Supplementary information

Role of ubiquitin-protein ligase UBR5 in the disassembly of mitotic checkpoint complexes.

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Supplementary Information Text

SI Material and Methods.

Antibodies. Rabbit polyclonal antibodies directed against the human proteins were used for immunoprecipitation and immunodepletion. These were raised using the following antigens: α-Cdc27, 17-aa C-terminal peptide of Cdc27; α-UBR5, the corresponding his6-tagged 1- to 1109-aa fragment of UBR5; α-BubR1, his6-tagged 38- to 468-aa fragment of BubR1. All antibodies were purified by affinity chromatography on their respective antigens. In addition, the following commercial antibodies were used for immunoblotting: Mad2, Cdc20, APC4 and EDD/UBR5 (Santa Cruz Biotechnology sc4774, sc-13162, sc-514895 and sc-515494, respectively); Myc-tag (Cell Signaling 2276S); BubR1 and Bub3 (BD Transduction Laboratories 612053 and 611731, respectively). Immunoblots were detected and quantified with fluorescently labeled secondary antibodies using Odyssey (Li-Cor) scanner.

Immunoprecipitation. Extracts from nocodazole-arrested HeLa cells were prepared as described (1). Immunoprecipitation of extracts was carried out as described previously (2). Briefly, antibodies were bound to Affi-Prep Protein A beads (Bio-Rad) (0.5 mg antibody per ml of packed beads), beads were mixed with extracts at a ratio of 5:1 (extracts/beads, v/v), and were rotated at 4 °C for 2 h. The beads were then washed three times with a buffer consisting of 50 mM Tris·HCl (pH 7.2), 20% (v/v) glycerol, 1 mg/ml BSA, 1 mM DTT, 300 mM NaCl, and 1% (v/v) Nonidet P-40 and once with the same buffer without NaCl and Nonidet P-40. Subsequently, immunoprecipitates were subjected to phosphatase treatment for better quantitation of phosphorylated proteins.

Samples were incubated with shaking (1400 rpm) for 1 h at 30°C with 6 units of lambda phosphatase (New England Biolabs), in a reaction mixture consisting of 50 mM Tris·HCl (pH 7.2), 20% (v/v) glycerol, 1 mg/ml BSA, 1 mM DTT and 5 mM $MnCl_2$. Immunoprecipitated material was resolved by SDS/PAGE and immunoblotted for proteins indicated in Figure legends.

Immunodepletion. For immunodepletion of UBR5 from HeLa cell extracts, polyclonal anti-UBR5 antibody was bound to Affi-Prep Protein a beads (Bio-Rad) at a concentration of 0.5 mg/mL packed beads. For sham depletions, purified rabbit non-immune IgG (Sigma 12-370) was bound to Protein A beads at a similar concentration. Beads bound to antibodies were washed twice with Tris-buffered saline (20 mM Tris-HCl, pH 7.6, 150 mM NaCl) containing 1 mg/mL BSA. Samples of 10 µl (packed volume) of beads were mixed with 2 mg protein of extract by rotation at 50 rpm 4 °C for 2 h. Supernatants were collected following removal of beads by three consecutive centrifugations (10,000 g for 1 min). In all preparations, completeness of immunodepletion was examined by subjecting samples of 30 µg of supernatants to immunoblotting for UBR5.

Mass spectrometric analysis of immunopurified APC/C∗MCC. APC/C with MCC and other associated proteins were immunoprecipitated from extract of nocodazole-arrested HeLa cells (~ 30 mg of protein) with anti-Cdc27 beads as described above, except that following immunoprecipitation, beads were washed more extensively and samples were eluted from antibody with C-terminal peptide of Cdc27, as follows: Beads were washed twice with TBST [Tris-Buffered Saline-Tween, consisting of 20 mM Tris-HCl (pH 7.20), 150 Mm NaCl and 0.04% Tween-20], then 3 times with shaking for 5 minutes at 0°C with TBST containing 400 mM NaCl, and then twice with TBST. Subsequently, APC/C and associated proteins were eluted with 17-aa C-terminal peptide of Cdc27 (1 mM peptide in

TBST), for 2 hrs. at 4°C with shaking at 1,400 rpm. Supernatants were separated from beads, concentrated by ultrafiltration, resolved by SDS-PAGE and stained with Brilliant Blue G. Control samples of mitotic extracts adsorbed to non-immune rabbit IgG beads were subjected to similar treatment and were used to correct for non-specific adsorption of proteins to beads. Material eluted from gels was analyzed by mass spectrometry of tryptic peptides at the Smoler Proteomics Center of the Technion-Israel Institute of Technology, as described previously (3).

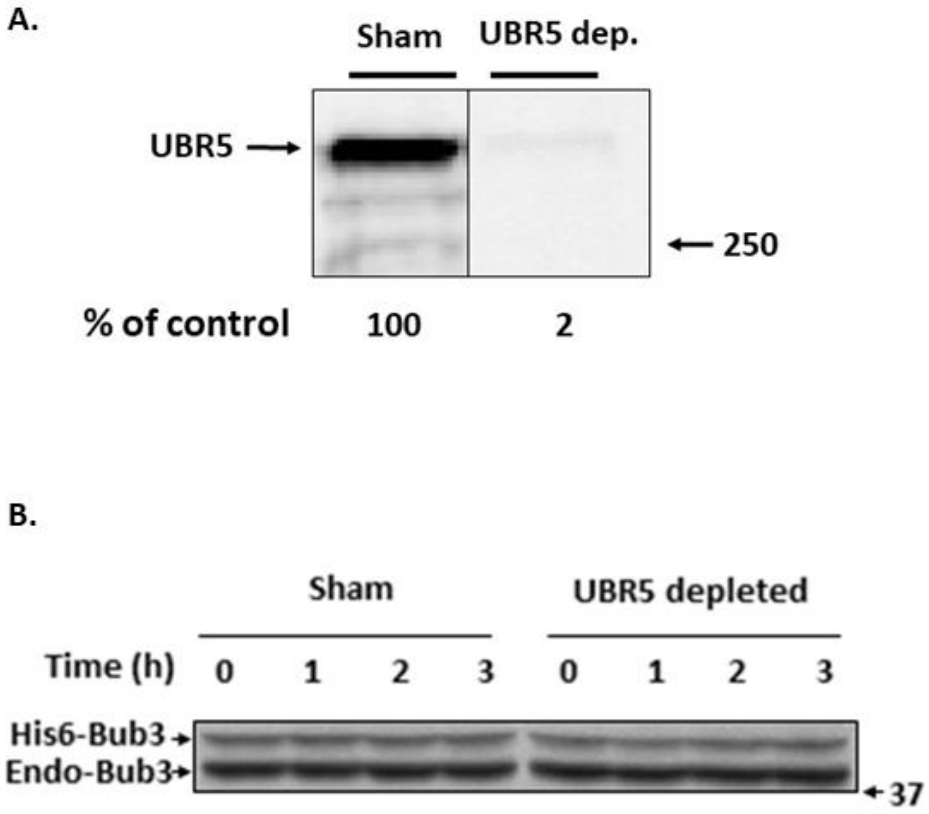

Fig S1. *(A)* Efficiency of immunodepletion of UBR5 from extracts of mitotic HeLa cells. Immunodepletion of UBR5 from mitotic HeLa cell extracts and sham treatment were carried as described in *SI Materials and Methods*. Equal amounts of samples (30 μg of protein) were subjected to immunoblotting with anti-UBR5. The amount of UBR5 that remained following immunodepletion was expressed as the percentage of the amount in the sham treatment. Number on right indicates the electrophoretic migration position of 250-kDa marker protein. *(B)* Total levels of recombinant and endogenous Bub3 proteins in incubated extracts.

Sham-treated or UBR5-depleted extracts were supplemented with recombinant his6-Bub3∗BubR1 sub-complex and were incubated as described under Fig. 2A. Samples were taken prior to immunoprecipitation and were immunoblotted for Bub3. The position of endogenous Bub3 (“Endo-Bub3”) is indicated.

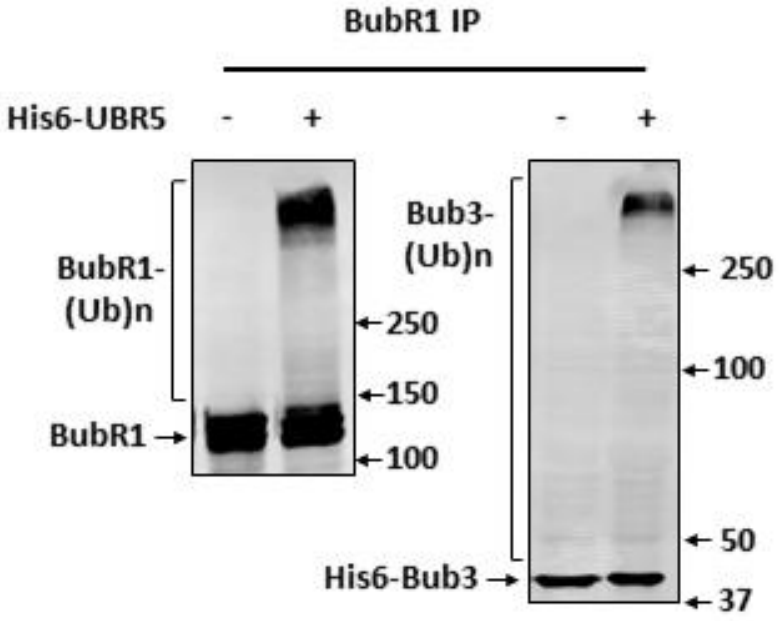

Fig. S2. Ubiquitylation of components of Bub3∗BubR1 sub-complex by recombinant UBR5. Incubation of his6-Bub3∗BubR1 with ubiquitylation system in the presence or absence of recombinant his6-UBR5 was carried out as described in Fig. 2D. Reaction products of unfractionated mixtures prior to immunoprecipitation were subjected to immunoblotting for the indicated proteins. Electrophoretic migration positions of marker proteins (kDa) are indicated on the right.

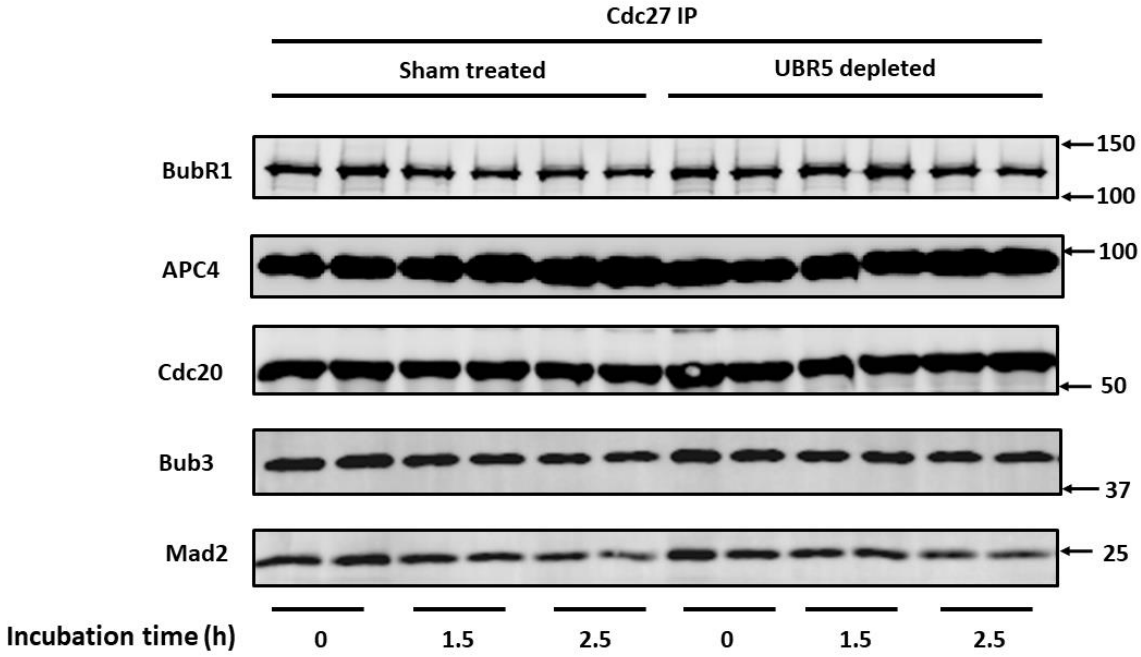

Fig. S3. Influence of UBR5 depletion on the disassembly of APC/C-bound MCC. Sham treated or UBR5-depleted checkpoint extracts (1.6 mg of protein) were incubated at 23 °C for the indicated time periods in the presence of ATP, in a reaction mixture similar to that in Fig 4A. Subsequently, duplicate samples were subjected to immunoprecipitation with anti-Cdc27 beads (10 µl packed beads), followed by washing and lambda phosphatase treatment as described in *SI Materials and Methods*. Finally, the release of checkpoint proteins from anti-Cdc27 beads was determined by immunoblotting of immunoprecipitated material for the indicated proteins. Immunoblots for APC4 subunit of APC/C served as loading controls. Numbers on the right represent the electrophoretic migration of marker proteins (kDa).

Table S1. Mass spectrometric analysis of selected proteins in anti-Cdc27 immunoprecipitates of mitotic HeLa cell extracts.

Gene name	Description	# Unique peptides
ANAPC1	Anaphase Promoting Complex Subunit 1	101
ANAPC4	Anaphase Promoting Complex Subunit 4	52
CDC20	Cell Division Cycle Protein 20	34
BUB3	Mitotic Checkpoint Protein BUB3	24
MAD2L1	Mitotic Spindle Assembly Protein MAD2A	10
UBR5	E3 ubiquitin ligase UBR5	14

Table S1. Immunopurification of APC/C-associated proteins for extracts of nocodazole-arrested cells and mass spectrometry analysis were carried out as described in *SI Materials and Methods*.

SI References.

1. I. Braunstein, S. Miniowitz, Y. Moshe, A. Hershko, Inhibitory factors associated with anaphase-promoting complex/cyclosome in mitotic checkpoint. *Proc. Natl. Acad. Sci. USA* **104**, 4870-4875 (2007).
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3. S. Kaisari *et al*., Role of Polo-like kinase 1 in the regulation of the action of p31^{comet} in the disassembly of mitotic checkpoint complexes. *Proc. Natl. Acad. Sci. USA,* **116,** 11725-11730 (2019).