Mattie et al., https://doi.org/10.1083/jcb.201611194

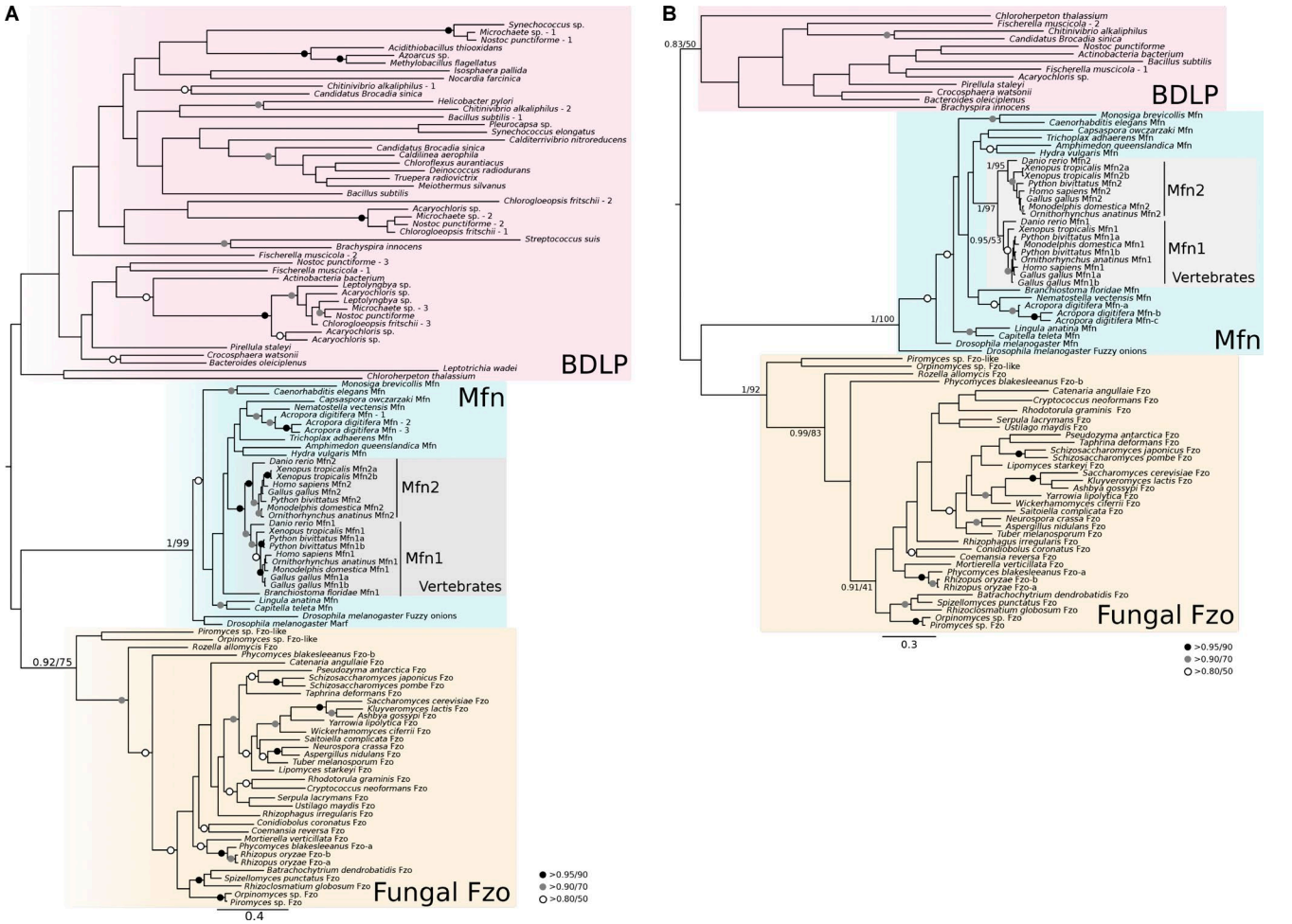

Figure S1. **Preliminary phylogenetic reconstructions of Mfns, Fzo1, and BDLPs. (A)** Phylogenetic reconstruction of the complete dataset. All sequences collected from bacterial, animal, and fungal genomes were aligned and subjected to phylogenetic reconstruction as described in Materials and methods and the legend to Fig. 1. **(B)** Phylogenetic reconstruction with long branches removed. As in A, but with long-branching bacterial sequences removed. Tree topologies are from MrBayes analyses. Support as depicted.

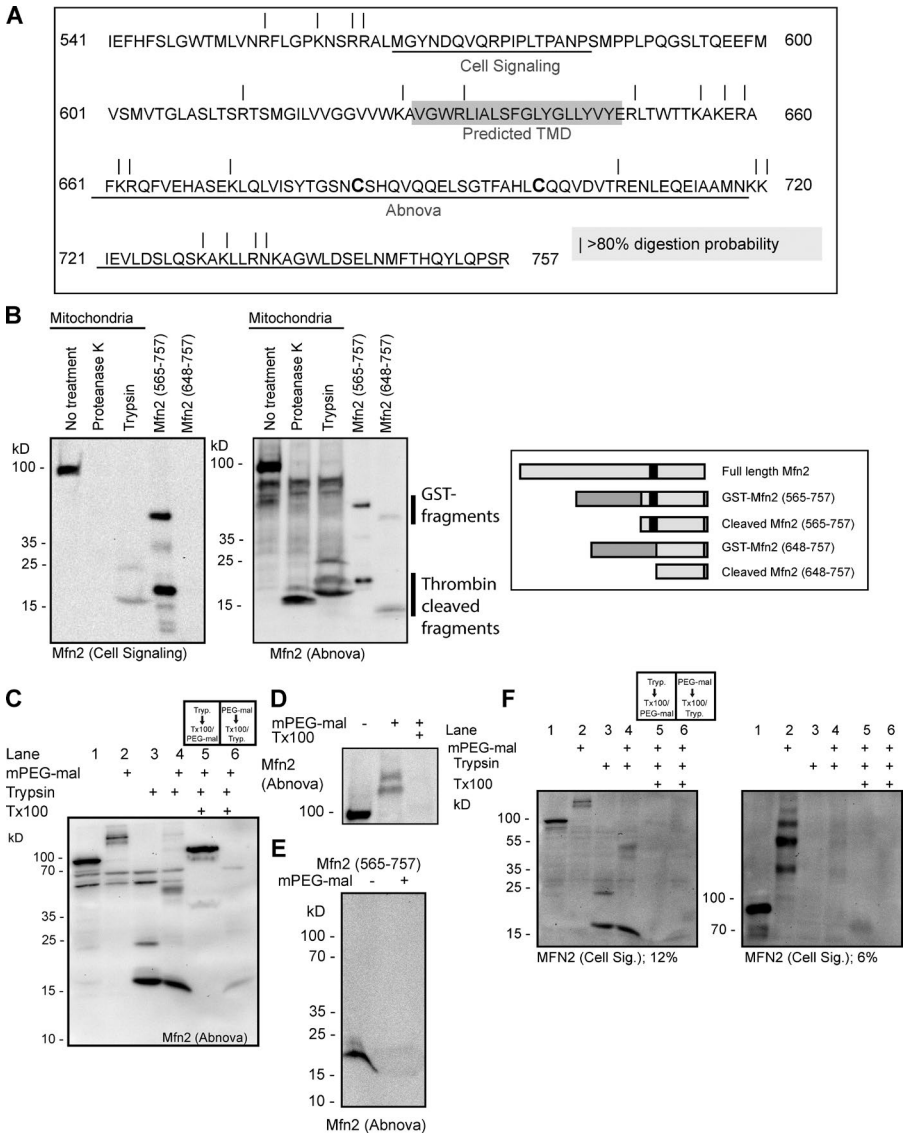

Figure S2. **Extended analysis of Mfn2 topology and epitope mapping. (A)** Amino acid sequence of Mfn2 541–*757* showing predicted trypsin digestion sites, anti-Mfn2 Cell Signaling Technology and Abnova binding sites, and predicted TMD. **(B)** To confirm the binding sites of the two anti-Mfn2 antibodies, two GST-tagged Mfn2 truncated forms were expressed in *Escherichia coli* and purified using GSH Sepharose. Then the GST tag was cleaved with thrombin. The thrombin digestion was not complete; therefore, both the GST-tagged (top bands indicated) and the cleaved truncated Mfn2 (bottom bands indicated) appear in the blot. One of the constructs consisted of aa 565–*757*, predicted molecular weight of ∼20 kD. Mfn2 565–*757* is recognized by both antibodies and runs slightly above the trypsin-protected endogenous Mfn2 fragment (lane 3 vs. 4), consistent with the inclusion of additional residues N-terminal to the predicted cleavage site in the recombinant fragments. Mfn2 648–*757* includes the amino acid residues just after the predicted TMD, predicted molecular weight of ∼14 kD. This recombinant fragment was recognized by the anti-Mfn2 Abnova antibody as expected (right panel, lane 5), but not the Cell Signaling Technology antibody (left panel, lane 5). Mitochondria were incubated with trypsin or proteinase K (which has broad cleavage specificity). The Cell Signaling Technology antibody recognized a ∼20-kD fragment upon digestion with trypsin, which was lost upon digestion with proteinase K (left panel, lane 2 vs. 3). In contrast, the Abnova antibody binding site located C-terminal to the TMD was not affected by proteinase K digestion, and as expected, the protected fragment is smaller than the trypsin-cleaved fragment (right panel, lane 2 vs. 3). **(C–E)** PEGylation experiments probed with the Abnova antibody. In C, isolated mitochondria from HEK293 cells were treated with 10 kD mPEG-mal, which reacts with cysteine residues and shifts full-length Mfn2 (lane 2). Mitochondria were digested with 100 µg/ml trypsin, showing a protected fragment (lane 3). The addition of trypsin in the presence of detergent leads to digestion of the fragment (lane 6). This trypsin-protected fragment was not modified upon addition of mPEG-mal, indicating the inaccessibility of this fragment to the mPEG-mal (lane 4). To confirm that the protected fragment can be modified with mPEG-mal, mitochondria were first digested with trypsin, followed by the inactivation of this trypsin with SBTI and PMSF. The trypsin-treated mitochondria were solubilized with Triton X-100 and incubated with mPEG-mal (lane 5). As observed with the Cell Signaling Technology antibody and upon conjugation of full-length Mfn2 in detergent (Fig. 2, F and G), conjugation of these cysteine residues led to the loss of antigenicity against Abnova antibody as well. This is seen further in D, where isolated mitochondria were incubated with 10 kD mPEG-mal in the presence or absence of detergent and separated using a 10% SDS-PAGE. Mfn2 shifted up when modified with mPEG-mal (lane 2). Incubation of Triton X-100–solubilized mitochondria with PEG-mal, however, led to a reduction in immunogenicity against the Abnova antibody (lane 3), as was observed with the Cell Signaling Technology antibody in Fig. 2. **(G)** Addition of PEG-mal to the recombinant fragment of Mfn2 (565–*757*) again reveals a loss in antigenicity upon conjugation. **(F)** A repeat of the PEGylation assay with Mfn2 products revealed using the Cell Signaling Technology antibody, as in C and D.

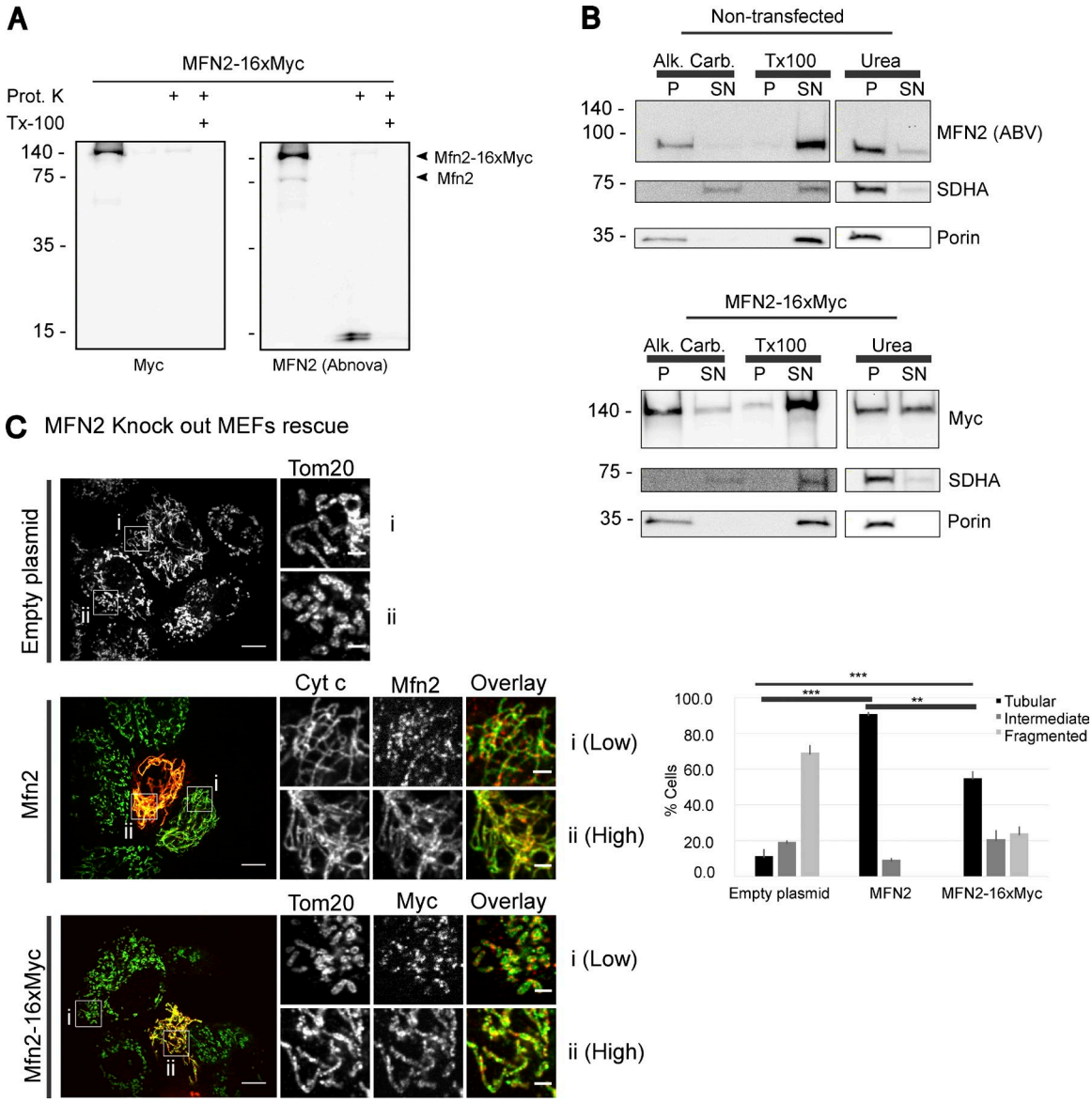

Figure S3. **Biochemical and functional characterization of C-terminally tagged Mfn2-16xMyc1x6His. (A)** Proteinase K digestion of mitochondria isolated from Mfn2-16xMyc1xHis–transfected HEK293 cells in the presence or absence of Triton X-100. Samples were processed for Western blotting and immunoblotted with anti-Myc antibodies followed by reblotting with anti-Mfn2 (Abnova) without stripping the membrane. This revealed that the Myc epitope tags were completely protease accessible, unlike the endogenous Mfn2. **(B)** To determine whether the tagged Mfn2 was inserted into the outer membrane, we performed alkaline carbonate extraction, urea extraction, and Triton X-100 solubilizations. 25 µg mitochondria isolated from nontransfected or Mfn2-16xMyc1x6His–transfected HEK293 cells was extracted with 100 mM alkaline carbonate, pH 12, 1% Triton X-100 (in isolation buffer), or urea buffer (4.5 M urea, 150 mM KCl, 1 mM DTT, and 20 mM Hepes, pH 7.4). After extraction, the samples were centrifuged, and the pellets and supernatants were processed for Western blotting. SDHA and Porin were used as controls. The data show that Mfn2-16xMyc1xHis, similar to endogenous Mfn2, was efficiently extracted with buffered Triton X-100, suggesting that the protein is not forming aggregates. Although much of Mfn2-16xMyc1xHis appeared in the pellet fraction of an alkali extraction, nearly half of the protein was extracted with a 4-M urea salt wash. These urea conditions were not enough to strip the membrane-associated SDHA, indicating that Mfn2-16xMyc1xHis is mainly peripherally associated, and not properly inserted into the membrane. **(C)** This tagged Mfn2 was previously shown to rescue mitochondrial morphology in Mfn2-deficient MEFs (Chen et al., 2003), which prompted us to revisit the functional capacity of Mfn2-16xMyc1xHis in comparison with untagged Mfn2, both under the same promoter. MFN2-KO MEFs were therefore transfected with empty plasmid, untagged Mfn2, or Mfn2-16xMyc1xHis. After 17 h, cells were fixed and processed for IF. Transfected cells were imaged, and representative images are shown. Mitochondrial morphology was quantified into "tubular," "intermediate," or "fragmented" categories. The plot presents quantification of three independent experiments. The total numbers of cells counted are 191, 153, and 171 for empty plasmid, Mfn2, and Mfn2-16xMyc1x6His, respectively. Bar: 10 µm; (insets) 2 µm. Statistical significance was analyzed using unpaired Student's *t* test. **, $P < 0.01$; ***, $P < 0.005$. Mfn2-deficient MEFs transfected with an empty plasmid showed fragmented morphology in ~70% of cells. Untagged Mfn2 rescued the phenotype, with >90% of cells showing tubular morphology at both low and high expression levels (Fig. S3 C). Mfn2-16xMyc1xHis showed a partial rescue, with ~55% of cells with tubular morphology similar to the untagged rescue conditions, ~24% remaining completely fragmented, and some with intermediate morphologies. The ability to rescue appeared to correlate with very high expression of the protein (two cell insets), which was in contrast to the untagged Mfn2 condition. This suggests that the localization of the C-terminal domain within the IMS is not absolutely essential to drive fusion; rather, it may play a regulatory function in the activation of fusion, presumably through redox-regulated oligomeric assemblies.

Provided online is Table S1 in Excel, showing protein accessions retrieved in this study.

Reference

Chen, H., S.A. Detmer, A.J. Ewald, E.E. Griffin, S.E. Fraser, and D.C. Chan. 2003. Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *J. Cell Biol.* 160:189–200. https://doi.org/10.1083/jcb.200211046