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Supplemental information

Mechanism of ribosome-associated

mRNA degradation during tubulin autoregulation

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fusions

fusions

TurboID-TTC5

491P Ŋ, kDa 250 130 95 72 55 43 34 26 17 11 Biotinylated proteins (SYPRO Ruby)

Figure S1. The TTC5 K97A mutation abolishes tubulin autoregulation, related to Fig. 1.

(A) Model of TTC5 bound to the ribosome containing a 64 amino acid β-tubulin nascent chain from a previously published structure [S1] (PDB: 6T59) with the 40S subunit docked for reference. ConSurf [S2] was used to display the residue conservation of TTC5 on the surface. The highly conserved surface patch containing the K97 residue is indicated. This patch is not involved in either ribosome binding or nascent tubulin recognition, leading us to a hypothesis in which it is a docking site for downstream factors. (B) Tubulin autoregulation assay as described in Fig. 1B for TUBB, with TUBA1B reproduced from Fig. 1B for comparison. The red dashed line indicates the starting tubulin mRNA level prior to colchicine, arbitrarily set to a value of 1. The black dashed lines indicate the fold change in wild type (WT) cells for each tubulin subunit. Both TUBA1B and TUBB mRNA were quantified in all experiments in the manuscript. Because the results were comparable for both mRNAs throughout, we display only one tubulin gene in most figures for brevity and clarity. TTC5-KO cells were complemented by stable inducible expression with either GFPtagged wild type (WT) TTC5 or the K97A mutant. Data show the mean from 2 independent experiments, one of which contained 2 replicates for the TTC5 K97A cell line. Error bars denote standard deviation (SD). Single asterisks denote significant stabilization of tubulin mRNA in KO + K97A cells compared to WT (p<0.05) (C) The complemented GFP-tagged cell lines from panel B were imaged using a wide-field microscope (Life Technologies EVOS FL) to verify that expression and localization were similar for both constructs. The same microscope settings were used for both images. (D) Reconstitution of TTC5 recruitment to tubulin ribosome nascent chains (RNCs). 94-residue β -tubulin (TUBB) nascent chains were produced in rabbit reticulocyte lysates in the presence of recombinant WT or K97A mutant Twin-Streptagged TTC5 (Strep-TTC5) as indicated. "MHQV" indicates a β-tubulin construct in which its TTC5interacting MREI motif has been mutated. TTC5-associated proteins were enriched via its Strep tag, separated by SDS-PAGE and visualized by SYPRO Ruby staining for total protein (top) or autoradiography for the β -tubulin nascent chain (Tub. NC, bottom). (E) Autoregulation assay with the indicated WT, TTC5-KO, or TurboID-TTC5 rescue cell lines. Data show the mean ± SD from 2 independent experiments, one of which contained 2 replicates. Single and double asterisks indicate p<0.05 and p<0.01, respectively; "ns" indicates not significant. (F) Western blot analysis of cell lines used in panel E. Cells were pre-treated with 10 μ M colchicine (colch.) for 30 minutes as indicated by filled circles, and 50 μ M biotin was added for 2.5 h (all samples). (G) Biotinylated proteins enriched from parental, TurboID-TTC5 WT or K97A cell lines as detected by SYPRO Ruby staining. Samples prepared in the same way were used for the mass spectrometry experiment in Fig. 1C. 50 µM biotin was added 2.5h prior to harvesting of cells. To avoid overexpression of TurboID-TTC5 constructs in biotinylation experiments, we exploited the basal leaky expression from the tetracycline-regulated CMV promoter seen in the absence of added doxycycline.



Figure S2. TTC5 recruits SCAPER to tubulin ribosome nascent chain complexes, related to Fig. 2. (A) Top-ranked AlphaFold2 (AF2) multimer prediction of the interaction between TTC5 and SCAPER. The SCAPER C-terminal domain (CTD, residues 857–1400) is shown, with the rest of the molecule omitted for clarity. The TTC5 K97 residue is highlighted in red spheres. (B) Predicted Aligned Error (PAE) matrix of the TTC5-SCAPER AF2 multimer prediction shown in panel A. Lower PAE values (blue) indicate higher confidence in the relative positions and orientation between residue pairs in the model. Note that a strong signal is observed between TTC5 and the CTD of SCAPER. (C) 64-residue β -tubulin (TUBB) nascent chain complexes were produced by in vitro translation in the presence of SCAPER and TTC5 as in Fig. 2C. Reactions were then centrifuged through 10–50% sucrose gradients and fractions were analysed by SDS-PAGE, western blotting (WB), and autoradiography (autorad). An aliquot of the total reaction is analyzed in the first lane of each gradient. The peak ribosome-containing fractions, predominantly monosomes, are indicated by dashed boxes. Both the WT β -tubulin sequence and WT TTC5 are required for efficient recruitment of SCAPER to RNCs.



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Figure S3. SCAPER is required for tubulin autoregulation, related to Fig. 2.

(A) Tubulin mRNA levels were determined in the gPCR-based autoregulation assay for cells transfected with the indicated siRNAs for 72h. Samples from colchicine-treated cells (10 μ M for 3h) were normalized to untreated control samples, arbitrarily set to a value of 1 (red dashed line). The black dashed lines indicate the fold change in wild type (WT) cells for each set of samples. Data show the mean from 2 independent experiments. (B) Autoregulation assays were performed using the indicated HEK293 T-REx cell lines or four different SCAPER KO clones (cl.) obtained from two different soRNAs. Data show the mean from 2 independent experiments. Single, double, and triple asterisks indicate p<0.05, p<0.01, and p<0.001, respectively. (C) Whole cell lysates from HEK T-REx WT and SCAPER KO (sgRNA1 cl.1) were analyzed by SDS-PAGE and western blotting using anti-SCAPER and anti-β-actin antibodies. (D) Indel analysis for SCAPER KO sgRNA1 cl.1 using genomic DNA sequencing followed by TIDE analysis [S3]. This clone shows a 1-nucleotide insertion and a 2-nucleotide deletion for the two alleles. sgRNA1 targets exon 5 around codon 127. The same clone was used throughout the rest of the study and to generate rescue cell lines. (E) Quantification of mRNA decay rates in HEK T-REX WT and SCAPER KO cells after transcription shut-down with actinomycin D (ActD) for α -tubulin (TUBA1B, top) and β -tubulin (TUBB, bottom). Cells were treated with 5 μ g/ml ActD or 100 nM combretastatin A4 (CA4) from t = 0 as indicated. TUBA1B and TUBB mRNAs were quantified by RT-gPCR and normalized to GAPDH (a transcript that was stable for the observed period) and the t = 0 value. Statistical analysis was performed using a one-sample t-test. Values significantly different from 1 (t = 0 value) are indicated by single, double, and triple asterisks (p<0.05, p<0.01, and p<0.001, respectively; "ns" indicates not significant). For the WT + ActD + CA4 samples, an exponential decay curve was fitted to the data (blue curve and parameters). Data show mean ± SD from 3 replicates.



Figure S4. The SCAPER α -helical and C-terminal domains are critical for tubulin autoregulation, related to Fig. 2.

(A) Top: Structural model of SCAPER predicted by trRosetta [S4]. The displayed model was stitched from two overlapping predictions. Subdomains are color-coded as in the schematic below. Bottom: Schematic of the SCAPER domain architecture with annotated and predicted features indicated (drawn to scale). Pathologic mutations [S5-S7] characterized in autoregulation assays in this study are indicated by red arrow heads. Positions of pathologic frameshift and splice site mutations are indicated by black and green asterisks, respectively. RSL: cyclin A binding motif (Arg¹⁹⁹-Ser²⁰⁰-Leu²⁰¹); ZnF: Zinc finger; CTD: Carboxyterminal domain. (B) Tubulin autoregulation was tested by pulse-labelling of newly synthesized proteins with ³⁵S-methionine. Indicated cell lines were transiently transfected with FLAG-SCAPER encoding plasmids. Plotted is the ratio of ³⁵S-labelled tubulins from cells after 3h combretastatin A4 (CA4) treatment versus untreated control conditions as quantified by autoradiography. RSL-AAA: mutation of the cyclin A binding site (Arg¹⁹⁹-Ser²⁰⁰-Leu²⁰¹) to alanines. Numbers refer to SCAPER amino acid (aa) positions. Mean and individual data points from two independent experiments are plotted. We note that pathologic alleles with truncations after residue 726 and 935 have been reported to cause disease phenotypes such as intellectual disability, a Bardet-Biedl syndrome-like illness, and male infertility [S6, S8]. (C) Total protein analysis by western blotting of cells used in panel B. (D) Autoregulation assay with WT, SCAPER-KO and the indicated stable rescue cell lines. Note that part of the data is reproduced from Fig. 2D for comparison. Data show the mean ± SD from 2 independent experiments, one of which contained 2 replicates. For statistical comparisons against the WT cell line for each tubulin isoform, single, double, and triple asterisks indicate p<0.05, p<0.01, and p<0.001, respectively; "ns" indicates not significant. (E) Western blot analysis of SCAPER expression levels of cell lines used in panel D and Fig. 2D. Note that the SCAPER-S1219N variant shows lower expression levels. This indicates that the mutation might destabilize the protein, a potential explanation for the pathologic phenotype it has in a heterozygous patient with the Δ E620 mutation on the second allele [S5]. Under overexpression conditions, SCAPER-S1219N is functional for autoregulation (see panel D).



Figure S5. Cryo-EM analysis of SCAPER bound to TTC5 and 80S ribosomes, related to Fig. 3.

(A) RELION 4.0 classification and processing workflow for reconstruction of ribosomal particles from cryo-EM micrographs. FCwSS: Focussed classification with signal subtraction. (B) Map coloured according to the local resolution after final FCwSS around SCAPER and subtraction of the 40S subunit. (C) Goldstandard Fourier shell correlation (FSC) curve (blue) of the final 60S-TTC5-SCAPER map illustrating an overall resolution of 2.8 Å. The phase-randomized, masked FSC curve (orange) is also shown.



Figure S6. SCAPER contacts with 28S rRNA expansion segment ES27L, related to Fig. 3.

(A) View of the cryo-EM map with electrostatic surface potential [in kcal/(mol**e*)] displayed on the SCAPER CTD model. Note the two highly basic surface patches on SCAPER (dashed outlines) that contact the 60S body and ES27L rRNA. (B) Side-view of the SCAPER-CTD model from panel A with the path of the density for the rRNA expansion segment ES27L marked by dashed lines. Positively charged residues that were targeted by mutagenesis are indicated. (C, D) Western blot analysis of expression levels of FLAG-SCAPER constructs used in Fig. 3E and 3F, respectively. EMS-AAA: E1338A, M1339A, S1340A; RK-EE: R907E, K910E; RR-AA: R934A, R941A. SCAPER mutations targeting expansion segment contacts were as follows: ES*-4E: K867E, K870E, K873E and K874E; ES*-7E: as ES*-4E plus K869E, K871E and R878E.



Figure S7. The CCR4-NOT complex is required for tubulin autoregulation, related to Fig. 4 and 5. (A) Proximity labelling using HEK T-REX TurboID-SCAPER WT cells was performed in control conditions or after microtubule (MT) depolymerization with 100 nM Combretastatin A4 (CA4). Biotinylated proteins were enriched and analyzed by quantitative mass spectrometry. See also Supplementary Table S3. 3 replicates were analyzed for both conditions. (B) Western blot analysis of samples as used in Fig. 4D confirming knockdown of TTC5 and CNOT1. Note that depletion of the CNOT1 scaffolding subunit also leads to destabilization of other CCR4-NOT subunits (CNOT6, CNOT7). Specific bands are indicated by triangles for TTC5 and CNOT1; non-specific bands are indicated by asterisks. (C) Western blot analysis of samples used in Fig. 4E. Signals from blots with an antibody against endogenous CNOT8 (second panel) show both CNOT8 depletion after knockdown and expression of siRNA-resistant rescue constructs CNOT8-WT and CNOT8-CD. Specific bands are indicated by triangles for CNOT8 and CNOT6L; non-specific bands are indicated by asterisks. (D) Western blot analysis of knockdown efficiency for selected samples used in Fig. 5A. Although we did not have access to a functional CNOT10 antibody, we note that CNOT10-KD leads to destabilization of CNOT11, corroborating that they might act as a functional unit. (E) Western blot analysis of expression levels of FLAG-SCAPER constructs used in Fig. 5D. FL-SS: F628S, L632S; FIL-SKS: F628S, I629K, L632S; EE-KK: E618K, E625K; ED-KK: E633K, D640K. (F) Western blot analysis of expression levels of CNOT11 and rescue constructs used in Fig. 5E. Membranes were probed with an antibody raised against a peptide of endogenous CNOT11. The epitope was not affected by the indicated mutations. LV-QQ: L405Q, V454Q; LV-SS: L405S, V454S; LLV-SSS: L405S, L451S, V454S; RR-EE1: R447E, R450E; RR-EE2: R461E, R485E.







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- FL-SS: F628S, L632S
- FIL-SKS: F628S, I629K, L632S
- EE-KK: E618K, E625K
- ED-KK: E633K, D640K

CNOT11 mutagenesis:

- LV-QQ: L405Q, V454Q
- LV-SS: L405S, V454S LLV-SSS: L405S, L451S, V454S
- RR-EE1: R447E, R450E
- RR-EE2: R461E. R485E





Figure S8. Structural prediction of a putative SCAPER-CNOT11 complex, related to Fig. 5.

(A) Overview of an AF2 multimer predicted complex between CNOT11 and SCAPER coloured by predicted local distance difference test score (pLDDT, top, dark blue indicates high confidence), or per residue conservation (bottom). For SCAPER, only residues 604–666 of the α -helical domain are displayed for clarity. (B) PAE matrix of the model displayed in panel A. (C) Multiple sequence alignment of SCAPER homologs across the indicated species generated using Clustal Omega and displayed using Jalview software [S9, S10]. Highly conserved residues are shaded in blue. Residues in close proximity to CNOT11 in the AF2 predicted model are marked with black dots, and residues targeted in mutagenesis experiments are marked with color-coded dots. The E620 residue is highlighted by a red box. (D) Multiple sequence alignment of CNOT11 homologs generated as in panel C. Residues in close proximity to SCAPER in the AF2 model are marked dots, and residues targeted by mutagenesis are shown with coloured dots. Residue R485 is not shown in this alignment.



SiR-DNA stain





Figure S9. SCAPER is required for faithful mitosis, related to Fig. 6.

(A) Examples from time-lapse imaging of HeLa T-REx cells of the indicated genotype going through mitosis. DNA was visualized using SiR-DNA stain and maximum intensity projections are shown. Frames were aligned to nuclear envelope breakdown (t = 0). Misaligned chromosomes and segregation errors are highlighted by green and magenta arrows, respectively. (B) Autoregulation assay for HeLa cell lines used in panel A and Fig. 6B and 6C. Data show the mean from 2 independent experiments. For statistical comparisons against the WT cell line for each tubulin isoform, single and double asterisks indicate p<0.05 and p<0.01, respectively; "ns" indicates not significant. See also Fig. 2D for data from HEK293 T-REx cells. (C) Total protein analysis by western blotting for FLAG-tagged SCAPER in the HeLa cell lines used in panels A, B, and Fig. 6B and 6C. (D–E) Quantification of steady state tubulin mRNA levels in the indicated HEK T-REx cell lines. Tubulin mRNA levels were normalized to a reference gene (RPLP1) and to the WT cell line, and data from all relevant experiments in the manuscript were compiled. Data show mean \pm SD. Statistical analysis for KO and rescue cell lines was performed using a one-sample *t*-test. Values significantly different from 1 (WT levels) are indicated by single asterisks (p<0.05; "ns" indicates not significant). (D) n = 5, data reanalyzed from Fig. S1B and S1E. (E) n = 3, data reanalyzed from Fig. 5E.

Table S1. Cryo-EM data collection, processing, refinement and model statistics. Related to Fig. 3.

Data Collection	Dataset 1
Voltage (keV)	300
Pixel size (Å)	0.829
Detector	Gatan K3
Defocus range (µm)	-1.2 to -2.7
Electron dose (e ⁻ frame ⁻¹ Å ⁻²)	1
Data Processing	
Useable micrographs	19176 of 20932
Particles picked	1227269
Final particles	18949
Map sharpening B-factor (Å ²)	-20
Map resolution (Å)	2.8 (0.143 FSC)
EMDB accession code	EMD-16155
PDB accession code	8BPO
Model Composition	
Chains	62
Non-hydrogen atoms	143326
Protein residues	7633
RNA bases	3814
Metals (Mg ²⁺ /Zn ²⁺)	214/5
Refinement	
Model resolution (Å)	2.9 (0.5 FSC)
	2.4 (0.143 FSC)
CC (mask)	0.85
R.M.S deviations	
Bond lengths (Å)	0.004
Bond angles (°)	0.851
Validation	
Molprobity score	1.40
Clashscore, all atoms	3.79
Rotamers outliers (%)	0.03
Cβ outliers (%)	0.03
Ramachandran plot	
Favored (%)	96.47
Allowed (%)	3.50
Outliers (%)	0.03

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