Figures and figure supplements

Cdc48 regulates a deubiquitylase cascade critical for mitochondrial fusion

Tânia Simões et al
**Figure 1.** Cdc48 regulates Fzo1 and mitochondrial fusion. (A) Mitochondrial morphology of CDC48 mutant cells. Wild-type (wt) or cdc48-2 mutant cells were analyzed for mitochondrial tubulation after expressing a mitochondrial-targeted GFP plasmid. Cellular (Nomarski) and mitochondrial (GFP) morphology were visualized by fluorescence microscopy. Bottom panel, quantification of four independent experiments (with more than 200 cells each) including mean and standard deviation (SD), as described (Cumming et al., 2007). (B) Ubiquitylation of Fzo1 upon mutation of CDC48. Crude mitochondrial extracts from wt or cdc48-2 mutant cells expressing HA-Fzo1, or the corresponding empty vector, were solubilized and analyzed by SDS-PAGE and immunoblotting using HA-specific antibodies. Unmodified and ubiquitylated forms of HA-Fzo1 are indicated by a black arrowhead or black arrows, respectively. Ubiquitylated forms of Fzo1 are labeled with Ub. Bottom panel, quantification of three independent experiments, normalized to PoS and including SD. **, p<0.01 (paired t-test). (C) Steady state levels of Fzo1 upon mutation of CDC48. Total cellular extracts of wt or cdc48-2 mutant cells were analyzed by SDS-PAGE and immunoblotting using Fzo1- or Ubc6-specific and, as a loading control, Tom40-specific antibodies. Bottom panels, quantification of three independent experiments, including SD. (D) Proteasome dependence of Fzo1 degradation in cdc48-2 mutant cells. The turnover of endogenous Fzo1 expressed in Δpdr5 Δsnq2 and Δpdr5 Δsnq2 cdc48-2 cells was assessed after inhibition of cytosolic protein synthesis with cycloheximide (CHX), for the indicated time points in exponentially growing cultures in absence or presence of the proteasomal inhibitor MG132. Samples were analyzed by SDS-PAGE and immunoblotting using Fzo1-specific, Ubc6-specific (as an unstable protein control) and Sec61-specific (as a loading control) antibodies. Right panel, quantification of five independent experiments, including SD. PoS, PonceauS staining.

DOI: https://doi.org/10.7554/eLife.30015.003
Figure 1—figure supplement 1. Cdc48 regulates Fzo1 and mitochondrial fusion. (A) Steady state levels of Fzo1 upon mutation of CDC48. Total cellular extracts of Δfzo1 or wt cells or different CDC48 mutant cells were analyzed by SDS-PAGE and immunoblotting using Fzo1-, Ubc6- and Tom40-specific antibodies. Bottom panels, quantification of five independent experiments, including SD. ns, p>0.05; *, p≤0.05; ***, p≤0.001 (One-way ANOVA, Tukey’s multiple comparison test). (B) Role of Cdc48 cofactors in the steady state levels of Fzo1. Total cellular extracts of wt cells or ufd1-2 and npl4-1 mutant cells were analyzed by SDS-PAGE and immunoblotting using Fzo1- or Ubc6-specific antibodies. Bottom panels, quantification of seven (ufd1-2) or nine (npl4-1) independent experiments, including SD. **p≤0.01; ***p≤0.001 (paired t-test). (C) Steady state levels of Fzo1 upon deletion of DOA1. Total cellular extracts of Δfzo1, wt or Δdoa1 cells were analyzed by SDS-PAGE and immunoblotting using Fzo1-, Ubc6- and Tom40-specific antibodies. Bottom panel, quantification of five independent experiments, including SD. *p≤0.05 (paired t-test). PoS, PonceauS staining.

DOI: https://doi.org/10.7554/eLife.30015.004
Figure 1—figure supplement 2. Cdc48 regulates Fzo1 and mitochondrial fusion. (A) Rescue analysis of Fzo1 steady state levels in cdc48-2 cells. Total cellular extracts of wt or cdc48-2 mutant cells expressing Cdc48, Cdc48<sup>A547T</sup> or the corresponding empty vector were analyzed by SDS-PAGE and immunoblotting using an HA-specific antibody. (B) Rescue analysis of Fzo1 ubiquitylation in cdc48-2 cells. Crude mitochondrial extracts from wt or cdc48-2 mutant cells, additionally expressing HA-Fzo1 and Cdc48, Cdc48<sup>A547T</sup> or the corresponding empty vector, as indicated, were lysed and HA-tagged Fzo1 was precipitated using HA-coupled beads. Samples were analyzed by SDS-PAGE and immunoblotting using an HA-specific antibody. Unmodified and ubiquitylated forms of HA-Fzo1 are indicated as in Figure 1B. (C) Rescue analysis of mitochondrial morphology in cdc48-2 cells. Wt or cdc48-2 mutant cells expressing Cdc48 or Cdc48<sup>A547T</sup> or the corresponding empty vector as indicated were analyzed for mitochondrial tubulation after expressing a mitochondrial-targeted GFP plasmid, as in Figure 1A. Quantification from three different experiments (with more than 200 cells each), including SD, as described (Cumming et al., 2007). IP, immunoprecipitation. PoS, PonceauS staining.

DOI: https://doi.org/10.7554/eLife.30015.005
**Figure 2.** Cdc48 specifically affects ubiquitylated Fzo1. (A) Physical interaction between Cdc48 and ubiquitylated Fzo1. HA-Fzo1, HA-Fzo1\(^{K464R}\) or the corresponding vector were expressed in \(\Delta fzo1\) cells. Crude mitochondrial extracts were lysed and HA-tagged Fzo1 was precipitated using HA-coupled beads and analyzed by SDS-PAGE and immunoblotting using HA- and Cdc48-specific antibodies. Unmodified and ubiquitylated forms of HA-Fzo1 are indicated as in 1B. (B) Effect of the anti-fusion ubiquitylation of Fzo1 on its interaction with Cdc48. HA-Fzo1 or HA-Fzo1\(^{K464R}\), expressed in the presence of Ubp2 (\(\Delta fzo1\) cells plus empty vector) or Ubp2\(^{C745S}\) (\(\Delta ubp2\) \(\Delta fzo1\) cells plus Ubp2\(^{C745S}\)-Flag), or the corresponding vector control (the empty vectors corresponding to HA-Fzo1 and Ubp2\(^{C745S}\)-Flag, expressed in \(\Delta ubp2\) \(\Delta fzo1\) cells), were analyzed for Cdc48 interaction, as in 2A. Unmodified and ubiquitylated forms of HA-Fzo1 are indicated by a black arrowhead or black arrows, respectively. Red arrows with no fill indicate Fzo1 ubiquitylated species specifically accumulating upon expression of Ubp2\(^{C745S}\). PoS, PonceauS staining; IP, immunoprecipitation; WB, western blot.

DOI: https://doi.org/10.7554/eLife.30015.006

---

**Figure 2.** Cdc48 specifically affects ubiquitylated Fzo1. (A) Physical interaction between Cdc48 and ubiquitylated Fzo1. HA-Fzo1, HA-Fzo1\(^{K464R}\) or the corresponding vector were expressed in \(\Delta fzo1\) cells. Crude mitochondrial extracts were lysed and HA-tagged Fzo1 was precipitated using HA-coupled beads and analyzed by SDS-PAGE and immunoblotting using HA- and Cdc48-specific antibodies. Unmodified and ubiquitylated forms of HA-Fzo1 are indicated as in 1B. (B) Effect of the anti-fusion ubiquitylation of Fzo1 on its interaction with Cdc48. HA-Fzo1 or HA-Fzo1\(^{K464R}\), expressed in the presence of Ubp2 (\(\Delta fzo1\) cells plus empty vector) or Ubp2\(^{C745S}\) (\(\Delta ubp2\) \(\Delta fzo1\) cells plus Ubp2\(^{C745S}\)-Flag), or the corresponding vector control (the empty vectors corresponding to HA-Fzo1 and Ubp2\(^{C745S}\)-Flag, expressed in \(\Delta ubp2\) \(\Delta fzo1\) cells), were analyzed for Cdc48 interaction, as in 2A. Unmodified and ubiquitylated forms of HA-Fzo1 are indicated by a black arrowhead or black arrows, respectively. Red arrows with no fill indicate Fzo1 ubiquitylated species specifically accumulating upon expression of Ubp2\(^{C745S}\). PoS, PonceauS staining; IP, immunoprecipitation; WB, western blot.

DOI: https://doi.org/10.7554/eLife.30015.006
Figure 2—figure supplement 1. Cdc48 specifically affects ubiquitylated Fzo1. Steady state levels of HA-Fzo1<sup>K464R</sup> upon mutation of CDC48. Total cellular extracts of Δfzo1 or Δfzo1<sup>cdo48-2</sup> mutant cells expressing HA-Fzo1 or HA-Fzo1<sup>K464R</sup> were analyzed by SDS-PAGE and immunoblotting using Fzo1-, Ubc6- and Tom40-specific antibodies. Bottom panel, quantification of four independent experiments, including SD. PoS, Ponceau S staining.

DOI: https://doi.org/10.7554/eLife.30015.007
**Figure 3.** Cdc48 supports ubiquitin-dependent turnover of Ubp12. (A) Stability of the Ubp12 protein. The turnover of Ubp12 endogenously Flag tagged (Ubp12-Flag\textsuperscript{int}), in wt or cdc48-2 cells, was assessed with CHX.
Figure 3 continued

chase, as in 1D. Samples were analyzed by SDS-PAGE and immunoblotting using a Flag-, Tom40- and, as an unstable protein control, a Ubc6-specific antibody. Bottom panel, quantification of three independent experiments, including SD. (B) Ubiquitylation of Ubp12. The Ubp12\(^{C372S}\)-Flag inactive variant, expressed from an episomal plasmid, was immunoprecipitated from total soluble extracts using Flag-coupled beads. After elution, Ubp12 was analyzed by western blot using Flag- or ubiquitin (Ub - P4D1)-specific antibodies. Ubiquitylated forms of Ubp12\(^{C372S}\)-Flag are labeled with Ub. (C) Physical interaction between Cdc48 and Ubp12. The catalytically inactive Ubp12\(^{C372S}\)-Flag variant, expressed from an episomal plasmid, or the corresponding empty vector, were expressed in \(\Delta\)ubp12 (CDC48) or \(\Delta\)ubp12 cdc48-2 (cdc48-2) mutant cells and analyzed for Cdc48 interaction. Crude mitochondrial extracts were lysed, Flag-tagged Ubp12 was precipitated using Flag-coupled beads, and the eluate analyzed by SDS-PAGE and immunoblotting using Flag- and Cdc48-specific antibodies. PoS, Ponceau S staining, IP, immunoprecipitation; WB, western blot.

DOI: https://doi.org/10.7554/eLife.30015.008
Figure 3—figure supplement 1. Cdc48 supports ubiquitin-dependent turnover of Ubp12. (A) Turnover of episomal Ubp12 in wt or cdc48-2 cells. Ubp12-Flag stability was assessed after inhibition of cytosolic protein synthesis with cycloheximide (CHX), for the indicated time points in exponentially growing cultures. Samples were analyzed by SDS-PAGE and immunoblotting using Flag-, Ubc6- and Tom40-specific antibodies. Bottom panel, quantification of three independent experiments, including SD. (B) Proteasome dependence of Ubp12-Flag degradation. The turnover of Ubp2-Flag, expressed from an episomal plasmid, was assessed as in 1D. Samples were analyzed by SDS-PAGE and immunoblotting using Flag-, Ubc6- and Ssc1-specific antibodies. (C) Ubp12 expression levels. Expression levels of endogenously Flag-tagged Ubp12 (Ubp12-Flagint), Ubp12-Flag expressed from an episomal plasmid and endogenously Flag-tagged Ubp12 under the control of a pGAL promoter (pGAL-Ubp12-Flagint) (grown in glucose or galactose as indicated) were analyzed by SDS-PAGE and immunoblotting using Flag- and Ssc1-specific antibodies. Pos, PonceauS staining.

DOI: https://doi.org/10.7554/eLife.30015.009
**Figure 4.** Interdependence of Cdc48 and Ubp12 for Fzo1 regulation. (A) Mitochondrial morphology upon deletion of UBPl2 and/or mutation of CDC48. The indicated mutant cells were analyzed for mitochondrial tubulation after expressing a mitochondrial-targeted GFP plasmid, as in Figure 1A. Right panel, quantification from three different experiments (with more than 200 cells each), including SD, as described (Cumming et al., 2007) (B) Respiratory capacity of cells upon deletion of UBPl2 and/or mutation of CDC48. Fivefold serial dilutions of exponentially growing cells of wt or the mutant strains Δubp12, cdc48-2, and Δubp12 cdc48-2 were spotted on YP media supplemented with lactate (YP Lac) and incubated at 30°C for two days or 37°C for five days. (C) Ubiquitylation levels of Fzo1 upon deletion of UBPl2 and/or mutation of CDC48. Crude mitochondrial extracts from the indicated strains additionally expressing HA-Fzo1, or the corresponding empty vector, were analyzed by SDS-PAGE and immunoblotting using an HA-specific antibody. Unmodified and ubiquitylated forms of HA-Fzo1 are indicated as in Figure 1B. Bottom panel, quantification of four independent experiments, normalized to PoS and including SD. ns, p>0.05. *, p≤0.05; **, p≤0.01 (One-way ANOVA, Tukey’s multiple comparison test). PoS, PonceauS staining.

DOI: https://doi.org/10.7554/eLife.30015.010
Figure 4—figure supplement 1. Interdependence of Cdc48 and Ubp12 for Fzo1 regulation. (A) Mitochondrial morphology upon deletion of UBP12 in Δfzo1 cells. The indicated mutant cells were analyzed for mitochondrial tubulation after expressing a mitochondrial-targeted GFP plasmid, as in Figure 1A. Quantification from three different experiments (with more than 200 cells each), including SD, as described (Cumming et al., 2007). (B) Mitochondrial morphology upon expression of HA-Fzo1 in Δfzo1 Δubp12 cells. The indicated mutant cells were analyzed for mitochondrial tubulation after expressing a mitochondrial-targeted GFP plasmid, as in Figure 1A. Quantification from three different experiments (with more than 200 cells each), including SD, as described (Cumming et al., 2007). (C) Mitochondrial morphology upon endogenous expression of HA-Fzo1 or HA-Fzo1K464R in Δubp12 cells. The indicated mutant cells were analyzed for mitochondrial tubulation after expressing a mitochondrial-targeted GFP plasmid, as in Figure 1A. Quantification from one experiment (with more than 200 cells each). (D) Mitochondrial morphology upon deletion of UBP12 in Δfzo1 Δdnm1 cells. The indicated mutant cells were analyzed for mitochondrial tubulation after expressing a mitochondrial-targeted GFP plasmid, as in Figure 1A. Quantification from three different experiments (with more than 200 cells each), including SD, as described (Cumming et al., 2007). DOI: https://doi.org/10.7554/eLife.30015.011
Figure 4—figure supplement 2. Interdependence of Cdc48 und Ubp12 for Fzo1 regulation. (A) Analysis of mtDNA content in cdc48-2 cells using RT-PCR. mtDNA content in Δfzo1, wt and cdc48-2 cells was analyzed by RT-PCR.
measuring COX3 and ACT1 (as housekeeping gene) RNA levels using RT-PCR. Quantification of six independent experiments, including SD. *p ≤ 0.05 (paired t-test). Figure 4B—Analysis of mtDNA content in cdc48-2 cells using the Cox2 protein amount. Total cellular extracts of Δfzo1, wt and cdc48-2 cells were analyzed by SDS-PAGE and immunoblotting using Cox2- (as mtDNA marker) or Ubc6-specific antibodies. Bottom panel, quantification of five independent experiments, including SD. *p ≤ 0.05 (paired t-test). (C) Respiratory capacity of cdc48-2 cells upon expression of wt or mutant Cdc48. A spot assay was performed as described in Figure 4B with the indicated cells but using YPLac, grown at 30°C for 1 day and at 37°C for 3 days. (D) Physical interaction between Cdc48 and Fzo1 in Δubp12 cells. HA-Fzo1 or the corresponding empty vector was expressed in wt or Δubp12 cells and analyzed for Cdc48 interaction, as in 2A. Crude mitochondrial extracts were lysed, HA tagged Fzo1 was precipitated using HA-coupled beads, and the eluate was analyzed by SDS-PAGE and immunoblotting using HA- and Cdc48-specific antibodies. Unmodified and ubiquitylated forms of HA-Fzo1 are indicated as in Figure 1B. (E) Steady state levels of Fzo1 upon deletion of UBP2 and/or mutation of CDC48. Total cellular extracts of wt cells or Δubp12, cdc48-2 and Δubp12 cdc48-2 mutant cells were analyzed by SDS-PAGE and immunoblotting using HA-, Ubc6- and Tom40-specific antibodies. Bottom panel, quantification of six independent experiments, including SD. ns, p > 0.05 (One-way ANOVA; Tukey’s multiple comparison test). PoS, Ponceau S staining; IP, immunoprecipitation; WB, western blot.

DOI: https://doi.org/10.7554/eLife.30015.012
Figure 5. Ubp12 modulates Ubp2 ubiquitylation and turnover. (A) Interdependent role of Ubp2 and Ubp12 for the steady state levels of Fzo1. Total cellular extracts of wt or Δubp2, Δubp12, and Δubp2 Δubp12 mutant cells expressing HA-Fzo1 and also expressing either Ubp2-Flag or the corresponding empty vector, as indicated, were analyzed by SDS-PAGE and immunoblotting using HA- and Tom40-specific antibodies. Bottom panel, quantification of four independent experiments, including SD. (B) Turnover of endogenous Ubp2 in wt or Δubp12 cells. The turnover of endogenously 3xHA-tagged Ubp2 (Ubp2-3xHAint) was assessed as in 3A. Samples were analyzed by SDS-PAGE and immunoblotting using antibodies against HA, Ubc6 and Ssc1. Right panel, quantification of four independent experiments, including SD. For the statistical analysis of the degradation kinetics of each strain, a paired t-test was used; for the statistical analysis of the difference in steady state levels of both strains at the indicated time points (t1h, t3h) an unpaired t-test was used. ns, p>0.05; *, p≤0.05; **, p≤0.01. (C) Ubiquitylation of Ubp2. The Ubp2C745S-Flag inactive variant, expressed in wt or Δubp12 cells, was immunoprecipitated from total soluble extracts using Flag-coupled beads. Eluted Ubp2 was analyzed by western blot using Flag- or ubiquitin (Ub - P4D1)-specific antibodies. Ubiquitylated forms of Ubp2C745S-Flag are labeled with Ub. PoS, Ponceau S staining; IP, immunoprecipitation; WB, western blot.

DOI: https://doi.org/10.7554/eLife.30015.013
Figure 5—figure supplement 1. Ubp12 modulates Ubp2 ubiquitylation and turnover. (A) Opposing roles of Ubp2 and Ubp12 for CHX resistance. A spot assay was performed, as described in Figure 4B, but on synthetic media supplemented with glucose (SCD) in the absence or presence of 0.5 µg/ml CHX and incubated at 30°C for one or five days, respectively. (B) Distinct roles of Ubp2 and Ubp12 for cellular ubiquitylation. Total cellular extracts of the indicated strains were analyzed by SDS-PAGE and immunoblotting using ubiquitin (Ub; αP4D1) and Tpi1-specific antibodies, used as loading control. Free ubiquitin or ubiquitylated conjugates are labeled with Ub. Right panels, quantification of three independent experiments showing the levels of free Ub or Ub conjugates, including SD.
DOI: https://doi.org/10.7554/eLife.30015.014
Figure 5—figure supplement 2. Ubp12 modulates Ubp2 ubiquitylation and turnover. (A) Proteasome dependence of Ubp2-Flag degradation in Δpdr5 Δsnq2 mutant cells. The turnover of ectopically expressed Ubp2-Flag was assessed as in Figure 1D. Samples were analyzed by SDS-PAGE and immunoblotting using Flag- and Ubc6-specific antibodies. (B) Physical interaction between Ubp2 and Ubp12. Catalytically inactive variants ectopically expressed Ubp2C745S-Flag and non-tagged Ubp12C372S, or their corresponding empty vectors, were expressed in Δubp2 Δubp12 cells. Total soluble extracts were prepared and Ubp12C372S was precipitated using Sepharose beads in the presence or absence of a Ubp12-specific antibody, as indicated. The eluates were analyzed by SDS-PAGE and immunoblotting using Flag- and Ubp12-specific antibodies. (C) Ubiquitylation of Ubp2. The Ubp2C745S-Flag inactive variant, expressed in wt, Δubp12 and Δubp12Δmdm30 cells, was immunoprecipitated from total soluble extracts using Flag-coupled beads. Eluted Ubp2 was analyzed by western blot using antibodies specific for Flag or ubiquitin (Ub; αP4D1). Ubiquitylated forms of Ubp2C745S-Flag are labeled with Ub. PoS, PonceauS staining; IP, immunoprecipitation; WB, western blot.

DOI: https://doi.org/10.7554/eLife.30015.015
Figure 6. Characterization of the deubiquitylation reaction by Ubp12. (A) Analysis of ubiquitin chain-type composition of Fzo1. Crude mitochondrial extracts from wt or Δubp12 mutant cells expressing HA-Fzo1, and over-expressing either wt ubiquitin (Ub) or ubiquitin with a K48R mutation (UbK48R).

Figure 6 continued on next page
were solubilized, subjected to HA-immunoprecipitation and analyzed by SDS-PAGE and immunoblotting using an HA-specific antibody. Unmodified and ubiquitylated forms of HA-Fzo1 are indicated as in 1B. (B) Ubiquitin chain-type analysis of Fzo1 upon Ubp2<sup>C745S</sup> expression. Crude mitochondrial extracts from wt or ∆ubp2 (expressing Ubp2<sup>C745S</sup>) cells expressing HA-Fzo1 endogenously, and overexpressing either wt ubiquitin (Ub) or Ub<sup>K48R</sup>, were analyzed as in A. Unmodified and ubiquitylated forms of HA-Fzo1 are indicated as in 2B (C) Analysis of Ubp2 ubiquitin chain composition in ∆ubp12 cells. Soluble extracts from ∆ubp12 cells expressing Ubp2<sup>C745S</sup>-Flag and different ubiquitin variants (as indicated) were prepared and Flag-tagged Ubp2<sup>C745S</sup> was precipitated using Flag-coupled beads. The eluate was analyzed by SDS-PAGE and immunoblotting using antibodies against Flag and ubiquitin (Ub; αP4D1). (D) Deubiquitylation (DUB) assay using Ub<sub>2</sub> chains. Purified di-ubiquitin chains (Ub<sub>2</sub>) composed of either only K48- or K63-linkages were treated with the purified DUBs Ubp12, USP21 and USP2. Treated chains were analyzed by SDS-PAGE and immunoblotting using a ubiquitin-specific antibody (Ub; αP4D1). Mono-ubiquitin or di-ubiquitin chains are labeled with Ub<sub>1</sub> or Ub<sub>2</sub>, respectively. (E) DUB assay using Ub-chains. Purified poly-ubiquitin chains (Ub-chains) composed of either only K48- or K63-linkages were treated with the purified DUBs Ubp12, USP21 or USP2. Treated chains were analyzed by SDS-PAGE and immunoblotting as in C. Ubiquitin chains were labeled as in D with the subscript value indicating the amount of ubiquitin moieties in the respective chain. (F) Ubiquitylation pattern of Fzo1. Wt cells expressing HA-Fzo1 were analyzed for Fzo1 ubiquitylation upon the expression of Myc-ubiquitin, or the respective empty vector. HA-Fzo1 was immunoprecipitated from mitochondrial extracts using HA-coupled beads. Eluted Fzo1 was split into two and samples were analyzed by SDS-PAGE and immunoblotting using HA- or Myc-specific antibodies. Unmodified and ubiquitylated forms of HA-Fzo1 are indicated as in 1B. The composition of the additional species apparent upon co-expression of Myc-tagged ubiquitin is explained in the inset. PoS, PonceauS staining.

DOI: https://doi.org/10.7554/eLife.30015.016
Figure 6—figure supplement 1. Characterization of the deubiquitylation reaction by Ubp12. Opposite effects of Ubp12 and Ubp2 in Fzo1 stability. The turnover of HA-Fzo1 in wt, Δubp12, Δubp2 or Δubp12 Δubp2 cells was assessed after inhibition of cytosolic protein synthesis with cycloheximide (CHX), for the indicated time points in exponentially growing cultures. Samples were analyzed by SDS-PAGE and immunoblotting using a HA- and Hsp70-specific antibodies. Left panel, quantification of three independent experiments, including SD.
DOI: https://doi.org/10.7554/eLife.30015.017
Figure 7. Interdependent roles of Ubp2 and Ubp12. (A) Effect of Ubp2\textsuperscript{C745S} on Fzo1\textsuperscript{K464R} ubiquitylation. HA-Fzo1 or HA-Fzo1\textsuperscript{K464R} were expressed in the presence of Ubp2 (Δfzo1 cells plus empty vector) or instead in the presence of Ubp2\textsuperscript{C745S} (Δubp2 Δfzo1 plus Ubp2\textsuperscript{C745S}-Flag), as indicated. Crude mitochondrial extracts were solubilized and HA-tagged Fzo1 was analyzed by SDS-PAGE and immunoblotting using an HA-specific antibody.

Figure 7 continued on next page
Figure 7 continued

Unmodified and ubiquitylated forms of HA-Fzo1 are indicated as in 2B. (B) Effect of UBP2 deletion on the steady state levels of Fzo1<sup>K464R</sup>. Total cellular extracts of indicated strains expressing HA-Fzo1 or HA-Fzo1<sup>K464R</sup> as indicated were analyzed by SDS-PAGE and immunoblotting using HA- and Tom40-specific antibodies. Bottom panel, quantification of five independent experiments, including SD. (C) Effect of Ubp2 and Mdm30 on the steady state levels of Fzo1. Total cellular extracts of wt, Δubp2 and Δubp2Δmdm30 cells expressing HA-tagged Fzo1 endogenously (HA-Fzo1<sup>int</sup>) were analyzed by SDS-PAGE and immunoblotting using HA- and Tom40-specific antibodies. Bottom panel, quantification of three independent experiments, including SD. PoS, Ponceau S staining.

DOI: https://doi.org/10.7554/eLife.30015.018
**Figure 8.** Cdc48 regulates mitochondrial fusion via Ubp12 and Ubp2. (A) Steady state levels of Fzo1 in \( \Delta \text{ubp2} \Delta \text{ubp12} \) upon mutation of CDC48. Total cellular extracts of wt, \( \text{cdc48-2} \), \( \Delta \text{ubp2} \Delta \text{ubp12} \) and \( \Delta \text{ubp2} \Delta \text{ubp12} \text{cdc48-2} \) cells were analyzed by SDS-PAGE and immunoblotting using Fzo1- and αFzo1. Figure 8 continued on next page.

C.

D.

Simoès et al. eLife 2018;7:e30015. DOI: https://doi.org/10.7554/eLife.30015
Tom40-specific antibodies. Bottom panel, quantification of five independent experiments, including SD. (B) Steady state levels of Fzo1 in Δubp2 cells upon deletion of CDC48. Total cellular extracts of wt, cdc48-2, Δubp2 and Δubp2 cdc48-2 cells were analyzed by SDS-PAGE and immunoblotting using Fzo1- and Tom40-specific antibodies. Bottom panel, quantification of five independent experiments, including SD. (C) Mitochondrial morphology of cdc48-2 cells upon overexpression of Ubp2. Wt or cdc48-2 mutant cells expressing Ubp2 or the corresponding empty vector were analyzed for mitochondrial tubulation after expressing a mitochondrial-targeted GFP plasmid, as in Figure 1A. Quantification from three different experiments (with more than 200 cells each), including SE, as described (Cumming et al., 2007). ns, p>0.05, **p<0.01, ***p<0.001 (One-way ANOVA, Tukey’s multiple comparison test). (D) Role of Ubp2 overexpression on the respiratory capacity of CDC48-deficient cells. A spot assay was performed as described in Figure 4B with the indicated cells but using synthetic media supplemented with lactate (SCLac) and incubated for 4 days. PoS, Ponceau S staining.

DOI: https://doi.org/10.7554/eLife.30015.019
Cdc48 regulates mitochondrial fusion via Ubp12 and Ubp2. Physical interaction between Ubp2 and Cdc48. The catalytically inactive variant Ubp2C745S-Flag or the corresponding empty vector were expressed in Δubp12 cells and analyzed for Cdc48 interaction, as in 2A. Crude mitochondrial extracts were lysed and Flag-tagged Ubp2C745S was precipitated using Flag-coupled beads. The eluate was analyzed by SDS-PAGE and immunoblotting using Flag- and Cdc48-specific antibodies. PoS, Ponceau S staining; IP, immunoprecipitation; WB, western blot.

DOI: https://doi.org/10.7554/eLife.30015.020

Figure 8—figure supplement 1.
Cdc48

Ubp12

Fzo1 → OM fusion

Ubp2

Fzo1 → OM fusion

Figure 9. Synergistic regulation of mitochondrial fusion by the Cdc48 cascade. Cdc48 supports turnover of Ubp12, stabilizing ubiquitylation on Fzo1 that promotes mitochondrial fusion (green ubiquitins). Moreover, degradation of Ubp12 stabilizes Ubp2, facilitating the removal of ubiquitin chains on Fzo1 inhibiting mitochondrial fusion (red ubiquitins). Thereby, Cdc48 activates mitochondrial fusion via Ubp12 and Ubp2. In contrast, Cdc48 impairment blocks progression of mitochondrial fusion by actively preventing Ubp12 turnover. Ubp12 then leads to a cascade of events inhibiting mitochondrial fusion: A) removal of the pro-fusion ubiquitylated forms and B) inhibition of Ubp2, consequently leading to the accumulation of the anti-fusion ubiquitylated forms. This cascade allows a synergistic effect of Cdc48, via a DUB regulatory cascade, to effectively promote or inhibit mitochondrial fusion.

DOI: https://doi.org/10.7554/eLife.30015.021