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1 **Pet191 is a cytochrome *c* oxidase assembly factor in *Saccharomyces cerevisiae***

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8 Running title: Role of Pet191 in Assembly of Complex IV

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12 Abstract

13

14 The twin Cx₉C motif protein Pet191 is essential for cytochrome *c* oxidase maturation. The motif
15 Cys residues are functionally important and appear to be present in disulfide linkages within a large
16 oligomeric complex associated with the mitochondrial inner membrane. The import of Pet191
17 differs from other twin Cx₉C motif class of proteins in being independent of the MIA pathway.

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22 Cytochrome *c* oxidase (CcO), the terminal enzyme of the respiratory chain in mitochondria,
23 consists of 12-13 subunits, with the three core enzyme subunits (Cox1-Cox3) being encoded by the
24 mitochondrial genome (3). The assembly of CcO requires a myriad of steps including the insertion
25 of heme *a* and copper cofactors. Copper insertion into newly synthesized Cox1 and Cox2 chains
26 occurs on the IMS side of the inner membrane (IM) as the accessory molecules are localized within
27 this compartment (10). Two proteins, Cox11 and Sco1, are associated with the IM, and mediate the
28 copper metallation of the Cu_B and Cu_A sites in Cox1 and Cox2, respectively (7, 18). Cu(I) ions
29 transiently bound by Cox11 and Sco1 are provided by Cox17 within the IMS (12). Cox17 contains a
30 twin Cx₉C structural motif that adopts a helical hairpin conformation, stabilized by two disulfide
31 bonds with a single Cu(I) ion bound by vicinal Cys residues outside the twin Cx₉C motif (1, 2, 5) .
32 Two other IMS proteins, Cox19 and Cox23, are structurally related to Cox17 in containing a twin
33 Cx₉C structural motif and function in an undefined step in CcO assembly (19, 21).

34 The uncharacterized Pet191 protein is a variant of the twin C_x9C motif family. The
35 conservation of the twin C_x9C motif in Pet191 (Supplemental Fig. 1) and its importance in
36 respiration motivated us to investigate the role of Pet191 in CcO assembly (15). Yeast lacking
37 Pet191 are known to be respiratory deficient and fail to propagate on growth medium containing
38 glycerol as the sole carbon source (15) (Fig. 1A). The mutant cells are *rho*⁺, as a 3' Myc tagged
39 *PET191* gene can restore respiratory function. CcO activity was absent in cells cultured on glucose
40 or raffinose, but succinate cytochrome *c* reductase activity was elevated above wild-type levels (Fig.
41 1B). Complex III activity is elevated in certain CcO assembly mutants (14). The specific defect in
42 CcO was demonstrated by BN-PAGE, as dimeric complex III and both monomeric and dimeric
43 complex V species were observed in *pet191Δ* cells (Fig. 1C). Complex IV was absent as visualized
44 by Cox2 immunoblotting. Steady state levels of Cox1, Cox2 and Cox3 were undetectable in
45 *pet191Δ* cells (Fig. 1D). The diminution in Cox1-Cox3 protein levels in *pet191Δ* cells arises from
46 impaired stability of CcO, since mitochondrial translation is comparable to other CcO assembly
47 mutants (4).

48 Whereas the twin C_x9C protein Cox17 has a role in copper metallation of CcO during
49 biogenesis, Pet191 does not appear to have a prominent role in this process. The addition of
50 supplemented copper salts to the growth medium of *pet191Δ* cells does not reverse the respiratory
51 deficient phenotype as occurs with *cox17Δ* cells. Copper ions used in the metallation of CcO and
52 Sod1 in the IMS derive from matrix copper ligand complex (9). Cells lacking Pet191 have normal
53 mitochondrial copper levels and normal Sod1 activity in mitochondria, suggesting that Pet191 does
54 not perturb mitochondrial copper metallation processes or Sod1 activation within the IMS.

55 Immunoblotting of Myc tagged Pet191 revealed that it localizes to the mitochondria (Fig.
56 1E) and was tightly associated with a membrane (Fig. 1F). Pet191 was not solubilized by sonication

57 of the mitoplasts and was not released from the IM by sodium carbonate extraction at pH 10.5.
58 However at pH 11.5, sodium carbonate buffer was sufficient to solubilize Pet191. Pet191 remained
59 associated with mitoplasts after hypotonic swelling, but was degraded with the addition of
60 proteinase K (Fig. 1G). The release of the IMS Cyb2, but not Pet191, upon hypotonic swelling
61 suggested that Pet191 is not a soluble IMS protein. Thus, Pet191 is tightly associated with the IM
62 facing the IMS side of the membrane.

63 Chromosomally HA-tagged Pet191 solubilized in digitonin migrated on blue native PAGE
64 as a complex of approximately 500 kDa (Fig. 2A). Deoxycholate solubilized Pet191-Myc eluted on
65 size permeation chromatography in a volume corresponding to approximately 530 kDa (Fig. 2C).
66 However, extraction of Pet191 with 0.1% DOC in the presence of 100 mM DTT resulted in elution
67 of Pet191 in a volume closer to the predicted monomeric mass (Fig. 2C). These results are
68 consistent with Pet191 existing in an oxidized conformer in mitochondria. Cco deficient cells
69 contain a more reducing IMS as assessed by the Mia40 redox state (6). To determine whether the
70 Pet191 oligomer was sensitive to perturbations in the redox state of the IMS, BN-PAGE analysis
71 was carried out on Pet191-HA in respiratory deficient *cox11Δ* cells cultured in raffinose. The
72 Pet191 oligomer persists albeit at lower levels in *cox11Δ* cells (Fig. 2B).

73 Mutational analysis of Pet191 was carried out to assess whether the cysteine residues are
74 functionally important. Cysteiny residues within the twin C_x9C motif as well as the linker motif
75 were singly substituted with alanine residues (Fig. 3B). Mutant alleles of *PET191* were transformed
76 into *pet191Δ* cells and tested for their ability to support growth on glycerol-containing medium.
77 Cells harboring mutant alleles C5A and C56A were respiratory deficient, whereas three additional
78 alleles, C15A, C32A and C46A, were partially compromised in growth at 30°C or 37°C (Fig. 3A). If
79 Pet191 folds in a helical hairpin analogous to Cox17 or Cox12, then Cys5 and Cys56 may be an

80 aligned pair existing as a disulfide bridge (Fig. 3B). All mutant proteins were equivalently
81 expressed as shown by immunoblot analysis (Fig. 3C).

82 Transformation of wild-type cells with the mutant *PET191* alleles revealed that the presence
83 of either C5A or C56A Pet191 had a slight dominant negative effect on respiratory growth (Fig.
84 3D). In contrast, the C15A mutant that was only weakly compromised in supporting glycerol
85 growth of *pet191* Δ cells lacked any negative effects on the growth of wild-type cells on glycerol
86 medium. The C5A mutant protein existed in a smaller complex relative to the wild-type protein by
87 gel filtration (Fig. 3E). Thus, the non-functionality of the C5A protein may result from an abnormal
88 Pet191 complex. To determine whether the dominant negative effect of C5A Pet191 influenced the
89 endogenous Pet191, we carried out gel filtration studies on wild-type cells harboring the C5A
90 Pet191 mutant. The presence of C5A Pet191 in wild-type cells led to an attenuation in the size of
91 the solubilized wild-type protein (Fig. 3E).

92 The dominant negative effect of the mutant Pet191 on the wild-type protein suggested that
93 the two proteins interact. This was confirmed by immunoprecipitation (IP) studies. Mitochondria
94 isolated from cells harboring a vector encoded Pet191-Myc and chromosomal Pet191-HA were
95 used for IP with anti-Myc beads. Pet191-HA co-IP with Pet191-Myc (Fig. 3F). Thus, Pet191
96 complex is a homo-oligomer, but the large size ~500 kDa of the complex may suggest that
97 additional proteins are present.

98 Two of the six conserved Cys residues (Cys5 and Cys56) important for Pet191 function may
99 participate in the disulfide stabilization of the complex, since the C5A Pet191 allele fails to
100 assemble into the wild-type complex. Structures of three twin Cx₉C motif proteins, Cox12, Cox17
101 and Qcr6, reveal disulfide-bonded helical hairpin conformations. Cox12 and Qcr6 are IMS-facing
102 subunits of the CcO and *bc*₁ complexes, respectively. In *S. cerevisiae*, only one of the two Cys pairs

103 exists in Qcr6. If Pet191 adopts a related helical hairpin conformation, the functionally important
104 Cys residues Cys5 and Cys56 may form a disulfide pair.

105 Twin Cx₉C motif proteins like Cox17 are imported into the IMS by the MIA import pathway
106 through an oxidative folding mechanism involving Mia40 and Erv1 (8, 16, 22, 24). Since Pet191
107 has a related twin Cx₉C motif, we addressed if Pet191 was imported through the MIA pathway.
108 Temperature-sensitive *erv1-2ts* mutant (13) cells cultured at 22°C import IMS proteins normally,
109 but import is attenuated upon a shift of cells to the non-permissive temperature (16, 22). We
110 observed that *erv1-2ts* cells cultured at 22°C have normal levels of Sod1, Ccs1, Cox23 and Pet191
111 within the mitochondria (Fig. 4A). However, cells shifted to 37°C have attenuated levels of Sod1,
112 Ccs1 and Cox23, but not Pet191-Myc suggesting that Pet191 is imported in a MIA-independent
113 pathway. The attenuated levels of Sod1, Ccs1 and Cox23 are consistent with their dependency on
114 the MIA complex for IMS import. An independent assessment of the role of Erv1 in Pet191 uptake
115 was conducted using *in vitro* mitochondrial import of Pet191 translated in a rabbit reticulocyte
116 lysate. Mitochondria were isolated from wild-type or *erv1-2ts* cells and tested for [³⁵S]-Pet191
117 import. After treatment with proteinase K, Pet191 was observed in both wild-type and *erv1-2ts*
118 mitochondria incubated at the nonpermissive temperature in which Erv1 is inactive (Fig. 4B).
119 Whereas import of Pet191 into *erv1-2ts* mitochondria was normal, the import of radiolabeled
120 Cox19, a known MIA substrate, was impaired in the respective mutant. Import of Cox19 was also
121 normal in mitochondria isolated from *pet191Δ* cells (Fig. 4C). Thus, we conclude that Pet191 is
122 imported into the mitochondria independent of Mia40/Erv1. The actual mechanism of Pet191
123 import is unclear as it lacks a N-terminal mitochondrial import motif as deduced by either
124 MITOPROT or PSORT algorithm.

125 Pet191 joins the list of twin Cx₉C motif proteins consisting of Cox17, Cox19 and Cox23
126 involved in CcO biogenesis. A series of other twin Cx₉C proteins exist within the IMS whose
127 functions are unknown. These proteins include Mic14 and Mic17 (11). Although Mic14 contains a
128 duplicated twin Cx₉C structural motif, it has no role in CcO biogenesis. Cells lacking Mic14 show
129 no growth defect on glycerol/lactate medium, consume oxygen at wild-type levels and have normal
130 CcO activity. Thus, only a subset of soluble twin Cx₉C proteins in the IMS have a role in CcO
131 biogenesis.

132 The conservation of Pet191 in mammalian cells suggests that Pet191 may have a significant
133 role in mammalian mitochondria. Functional studies have not appeared on the human Pet191
134 ortholog, nor have human mutations in *PET191* been identified in patients with CcO deficiency
135 (23).

136

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142

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214

215

216

Figure legends

217

218 **Fig. 1. Pet191 is required for CcO biogenesis.** (A) Serial dilutions of *pet191*Δ cells transformed
219 with an empty vector or *PET191* were plated onto complete medium containing 2% glucose or 2%
220 lactate/glycerol as a carbon source. The wild-type (WT) and *pet191*Δ diploid strains of
221 *Saccharomyces cerevisiae* (BY4743) were used. (B) CcO and SDH/*bc*₁ activities. Activities of the
222 wild-type mitochondria were set as 100%. Mitochondria were isolated from cells cultured on
223 raffinose medium. (C) Mitochondria were solubilized in a lysis buffer containing 1.5% digitonin
224 and protein complexes were separated by BN-PAGE. The respiratory chain complexes (III, IV and
225 V) were analyzed by immunoblotting with respective antibodies. (D) Steady-state levels of
226 mitochondrial proteins were analyzed by SDS-PAGE followed by Western blotting. (E) Cells
227 expressing Pet191-Myc were fractionated and cytosolic (cyto.) and mitochondrial (mito.) fractions

228 were collected, resolved by SDS-PAGE and analyzed by immunoblotting with the anti-Myc, anti-
229 Pgc1 and anti-porin antibodies. The Pet191-Myc was expressed on a pRS426 vector under the
230 *MET25* promoter and *CYCI* terminator (17). (F) Mitochondria of a strain expressing Pet191-Myc
231 (M, lane 1) were treated with either 0.1 M Na₂CO₃ (pH 10.5 or 11.5) or sonicated (sonic.) and
232 fractionated by high-speed centrifugation. Obtained pellet (P, lanes 2, 4 and 6) and supernatant (S,
233 lanes 3, 5 and 7) fractions were analyzed by immunoblotting using antibodies against Myc-epitope,
234 matrix protein Sod2 and IM-anchored protein Cox2. (G) Intact (lanes 1, 2 and 4) or swollen (lane 3)
235 mitochondria were incubated with (lanes 2, 3 and 5) or without (lanes 1 and 4) proteinase K (PK).
236 In the lanes 4 and 5, mitochondria were lysed with 1% Triton X-100 (TX-100) prior to incubation
237 with PK. Western blot analysis was performed using antibodies specific for Myc-tag, matrix protein
238 Sod2 and intermembrane space protein Cyb2.

239

240 **Fig. 2. Molecular size of Pet191.** (A) Mitochondria isolated from wild-type (WT) cells and the
241 strain containing genomically tagged Pet191 (*PET191::3HA*) were solubilized and subjected to BN-
242 PAGE followed by immunoblot analysis with an antibody against the HA-epitope. A genomically
243 HA-tagged variant of *PET191* was generated by homologous recombination inserting the triple HA
244 tag 3' to the ORF. (B) Mitochondria prepared from WT and *cox11Δ* cells carrying *PET191::3HA*
245 were analyzed by BN-PAGE as described (25) using anti-HA and porin antibodies. (C) Cells
246 expressing Pet191-Myc or its mutant form (C5A) were used for mitochondrial preparation. Isolated
247 mitochondria were lysed in a buffer containing 0.1% deoxycholate (DOC) in the presence (+DTT)
248 or absence (-DTT) of 100 mM DTT and clarified lysates were loaded onto the size-exclusion
249 column (10/30 G-200 Superdex) equilibrated with buffer containing 0.05% DOC ± 5 mM DTT.
250 Fractions were directly assayed by slot immunoblotting with anti-Myc antibodies. Column void

251 (V_E) and internal (V_I) volumes are marked by arrows.

252

253 **Fig. 3. Conserved cysteine residues are important for Pet191 function.** (A) Serial dilutions of

254 *pet191*Δ cells transformed with an empty vector, *PET191* or its mutant forms were plated onto

255 complete medium containing 2% glucose or 2% lactate/glycerol as a carbon source and grown for 4

256 days at 30°C or 37°C. Mutant forms of Pet191 (C5A, C15A, C21A, C32A, C46A, C56A and C86A)

257 were generated by site-directed mutagenesis using pRS426-*PET191*-Myc as a template. (B).

258 Schematic demonstrating possible conformation of Pet191 molecule where two Cx₉C motifs face

259 each other. Positions of the most critical cysteine residues depicted by light gray circles. Other Cys

260 residues are shown in black. (C) Mitochondria isolated from *pet191*Δ cells carrying an empty

261 vector, *PET191* or its mutant forms were subjected to SDS-PAGE followed by Western blot

262 analysis with the antibodies against Myc-epitope and porin. (D) Wild-type (WT) cells over-

263 expressing *PET191* or its C5A, C15A or C56A mutant versions were serially diluted, plated as in

264 panel A and grown for 3 days at 30°C. (E) Mitochondria from the cells co-expressing Pet191-HA

265 and Pet191-Myc or its mutant form C5A were solubilized and analyzed as described above, except

266 of DTT treatment. Collected fractions were analyzed using anti-Myc and anti-HA antibodies. (F)

267 Mitochondria isolated from WT *PET191::3HA* strain with (lanes 1-3) or without (lanes 4-6)

268 Pet191-Myc were lysed and clarified extracts were immunoprecipitated with agarose-coupled anti-

269 Myc antibodies. Co-immunoprecipitation of Pet191-Myc and Pet191-HA was performed as

270 described (20), using anti-Myc-agarose coupled beads (Santa Cruz Biotechnology). The load

271 (represents 5% of the extracts, lanes 1 and 4), entire wash (lanes 2 and 5) and eluate (lanes 3 and 6)

272 fractions were analyzed by immunoblotting.

273

274 **Fig. 4. *erv1* mutant mitochondria have normal levels of Pet191-Myc.** (A) Wild-type (WT) and
275 temperature-sensitive *erv1-2ts* strains expressing Pet191-Myc or Cox23-Myc were grown at 22°C.
276 The temperature-sensitive strain *erv1-2ts* (13) and corresponding WT strain were a kind gift from
277 Dr. Roland Lill. For mitochondrial isolation from *erv1-2ts* cells, the cells were pre-grown in
278 supplemented synthetic medium containing 2% raffinose at 22°C till the early logarithmic phase.
279 Cultures were split into two halves, one of which was subjected to a temperature shift (22°C to
280 37°C) and another left at 22°C. After 7-10 h incubation cells were harvested and mitochondria
281 isolated as described above. Mitochondria were isolated, separated by SDS-PAGE and analyzed by
282 immunoblotting with antibodies recognizing Myc-tag, Ccs1, Sod1 and porin. (B) Pet191 was
283 synthesized in rabbit reticulocyte lysate in the presence of [³⁵S]-methionine and incubated for 20
284 min at 30°C with mitochondria isolated from either wild-type (WT) or *erv1-2ts* cells. After
285 incubation mitochondria were reisolated, washed and either swollen in hypotonic medium (SW) or
286 incubated with proteinase K (PK). Samples were then subjected to SDS-PAGE followed by
287 autoradiography. (C) Radiolabeled Cox19 was imported into the WT, *pet191Δ* or *erv1-2ts*
288 mitochondria as described above.

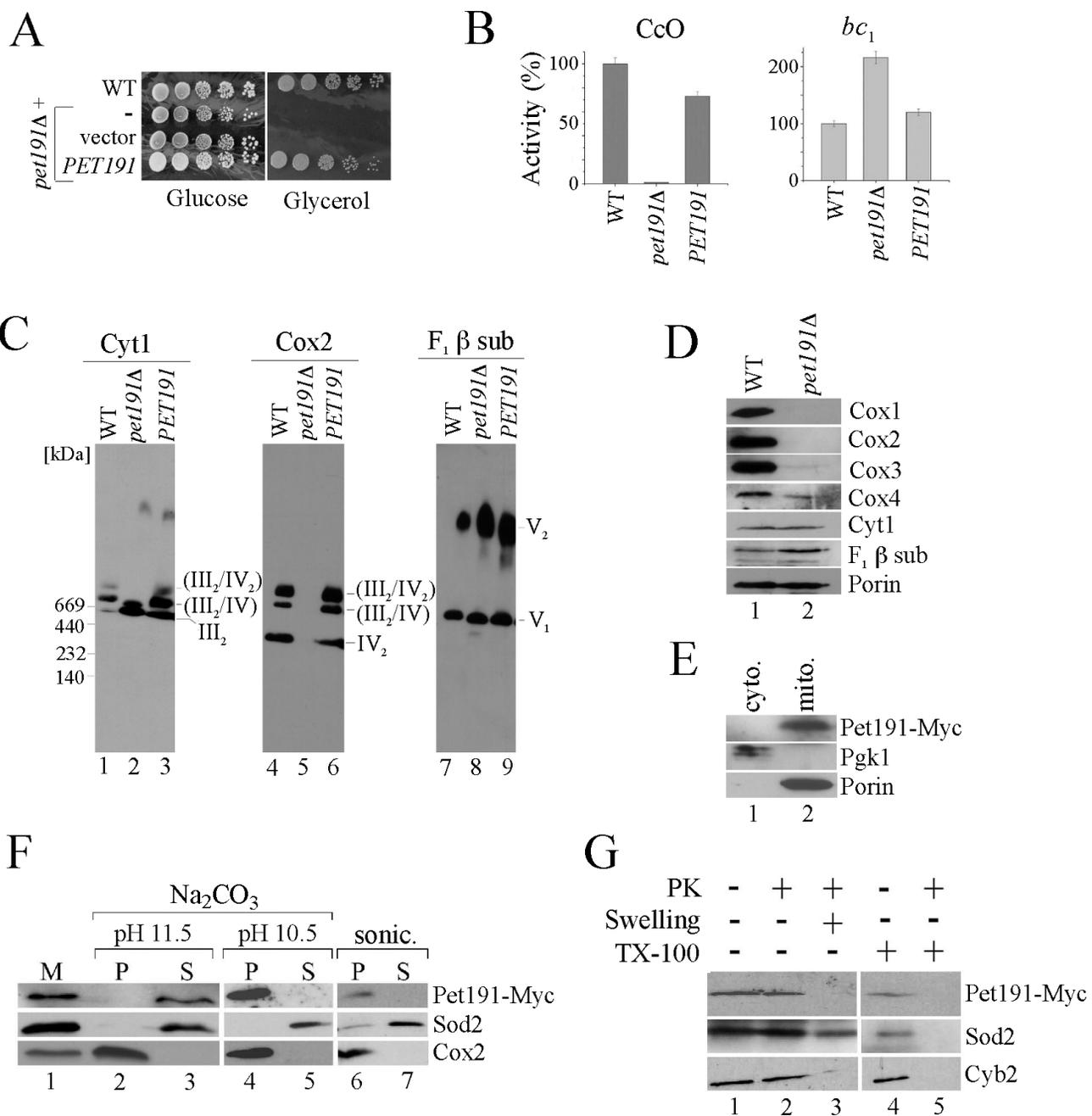


Figure 1

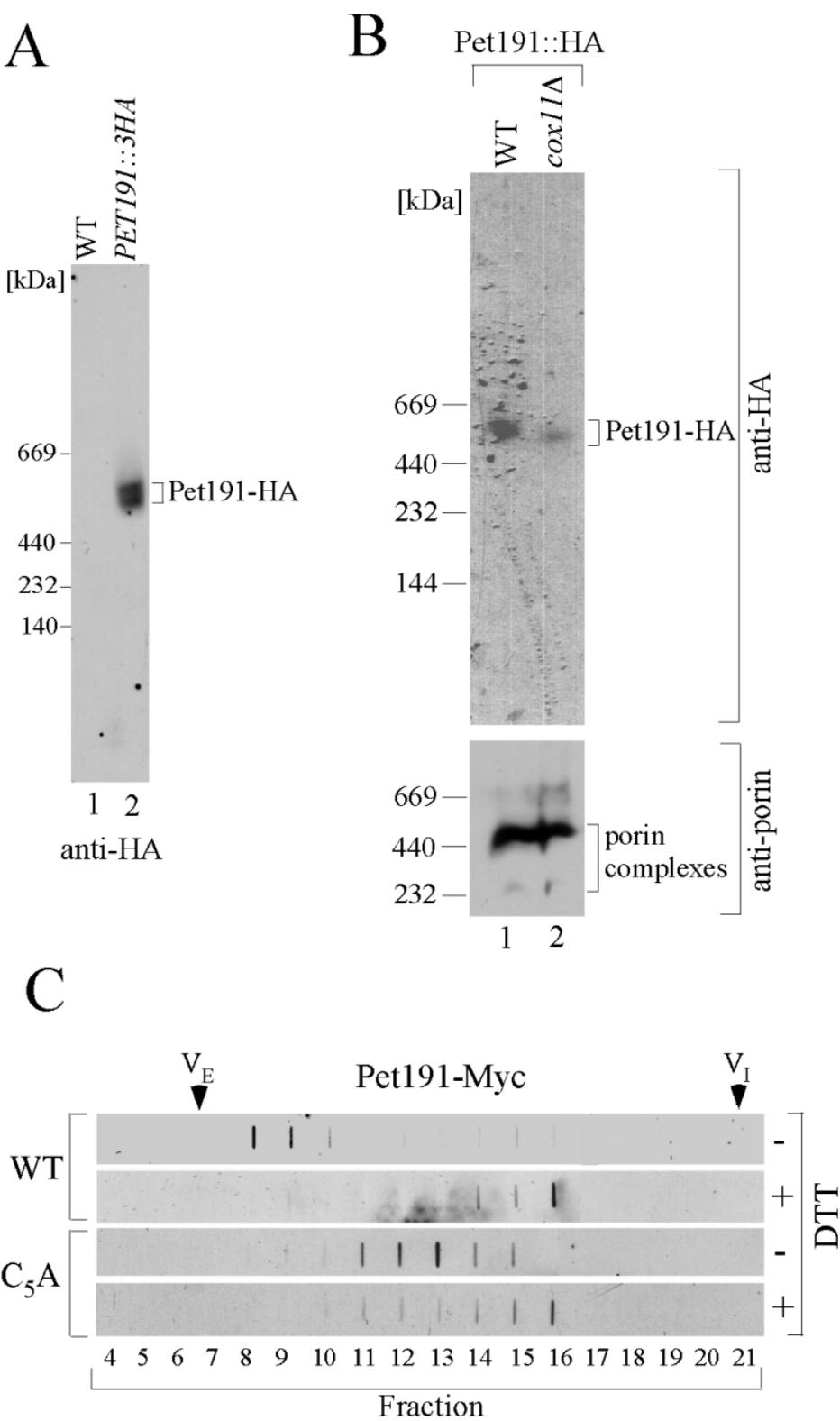


Figure 2

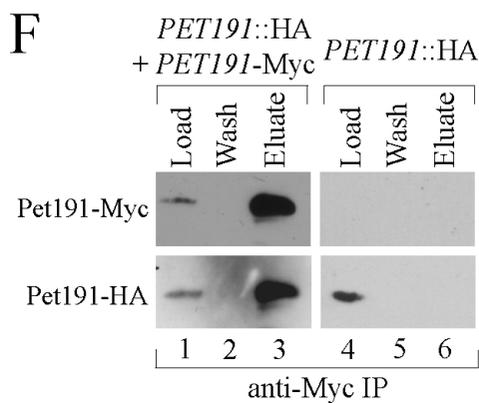
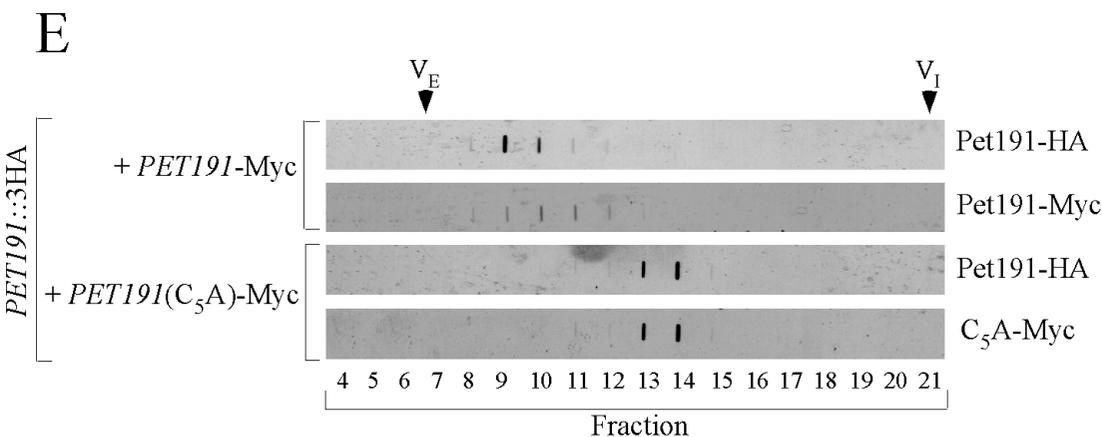
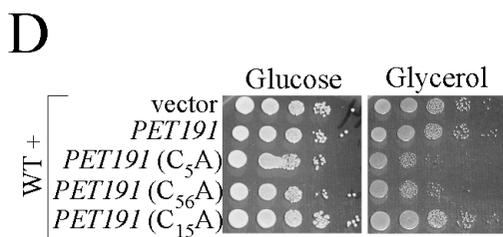
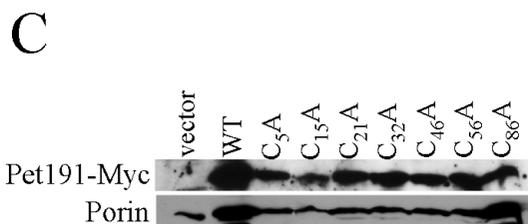
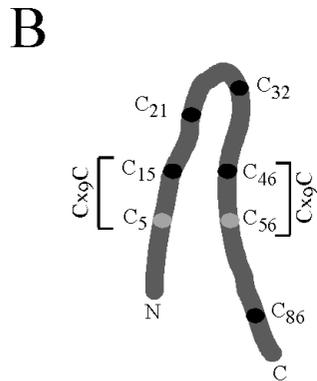
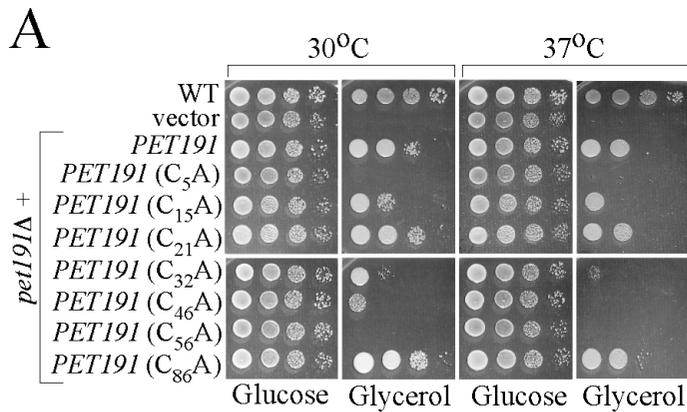
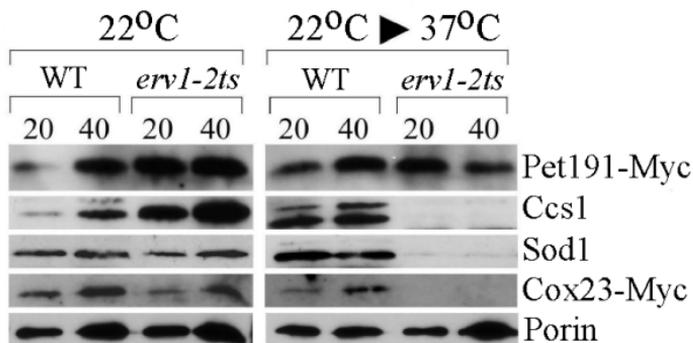
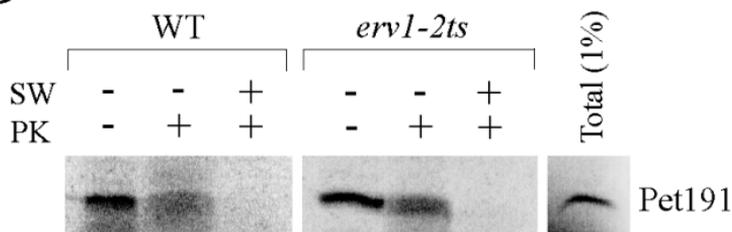
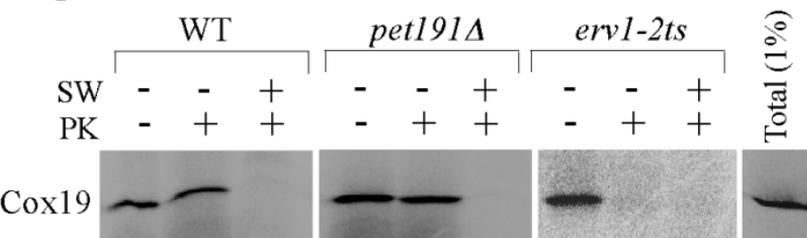


Figure 3

A**B****C****Figure 4**