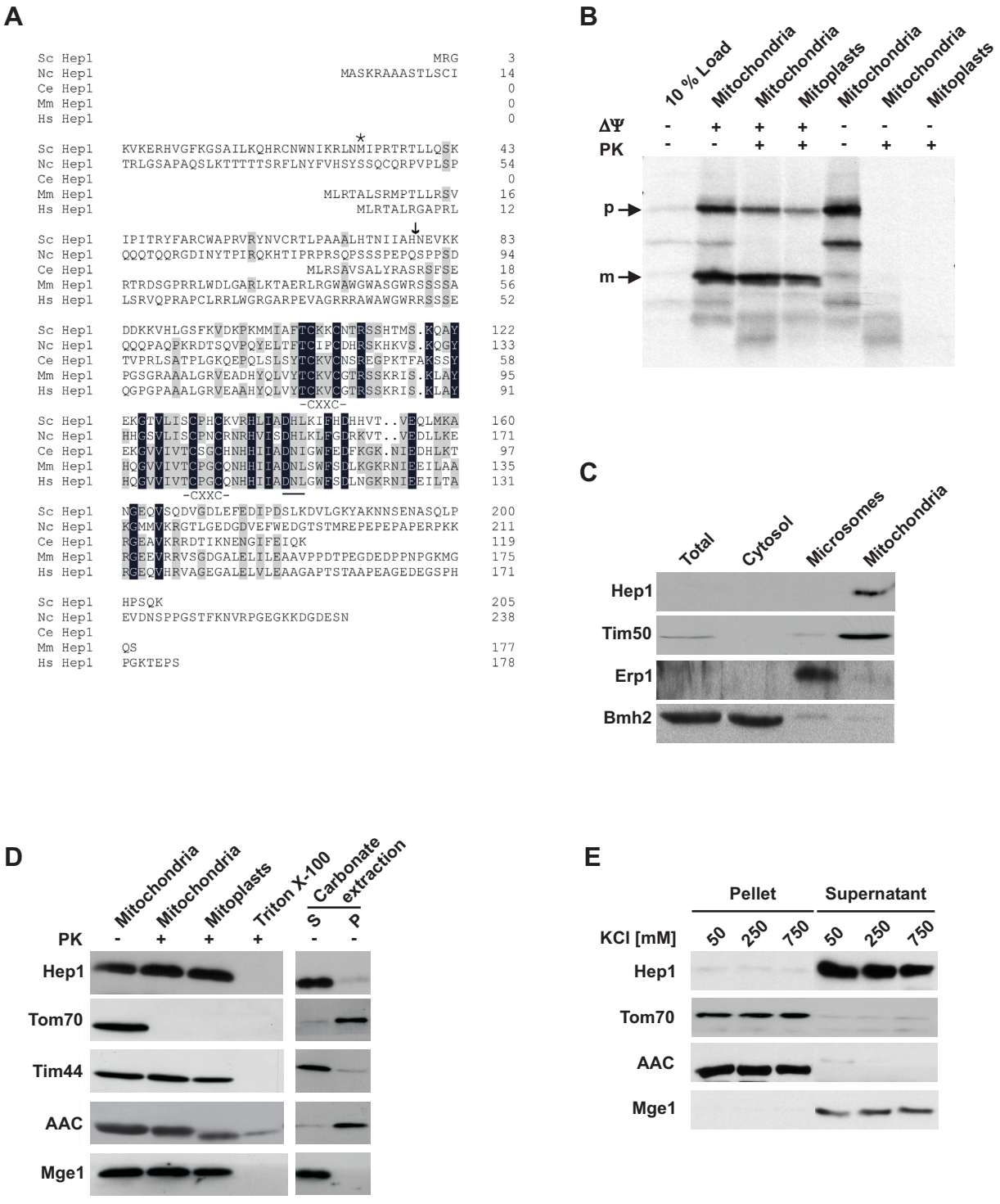

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Figure S1. Hep1 is a mitochondrial matrix protein. (**A**) Alignment of Hep1 proteins of various organisms. Sc, *Saccharomyces cerevisiae*; Nc, *Neurospora crassa* (CAE76386); Ce, *Caenorhabditis elegans* (AAF02170); Mm, *Mus musculus* (XP_484981); Hs, *Homo sapiens* (AL110466). The alignment was generated using the software DNAMAN 4.22, Lynnon Biosoft. Identical residues are shown in black, similar residues in grey. In all species the presence of a mitochondrial targeting signal (MTS) is predicted. There is a second methionine in position 32 in yeast, indicated by an asterisk, which was suggested to function as a start site (Burri *et al*., 2004). The following sequence has properties of an MTS. Following processing and removal of the MTS, the mature protein will be the same, whatever start methionine is taken. We determined the N terminus of the mature protein by Edman degradation. It is marked by an arrow in the sequence. The two CXXC and the typical D(H/N)L motifs are underlined. (**B**) Hep1 is imported into isolated mitochondria in a membrane potential manner and processed to the mature species (m) of ca. 17 kDa. Reticulocyte lysate containing ^{35}S-labeled Hep1 was incubated with mitochondria in the presence or absence of membrane potential ($\Delta\Psi$). Mitochondria were reisolated, aliquots were converted to mitoplasts and treated with proteinase K. Samples were subjected to SDS-PAGE and autoradiography. p, precursor of Hep1, starting with the first methionine of the open reading frame. (**C**) Hep1 is located to mitochondria. It fractionates together with the mitochondrial protein Tim50. Equal amounts of protein of subcellular fractions were subjected to SDS-PAGE and immunoblotting with antibodies against Hep1 and marker proteins of mitochondria (Tim50), microsomes (Erp1) and Cytosol (Bmh2). (**D**) Hep1 is located to the mitochondrial matrix. When mitochondria and mitoplasts were prepared and treated with proteinase K, Hep1 was not degraded. After disruption of the inner membrane by the detergent Triton X-100, the protein was degraded by the added protease. Upon alkaline extraction with

carbonate, Hep1 was recovered in the supernatant indicating that it is not an integral membrane protein. Left panel: Mitochondria, mitoplasts and Triton X-100 solubilized mitochondria were incubated with or without proteinase K (PK, 100 µg/ml). Right panel: supernatant (S) and pellet (P) of the carbonate extraction. Samples were subjected to SDS-PAGE and immunoblotting with antibodies against Hep1 and various marker proteins of mitochondria: Tom70, integral outer membrane protein; Tim44; matrix protein attached to the matrix side of the inner membrane; AAC, ADP/ATP carrier, integral inner membrane protein; Mge1, soluble matrix protein. (**E**) Hep1 is found in the soluble fraction after sonication of mitochondria. This indicates that Hep1 is not tightly associated with the inner membrane of mitochondria, but rather a soluble matrix protein. Isolated mitochondria were opened by sonication in the presence of increasing amount of KCl, as indicated. Samples were subjected to SDS-PAGE and immunoblotting as in (**D**). (**F**) The 5'-untranslated region of the *HEP1* gene contains a HSE-like sequence. The HSE-like sequences are boxed. The start codon is underlined. HSE, heat shock element.

References

Burri, L., Vascotto, K., Fredersdorf, S., Tiedt, R., Hall, M.N. and Lithgow, T. (2004) Zim17, a novel zinc-finger protein essential for protein import into mitochondria. *J. Biol. Chem.* **279**, 50243-50249.