Supplemental material for

THE ACTIVE SITE OF YEAST PHOSPHATIDYLINOSITOL SYNTHASE PIS1 IS FACING THE CYTOSOL

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Supplemental materials and methods

Materials

Anti-V5-HRP antibody was from Invitrogen, anti-FLAG, anti-HA and anti-mouse IgG antibodies were from Sigma, anti-Kar2 from Santa Cruz Biotech. HRP-labeled antibodies were visualized with Advansta WesternBright ECL (K-12045-D50).

Western blotting

Samples were separated on 10% SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore) in transfer buffer (10 mM Tris, 100 mM glycine, 10% methanol)(35) and Western blotting was performed using first anti-FLAG antibodies (1/2000 in TBS with 1% milk powder) to detect the luminal control Gpi8. After having dried on the bench overnight, blots (without stripping) were rpobed by anti-V5-HRP antibodies (1/10'000 in PBS with 5% milk powder) to detect Pis1. Similarly, for Pis1 quantification, blots were first probed by anti-Kar2, dried and then probed with anti-V5-HRP.

Calculation of PI synthase activity of microsomes.

For all enzyme assays, routinely done with freshly prepared microsomes, the amount of Pis1-V5-His6 present in these microsomes was quantitated on a frozen sample in a semi-quantitative way by Western blotting (Fig. 3C). For this quantification, a large batch of microsomes from *pis1*Δ cells containing wt Pis1-V5-His6 on a plasmid, induced for 30 min, was prepared and identical aliquots thereof were added as “the” reference sample in all SDS-PAGE gels to be used for blots. All extracts and the reference sample were run in duplicate on SDS-PAGE gels. The Western blots were probed with anti-V5-HRP to detect Pis1 and by anti-Kar2. The western blot signals were normalized, first by averaging the duplicate samples, then by normalizing signals for loading by taking the Kar2 signal of the reference sample as the reference. Once the averaged signals of mutant microsomes were corrected for loading errors, the Pis1 signals of mutants were compared to the Pis1 signal of the reference sample and a correction factor calculated. E.g., if the Pis1 signal of a mutant was 80% of the Pis1 signal in the reference sample, that factor was set as 1.25. This correction factor was used to correct the result of the enzyme assay (nmol of Ino

incorporated into lipids/min). These correction factors were always between 1 and 2. Enzyme assays on a given microsomal preparation were done in duplicate, and standard deviations of these duplicate assays are routinely reported.
Thus the reported activities ought to be comparable in the sense that they all correspond to roughly the same but unknown amount of Pis1 protein. Thus, for brevity figures report Pis1 activity as nmol of PI formed per minute and per mg of microsomal protein, but for normalized samples the term nmol Ino/min/mg is slightly incorrect and represents an abbreviation for what more precisely is described as nmol of Ino incorporated per minute per an amount of microsomal protein containing the same amount of Pis1 as 1 mg of our reference sample. As for the empty vector control sample, it was not normalized and added just to show that the 30 min induction leads to a higher than physiological level of activity.

Estimation of detergent:lipid ratios and mol% values of lipid components
We arbitrarily assumed that the protein:lipid ratio (w/w) in washed microsomes is 1. Molecular weights of yeast type phospholipids PC, PE, PI and PS are 760, 718, 854 and 783 Da. Given their relative abundances, we estimate the average MW of phospholipids to be 770 Da. DDM has a molecular mass of 510 Da. Based on these estimates we calculate the assays to contain 130 nmol of membrane lipids, 196 nmol of DDM and from 1.92 to 61.5 nmol CDP-DAG resulting in DDM:lipid ratios of 1.5 – 1 (CDP-DAG calculated as a lipid). Assuming a 1:1 ratio of protein:lipids in membranes enabled us also to calculate the mol% of CDP-DAG in assays. The 4-64 µg CDP-DAG added depending on assay correspond to 1.2 – 16 % of the sum of [nmol of microsomal lipids + nmol of detergent + nmol of CDP-DAG]. The exact mol% of CDP-DAG 18:1 thus were 1.19%, 4.59 and 16.2 mol% which in figures, for the sake of gaining space, were indicated as 1.2, 4.5 and 16 mol%.

Measuring hydrophobicity of PEG-mal
Duplicate samples of 5 - 10 mg of PEG-mal was diluted in 500 µl water, 500 µl of solvent was added and mixed by vortexing. The samples were incubated 30 min at RT, then centrifuged for 5 min at 16'000 x g. The two phases were transferred to new, weighed tubes and dried in a rotary evaporator. The samples from the organic phases were re-suspended in 500 µl of water and dried again, to obtain samples with a similar residual hydration levels. The presence of PEG-mal in each phase was determined by weighing the tubes.

Building of heatmap with R 3.1.0
The derivatized fraction measured from Western blots were exported from a spreadsheet to a text file with tab-separated values. The file was uploaded to R Studio 3.1.0 and the heatmap was build with the following commands:

```
#load pheatmap package; the data frame must be changed to a numeric matrix.
data.frame<-read.table("data.txt", sep= "\t", header=TRUE,row.names=1)
Mat<-as.matrix(data.frame) #to turn it into a matrix
mode(Mat) #to check if the matrix is numeric
pheatmap(Mat,
color = colorRampPalette(c("#fcfeff", "#0452bf"))(20), #(20) to get a 20 steps color scale
kmeans_k = NA, breaks = NA, border_color = "#281d1dff",
cellwidth = 15, cellheight = 15, scale = "none",
cluster_rows = FALSE, cluster_cols = FALSE,
legend = TRUE, legend_breaks = c(0,20,40,60,80,100), legend_labels = c(0,20,40,60,80,100),
annotation = NA, annotation_colors = NA,
annotation_legend = TRUE, drop_levels = FALSE,
show_rownames = TRUE, show_colnames = TRUE, main = NA,
fontsize = 10, display_numbers = TRUE,
number_format = "%.0f",
fontsize_number = 5, filename = NA,
width = NA, height = NA,
lwd = 0.8, lineheight = 0.8)
```

Supplemental Tables S1-S3

Table S1. Yeast strains

Name	Genotype	Reference
BY4742	S288C background *MAT***alpha** *his3Δ1 leu2Δ0 ura3Δ0 MET15 lys2Δ0*	Euroscarf
Y25529 pis1Δ/PIS1	BY4743; *MAT***a**/**alpha** *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 YPR113w::kanMX4/YPR113w*	Euroscarf
FBY4413 pis1Δ.PIS1	*MAT***a** *pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0 MET15 lys2Δ0* containing plasmid 855 (PIS1-V5-H6)	this study
FBY4417 pis1Δ.Pis1ΦC	*MAT***a** *pis1::kanMX his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0* containing plasmid pBF727 (pis1ΦC-V5-H6)	this study
Y8205	S288C background. *MAT***alpha** *can1Δ::STE2pr-Sp_his5 lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0*	(33)
FBY4457 pis1Δ/PIS1*	*MAT***a**/alpha *pis1Δ::kanMX4/PIS1 CAN1/can1Δ::STE2pr-Sp_his5 LYP1/lyp1Δ::STE3pr-LEU2 his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/MET15, lys2Δ0/LYS2*	this study
Y17202 trp1Δ	BY4742 *MAT***alpha** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp1Δ::kanMX4*	Euroscarf
FBY2280	as Y17202 but containing plasmid pBF649 with FLAG-Gpi8	this study
STY50 DTR strain	*MAT***alpha** *his4-401 leu2-3,-112 trp1-1 ura3-52 HOL1-1 SUC2::LEU2*	(36)
FBY7370	STY50 harboring pJK90 with Pis1(1-60), DTR after G60	this study
FBY7371	STY50 harboring pJK90 with Pis1(1-81), DTR after D81	this study
FBY6165	STY50 harboring pJK90 with Pis1(1-137), DTR after S137	this study
FBY6164	STY50 harboring pJK90 with full length Pis1, DTR after Y220	this study
FBY4414	*PIS1 his3Δ1 leu2Δ0 ura3Δ0* containing empty pYES2NT/B	this study
FBY4440 Pis1ΦC-S3C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF739	this study
FBY4443 Pis1ΦC-N40C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF764	this study
FBY4483 Pis1ΦC-T43C pis1Δ	*MAT***a** *pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0 can1Δ::STE2pr-Sp_his* harboring pBF440	this study
FBY4463 Pis1ΦC-K65C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF443	this study
FBY4454 Pis1ΦC-Y66C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF442	this study

FBY4467 Pis1ΦC-N67C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF444	this study
FBY4469 Pis1ΦC-Q68C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF445	this study
FBY4458 Pis1ΦC-V69C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF446	this study
FBY4484 Pis1ΦC-S70C pis1Δ	*MAT***a** *pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0 can1Δ::STE2pr-Sp_his* harboring pBF447	this study
FBY4427 Pis1ΦC-S71C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF734	this study
FBY4421 Pis1ΦC-L72C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF448	this study
FBY4452 Pis1ΦC-S83C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF441	this study
FBY4446 Pis1ΦC-G108C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF766	this study
FBY4485 Pis1ΦC-V132C pis1Δ	*MAT***a** *pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0 can1Δ::STE2pr-Sp_his* harboring pBF449	this study
FBY4502 Pis1ΦC-G133C pis1Δ	*MAT***a** *pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0 can1Δ::STE2pr-Sp_his* harboring pBF454	this study
FBY4492 Pis1ΦC-E134C pis1Δ	*MAT***a** *pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0 can1Δ::STE2pr-Sp_his* harboring pBF450	this study
FBY4493 Pis1ΦC-G135C pis1Δ	*MAT***a** *pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0 can1Δ::STE2pr-Sp_his* harboring pBF451	this study
FBY4486 Pis1ΦC-E136C pis1Δ	*MAT***a** *pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0 can1Δ::STE2pr-Sp_his* harboring pBF452	this study
FBY4435 Pis1ΦC-S137C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF735	this study
FBY4431 Pis1ΦC-S172C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF765	this study
FBY4424 Pis1ΦC-A213C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF728	this study
FBY4478 WT Pis1 & Gpi8	as Y17202 but containing plasmids pBF649, 855	this study
FBY2281 Pis1ΦC & Gpi8	As Y17202 but containing plasmids pBF649, pBF727	this study
FBY2284 Pis1ΦC-S3C & Gpi8	As Y17202 but containing plasmids pBF649, pBF739	this study
FBY2285 Pis1ΦC-N40C & Gpi8	As Y17202 but containing plasmids pBF649, pBF764	this study
FBY2278 Pis1ΦC-T43C & Gpi8	As Y17202 but containing plasmids pBF649, pBF440	this study
FBY4403 Pis1ΦC-K65C & Gpi8	As Y17202 but containing plasmids pBF649, pBF443	this study
FBY4404 Pis1ΦC-Y66C & Gpi8	As Y17202 but containing plasmids pBF649, pBF442	this study

FBY4405 Pis1ΦC-N67C & Gpi8	As Y17202 but containing plasmids pBF649, pBF444	this study
FBY4406 Pis1ΦC-Q68C & Gpi8	As Y17202 but containing plasmids pBF649,, pBF445	this study
FBY4407 Pis1ΦC-V69C & Gpi8	As Y17202 but containing plasmids pBF649, pBF446	this study
FBY4408 Pis1ΦC-S70C & Gpi8	As Y17202 but containing plasmids pBF649, pBF447	this study
FBY2276 Pis1ΦC-S71C & Gpi8	As Y17202 but containing plasmids pBF649, pBF734	this study
FBY4409 Pis1ΦC-L72C & Gpi8	As Y17202 but containing plasmids pBF649, pBF448	this study
FBY2279 Pis1ΦC-S83C & Gpi8	As Y17202 but containing plasmids pBF649, pBF441	this study
FBY2286 Pis1ΦC-G108C & Gpi8	As Y17202 but containing plasmids pBF649, pBF766	this study
FBY4473 Pis1ΦC-V132C & Gpi8	As Y17202 but containing plasmids pBF649, pBF449	this study
FBY4477 Pis1ΦC-G133C & Gpi8	As Y17202 but containing plasmids pBF649, pBF454	this study
FBY4474 Pis1ΦC-E134C & Gpi8	As Y17202 but containing plasmids pBF649, pBF450	this study
FBY4475 Pis1ΦC-G135C & Gpi8	As Y17202 but containing plasmids pBF649, pBF451	this study
FBY4476 Pis1ΦC-E136C & Gpi8	As Y17202 but containing plasmids pBF649, pBF452	this study
FBY2283 Pis1ΦC-S137C & Gpi8	As Y17202 but containing plasmids pBF649, pBF735	this study
FBY2277 Pis1ΦC-S172C & Gpi8	As Y17202 but containing plasmids pBF649, pBF765	this study
FBY2282 Pis1ΦC-A213C & Gpi8	As Y17202 but containing plasmids pBF649 & pBF728	this study
FBY4521 Pis1-C53A & Gpi8	As Y17202 but containing plasmids pBF461 & pBF649	this study
FBY4522 Pis1-C53A pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF461	this study
FBY4503 Pis1-C154A & Gpi8	As Y17202 but harboring pBF458 & pBF649	this study
FBY4505 Pis1-C154A pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF458	this study
FBY4504 Pis1-C100A-C154A & Gpi8	As Y17202 but harboring pBF459 & pBF649	this study
FBY4517 Pis1-C100A-C154A pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF459	this study
FBY4536 Pis1-K & Gpi8	As Y17202 but harboring pBF464 & pBF649	this study
FBY4544 Pis1-K pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring	this study

	pBF464	
FBY4527 Pis1ΦC-K & Gpi8	As Y17202 but harboring pBF463 & pBF649	this study
FBY4529 Pis1ΦC-K pis1Δ	p*is1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF463	this study
FBY4542 Pis1-K-Y66C & Gpi8	As Y17202 but pBF649 harboring pBF467	this study
FBY4566 Pis1-K-Y66C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF467	this study
FBY4537 Pis1-K-Q68C & Gpi8	As Y17202 but harboring pBF466 & pBF649	this study
FBY4549 Pis1-K-Q68C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF466	this study
FBY4543 Pis1-K-V69C & Gpi8	As Y17202 but harboring pBF468 & pBF649	this study
FBY4554 Pis1-K-V69Cpis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF468	this study
FBY4538 Pis1-K-S71C & Gpi8	As Y17202 but harboring pBF465 & pBF649	this study
FBY4547 Pis1-K-S71C pis1Δ	*pis1Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0* harboring pBF465	this study
FBY4501 Pis1-V69S	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 can1Δ::STE2pr-Sp_his* harboring pBF453	this study
FBY4496 Pis1-V69N	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 can1Δ::STE2pr-Sp_his* harboring pBF452	this study
FBY4490 Pis1-G133N-G135S	*MAT***alpha**, *pis1Δ::kanMX, his3Δ1, leu2Δ0, ura3Δ0, lyp1Δ::STE3pr-LEU2* harboring pBF455	this study
FBY4494 Pis1-G135N	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 can1Δ::STE2pr-Sp_his* harboring pBF456	this study
FBY4499 Pis1-E134N-G135E-E136S	*MAT***alpha**, *pis1Δ::kanMX, his3Δ1, leu2Δ0, ura3Δ0, lyp1Δ::STE3pr-LEU2* harboring pBF457	this study

Table S2. Plasmids

Construct	Name	Description
Empty pYES2NT/B		pYES2NT/B vector from Invitrogen (V8252-20) 2µ, *GAL1* promoter, *URA3* and amp^R, C-terminal V5 epitope and poly-His tags.
PIS1-V5-H6	855	*PIS1* with C-terminal V5-H6 tags in pYES2NT/B
pis1ΦC	pBF727	As 855 but all five native Cys of *PIS1* changed to alanine
pis1ΦC-S3C	pBF739	Serine 3 mutagenized to Cys in pBF727
pis1ΦC-N40C	pBF764	Asparagine 40 mutagenized to Cys in pBF727
pis1ΦC-T43C	pBF440	Threonine 43 mutagenized to Cys in pBF727
pis1ΦC-K65C	pBF443	Lysine 65 mutagenized to Cys in pBF727
pis1ΦC-Y66C	pBF442	Tyrosine 66 mutagenized to Cys in pBF727
pis1ΦC-N67C	pBF444	Asparagine 67 mutagenized to Cys in pBF727
pis1ΦC-Q68C	pBF445	Glutamine 68 mutagenized to Cys in pBF727
pis1ΦC-V69C	pBF446	Valine 69 mutagenized to Cys in pBF727
pis1ΦC-S70C	pBF447	Serine 70 mutagenized to Cys in pBF727
pis1ΦC-S71C	pBF734	Serine 71 mutagenized to Cys in pBF727
pis1ΦC-L72C	pBF448	Leucine 72 mutagenized to Cys in pBF727
pis1ΦC-S83C	pBF441	Serine 83 mutagenized to Cys in pBF727
pis1ΦC-G108C	pBF766	Glycine 108 mutagenized to Cys in pBF727
pis1ΦC-V132C	pBF449	Valine 132 mutagenized to Cys in pBF727
pis1ΦC-G133C	pBF454	Glycine 133 mutagenized to Cys in pBF727
pis1ΦC-E134C	pBF450	Glutamic acid 134 mutagenized to Cys in pBF727
pis1ΦC-G135C	pBF451	Glycine135 mutagenized to Cys in pBF727
pis1ΦC-E136C	pBF452	Glutamic acid 136 mutagenized to Cys in pBF727
pis1ΦC-S137C	pBF735	Serine 137 mutagenized to Cys in pBF727
pis1ΦC-S172C	pBF765	Serine172 mutagenized to Cys in pBF727
pis1ΦC-A213C	pBF728	Alanine 213 mutagenized to Cys in pBF727
YCPlac22		CEN4, *TRP1* and amp^R
2xFlag-Gpi8	pBF649	*GPI8* with two N-terminal FLAG tags (DYKDDDDK) under its endogenous promoter in YCPlac22
pJK90		*TPI*$_{UAS}$-HA-*SUC2-HIS4C*, 2µ, *URA3* (34)
PIS1-C53A	pBF461	C53A in 855
PIS1-C154A	pBF458	C154A in 855
PIS1-C100A-C154A	pBF459	C100A and C154A in 855
PIS1-K	pBF464	WT Pis1 with natural ER retention signal KNKTY added at the end, after V5-His6 tag of Pis1-V5-H6
pis1ΦC-K	pBF463	Cys-free Pis1 with natural ER retention signal KNKTY added at the end, after V5-His6 tag pYES2NT/B
PIS1-K-Y66C	pBF467	Y66C in Pis1-K, construct having a total of 6 Cys
PIS1-K-Q68C	pBF466	Q68C in PIS1-K, construct having a total of 6 Cys
PIS1-K-V69C	pBF468	V69C in PIS1-K, construct having a total of 6 Cys
PIS1-K-S71C	pBF465	S71C in PIS1-K, construct having a total of 6 Cys
PIS1-V69S	pBF453	V69S in PIS1-V5-H6
PIS1-V69N	pBF452	V69N PIS1-V5-H6
PIS1-G133N-G135S	pBF455	G133N and G135S mutations in PIS1-V5-H6
PIS1-G135N	pBF456	G135N in PIS1-V5-H6
PIS1-134-6/NES	pBF457	E134N, G135E and E136S mutations in PIS1-V5-H6

Table S3. Primers

Underlined: restriction sites; bold: mutation; s: sense; a: antisense.

No°	**name (aim)**	**5' → 3' sequence**
1082	PIS1.F1 (YPR113W cloning, incl. HindIII)	AAAAGCTTATGAGTTCGAATTCAACACC
1083	PIS1.R1 (YPR113W cloning, incl. XbaI, no stop codon)	AATCTAGAGTAAGTCTTGTTCTTCTCG
1084	PIS1 C53A (substitution of native Cys 53)	CATGGTTGTATAGTACATCA**GCA**CTACTGGATGCG CTAGACGG
1085	PIS1 C90A C93A C100A (substitution of native Cys 90, 93, 100)	TGGCTTGATG**GCA**TTCCTT**GCA**GTGCAGTATCCCC AATGG**GCA**GTTTTCTTCC
1086	PIS1 C154A (substitution of native Cys 154)	GAGACGTACTGTTCACTATC**GCA**GCGTTTAACGAA CTATTTTA
1161	PIS1conf.F (269bp upstream of YPR113W ORF)	AAGTACCGATGAGATGAGATG
1162	PIS1conf.R1 (319bp downstream of YPR113W stop codon)	TTGAGATAGAGGAGAGGTCTTC
858	kanMX4ver.F2 (100 to 118 of KanMX4 cassette, in TEF promoter)	CGAGGGGAAATTAATAGGTTGT
482	kanMX-Ver.R1 (376bp upstream of kanMX4 stop codon)	CAAGGAGGGTATTCTGGGC
1087	PIS1 S3C (substitution to Cys)	AATATTAAGCTTATGAGT**TGT**AATTCAACACCAG AAAAGGTTAC
1354	PIS1 N40C (substitution to Cys)	CTTTCTTTTTTCGTTATGAAG**TGT**CATCCTACGGC CTTTACATG
1394 (s)	PIS1 T43C (substitution to Cys)	GCCCTTTCTTTTTTCGTTATGAAGAATCATCCT**TG C**GCCTTTACATGGTTGTATAG
1395 (a)		CTATACAACCATGTAAAGGCGCAAGGATGATTCTT CATAACGAAAAAAGAAAGGGC
1419 (s)	PIS1 K65C (substitution to Cys)	CTAGACGGAACCATGGCAAGA**TGC**TACAATCAGGT TTCCAGTCTG
1420 (a)		CAGACTGGAAACCTGATTGTAGCATCTTGCCATGG TTCCGTCTAG
1423 (s)	PIS1 Y66C (substitution to Cys)	GAACCATGGCAAGAAAG**TGC**AATCAGGTTTCCAGT CTG
1424 (a)		CAGACTGGAAACCTGATTGCACTTTCTTGCCATGG TTC
1421 (s)	PIS1 N67C (substitution to Cys)	AACCATGGCAAGAAAGTAC**TGT**CAGGTTTCCAGTC TGGGTG
1422 (a)		CACCCAGACTGGAAACCTGACAGTACTTTCTTGCC ATGGTT
1425 (s)	PIS1 Q68C (substitution to Cys)	CCATGGCAAGAAAGTACAAT**TGC**GTTTCCAGTCTG GGTGCCG
1426 (a)		CGGCACCCAGACTGGAAACGCAATTGTACTTTCTT GCCATGG
1427 (s)	PIS1 V69C (substitution to Cys)	GGCAAGAAAGTACAATCAG**TGT**TCCAGTCTGGGT GCCGTTC
1428 (a)		GAACGGCACCCAGACTGGAACACTGATTGTACTTT CTTGCC
1431 (s)	PIS1 S70C (substitution to Cys)	AAAGTACAATCAGGTT**TGC**AGTCTGGGTGCCGTTC
1432 (a)		GAACGGCACCCAGACTGCAAACCTGATTGTACTTT
1088	PIS1 S71C (substitution to Cys)	AAAGTACAATCAGGTTTCC**TGT**CTGGGTGCCGTTC TGGACATGG

1429 (s) 1430 (a)	PIS1 L72C (substitution to Cys)	GCAAGAAAGTACAATCAGGTTTCCAGT**TGC**GGTGC CGTTCTGGAC GTCCAGAACGGCACCGCAACTGGAAACCTGATTGT ACTTTCTTGC
1396 (s) 1397 (a)	PIS1 S83C (substitution to Cys)	GGACATGGTTACCGACAGA**TGC**AGTACCGCTG CAGCGGTACTGCATCTGTCGGTAACCATGTCC
1356	PIS1 G108C (substitution to Cys)	TTCTTCCAATTAATGCTG**TGC**TTGGATATTACTAG TCACTAC
1532	PIS1 V132C (substitution to Cys)	GTGCTGGTAAGACTTCTCATAAAAGT**TGC**GGCGAG GGTGAGTCC
1533	PIS1 G133C (substitution to Cys)	GACTTCTCATAAAAGTGTG**TGC**GAGGGTGAGTCCA G
1534	PIS1 E134C (substitution to Cys)	CTTCTCATAAAAGTGTGGGC**TGC**GGTGAGTCCAGA TTGTTACAC
1535	PIS1 G135C (substitution to Cys)	CATAAAAGTGTGGGCGAG**TGT**GAGTCCAGATTGT TAC
1536	PIS1 E136C (substitution to Cys)	CTTCTCATAAAAGTGTGGGCGAGGGT**TGC**TCCAGA TTGTTACACCTG
1089	PIS1 S137C (substitution to Cys)	AAAGTGTGGGCGAGGGTGAG**TGT**AGATTGTTACA CCTGTACTAC
1355	PIS1 S172C (substitution to Cys)	ACTTGCAGTTGTTCTCAAAT**TGT**GCAACCTTTGGT AAATGG
1090	PIS1 A213C (substitution to Cys)	AGACAACGATGCCAAGAAT**TGT**AACGAGAAGAAC AAGACTTAC
–	pYES420-441F (sequencing primer)	CCTCTATACTTTAACGTCAAGG
68 69 70	MATout (mating type determination) MATa (mating type determination) MATalpha (mating type determination)	AGTCACATCAAGATCGTTTATGG GCACGGAATATGGGACTACTTCG ACTCCACTTCAAGTAAGAGTTTG
914	Pis1 forward	5'-AGG TGG TTT GTT ACG CAT GCA AGC TTG ATA TCG AAa tga gtt cga att caa cac cag-3'
915	Pis1-D81-DTR	5'-GAT GGT CTA GAG GTG TAA CCA CTT GAG TTC TTA GGg tcg gta acc atg tcc aga acg-3'
916	Pis1-G60-DTR	5'-GAT GGT CTA GAG GTG TAA CCA CTT GAG TTC TTA GGt ccg tct agc gca tcc agt aga c-3'
1527	PIS1_V69S_a, to introduce a glycosylation site in PIS1, in NQVSS segment -> NQsSS	aacggcacccagactggaactctgattgtactttcttgcc
1528	PIS1_V69N, to introduce a glycosylation site in PIS1, in NQVSS segment -> NQnSS	ggcaagaaagtacaatcagaattccagtctgggtgccgttc
1529	PIS1_G133N_G135S, to introduce a glycosylation site in PIS1, in VGEGES sequence -> VnEsES	cataaaagtgtgggcgagtgtgagtccagattgttac
1530	PIS1_G135N, to introduce a glycosylation site in PIS1, in VGEGES sequence-> VGEnES,	ctcataaaagtgtgggcgagaatgagtccagattgttacacc
1531	E134Nes_a, to introduce a glycosylation site in PIS1, in VGEGES sequence-> VGnesS,	gctggtaagacttctcataaaagtgtgggcaatgagtcgtccagatt gttacacctgtactacacgag
1644	F1-PIS1extract, to amplify PIS1 from the pYES vector	GGGAATATTAAGCTTatgagttcgaattc
1651	PIS1-corr-R,, to add the KXKXX at the end, after the tag, and amplify the PIS1 constructs all in one.	CTAATTACATGATGCGGCCCTCTAGGATCAGCGGG TTTAAACTCAgtaagtcttgttcttATGG

1671	Pis1_vector.R2 to amplify the beginning of PIS1, up to C53	ctggatgcgctagacggaac
1668	Pis1_loop.F1, to amplify the CAPT loop, 77bp	ctggatgcgctagacggaac
1656	PIS1 R1-loop , to amplify the CAPT loop, after C53, before the KXKXX signal	GTAACCATGTCCAGAACGGC
1657	PIS1 F2-plasmid, to amplify the end of PIS1, after the CAPT loop	GCCGTTCTGGACATGGTTAC
1654	PIS1-corr-R2, to amplify the end of PIS1 and add the KXKXX signal at the end	GCGGGTTTAAACTCAgtaagtcttgttcttATGGTGATGGTGATGATGACC

References in supplemental material

33. Baryshnikova, A., Costanzo, M., Dixon, S., Vizeacoumar, F. J., Myers, C. L., Andrews, B. and Boone, C. (2010) Methods Enzymol 470, 145-179.
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35. Ursitti, J. A., Mozdzanowski, J. and Speicher, D. W. (2001) Curr Protoc Protein Sci Chapter 10, Unit 10.7.
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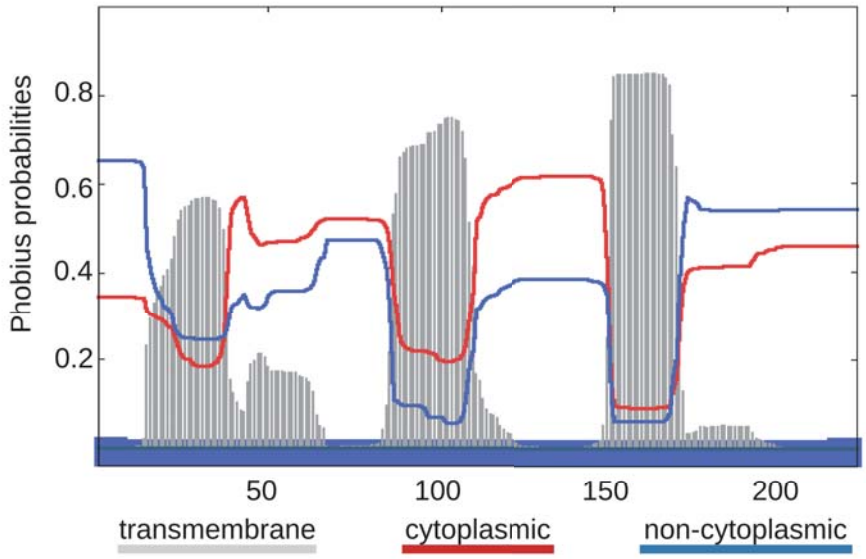


Figure S1. Topology prediction for Pis1 by Phobius (http://phobius.sbc.su.se). Positional probabilities for TMs are represented by grey vertical lines. Only the region between 150 and 170, which corresponds to TM5, has a high probability of being a TM, with a concomitant low probability for being a cytosolic (red) or luminal (blue) loop. In Phoebius, the TOPCONS TMs 2, 4 and 6 appear as being part of loops rather than as TMs. The N-terminus is considered to be rather luminal. The orientation of the CAPT region (between amino acids 56 - 81) and the C-terminus are not clearly predicted.

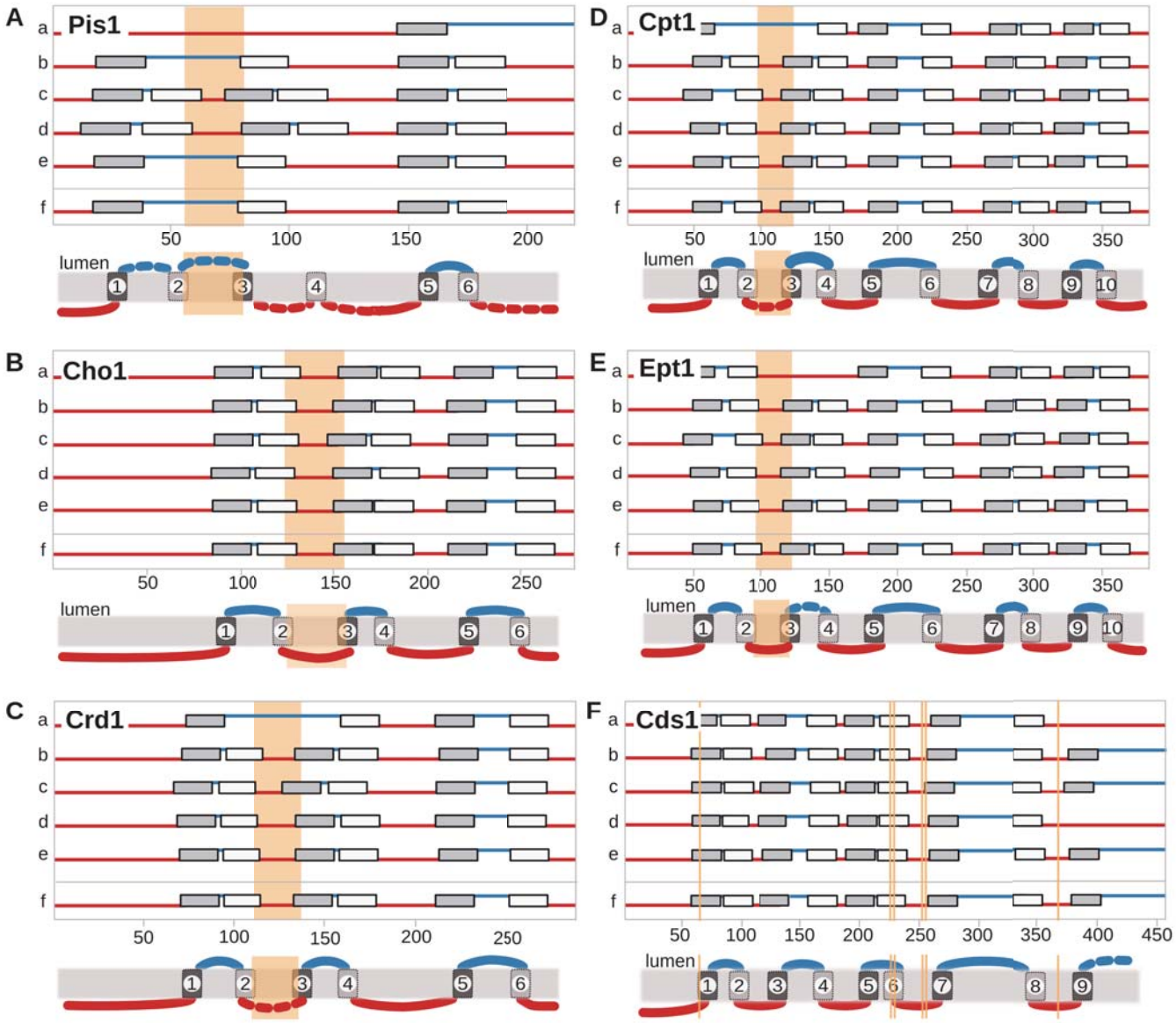


Figure S2. TOPCONS predictions for the pfam01066 members using CDP-DAG as a substrate (A – C), for Cpt1 (D) and Ept1 (E), which use CDP-activated choline and ethanolamine, and Cds1 (F), the CDP-DAG synthase, which does not have a CAPT motif, respectively. Schematic representations of final topology are shown below each TOPCONS prediction. Red: cytosolic; blue: luminal; grey and white boxes: TMs. Lines **a – e** represent topology predicted by SCAMPI-seq, SCAMPI-msa, PRODIV, PRO and OCTOPUS algorithms. Line **f** represents final TOPCONS prediction taking into account a – e plus two further algorithms. Orange rectangles contain the CAPT motif. Strongly conserved residues of Cds1 are dispersed over the entire protein (orange lines). In the schemes at the bottom of each panel, continuous lines indicate unanimous predictions, dashed lines contradictory predictions. Note that CAPT motifs usually encompass the end and the beginning of two vicinal predicted TMs.

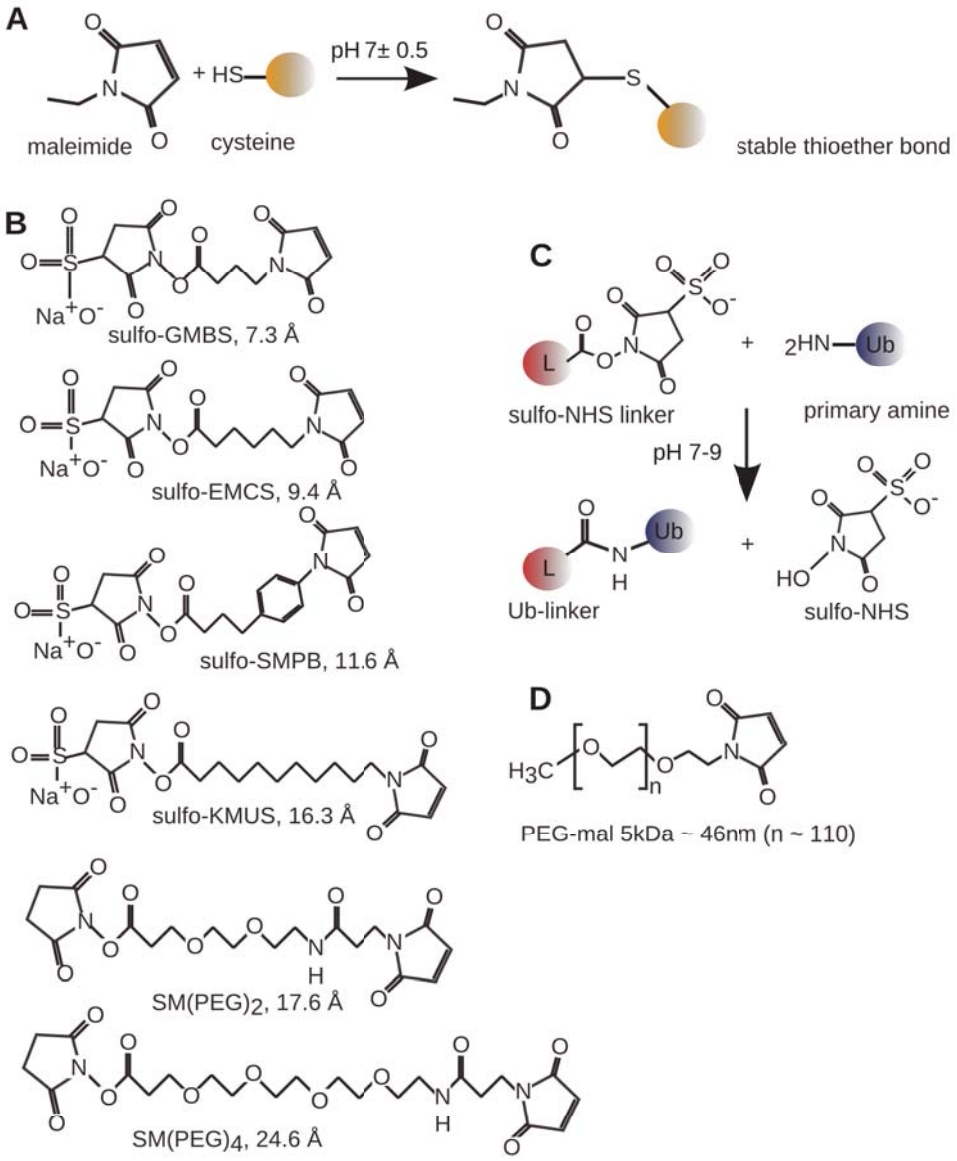


Figure S3. Cysteine reaction with maleimide. A, maleimide reacts with thiols at pH 6.5-7.5 to form a stable thioether bond. In this pH range maleimides, when added to microsomes, react specifically with the thiol group of Cys of proteins. **B**, heterobifunctional Cys and Lys specific crosslinkers with spacers of various lengths ranging from 7.3 Å to 24.6 Å were utilized. **C**, conjugation of crosslinkers to ubiquitin. The crosslinker (L) was conjugated to ubiquitin (Ub) by the N-hydroxysuccinimide ester group (NHS-ester), since ubiquitin does not contain any Cys residues. Sulfonated groups increase the solubility of linkers without interfering with the reaction. **D**, structure of PEG-mal (methoxypoly(ethyleneglycol)maleimide$_{5000}$). PEG-mal is a mixture of variable number of ethyleneglycol units. The lot used had an average of 5 kDa, with a polydispersity of 1.4 (Sigma).

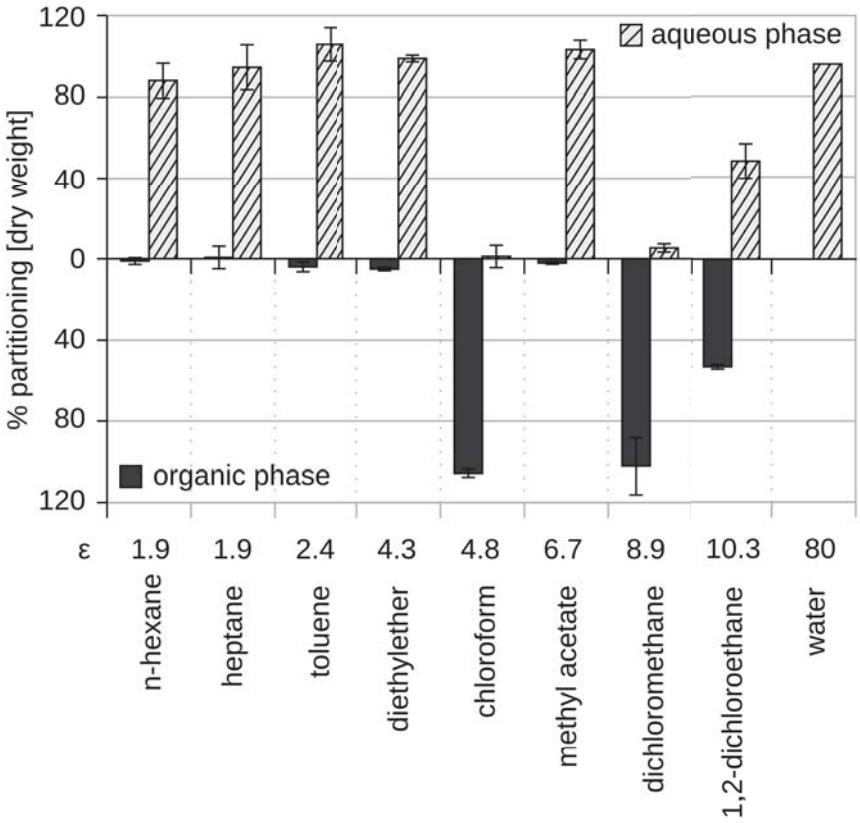


Figure S4. Partitioning of PEG-mal in various water - organic solvent (1:1) systems.
Percentage of PEG-mal recovered after phase separation in aqueous phase (top, hatched) and various organic phase (bottom, black) for solvents with dielectric constants from 1.9 to 10.3 determined as described in supplemental materials and methods. Total recovery of PEG-mal at the end was > 85% of initial amount.

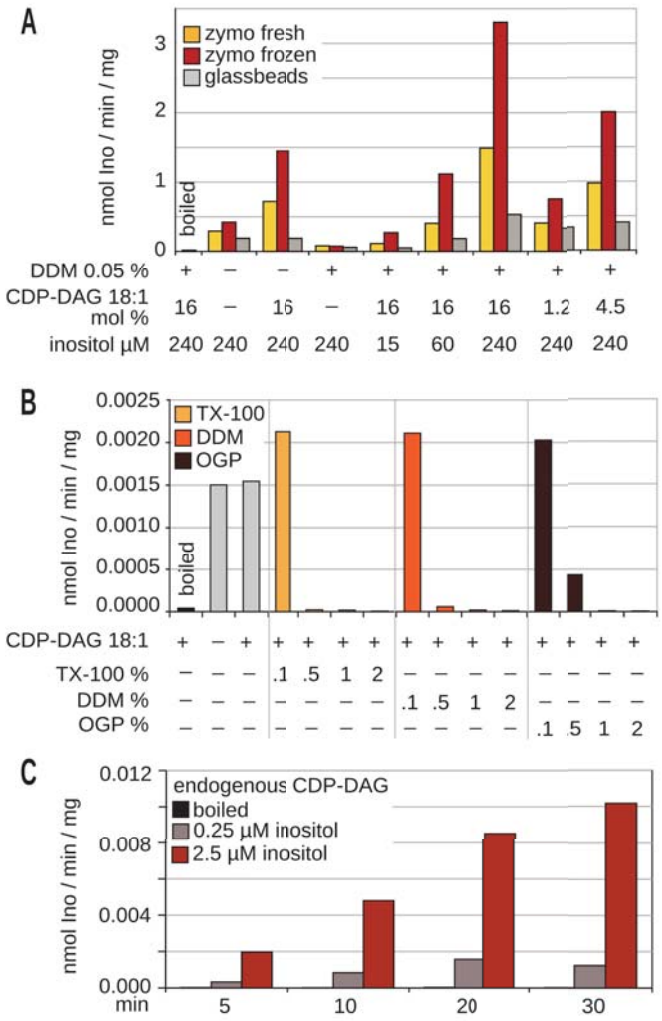


Figure S5. Preliminary PI synthase *in vitro* assays. A, enzymatic activity of *pis1*Δ + Pis1 microsomes, prepared from spheroplasts and used immediately (yellow) or after one freeze/thaw cycle (red), or obtained by glass bead disruption of cells (grey) with various substrate concentrations. The reactions were run at RT for 25 min. **B**, activity of microsomes freshly prepared from spheroplasts in presence of Triton X-100 (TX-100), DDM or octyl-glucopuranoside (OGP) (0.1 and 2%), with 0.6 mol% CDP-DAG 18:1 (10 µM) and 1 µCi of [^{3}H]Ino (0.25 µM). The reactions were run at RT for 25 min. **C**, activity of microsomes prepared as in B, with increasing Ino concentration and only endogenous CDP-DAG in 0.05% DDM. The reactions were run at RT, for 5, 10, 20 or 30 min.

Interpretation of data in panel B: Taking into account the molar amounts CDP-DAG, the variable amounts of detergent and of membrane lipids (assuming the lipid:protein ratio in microsomal membranes to be 1:1), we calculate the mol% of CDP-DAG in the assays with 0.1 and 0.5 % of DDM to be 0.382 mol% and 0.18 mol%, respectively. Thus, surface dilution of the CDP-DAG substrate should not reduce activity at 0.5% DDM to less than 47% of the activity at 0.1% DDM. Yet, going from 0.1 to 0.5 % DDM or Triton X-100 totally abolishes activity. Thus, we feel that the data clearly indicate that detergent changes the structure of Pis1 or acts as an inhibitor.

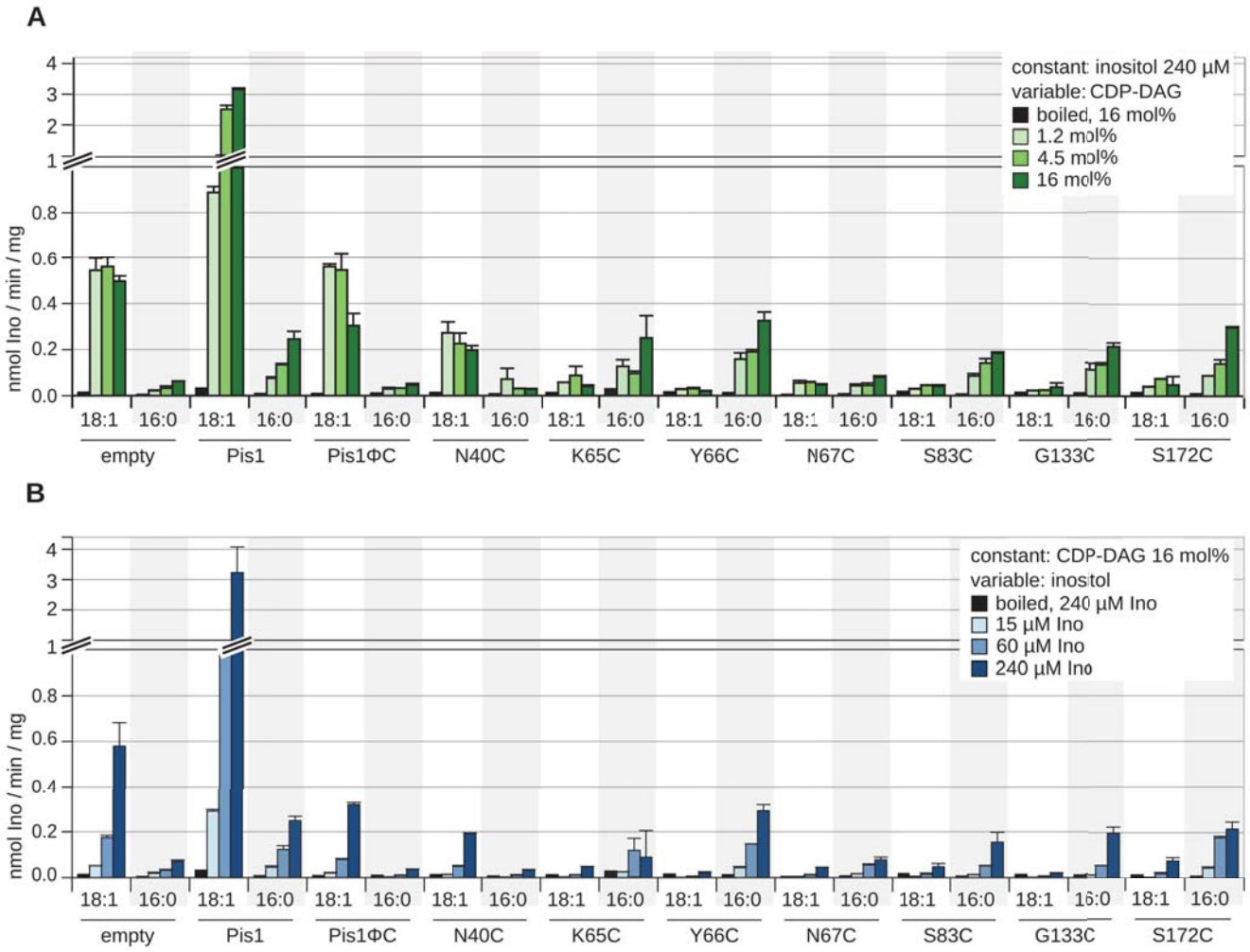


Figure S6. CDP-DAG 18:1 vs. CDP-DAG 16:0. Microsomal activity of wt cells (+ empty vector), *pis1*Δ + Pis1, *pis1*Δ + Pis1ΦC and Pis1ΦC-variants in presence of CDP-DAG 18:1 or CDP-DAG 16:0 as indicated at the bottom. **A**, assays run at constant Ino concentration (240 µM) and **B**, at constant CDP-DAG concentration (16 mol% = 307 µM). Black bars: boiled controls, containing the highest concentrations of both substrates.

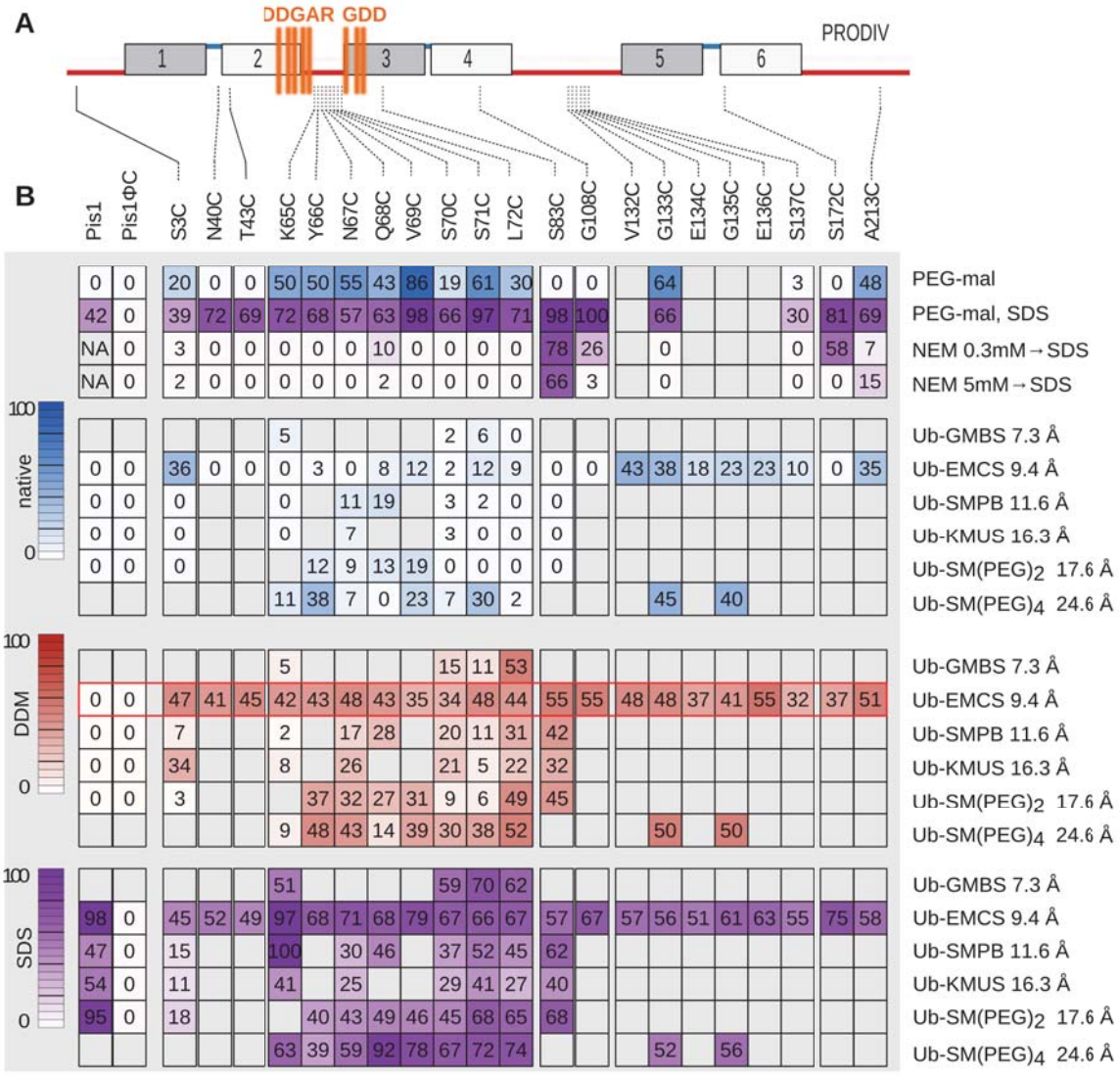
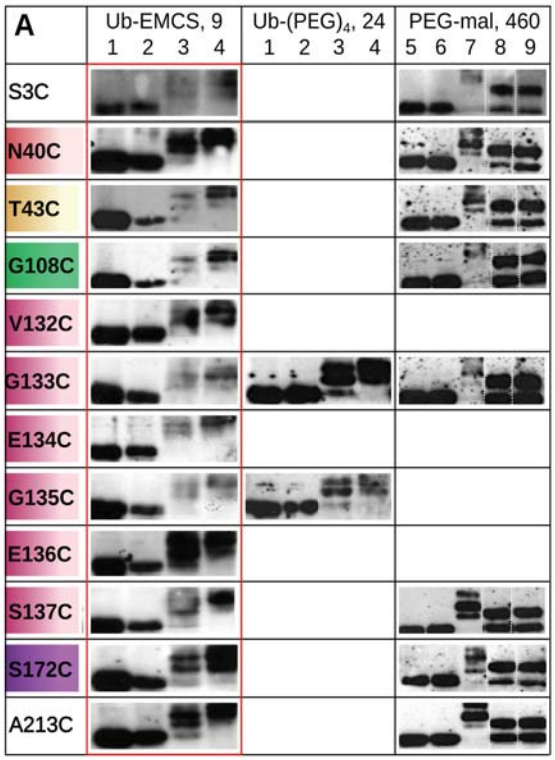


Figure S7. Quantification of SCAMs of Pis1ΦC variants. A, TOPCONS PRODIV topology prediction for Pis1 as in Fig. 1C. Absolutely conserved residues are represented by orange lines and dashed lines link relative positions in the sequence to the substituted residues in the Pis1ΦC variants. **B,** degree of derivatization in % of different alleles in different conditions. Top panel: PEG-mal/NEM method. Lower Panels, Ub-mal/DDM method. The squares color shaded in blue represent the derivatizations in absence of detergent, squares shaded in red show derivatizations in presence of DDM, those shaded in purple derivatizations in presence of SDS (1%). Grey cells: not tested or result discarded because microsomes were not tight. The experiments, in which 1% instead of 0.05% DDM was used are framed in red. Percentages were calculated according to ($Pis1_{deriv.}$ / [$Pis1_{deriv.}$ + $Pis1_{non\text{-}deriv.}$]) x 100 (see materials and methods) counting as derivatized only monosubstituted forms of Pis1, except for the sample of wt Pis1 derivatized in SDS: there, all derivatized forms were counted. NA, not applicable.


A
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1 2 3 4
Ub-(PEG)4, 24
1 2 3 4
PEG-mal, 460
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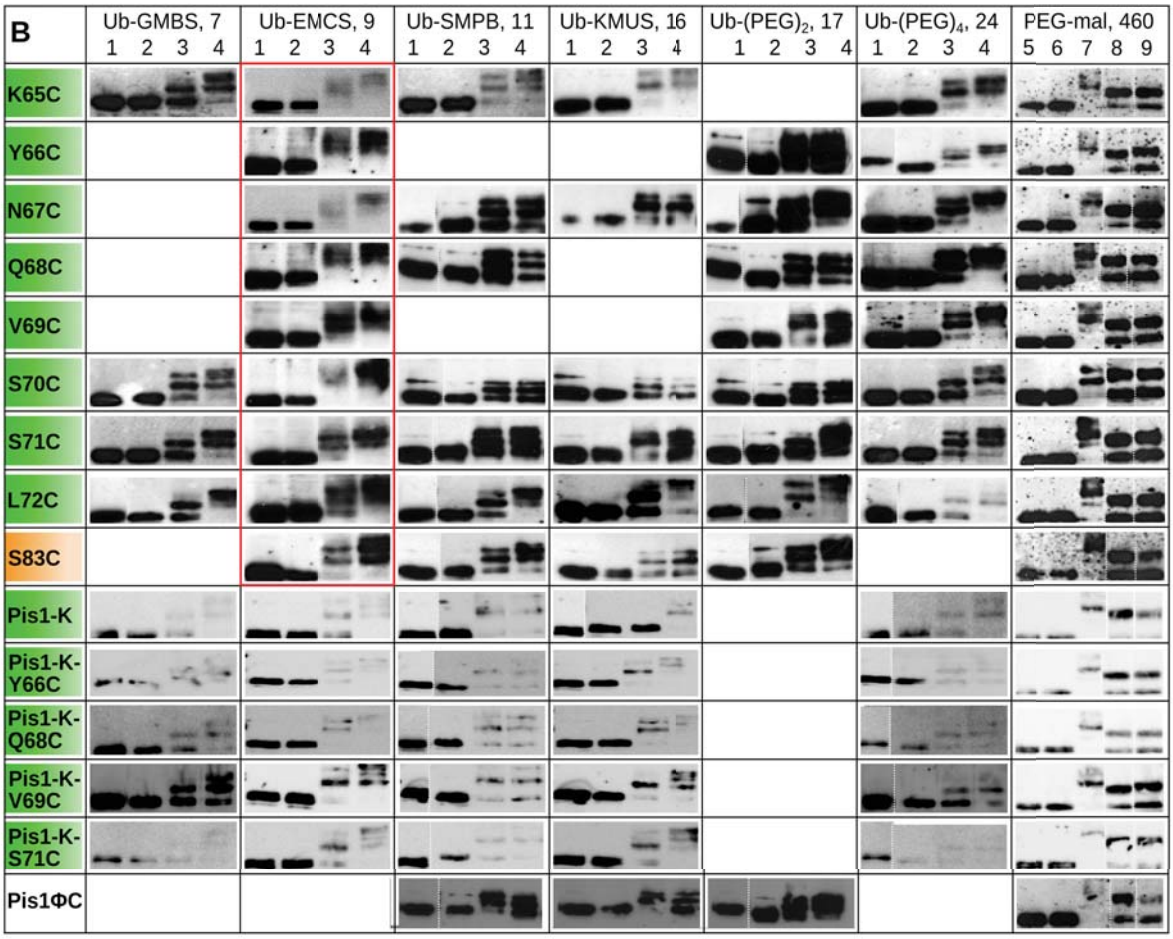
G133C
E134C
G135C
E136C
S137C
S172C
A213C


B
Ub-GMBS, 7
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Ub-EMCS, 9
1 2 3 4
Ub-SMPB, 11
1 2 3 4
Ub-KMUS, 16
1 2 3 4
Ub-(PEG)2, 17
1 2 3 4
Ub-(PEG)4, 24
1 2 3 4
PEG-mal, 460
5 6 7 8 9
K65C
Y66C
N67C
Q68C
V69C
S70C
S71C
L72C
S83C
Pis1-K
Pis1-K-Y66C
Pis1-K-Q68C
Pis1-K-V69C
Pis1-K-S71C
Pis1ΦC

Figure S8. Western blots of Ub-mal/DDM and PEG-mal/NEM SCAMs for Gpi8. Gpi8 control Western blots of the SCAMs assays presented in Figure 4. From left to right: smallest linker (GMBS, 7 Å) to the longest (PEG-mal, 460 Å), with the linker length given in Å next to the name of the linker. Color code is the one used in Fig. 4. Ub-mal/DDM method: Lanes 1: control, lanes 2: Ub-mal, lanes 3: Ub-mal/DDM, lanes 4: Ub-mal/SDS. PEG-mal/NEM method: Lanes 5: control, lanes 6: PEG-mal, lanes 7: PEG-mal in presence of SDS, lanes 8: 0.3 mM NEM, then PEG-mal/SDS, lanes 9: 5 mM NEM, then PEG-mal/SDS. In these last two lines, the microsomes were first derivatized by NEM (0.3 mM and 5 mM) without detergent to block surface Cys; subsequent treatment with PEG-mal in SDS derivatized buried Cys. Cropping of plots of Pis1-K and its variants removed the principle species, which contained 5 or 6 PEG-mals. **A**, FLAG-Gpi8 Western blots for the Pis1ΦC variants having a Cys substitution outside of the CAPT motif. **B**, FLAG-Gpi8 Western blots for the Pis1ΦC and Pis1-K variants having a Cys substitution within the CAPT motif.

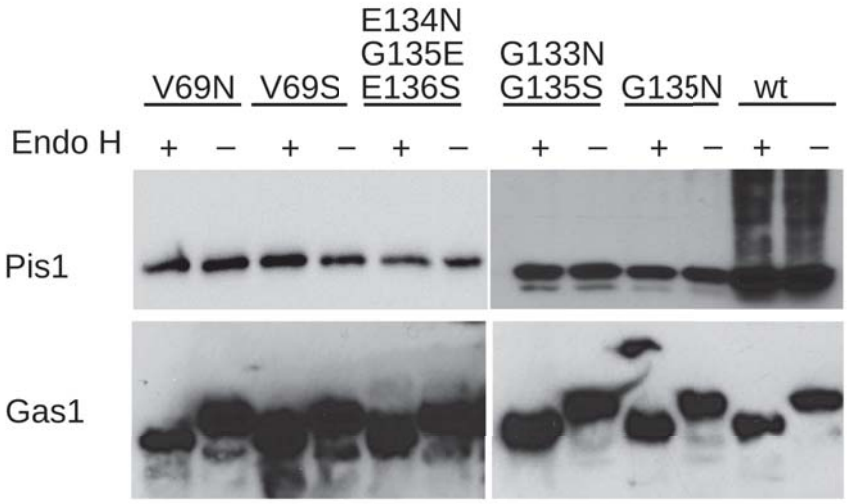
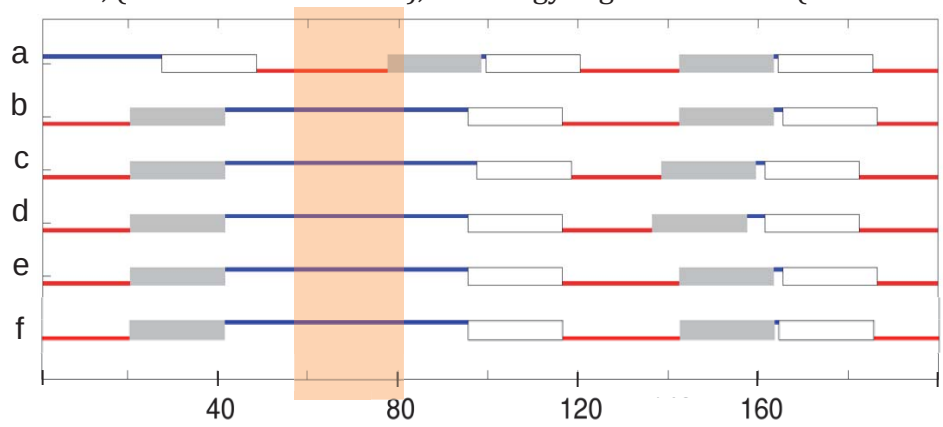


Figure S9. N-glycosylation sites. Microsomes of cells harboring Pis1 constructs with N-glycosylation sites introduced by site-directed mutagenesis in non-conserved residues were solubilized in SDS and treated with endoglycosidase H (endo H, +) or with endo H buffer (-). Western blots were probed with anti-V5-HRP (top) and anti-Gas1 antibodies (bottom). The constructs as well as the wt Pis1 (last two lanes) were not glycosylated and insensitive to endo H treatment, contrary to Gas1, which is a well known N-glycosylated GPI protein.

AfDIPPS, (PDB structure 4MND), homology region with Pis1 (amino acids 270-469)



Af2299, (PDB structure 4O6N), homology region with Pis1 (amino acids 196-372)

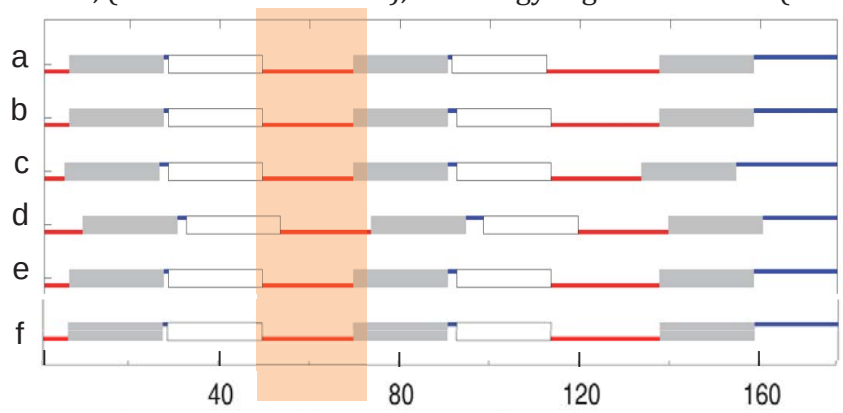


Figure S10. TOPCONS predictions for archeal CAPTs. The homology regions of the two crystallized *A.fulgidus* CAPTs (gi|661918374 and gi|635575527) with Pis1 were analyzed by TOPCONS. Annotation as in Fig. 1C. The integrated final predictions are shown in line f. The same topology is obtained when the entire sequences including the N-terminal hydrophilic regions are included in the TOPCONS query (not shown).