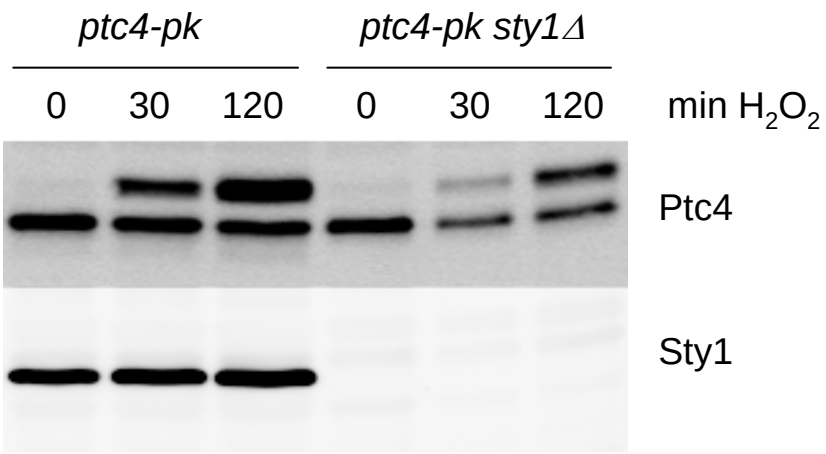
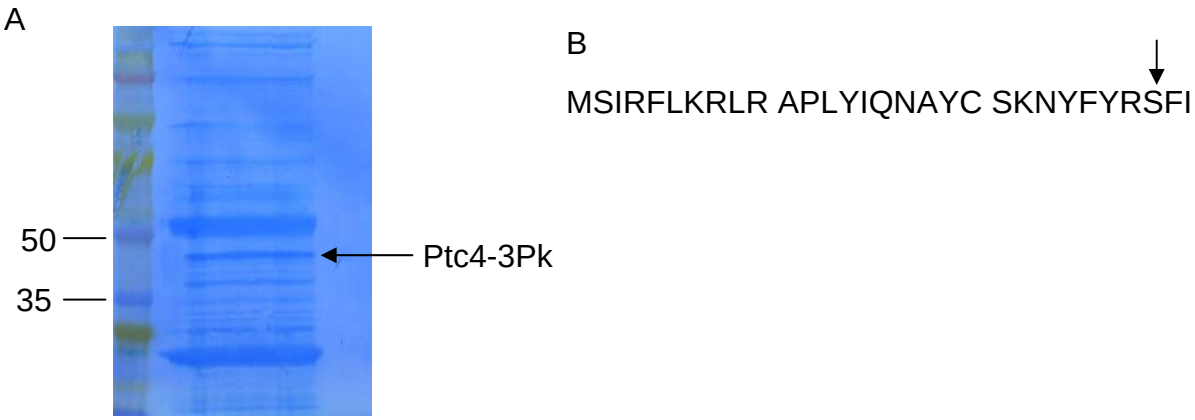


Supplementary Figure 1. The appearance of the oxidative stress-specific isoform of Ptc4 does not depend upon Sty1.

Whole cell extracts were isolated from cells treated with 1 mM H_2O_2 for the indicated times. These were examined by western blotting using anti-Pk antiserum to detect Ptc4 and anti-Hog1 antiserum to detect Sty1. Equal loading of protein was assessed by ponceau S staining of the membrane (data not shown). There is less Ptc4 protein in a *sty1Δ* because the expression of *ptc4* mRNA is dependent upon Sty1 (Chen et al., MBC, 2003).

Chen, D., Toone, W.M., Mata, J., Lyne, R., Burns, G., Kivinen, K., Brazma, A., Jones, N. and Bähler, J. (2003). Global transcriptional responses of fission yeast to environmental stress. Mol Biol Cell, 14, 214-229.



Supplementary Figure 2. Identification of the amino terminus of the mature form of Ptc4.

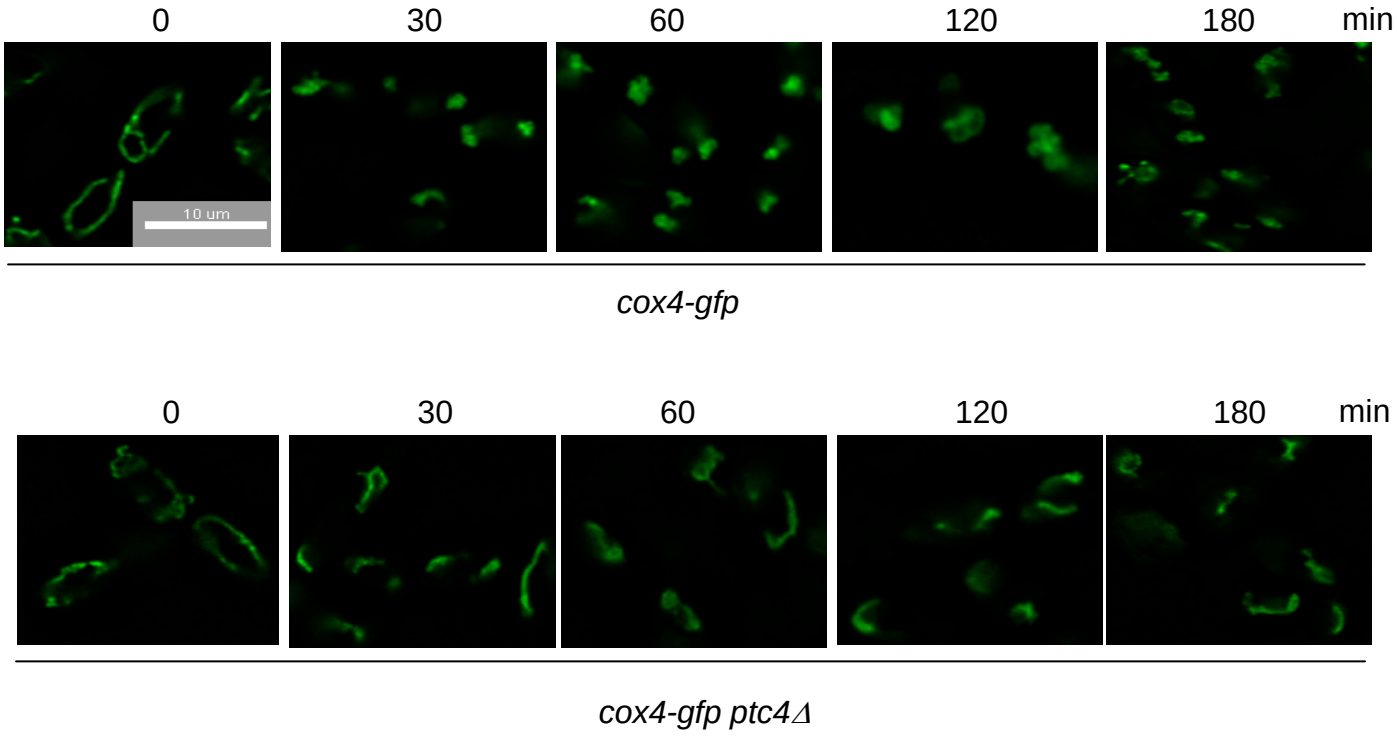
As described in the main text, H_2O_2 led to the appearance of a slower migrating isoform of Ptc4 (Figure 2A). This extra isoform was not due to an alternatively spliced version of Ptc4 or an alternative start site for transcription as the length of the *ptc4* message (as determined by 5'-RACE) did not change upon H_2O_2. Furthermore, cycloheximide treatment revealed that new protein synthesis was necessary for production of the slower migrating isoform. The upper isoform did not resolve upon treatment with lambda phosphatase (data not shown).

We hypothesized that the upper band might represent full length Ptc4 and that the faster migrating band might represent Ptc4 from which the mitochondrial targeting sequence has been cleaved. This was confirmed by purification of Ptc4 from unstressed cells followed by Edman sequencing to determine to N-terminal residue of the protein.

(A) Purification of Ptc4 protein for Edman degradation sequencing. Ptc4-3Pk protein was purified from 8 litres of *S. pombe* cells grown to mid-log phase in YE. Ptc4 protein was purified by immunoprecipitation using anti-Pk antiserum. The resulting immunoprecipitated protein was separated by SDS-PAGE, transferred to PVDF and the proteins identified by staining with Coomassie Blue. Molecular mass markers are shown in kiloDaltons. The band corresponding to Ptc4 was excised from the membrane and analyzed by Edman degradation. This revealed that the amino-terminal residue of the faster migrating isoform was phenylalanine which corresponded to residue 29 of the full length sequence. (B) The N-terminal MTS of Ptc4 is shown along with the cleavage site (between serine 28 and phenylalanine 29 –illustrated by an arrow).

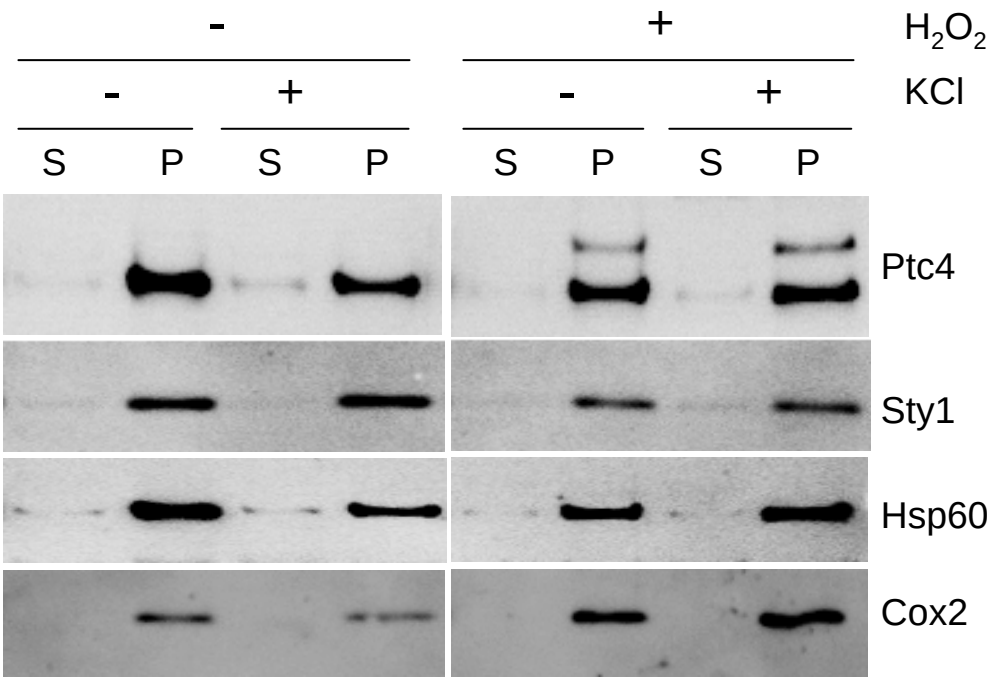
Mutating an arginine residue within the MTS that was predicted to be important for cleavage, namely R27, generated an isoform that migrated with the same apparent molecular mass as the H_2O_2-specific version of Ptc4 (Figure 5A). These data are consistent with oxidative stress inhibiting cleavage of Ptc4, resulting in the appearance of the full-length form of the protein.

Di et al., Supplementary Figure 2



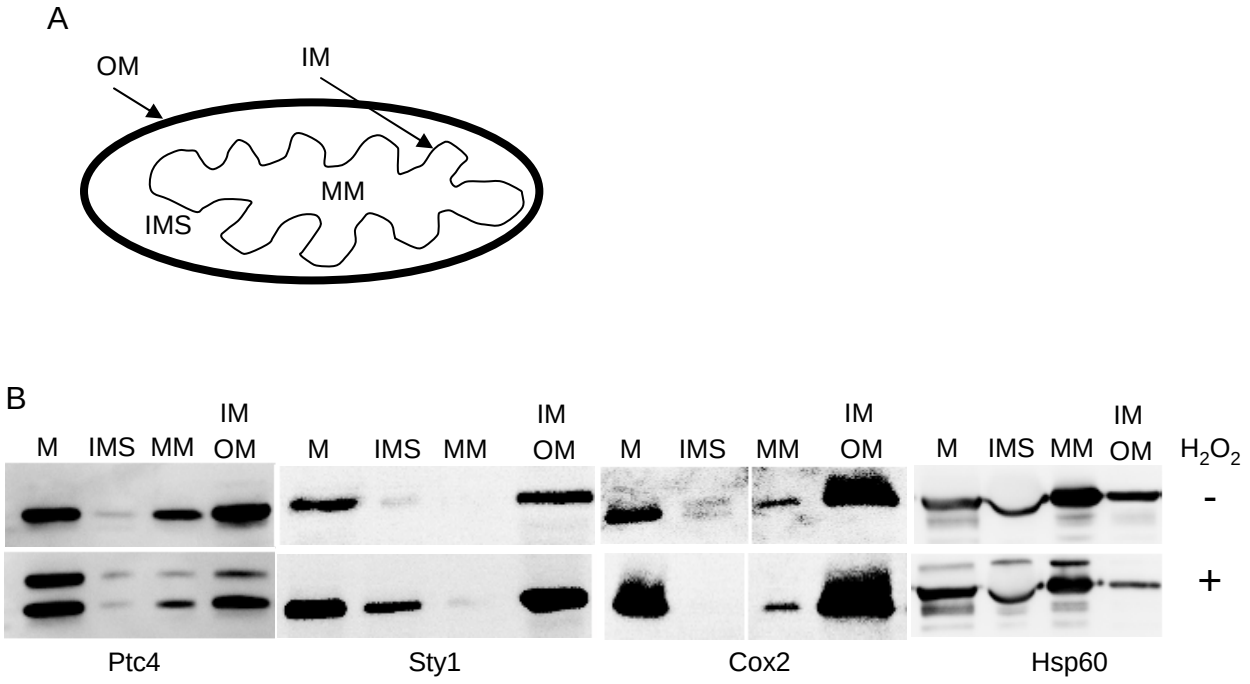
Supplementary Figure 3. The mitochondrial morphology change that is observed upon exposure to H_2O_2 is delayed in a *ptc4Δ* mutant.

Cells were exposed to 1 mM H_2O_2 for the times shown before being imaged by fluorescence microscopy. The scale bar represents 10μM. Strains used were:
NJ1046 *cox4-gfp:kanMX6 ade6-M210 ura4-D18 leu1-32 his7-366*
NJ1048 *cox4-gfp:kanMX6 ptc4::natMX6 ade6M210 ura4-D18 leu1-32 his7-366*



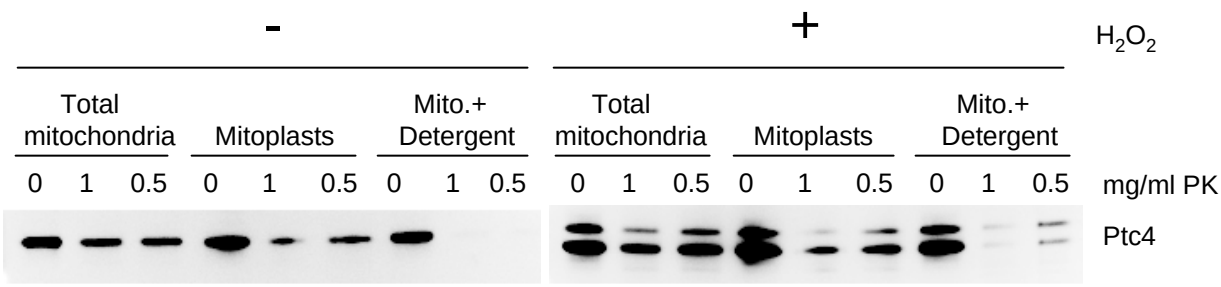
Supplementary Figure 4. Ptc4 and Sty1 are not peripherally associated with the cytoplasmic side of the outer mitochondrial membrane.

Mitochondria were purified from *ptc4-3Pk* cells grown in the presence or absence of 1mM H_2O_2 for 30 minutes then resuspended in 0.5M KCl and incubated for 20 minutes on ice. The mitochondria were pelleted by centrifugation and the resulting mitochondrial fraction (P) plus supernatant (S) were analysed by immuno-blotting. Ptc4 was detected using anti-Pk antiserum.



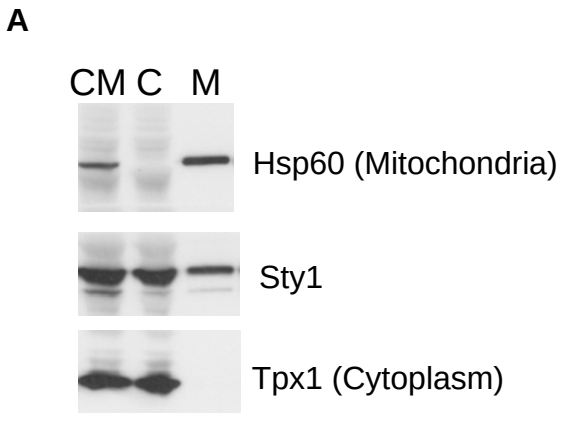
Supplementary Figure 5. Ptc4 associates with mitochondrial membranes to a greater extent than with the matrix.

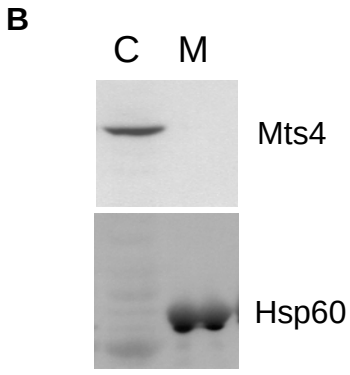
Sty1 also co-fractionates with the inter-membrane space, especially after H_2O_2 exposure.
(A) Diagram of a mitochondrion: MM – mitochondrial matrix fraction;
IM – inner mitochondrial membrane; IMS – inter-membrane space; OM – outer mitochondrial membrane.
(B) Purified mitochondria (M) were sub-fractionated into a matrix fraction (MM), an inter-membrane space fraction (IMS) and a mitochondrial membrane fraction consisting of inner and outer membranes (IMOM).
Fractionations were performed on mitochondria derived from cells that had been grown both in the presence and absence of H_2O_2 for 120 minutes and analyzed by immuno-blotting as indicated.



Supplementary Figure 6. Proteinase K treatment of purified mitochondria treated with detergent leads to complete digestion of Ptc4.

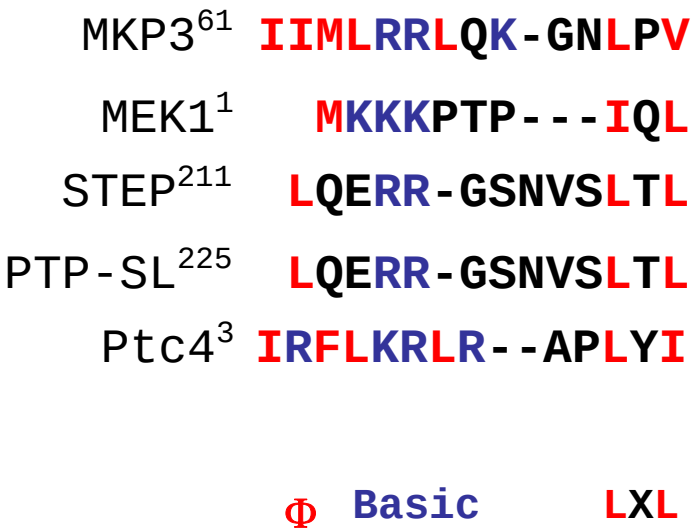
Mitochondria were purified from *ptc4-3Pk* cells grown in the presence or absence of 1mM H_2O_2 for 30 minutes. Mitoplasts were prepared from purified mitochondria by treating with hypotonic buffer. Mitochondria purified from an equal number of cells were treated with proteinase K (PK) at the indicated concentration for 30 minutes. Mito + detergent represents total mitochondria that have been resuspended in 0.4% triton X-100. The digested mitochondria were analyzed by immuno-blotting using anti-Pk antiserum to detect Ptc4.





Supplementary Figure 7. **Sub-cellular fractionation to isolate mitochondria from wild type cells.**

(A) Total whole cell extracts were separated by centrifugation into a post-nuclear fraction containing cytoplasm and mitochondria (CM). Further centrifugation resulted in a mitochondrial fraction (M) and the supernatant which consisted of the cytoplasm (C). Extracts corresponding to equivalent amounts of cells were examined by immuno-blotting using the indicated antiserum.
(B) Another fractionation using Mts4 as a control. This protein is found in both the cytoplasm and nucleus; Wilkinson et al., 1998, EMBO J, 17(22):6465-76.



Supplementary Figure 8. **Identification of a putative MAPK docking domain in the mitochondrial targeting sequence of Ptc4.**

Alignment of the N-terminal region of Ptc4 with sequences of MAP kinase docking domains found in various substrates that follow the hydrophobic – basic – LXL features (Sharrocks et al., 2000). The numbers represent the position in the protein of the first amino acid shown. Basic and hydrophobic residues found in a number of docking domains are shown in blue and red respectively. The features found in this subset of docking domains are indicated below the alignment (Φ) represents a hydrophobic residue.

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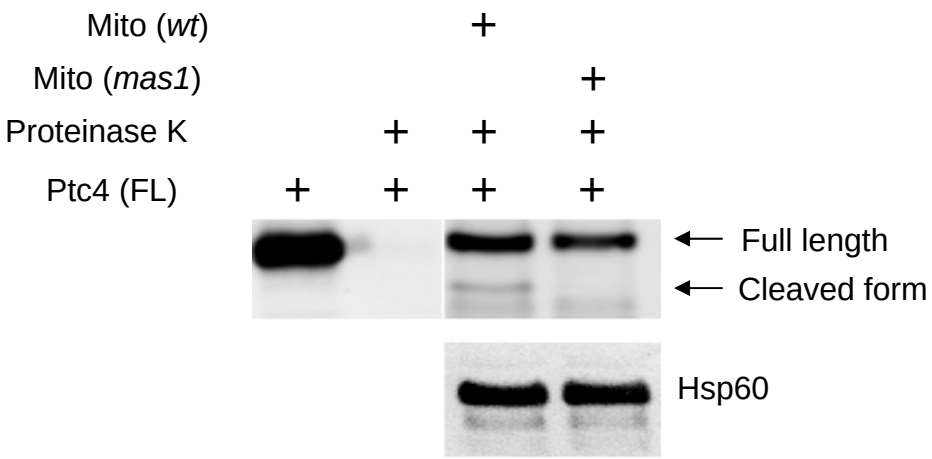

Supplementary Figure 9. **The cleavage of the amino-terminus of Ptc4 requires MPP.**

Full-length Ptc4 was expressed in and purified from *E.coli* and incubated with isolated budding yeast mitochondria (Mito) from either wild type or a *mas 1-1* mutant in import buffer. Proteinase K was added to remove non-imported protein after the import reaction. The precursor Ptc4 was imported into mitochondria and cleaved. Both non-cleaved and cleaved Ptc4 were protected from proteinase K. Only the non-cleaved form of Ptc4 was detected in the *mas1-1* mutant. Hsp60 protein shows the same amount of mitochondria were used for each reaction. Strains used were: *wt* (AH216) and *mas1* as described in Yaffe, M.P. and Schatz, G. (1984) Proc Natl Acad Sci U S A. 81(15):4819-23.