Supplemental information

Redox-sensitive E2 Rad6 controls cellular response to oxidative stress via K63-linked ubiquitination of ribosomes

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Figure S1. Rad6 ubiquitinates ribosomes in vitro and in vivo. Related to Figure 1.

A Episomal expression of HA-tagged Rad6 reverts rad6Δ growth defect. Growth assay details are provided in the Methods section.

B Immunoblot anti-HA (Rad6) shows that thioester bond between ubiquitin and wild-type Rad6 (WT) is reduced by incubation with 15 mM DTT. C88S mutant likely carries an oxyester bond with ubiquitin. Both C88A and C88G do not bind ubiquitin.

C Immunoblot anti-Rad6 for Rad6 charging assay. Affinity-purified Rad6 (500 nM) was incubated in the presence of E1 (21 nM), ubiquitin (1.25 μM), and ATP (10 mM) for 30 min at 30 °C.

D Immunoblot of in vitro ubiquitination assay using ribosomes extracted from rad6Δ, bre1Δ, and the double mutant rad6Δ bre1Δ cells.

E Immunoblot of in vitro ribosomal ubiquitination assay. Ribosomes isolated from rad6Δbre1Δ strain were incubated with ubiquitination system. Controls show that high molecular ubiquitin conjugates are not ubiquitinated forms of Rad6, Bre1, or E1.
F Immunoblot of cells treated in the presence or absence of 0.6 mM H$_2$O$_2$ for 30 min shows that accumulation of K63 ubiquitin chains in yeast cells depends on RAD6 and K63 residue of ubiquitin. Anti-actin was used as loading control.

G, H Immunoblot of in vitro ubiquitination assay using ribosomes extracted from ubc13Δ (G), or hel2Δ strains (H) to rule out Ubc13 and Hel2 contamination.

I, J Deletion of UBC13 or HEL2 does not prevent K63 ubiquitination under stress. Immunoblot anti-K63 ubiquitin chains of isolated ribosomes from rad6Δ and ubc13Δ strains (I) and of total lysate from rad6Δ and hel2Δ (J) after 30 min incubation with 0.6 mM H$_2$O$_2$. Anti-uL5 and anti-actin were used as loading control for ribosome isolates and total cell lysate, respectively.
Figure S2. Rad6 associates with ribosomes and polysomes fractions. Related to Figure 2.

A Immunoblot from sucrose density sedimentation analysis shows that Rad6 recruitment to polysome fraction is independent of oxidative stress induced by 0.6 mM H₂O₂ for 30 min.

B Immunoblot anti-Rad6 from sucrose density sedimentation analysis of wild-type (WT, SUB280) cells in the presence or absence of 0.6 mM H₂O₂ for 30 min. *Anti-Rad6 unspecific band.

C-E Rad6 recruitment to polysome fraction is independent of the C- or N-terminal location of the HA epitope (C), Rad6 catalytic cysteine Cys88 (D), or Rad6 known E3 partners (E). *Rad6⁸⁸C linked to ubiquitin likely through an oxyester bond.

F The E3 Bre1 is present in the polysome fraction. Immunoblot anti-HA (Bre1) from a sucrose sedimentation fractionation. uS3 and uL5 were used as markers for the 40S and 60S ribosome subunits, respectively.
224,217 particles

Ribo^{Rad6IP}

pixel size 1.8 Å
3D classification

Re

Refinement 68,538 particles

3.2 Å
4.5 Å
64,035 particles
3.3 Å

40S focus mask refinement

rotated PRE classic PRE 20%

40S beak and P stalk focus mask classification

rotated PRE 19%
3.3 Å
done

7,159 particles
25,259 particles
14,552 particles
7.159 particles

Reconstruction

Extended beak
Conventional beak

Conventional beak

Ribo^{Rad6mix}

458,977 polished particles

cisTEM classification (local alignment)

80S front view

40S bottom view

tRNA site

pre-translocation

A/P

P/E

E/P

E/P

E

P

tRNA site

post-translocation

superposed

329,217 particles

A/P, P/E 5.4 Å

E/P 5.0 Å

E/P 5.5 Å

E, P 6.0 Å

FSC

Resolution(1/Å)

Resolution(1/Å)

Conventional Beak 3.7 Å

Extended Beak 3.7 Å

FSC
**Figure S3. Cryo-electron microscopy analysis of Rad6-interacting ribosomes.** Related to Figure 2.

A 3D classification analysis of ribosomes isolated from rad6Δ cells incubated *in vitro* with purified Rad6 (RiboRad6mix). Superimposed maps aligned based on the 60S showing higher variation for the 40S subunit (front view and bottom view show the rotational movement of 40S for different classes). Close up view of the decoding center shows tRNA densities and the different translation states (pre-translocation to post-translocation), depicted from cyan to salmon.

B 3D classification analysis of ribosomes co-immunoprecipitated with FLAG-tagged Rad6 (RiboRad6IP). Superimposed maps show the rotated pre-translocation (PRE, cyan and purple) and the classic translocation states (yellow) obtained from global classification results without alignment. The final maps resulting from the focused classification experiments are colored in pink (extended 40S beak), magenta (bad class), and blue (conventional 40S beak).

C Assessment of overall map resolution according to the Fourier shell correlation (FSC) 0.143-cutoff criteria for RiboRad6mix (left) and RiboRad6IP (right) classification results with the conventional and extended 40S beak structures shown in Figure 2G.
Focus classification of ribosomes from Ub<sup>K63R</sup> mutant strain

fitting to extended 18S rRNA

Focus classification of K63-ubiquitinated ribosomes

fitting to extended 18S rRNA (class at pre-translocation stage)

Receptor lysine

Rad6 C88

Rad6 60-DEE-62

Rad6 Y82

Rad6 N84

Rad6 N94
Figure S4. Focused classification cryo-EM data analysis and Rad6 eS12 docking predictions. Related to Figure 3.

A The results from focused classification analysis of Ribο^{Rad6mix} are superimposed on the 40S masked consensus of refined ribosomes.

B 3D reconstruction of the Ribο^{Rad6mix} K6 class, with extended 18S rRNA colored in blue. Inset shows the 40S beak density.

C 3D reconstruction of Ribο^{Rad6IP-XL}. Inset shows the 40S beak density with extended 18S rRNA colored in blue.

D 3D reconstruction results of focused classification of ribosomes from the K63R ubiquitin mutant strain EMD-22196 (Zhou et al., 2020). The back part of ribosomes is dimmed to highlight the 40S head. Zoomed-in view of the class with tRNAs reveals the extended 18S rRNA fitted into the density.

E Corresponding 3D reconstruction results of K63 ubiquitinated ribosomes EMD-22198 (Zhou et al., 2020) after focused classification.

F Rad6 was docked to ribosome receptor sites eS12 at ubiquitinated lysine residues K85 and K90. Top ten predictions from ClusPro 2.0 positioned Rad6 (purple) C88 close to the putative ubiquitinated lysine residue on the ribosome receptor (orange), suggesting a catalytically competent configuration. Insets show placement of Rad6 60-DEE-62 (red) and additional amino acids (Y82, N84, N94, purple) on the surface of the ribosome receptor colored by hydrophobicity (cyan, hydrophilic; tan, hydrophobic).
Figure S5. Impact of Rad6 deletions and mutations on K63 ubiquitination and cellular growth. Related to Figure 3.

A,B Immunoblot anti-HA (Rad6) from sucrose density sedimentation analysis. Representative samples for 40S, 60S, 80S, and polysomes were loaded in the same gel for improved comparison. Co-sedimentation profile for Rad6<sup>1-149</sup> (A) and Rad6<sup>1-122</sup> (B) were compared to wild-type Rad6. Complete blots are present in Figure 3F and 3H, respectively.

C,D Yeast cells expressing Rad6<sup>1-149</sup> and Rad6<sup>C88S</sup> variants are able to accumulate K63 ubiquitin chains in the cell lysate (C) and in the ribosome pellet (D) in response to H<sub>2</sub>O<sub>2</sub> treatment. The mutant Rad6<sup>C88S</sup> was used as negative control. Cells were incubated for 30 min in the presence of 0.6 mM H<sub>2</sub>O<sub>2</sub>. Anti-actin and anti-U5 were used as loading control.

E Immunoblot of cell lysate from rad6Δ cells expressing HA-tagged wild-type (WT) Rad6, Rad6<sup>C88S</sup>, Rad6<sup>1-149</sup>, or Rad6<sup>1-122</sup>. Cells were incubated for 30 min in the presence of 0.6 mM H<sub>2</sub>O<sub>2</sub>.

F Episomal expression of Rad6<sup>1-149</sup> or Rad6<sup>C88S</sup> reverses rad6Δ growth defect determined by serial dilution assays.
G Expression of Rad6^{1-122} does not reverse rad6Δ growth defect determined by serial dilution assays.

H Rad6^{WT}, Rad6^{1-149}, and Rad6^{1-122} but not Rad6^{C88A} are able to be charged with ubiquitin in vivo (Rad6~ub). Treatment of the cell lysate with 15 mM DTT reduced the Rad6-ubiquitin thioester bond.

I Growth assay of rad6Δ strain supplemented with Rad6^{WT}, pYES (empty vector), Rad6^{1-149}, and Rad6^{1-122} in the presence or absence of 25 mM hydroxyurea (HU).
Figure S6. Rad6 redox regulation by disulfide formation with Uba1. Related to Figure 4.

A Rad6 forms hetero-disulfide complex following in vitro incubation of cellular lysate with 2 mM H$_2$O$_2$ for 30 min. Disulfides are reversed by 15 mM DTT. *Rad6 charged with ubiquitin through thioester bond.

B Immunoblot anti-Rad6 of in vitro oxidation assays conducted in lysates from yeast cells deleted for BRE1, RAD18, or UBR1 (S288c genetic background). Samples were oxidized for 30 min with 2 mM H$_2$O$_2$ and reduced with 25 mM TCEP or DTT for 20 min.

C Representative immunoblot from in vitro oxidation reactions for the E2 Ubc13 shows no formation of high molecular weight species. Reactions were performed using recombinant Ubc13 (100 nM) and UBA1 (100 nM) in the presence of 2 mM H$_2$O$_2$ for 30 min at RT. Samples were also alkylated with 20 mM IAM prior to H$_2$O$_2$ treatment or reduced with 10 mM DTT prior to immunoblot analysis.

D Immunoblot shows that Rad6$^{R7A/R11A}$ is present in the ribosome pellet but does not form disulfide upon H$_2$O$_2$ treatment. Cells were incubated with 0.6 mM H$_2$O$_2$ for 30 min prior to ribosome isolation and immunoblot analysis.

E Reversal of the Rad6-Uba1 complex is independent of proteasomal or lysosomal degradation. Yeast cells were grown into MPD medium (Liu et al., 2007) and pre-incubated for 1 h with the inhibitors (75 µM MG-132 of 5 mM 3-MA) prior to stress induction with 0.6 mM H$_2$O$_2$ for 30 min. After 30 min, cells were transferred to fresh medium without H$_2$O$_2$ still containing the respective inhibitors.

F Reversal of the Rad6-Uba1 complex is independent of autophagy demonstrated by the use of the atg7A strain, which is defective in autophagosome formation (Mizushima et al., 1998). Cells were incubated for 30 min with 0.6 mM H$_2$O$_2$ and then transferred to H$_2$O$_2$-free medium to recover for up to 60 min.

G Pulse-chase experiment shows that Rad6 is not degraded after stress induction. Yeast cells were pre-incubated in the presence or absence of 150 µg/ml of cycloheximide (CHX) for 30 min prior to oxidative stress induced by 0.6 mM H$_2$O$_2$. Cells were transferred to fresh medium containing or not CHX for up to 8 hours. Samples were DTT-reduced prior to western blot.

H CHX does not prevent reversal of Rad6-Uba1 disulfide. Cells were pre-incubated in the presence or absence of 150 µg/ml of cycloheximide (CHX) for 30 min prior to oxidative stress induced by 0.6 mM H$_2$O$_2$. Cells were transferred to H$_2$O$_2$-free medium to recover in the presence or absence of CHX for up to 60 min.

I Immunoblot shows that deletion of glutathione reductase (GLR1) slows down the reduction of the Rad6-Uba1 complex. Cells were incubated for 30 min with 0.6 mM H$_2$O$_2$ and then transferred to H$_2$O$_2$-free medium to recover or up to 60 min. Anti-actin was used as loading control.

J, K Immunoblot shows that reduction of the Rad6-Uba1 complex is not impacted by the deletion of genes of the thioredoxin family. Cells were incubated for 30 min with 0.6 mM H$_2$O$_2$ and allowed to recover in H$_2$O$_2$-free medium for 30 min.

L Sequence alignment of yeast E2s, highlighting the conservation of residues close to Rad6 catalytic cysteine. Alignment was performed with Clustal Omega (EMBL-EBI).
M Immunoblot of cells expressing Rad6-HA exposed to increasing concentrations of H₂O₂. Anti-GAPDH was used as loading control.

N Immunoblot of cells expressing Rad6-HA exposed to H₂O₂, hydroxyurea (HU), or methyl methanesulfonate (MMS) for 1h. Anti-GADPH was used as loading control.

O Deletion of DUBs that reduce the pool of free ubiquitin (Hanna et al., 2003) increases the formation of Rad6 disulfides. Cells were incubated in the presence or absence of 0.6 mM H₂O₂ for 30 min.
Figure S7. UBE2A functionality and Rad6 role in stress response. Related to Figure 6 and 7.

A Expression of human UBE2A in yeast cells reverts rad6Δ growth defect determined by serial dilution assays. pTDH3 represents the empty vector.

B Immunoblot anti-K63 ubiquitin chains shows that expression of UBE2A rescues rad6Δ K63 ubiquitin chain accumulation under 0.6 mM H₂O₂. pTDH3 represents the empty vector.

C HeLa cell lysate incubated in vitro with 20 mM cysteine alkylator iodoacetamide (IAM), oxidized with 2 mM H₂O₂, or reduced with 15 mM dithiothreitol (DTT). anti-Rad6 (UBE2A) immunoblot shows the formation of reversible disulfides. * Anti-Rad6 unspecific bands.

D Different exposures for anti-puromycin immunoblot presented in Figure 7A.

E Average time required for yeast cells from wild-type (WT) and rad6Δ strains to reach OD₆₀₀ of 0.2 and 1.2 starting from OD₆₀₀ of 0.05 in the presence or absence of H₂O₂ stress. Data was determined from three biological replicates. The complete growth curve is presented in Figure 7C.

F Cell cycle profile from flow cytometric analyses of DNA content. Cells were incubated with 0.6 mM H₂O₂ for 30 min. DNA labeling with SYTOX Green is described under Materials and Methods.

G Frequency distribution plots of forward scatter (FSC) measurements by flow cytometry for 10,000 WT and rad6Δ cells in the absence or presence of 0.6 mM H₂O₂ for 30 and 60 min. Forward scatter values allow for relative discrimination of cells by size. Bar graph shows mean FSC x 1,000 for each strain.