Supplementary Information for

Moonlighting Proteins Hal3 and Vhs3 Form a Heteromeric PPCDC with Ykl088w in Yeast CoA Biosynthesis

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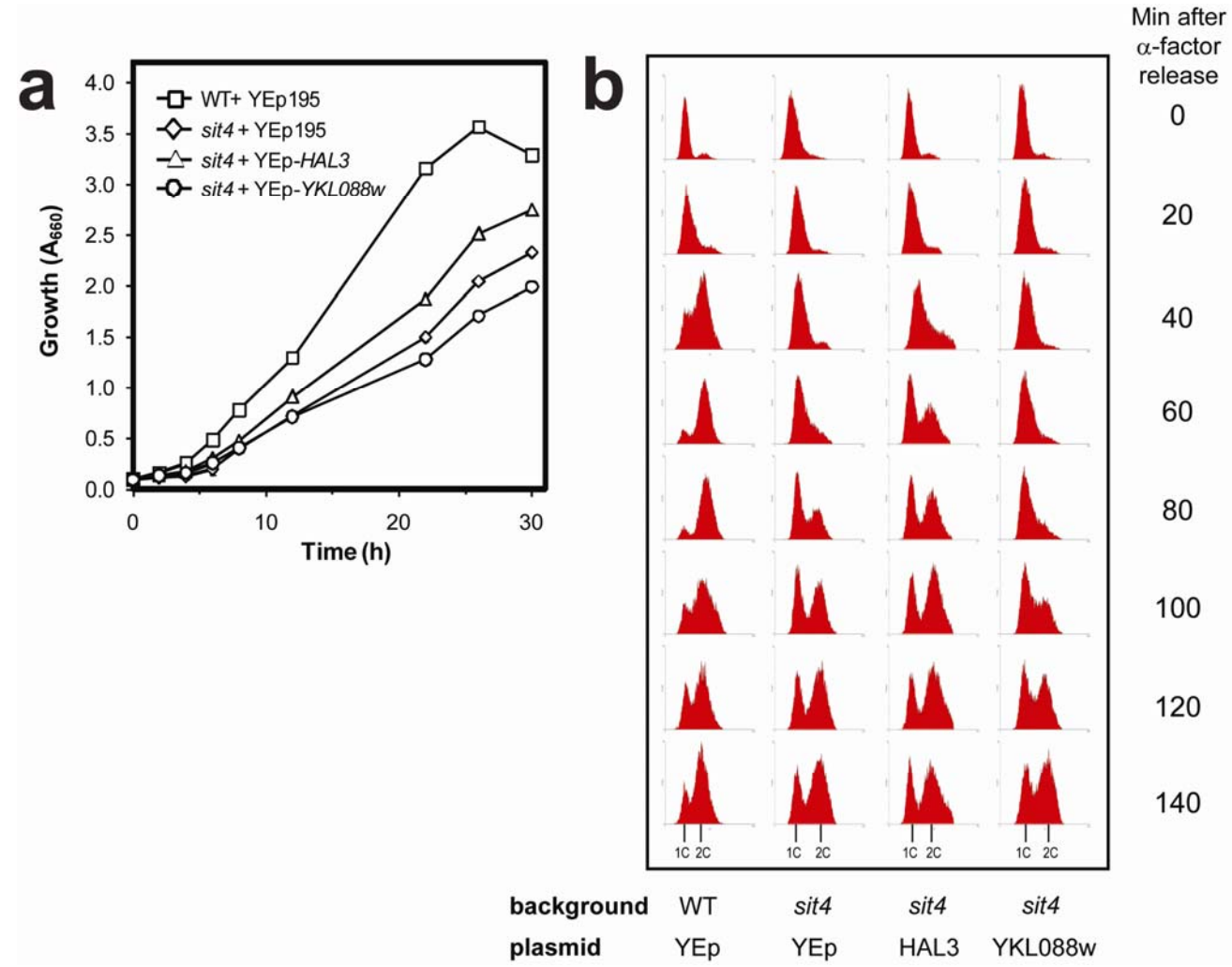
SUPPLEMENTARY RESULTS

Supplementary Figures



Supplementary Figure 1. Effect of high copy expression of *YKL088w* on wild type and *sit4* cells. (**a**) JA100 (wild type, WT) cells bearing an empty plasmid (YEp195), as well as JA110 (*sit4*) cells carrying the empty plasmid, YEp195-HAL3 or YEp195-YKL088w plasmids, were inoculated on synthetic minimal medium lacking uracil at a OD_{660} of 0.01, and growth was monitored for the indicated times. (**a**) Above-mentioned strains bearing the indicated plasmids were arrested in G_1 by incubation with α-factor. The pheromone was washed out and entry into cell cycle monitored by flow cytometry analysis of the DNA content. Experimental details can be found in Refs. 1,2.

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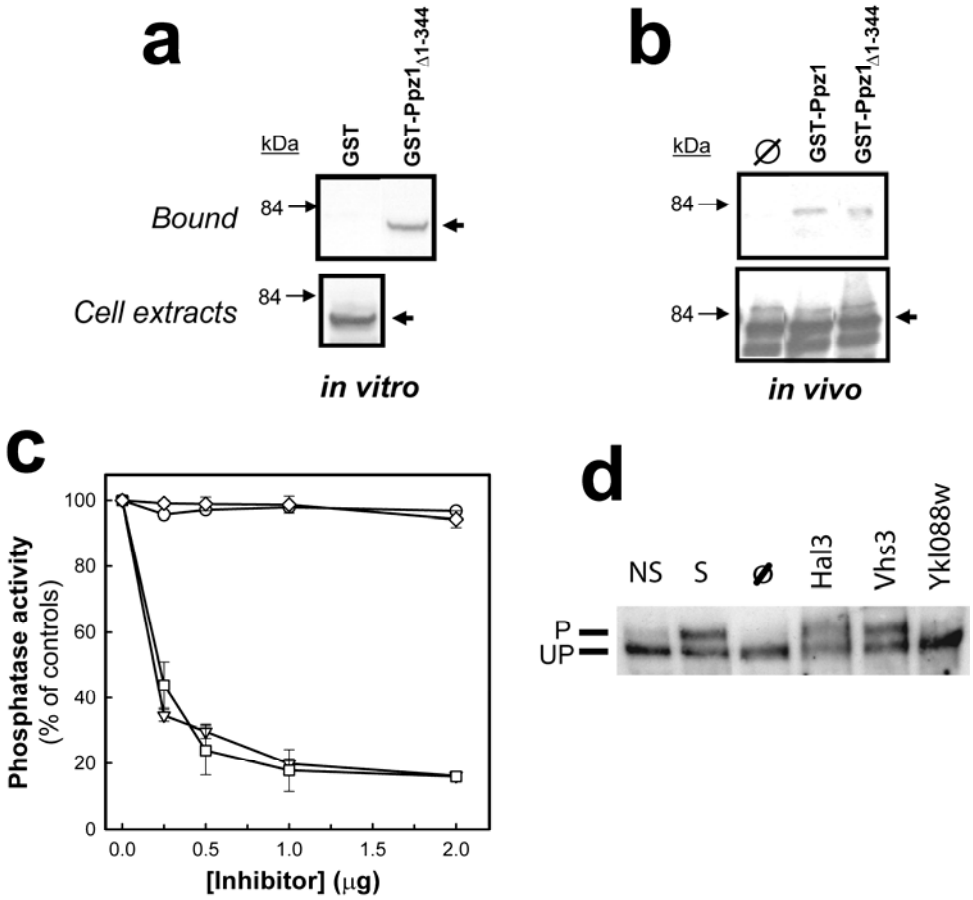

Supplementary Figure 2. Assessment of the capacity of Ykl088w to bind and inhibit Ppz1. (**a**) Cell extracts from strain EDN75 expressing a 3xFLAG tagged version of Ykl088w were mixed with bacterially expressed C-terminal half of Ppz1 (GST-Ppz1$_{\Delta 1\text{-}344}$) or with GST and the mixtures bound to glutathione-agarose beads. The presence of the tagged version of Ykl088w was assessed by SDS-PAGE, followed by immunoblot (using anti-FLAG antibodies) of the purified fraction as well as the original extract. (**b**) Strain EDN75 expressing the indicated forms of Ppz1 was transformed with the centromeric plasmid YCp22 bearing a 3xFLAG tagged version of Ykl088w, and the GST-fused versions of Ppz1 were affinity purified from total yeast extracts using glutathione-agarose beads. The presence of tagged Ykl088w bound to the protein phosphatase was monitored as in (a). Experimental details were as in Ref. 2. (**c**) Effect of Ykl088w on Ppz1 activity. One μg of purified GST- Ppz1$_{\Delta 1\text{-}344}$ was incubated with increasing amounts of GST (◇), GST-Ykl088w (○), GST-Hal3 (▽) or GST-Vhs3 (□) and the phosphatase activity measured using *p*-nitrophenyl phosphate (**9**) as substrate as in Ref. 23. (**d**) MCY3000 cells expressing HA-Reg1$_{1–443}$ were grown in high glucose (4%) media and shifted 20 min to low glucose media (0.05%). Crude extracts were obtained and the phosphorylation state of HA-Reg1$_{1–443}$ before (*NS*, not shifted) or after (*S*, shifted), monitored by SDS-PAGE and immunoblot with anti-HA antibodies. Two μg of purified GST-Ppz1$_{\Delta 1–344}$ (GST-Ppz1) were preincubated 5 min with buffer (Ø) or with 2 μg of either GST-Hal3, GST-Vhs3 or GST-Ykl088w, and then for 20 min at 30 °C with 1 μg of the extracts. Samples were analyzed by SDS-PAGE (10% polyacrylamide gels) and immunodetected with anti-HA monoclonal antibodies. *P*, phosphorylated; *UP*, unphosphorylated forms of HA-Reg1$_{1–443}$. For experimental details, see Refs. 2,3.

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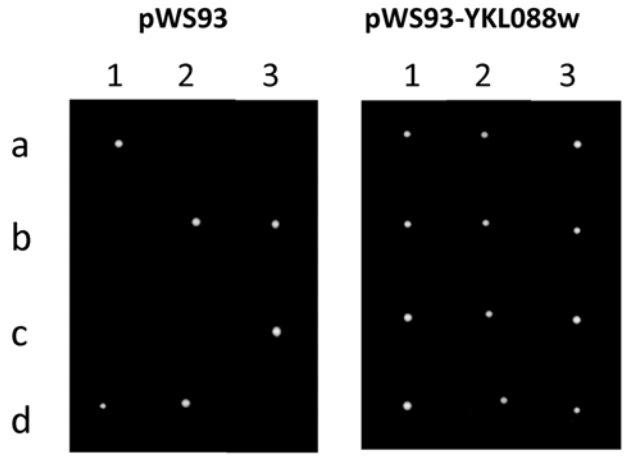
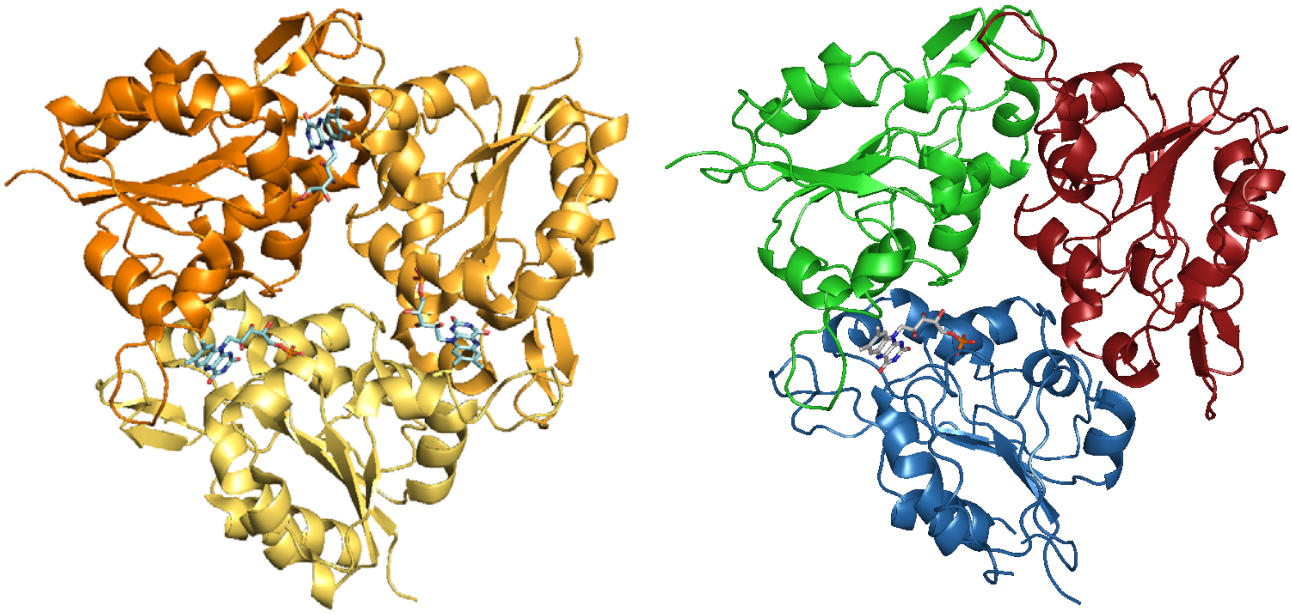


Supplementary Figure 3. *YKL088w* is an essential gene. The diploid strain MAR25, heterozygous for the *ykl088w* deletion was transformed with plasmid pWS93 or with the same plasmid carrying the *YKL088w* open reading frame. Cells were induced to sporulate and tetrad analysis performed. Three representative tetrads are shown for each case. As it can be observed, a 2:2 segregation of the lethal phenotype is found in cells carrying an empty vector, and none of the viable spores contained the disrupted allele. In contrast, expression of Ykl088w allowed the growth of all four spores from a tetrad.



Supplementary Figure 4. The structure of the *A. thaliana* PPCDC enzyme (AtHal3a) suggests a heteromeric model structure for the yeast PPCDC. On the left, the ribbon structure of the homotrimeric AtHal3a protein as determined by X-ray crystallography (PDB: 1MVN). The flavin cofactor is shown as a stick structure and identifies the active sites at the interfaces of the protomers. On the right, a model of the PPCDC domains of a heterotrimeric yeast protein based on the AtHal3a structure, with Hal3 shown in green, Vhs3 in red and Ykl088w in blue. The single predicted active site is identified by the presence of the flavin cofactor depicted as a stick structure. The relative position of the Hal3 and Vhs3 insertion sequences (see Fig. 1a and 1b) could not be modeled with certainty and has been omitted for clarity.

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SUPPLEMENTARY METHODS

Supplementary Table 1. *S. cerevisiae* strains used in this work.

Name	Relevant genotype	Source/Reference
JA100	*MAT**a*** *ura3-52 leu2-3,112 his4 trp1*-1 *can*-1r	(5)
JC010	JA100 *slt2*::*LEU2*	(4)
JA104	JA100 *hal3*::*LEU2*	(5)
EDN101	JA100 *slt2::URA3 hal3::LEU2*	(2)
EDN75	JA100 *ppz1*::*KanMX4*	(5)
1788	*MATa*/α *ura3-52 leu2-3,112 his4 trp1-1 can-1r*	D. Levin
MAR25	1788 *ykl088w*::*kanMX4/YKL088w*	This work
AGS4	1788 *hal3*::*LEU2/HAL3 vhs3*:: *kanMX4/VHS3*	This work
AGS31	1788 *hal3*::*LEU2/HAL3 vhs3*::*nat1/VHS3 ykl088w*::*kanMX4/YKL088w*	This work
DBY746	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*1 trp1-*Δ*239*	D. Botstein
EDN4	DBY746 *hal3*::*LEU2*	(6)

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Supplementary Table 2. Oligonucleotides employed in this work.

Oligonucleotide	Utilization	Sequence
5'-YKL088w_KanMX	ykl088w::kanMX4 interruption	5′-ATTTAAATAAAAGTCTAAAGGACCAGCAAAGA GTTATTAGGTGTGCGTACGCTGCAGGTCGAC-3′
3'-YKL088w_KanMX	ykl088w::kanMX4 interruption	5′-AAATAAAACATAAAACATAACACAAAATACTC TTTTCTAATGCATATCGATGAATTCGAGCTCG -3′
YKL088w_5'	YEp and YCplac-YKL088w plasmids	5′-GCGAATTCCAGAAGATCCCAACGAG-3′
YKL088w_3'	YEp and YCplac-YKL088w plasmids	5′-AAGGACGAATTCTGACAGAAAC-3′
5'-YKL088w_EcoRI	Construction of pGEX-YKL088w	5′-GGGAATTCATGACGGATGAAAAAGTGAAC-3′
3'-YKL088w_XhoI	Construction of pGEX-YKL088w	5′-CACGCTCGAGTTAAACTTCGGTTTTCACGT-3′
5'-YKL088w_NheI	Different versions of YKL088w	5′-CTGAACAAGTAAAAGACACGAAG-3′
3'-YKL088w_NdeI	Different versions of YKL088w	5′-GCGTTTGACTTGGGATTGAAG-3′
5'-YKL088w_SacI/STOP	Construction of YEp195-YKL088w_3FLAG	5′-AAGTTTAAATGCATTAGAAAAGAGT-3′
3'-YKL088w_SacI/STOP	Construction of YEp195-YKL088w_3FLAG	5′-CATTTAAACTTCGGTTTTCACGT-3′
5'-pCM220_SacI	Construction of YEp195-YKL088w_3FLAG	5′-GCTAGAGCTCACGCTGCAGGTGGACGG-3′
3'-pCM220_SacI	Construction of YEp195-YKL088w_3FLAG	5′-GCTAGACTCTAGAAGTGGCGCGCCTA-3′
5'-YKL_His391Ala	Construction of YEp195-YKL088w_H391A	5′-GATATTGGCCCATGAATTAAGAAAATGGG-3′
3'-YKL_His391Ala	Construction of YEp195-YKL088w_H391A	5′-TTCATGGGCCAATATCAAGTTCAGACTC-3′
5'-YKL_Cys478Ser	Construction of YEp195-YKL088w_C478S	5′-TATTAATATCCGGAGATATTGGTATGGGTG-3′
3'-YKL_Cys478Ser	Construction of YEp195-YKL088w_C478S	5′-ATATCTCCGGATATTAATACCTTTTCCACC-3′
5'-YKL_repI	Construction of YEp195-YKL088w_His^{Rep}	5′-GAAACAACGAACTGATCCTGTACTT CATATAGAATTAAGAAAATGGGCTG-3′
3'-YKL_repI	Construction of YEp195-YKL088w_His^{Rep}	5′-GATCAGTTCGTTGTTTCCATGCGTC CCATTCGTCTTCCTCCCTCCAAATT -3′
5'-pRS699-At	Different versions of AtHal3a	5′-GTCCTATCATTATCGTCTAAC-3′
3'-pRS699-At	Different versions of AtHal3a	5′-GTGAAGCTAAGAGTTGATGCC-3′
5'-AtHAL3_H90N	Construction of pRS699-AtHal3a_H90N	5′-TGTCCTTAACATCGAGCTTAGACGTTGG -3′
3'-AtHAL3_H90N	Construction of pRS699-AtHal3a_H90N	5′-AGCTACAATTCCTGTCCTAGTGGCTAGA -3′
5'-AtHAL3_C175S	Construction of pRS699-AtHal3a_C175S	5′-CTTGCCTCTGGAGACTACGGTAATGGAG -3′
3'-AtHAL3_C175S	Construction of pRS699-AtHal3a_C175S	5′-CAGAGGTCTCCGTTCAGAGAAGAACTAT -3′
5'-hPPCDC_XhoI	Construction of pRS699-HsHal3 and pRS699-HsHal3_C173S	5′-GCCCGCTCGAGATAATGAGTGAACCAAAGGCCTC-3′
3'-hPPCDC_XhoI	Construction of pRS699-HsHal3 and pRS699-HsHal3_C173S	5′-GCCCGCTCGAGTCAACTCTGCTGGAAGCCAC-3′
5'-dfp	Construction of pWS-*dfp*	5′-GCGAATTCATGAGCCTGGCCGGTA-3′
3'-dfp	Construction of pWS-*dfp*	5′-GCGCACGCGTCGACGGTGGCATAAGTCGGG-3′
5'-pGEX	Construction of pGEX mutants	5′-GGCAAGCCACGTTTGGTG-3′
3'-pGEX	Construction of pGEX mutants	5′-GAGCTGCATGTGTCAGAGG-3′
5'-Hal3_H378A	Construction of pGEX-Hal3_H378A	5′-GATCCTGTACTTGCTATAGAACTACG-3′
3'-Hal3_H378A	Construction of pGEX-Hal3_H378A	5′-CGTAGTTCTATAGCAAGTACAGGATC-3′
5'-Vhs3_H459A	Construction of pGEX-Vhs3_H459A	5′-GATCCTGTATTAGCCATAGAACTACG-3′
3'-Vhs3_H459A	Construction of pGEX-Vhs3_H459A	5′-GTAGTTCTATGGCTAATACAGGATC-3′
5'-Ykl088w_C478S	Construction of pGEX-Ykl088w_C478S	5′-GTATTAATATCCGGAGATATTG-3′
3'-Ykl088w_C478S	Construction of pGEX-Ykl088w_C478S	5′-CAATATCTCCGGATATTAATAC-3′

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Supplementary Table 3. Plasmids employed in this work.

Plasmids	Origin / Reference
YEplac195-YKL088w	This work
YEplac112-YKL088w	This work
YEplac181-YKL088w	This work
YCplac111-YKL088w	This work
YEplac195-YKL088w_3FLAG	This work
YCplac22-YKL088w_3FLAG	This work
pGEX-YKL088w	This work
YEp195-HAL3	(2)
YEp195-VHS3	(2)
pWS-HAL3	This work
pWS-YKL088w	This work
pRS699-AtHal3a	(7)
pRS699-MmHal3	R. Serrano
pRS699-HsHal3	This work
pWS93-*dfp*	This work
YEp195-HAL3_H378A	(2)
YEp195-VHS3_H459A	(2)
YEp195-YKL088w_H391A	This work
YEp195-YKL088w_C478S	This work
pRS699-AtHal3a_H90N	This work
pRS699-AtHal3a_C175S	This work
pRS699-HsHal3_C173S	This work
YEp195-HAL3_N466C	This work
YEp195-YKL088w_His^{Rep}	This work
pPHS_CoaC21 (pPROEX-HsCoaC)	(8)
pGEX-HAL3	(2)
pGEX-VHS3	(2)
pGEX-YKL088w_His^{Rep}	This work
pGEX-HAL3_H378A	This work
pGEX-VHS3_H459A	This work
pGEX-YKL088w_C478S	This work

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Plasmid construction

The construction of a version of *YKL088w* carrying a C-terminal 3xFLAG tag for high-copy expression in yeast was performed as follows. An artificial SacI site right in front of the stop codon (which introduces the residues EL) was created by sequential PCR using external oligonucleotides that encompassed both the NdeI and SnaBI sites. The amplification fragment was digested with these enzymes and used to replace the 1.29-kbp NdeI/SnaBI fragment of YEp195-YKL088w. A 3xFLAG tag, with added SacI sites, was amplified by PCR from plasmid pCM220 (a gift from M. Aldea, U. Lleida) and then cloned into the previous construct in the appropriate orientation to yield YEp195-YKL088w(3xFLAG). For low copy expression YEp195-YKL088w(3xFLAG) was digested with EcoRI and the insert cloned into this site of plasmid YCplac22. Mutations of Ykl088w His391 to Ala and Cys478 to Ser were made by sequential PCR. In a first step, the 1.29-kbp NdeI/SnaBI fragment of *YKL088w* gene was amplified in two separate reactions by using primers with the modification introduced to change His391 to Ala (*CA*C to *GC*C) or Cys478 to Ser (T*G*C to T*C*C). In the second step, the entire NdeI/SnaBI fragment was amplified, digested, and the product cloned into the NdeI/SnaBI sites of YEp195-YKL088w, to yield YEp195-YKL088w(H391A) and YEp195-YKL088w(C478S). Replacement of the PPC binding motif II of YKL088w by the corresponding Hal3 motif was also made by sequential PCR. In a first step, the 1.29-kbp NdeI/SnaBI fragment from *YKL088w* was amplified in two separate reactions by using primers with the modification introduced to substitute from nucleotides 1113 to 1188 of *YKL088w* with the equivalent region of *HAL3* (nucleotides 1095 to 1149). In the second step, the entire NdeI/SnaBI fragment was amplified, digested, and the product cloned into the NdeI/SnaBI sites of YEp195-YKL088w, to yield YEp195-YKL088w_His^{Rep}.

Expression of the *HAL3*-homolog genes from higher eukaryotes in *S. cerevisiae* was based on the use of two different plasmids, pRS699 which contains the *PMA1* promoter (from R. Serrano, U. Politécnica Valencia) and pWS93, containing the *ADH1* promoter[9]. Plasmids pRS699-AtHal3a[7] and pRS699-MmHal3 were a generous gift from R. Serrano. Plasmid pRS699-HsHal3 was prepared as follows. The human homolog of the AtHal3 gene (HsCoaC) was amplified by PCR, with XhoI sites added, using plasmid pPHS_CoaC21 (pPROEX-HsCoaC)[8] as template, then digested and cloned in the XhoI site of plasmid pRS699. An identical cloning strategy using plasmid pET28a-HsCoaC_C173S[10] as DNA template allowed construction of pRS699-HsHal3_C173S. Mutations of AtHal3a His90 to Asn and Cys175 to Ser were made by sequential PCR. In a first step, the whole AtHal3a gene was amplified in two separate reactions by using primers with the modification introduced to change His90 to Asn (*C*AC to *A*AC) or Cys175 to Ser (T*G*T to T*C*T). In the second step, the entire XhoI fragment was amplified, digested with XhoI, and the product cloned into the same site of pRS699, to yield pRS699-AtHal3a_H90N and pRS699-AtHal3a_C175S. The *E.coli* bifunctional CoaBC (*dfp*) gene was amplified by PCR, with EcoRI and SalI sites added, using plasmid pET28a-EcCoaBC[11] as template, then digested and cloned in the same sites of the plasmid pWS93.

For the expression of *HAL3* and *YKL088w* genes under the *ADH1* promoter, both ORFs were amplified by PCR, with EcoRI and XhoI sites added, digested and the product cloned into the EcoRI/SalI sites of pWS93 to yield pWS-HAL3 and pWS-YKL088w plasmids. Mutation of Hal3 Asn466 to Cys was made by sequential PCR. Firstly, the 0.70-kbp BamHI/HpaI fragment of *HAL3* gene was amplified in two separate reactions by using primers with the modification introduced to

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change Asn466 to Cys. Secondly, the entire BamHI/HpaI fragment was amplified, digested, and the product cloned into the BamHI/HpaI sites of YEp195-HAL3 to yield YEp195-HAL3_N466C.

For bacterial expression of the GST-tagged mutant proteins, the native pGEX plasmids were used as template[2]. Mutations were introduced by single overlap extension (SOE) PCR using using the pGEX sequencing primers (5'-pGEX and 3'-pGEX), as well as primers designed to introduce the relevant mutations, i.e. Hal3 His378 to Ala (*CA*T to *GC*T), Vhs3 His459 to Ala (*CA*C to *GC*C) and Ykl088w Cys478 to Ser (T*G*C to T*C*C). PCR products were digested and cloned into the EcoRI/XhoI site of pGEX6P-1 (Amersham Biosciences).

For bacterial expression of the GST-Ykl088w and GST- Ykl088w_His^{Rep} fusion proteins, the *YKL088w* and *YKL088w*_His^{Rep} genes were amplified by PCR, with added EcoRI/XhoI sites, using as template YEp195-YKL088w and YEp195-YKL088w_His^{Rep} plasmids respectively, and cloned into plasmid pGEX6P-1.

In Vitro and *In Vivo* Binding Assays

In vitro and *in vivo* binding assays of Ykl088w, Hal3 and Vhs3 with the entire or C-terminal Ppz1 protein were performed as previously described[2]. The interaction of Hal3 and Vhs3 with Ykl088w was assessed *in vitro* as follows. GST-Ykl088w, GST-Hal3 and GST-Vhs3 constructs (see above) were introduced into *E. coli* strain BL21 DE3 RIL (Stratagene). GST-Hal3 and GST-Vhs3 were expressed as described[2]. For GST-Ykl088w expression *E. coli* cultures were grown at 28 ºC and IPTG concentration reduced to 0.1 mM. Purification using glutathione-agarose beads (GE Healthcare) was performed as described[3]. In the case of Ykl088w, the GST-fused protein was eluted with 10 mM glutathione whereas in the case of GST-Hal3 and GST-Vhs3 the beads were treated with PreScission protease (GE Healthcare) to remove the GST moiety as recommended by the manufacturers, except that 10% glycerol was added to the eluted samples. For binding experiments, 30 μl (10 μg protein) of Hal3 was mixed with 15 μl (10 μg protein) of Vhs3 and 30 μl (20 μg protein) of GST-Ykl088w and incubated with gentle shaking at 4ºC for 60 min. A 50% slurry of glutathione-agarose beads (50 μl) was added to the mixture and incubation continued for 2 additional hours. Samples were centrifuged and the supernatant removed. Beads resuspended in 200 μl of buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol and 0.1% Triton X-100), transferred to MultiScreen filter plates (Millipore) and extensively washed with the resuspension buffer. Beads were then resuspended in 20 μl of washing buffer, 20 μl of 2x sample buffer added, boiled and samples subjected to SDS-PAGE in 10% polyacrylamide gels.

Protein phosphatase Ppz1 inhibition assays

The ability of Ykl088w to inhibit protein phosphatase Ppz1 activity was tested by two different assays using bacterially expressed proteins. The *p*-nitrophenyl phosphate (**9**)-based assay was carried out as previously described[2]. The second assay relied on the ability of recombinant Ppz1to dephosphorylate the N-terminal domain of Reg1. The assay is based in the lower mobility of phosphorylated Reg1 forms and was carried out essentially as previously described[3].

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PPCDC activity assays – Amounts of protein used

The amount of protein used in the various PPCDC activity assays depended on the specific experiment. For the first activity assays (Fig. 3c in the main text) 0.5 µg of each protein was used, whether individually or in mixtures. For the stoichiometric activity analysis (Fig. 4b in the main text), the activity of the Hal3/Vhs3/Ykl088w ternary mixture (containing 0.5 µg of each protein) was compared to binary mixtures of Hal3 and Ykl088w containing between 0.5 and 1.5 µg of each protein (exact amounts are indicated in Figure 4b), and to the Vhs3/Ykl088w binary mixture containing 1.0 µg Vhs3 and 0.5 µg Ykl088w. In the activity analysis of the mutant proteins based on product formation (Fig. 4c, closed bars) 0.35 µg of each protein was used, whether individually or in mixtures. For the assay of the mutant protein based on CO_2-release (Fig. 4c, open bars) the Hal3/Vhs3/Ykl088w ternary mixture contained 0.5 µg of each protein, and the Hal3/Ykl088w and Vhs3/Ykl088w binary mixtures contained 1.0 µg Hal3 or Vhs3 and 0.5 µg Ykl088w. Finally, to determine the activity of the engineered homomeric Ykl088wHis^{Rep} protein the amount of protein was increased incrementally from 0.5 µg to 3.0 µg; in the Hal3/Ykl088wHis^{Rep} binary mixture 1.0 µg of Hal3 and 0.5 µg of Ykl088w was used, and in the Hal3/Vhs3/Ykl088wHis^{Rep} ternary mixture each protein was present at 0.5 µg.

Statistical analysis

To determine whether the data points in the stoichiometric activity analysis (Fig. 4b) differed significantly, we compared the activity of the reference Hal3/Ykl088w sample with 0.5 µg of each protein with the activities of the samples in which the amount of either protein was increased to 1.0 µg. This was done by performing an unpaired t-test (assuming equal variance), which gave the following results:

H:0.5/Y:1.0 vs H:0.5/Y:0.5, P = 0.0067
H:0.5/Y:0.5 vs H:1.0/Y:0.5, P = 0.0441

This analysis shows that doubling the amount of either Hal3 or Ykl088w does alter the activity of the complex significantly. We did not perform an ANOVA analysis to compare all the data points with one another as the activity assays were only performed in duplicate (mainly due to the difficulty experienced in obtaining even minute amounts of Ykl088w).

To determine which data points in the activity analysis of the mutant proteins (Fig. 4c) and the engineered homomeric Ykl088w-His^{Rep} protein (Fig. 5c) showed significant activity (i.e. was significantly different from zero) a one sample t-test was performed, setting the expected mean at zero. These analyses gave the following results:

Fig. 4c	Product formation assay	CO_2-release assay
Hal3/Ykl088w	P = 0.0030	P = 0.0113
Hal3*/Ykl088w	P = 0.2342	P = 0.4569
Hal3/Ykl088w*	P = 0.3136	P = 0.0391
Vhs3/Ykl088w	n.d.	P = 0.0048
Vhs3*/Ykl088w	n.d.	P = 0.0840
Vhs3/Ykl088w *	n.d.	P = 0.0190
Hal3/Vhs3/Ykl088w	P = 0.0004	P = 0.0046
Hal3*/Vhs3/Ykl088w	P = 0.0028	n.d.
Hal3/Vhs3*/Ykl088w	P = 0.0031	n.d.
Hal3/Vhs3/Ykl088w*	P = 0.2537	P = 0.0476

Fig. 5c	Product formation assay
Hal3, 3.0	P = 0.4590
Ykl088w-His^{Rep}, 0.5	P = 0.2499
Ykl088w-His^{Rep}, 1.0	P = 0.0545
Ykl088w-His^{Rep}, 2.0	P = 0.0024
Ykl088w-His^{Rep}, 3.0	P = 0.0370
Hal3/Ykl088w-His^{Rep}	P = 0.0011
Hal3*/Ykl088w-His^{Rep}	P = 0.0189

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