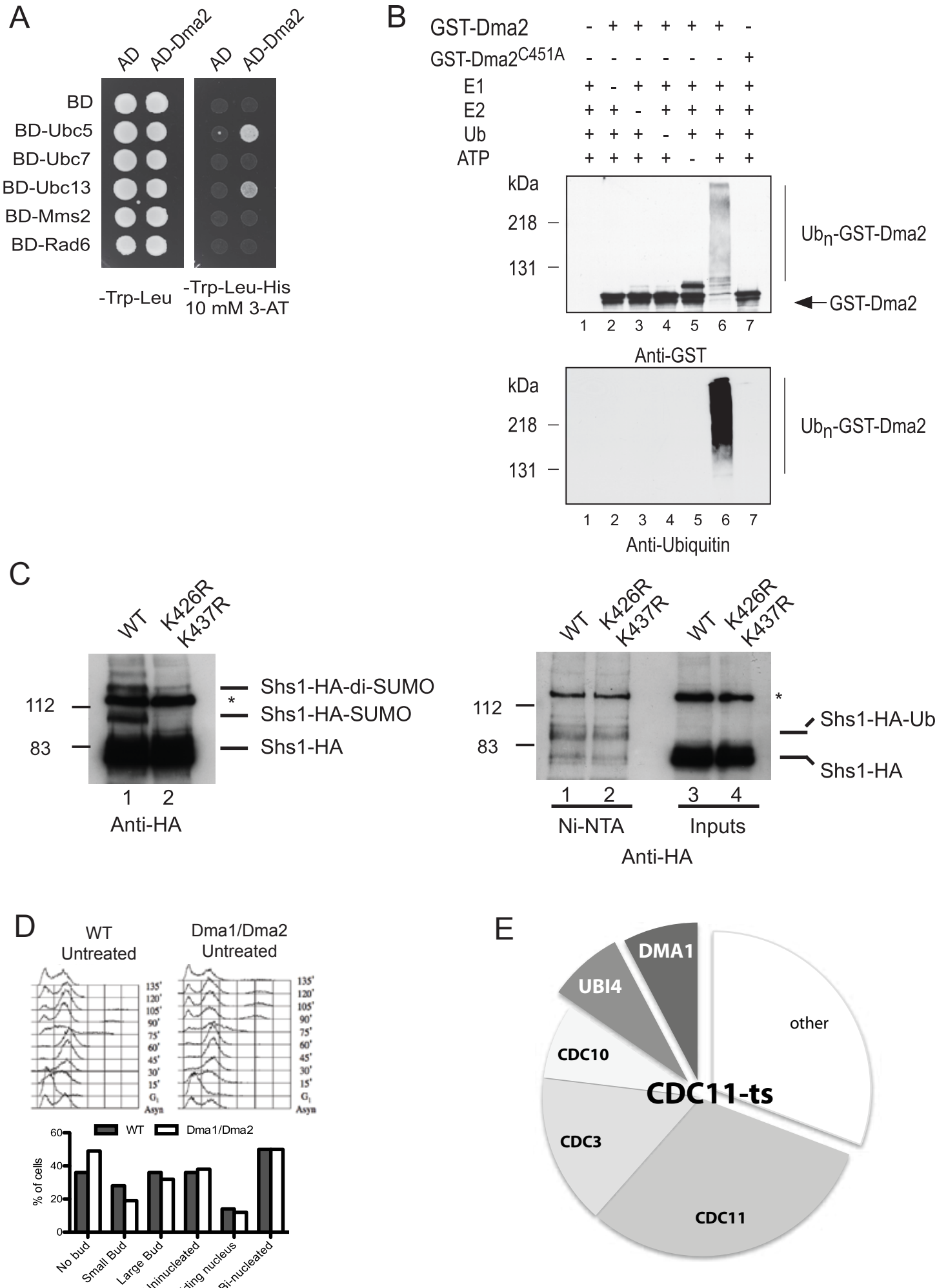
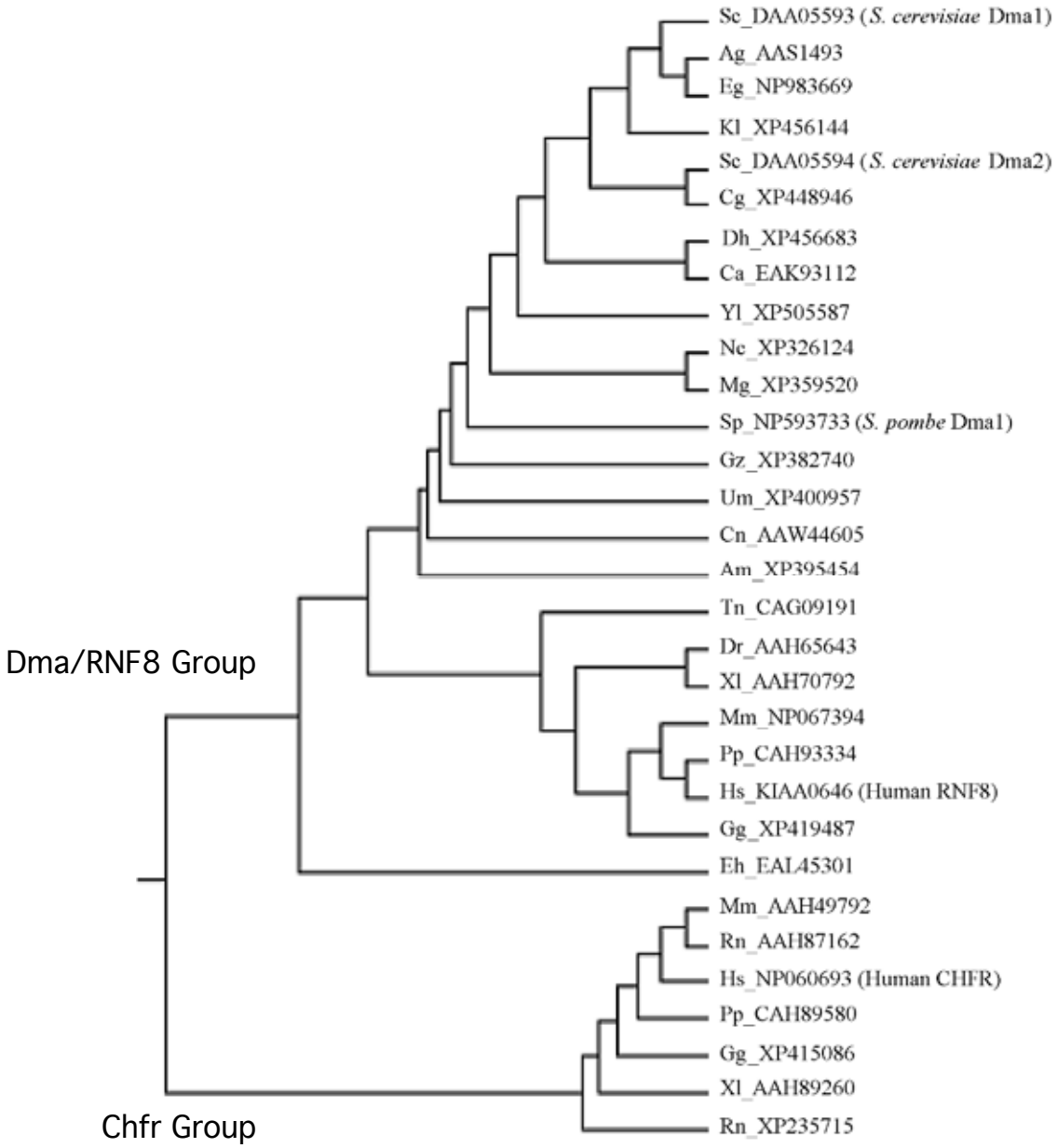


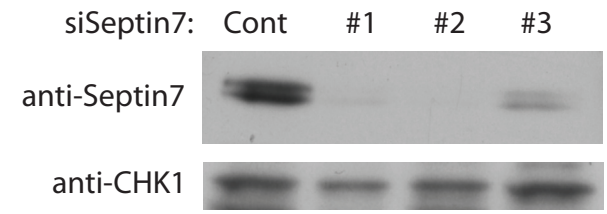
Supplementary Figure 1

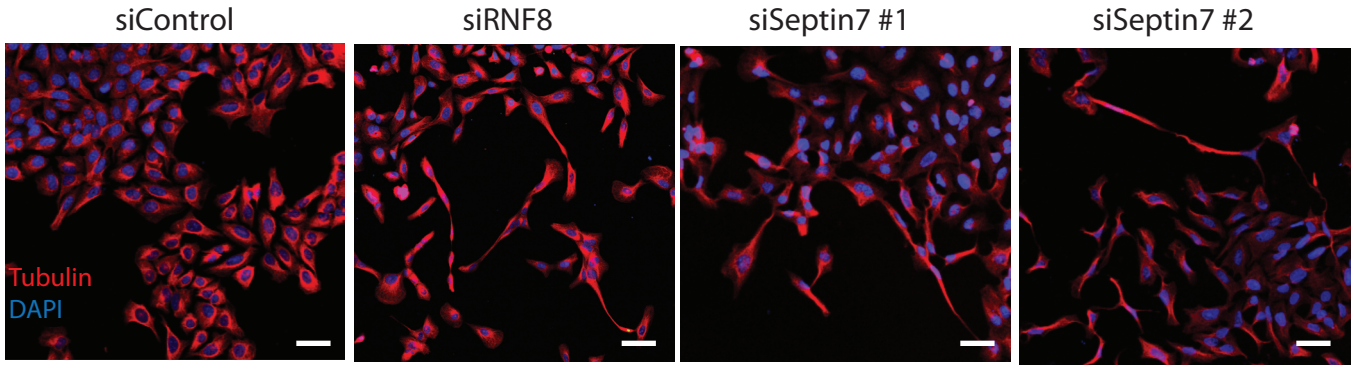
A ClustalW phylogenetic alignment of all annotated proteins with an FHA domain followed by a RING finger motif.



Ag: Ashbya gossypii; Am: Apis mellifera; Ca: Candida albicans; Cg: Candida glabrata;
Cn:Cryptococcus neoformans; Dh: Debaryomyces hansenii; Dr: Danio rerio;
Eg: Eremothecium gossypii; Eh: Entamoeba histolytica; Gg: Gallus gallus; Gz:Gibberella zeae;
Hs: Homo sapiens; Kl: Kluyveromyces lactis; Mg: Magnaporthe grisea; Mm: Mus musculus;
Nc:Neurospora crassa; Pp:Pongo pygmaeus; Rn:Rattus norvegicus;
Sc: Schaccaromyces cerevisiae; Sp: Schizoaccaromyces pombe; Tn: Tetraodon nigroviridis;
Um: Ustilago maydis; Xl: Xenopus laevis; Yl: Yarrowia lipolytica

Supplemental Figure 2





Supplementary Figure 3

Supplemental Data

Table I. Yeast strains used in this study

Strain	Relevant genotype	Source
ySP4155	*MAT a dma1ΔKlTRP1 dma2ΔKlLEU2 cla4ΔkanMX cla4-75 (on Ycplac22)*	(Fraschini et al. 2004)
Shs1TAP	*MATa SHS1-TAP::HIS3MX*	Open Biosystems
AH109	*MATa gal4Δ gal80Δ LYS2::GAL1UAS-GAL1TATA-HIS3 GAL2UAS-GAL2TATA-ADE2 URA3::MEL1UAS-MEL1TATA-lacZ*	Clonetech
SGY236	*MATa SHS1-3xHA::HIS3MX*	This study
SGY237	*MATa dma1ΔkanMX dma2ΔkanMX SHS1-3xHA::HIS3MX*	This study
SGY240	*MATa ura3::4x URA3::GAL1-DMA2 SHS1-3xHA::HIS3MX*	This study
SGY243	*MATa CDC11-3xHA::HIS3MX*	This study
SGY245	*MATa dma1ΔkanMX dma2ΔkanMX SHS1-CFP::spHIS5*	This study
SGY249	*MATa ura3::4x URA3::GAL1-DMA2 CDC11-3xHA::HIS3MX*	This study
SGY252	*MATa dma1ΔkanMX dma2ΔkanMX CDC11-3xHA::HIS3MX*	This study

Supplemental Figure 1. Dma2 interacts with E2 enzymes, has E3 ubiquitin ligase activity *in vitro* and colocalizes with septins. (A) The Ubc5, Ubc7, Ubc13, Mms2 and Rad6 E2 enzymes were expressed in yeast as fusions with the GAL4 DNA binding domain (BD) in combination with the Dma2 protein fused to the GAL4 transcription activation domain (AD) as indicated. Strains expressing empty binding and activating domains are represented as "BD" and "AD" respectively. Transformants were spotted on media selecting for the presence of plasmids (-Trp-Leu) or selecting for plasmids and for physical interactions between the E2s and Dma2 (-Trp-Leu-His, 10 mM 3-AT). Plates were incubated at 30ºC for 3 days. (B) Recombinant GST-Dma2 or a variant with a mutation in a conserved cysteine residue of the RING (GST-Dma2^{C451A}) was incubated with the indicated components for 1 h at 37°C and reactions were analyzed by Western blot with an anti-GST antibody. (C) Cells expressing WT Shs1-HA or a variant with mutations in the SUMO attachment sites (Shs1$^{K426R-K437R}$-HA) were treated with nocodazole for 2 h, proteins were prepared and analyzed by Western blotting with an anti-HA antibody (left

panel). Similarly, cells expressing WT Shs1-HA or the Shs1$^{K426R-K437R}$-HA mutant and over-expressing His-tagged ubiquitin (His-Ub) were grown to mid-log phase and lysates were prepared (right panel). One part of each lysate was incubated with Ni-NTA beads to purify proteins modified with His-Ub (Ni-NTA) and the bound material was analyzed by Western blotting with an anti-HA antibody. Another part of each lysate was directly loaded on the gel (Inputs). The asterisk (*) indicates a cross-reacting band detected by the HA-antibody. (D) Cell cycle analysis by flow cytometry (top) or microscopic quantification of budding (bottom). (E) A focused analysis of the genome-wide screen described by Magtanong *et al.*[18] showing expression of select proteins capable of compensating the lethality of Cdc11-ts alleles (Cdc11-1, Cdc11-2, Cdc11-4, Cdc11-5).

Supplemental Figure 2. ClustalW phylogenetic alignment of all annotated proteins with an FHA domain followed by a RING motif.

Supplemental Figure 3. Depleton of RNF8 and SEPT7. U2OS cells expressing control siRNA (siGFP), siRNF8 (UGGACAAUUAUGGACAACATT), or siSEPT7 (#1: AGACUGUACAGGUGGAACATT; #2: CGACTACATTGATAGTAAA; #3: GAAGAACCTTGAAGGCTATGT) were analysed 4 days following siRNA transfection. Cells were analysed by Western blot with an anti-SEPT7 antibody and anti-CHK1 for loading control. The same cells were analysed by immunofluorescence with anti-tubulin (red) and DAPI (blue) staining. More than 100 cells were counted from three independent fields from two indepedent experiments and the quantification could be found in Fig. 4D. Scale bar is 20 μm.

Video 1. CFP-RNF8, not YFP-Chfr localizes to the midbody.

Video 2. GFP-RNF8 localizes to the centrosomes and the midbody.

Video 3. The GFP-RNF8^{R42A} FHA mutant does not localize to centrosomes or the midbody.

Video 4. The GFP-RNF8^{C403S} RING mutant localizes to centrosomes and the midbody.

Video 5. YFP-RNF8 and CFP-SEPT7 significantly overlap in their localizations at the midbody.

Supplemental Materials and Methods

Yeast two-hybrid assay

Strain AH109 (Clontech) was co-transformed with combinations of activation (AD) and binding domain (BD) vectors and plated on SC-Trp-Leu. Transformants were then grown in liquid medium and spotted on SC-Trp-Leu or SC-Trp-Leu-His + 10 mM 3-AT (3-amino-1,2,4-triazole) and the plates were incubated at 30˚C for 3 days.

Antibodies

A rabbit polyclonal antibody against RNF8 was raised by using GST-RNF8 (residues 343-485) as antigen. Rabbits were injected with 400ug of protein with CovalAb standard procedures. Serum from the final bleed (day 80) was used for Western blot analysis. The anti-GFP antibody was obtained from Cancer Research UK. Other antibodies were purchased as indicated: Ubiquitin (Cell Signalling Technology), HA (Covance), Myc (Roche), GST (Santa Cruz Biotechnology), PAP (Sigma), Pgk1 (Molecular probes), Actin (Abcam).

Expression and purification of recombinant Dma2

Expression and purification of GST-DMA2 fusion proteins was done mainly as described.[33] Briefly, plasmids pGST-DMA2 and pGST-DMA2^{C451A} were transformed in E coli BL21/DE3, transformants were used to inoculate LB-AMP supplemented with 100 μM $ZnSO_4$, and GST fusion protein expression was induced for 3 h with 0.1 mM IPTG. Cells were resuspended in 50 mM TRIS pH 8, 120 mM NaCl, 1mM DTT, 1 mM AEBSF, 20 μg/ml leupeptin and lysed by sonication. Extracts were centrifuged and cleared supernatants were incubated with glutathione sepharose beads for 1 h at 4˚C. The beads were washed extensively with lysis buffer and bound proteins were eluted with 20 mM glutathione. Proteins were finally dialyzed against 20 mM Tris pH 8, 50 mM NaCl, 10 % glycerol and 1 mM DTT.

***In vitro* ubiquitylation assays**

For Fig. S1B, 1.5 μg of WT or mutant GST-Dma2 was incubated for 1 h at 37°C with 40 ng of yeast E1 (BostonBiochem) 200 ng of UbcH5b (BostonBiochem) and 3 μg of bovine ubiquitin (Sigma) in a buffer containing 50 mM Tris pH 7.5, 2.5 mM $MgCl_2$, 0.5 mM DTT and 2 mM ATP. Reactions were stopped by addition of 1 volume of 2x SDS gel loading buffer, proteins were separated on a 6% gel, and analyzed by immunoblotting with either an anti-GST or anti-ubiquitin antibody. For Fig. 1D, Shs1-TAP was purified from yeast using IgG-Sepharose beads. The beads were washed with the ubiquitylation buffer described above and the reactions (20 μl) were performed and analyzed as described above.