Supporting information for

Topology of the microsomal glycerol-3-phosphate acyltransferase Gpt2p/Gat1p of *Saccharomyces cerevisiae*

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Supplemental experimental procedures

Reagents. Zymolyase 20T was from Seikagaku Corporation (Tokyo, Japan), n-dodecyl-ß-D-maltoside, EDTA-free protease inhibitor cocktail and Pefabloc SC (AEBSF) from ROCHE (no. 11873580001 and 11429876001), anti-V5-HRP antibody from INVITROGEN, anti-HA from BabCO, anti-VSVG and anti-FLAG from SIGMA, anti-Kar2p from Santa Cruz Biotech. HRP-labeled antibodies were visualized by using the UptiLight HRP blot substrate (UPTIMA, France). Ubiquitin (from bovine erythrocytes), L-histidinol, picrylsulfonic acid (TNBS), palmitoyl coenzyme A lithium salt, (C16-CoA, P9716) and Agarose-poly-L-lysine were purchased from SIGMA, sulfo-EMCS (6-maleimidocaproic acid sulfo-n-succinimidyl ester) and sulfo-GMBS (N-[γ-maleimidobutyryloxy]-sulfosuccinimide ester) from Toronto Research Chemicals and Vivaspin 500 (MWCO 3 kDa) from GE Healthcare. [^{14}C]-L-glycerol-3-phosphate disodium salt (ARC 0316) was obtained from ANAWA Trading SA, Wangen, Switzerland.

Bioinformatics tools. Conserved amino acids and motifs in acyltransferase domains were obtained using http://pfam.sanger.ac.uk/family/PF01553#tabview=tab2) and http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml (Marchler-Bauer et al., 2011). Topology predictions were obtained from TOPCONS (http://topcons.net/index.php?about=octopus) and TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Calculations of ΔGs_{mi} for TMs were obtained from the ΔG predictor at http://dgpred.cbr.su.se/index.php?p=TMpred (full protein scan using default settings, *i.e*. length correction turned on, allowing for TMs from 19 to 23 amino acids in length). If the full protein scan option did not show any potential TM, we used the option "ΔG prediction" with options "length correction" and "allow subsequences" turned on. Surface exposure of amino acids was predicted using NetSurfP (http://www.cbs.dtu.dk/services/NetSurfP).

Plasmid construction. Plasmid pBF720 carrying Gpt2p-V5-His_6 (having a N719D mutation) was generated by PCR amplification of *GPT2* using genomic DNA from BY4742 as a template and in frame ligation into pYES2NT/B (INVITROGEN) containing a C-terminal V5-His_6 tag. Correction of the N719D mutation by the QuikChange multi site method (STRATAGENE) yielded pBF733. All cysteine-mutated alleles of Gpt2p-V5-His_6 were generated by the QuikChange multi site method (STRATAGENE) using pBF733 as a template with primers indicated in Table SII. For the construction of epitope tagged alleles of *GPT2* we started from plasmid pBF720. A BamHI site was inserted after amino acid 235 of *GPT2* using crossed PCR. For this, *GPT2* fragments 1 – 235 and 236 -743 were generated with PCR using primers 966 + 969 or 968 + 967 (Table SII), respectively, containing an additional BamHI site after amino acid 235. The two PCR fragments were cut with HindIII/BamHI or BamHI/NotI and ligated into the pYES2/NT vector opened with HindIII and NotI, pretreated with calf intestinal alkaline phosphatase. This triple ligation yielded a *GPT2* version with a V5-epitope and a His_6 tag at the C terminus and a BamHI site after amino acid 235. A double stranded HA-epitope and a double stranded VSVG-epitope, flanked by BamHI sites on each side were obtained through separate oligonucleotide synthesis of both strands, mixing equimolar amounts, heating to 94 °C and letting cool down. The vector and double stranded oligonucleotides were digested with BamHI; the vector was digested with shrimp alkaline phosphatase, mixed with 2-6-fold molar excess of digested double-stranded oligonucleotides and ligated. This yielded Gpt2p-235HA-V5-His_6 and Gpt2p-235VSVG-V5-His_6 (pBF223 and pBF225) having the respective epitopes flanked by Gly-Ser on each end, inserted between amino acids 235 and 236. The same strategy was used to insert tags after amino acid 412 of *GPT2* (pBF227 and pBF228), using primers 966 + 971 and 970 + 967 (Table SII). For all cysteine mutated alleles and tag insertion alleles, the entire reading frame was verified by sequencing; for DTR constructs the inserted N-terminal fragments of *GPT2* or *SCT1* were sequenced and errors were corrected using the QuikChange multi site method (STRATGENE) except for a conservative I53V mutation in Gpt2p-P467-DTR and a V248A mutation in Sct1p-P459-DTR. Sequencing was done by MICROSYNTH, Balgach, Switzerland.

Preparation of microsomes. Cells were grown to an A_{600} of 3-6. Cells were washed with ice-cold 10 mM NaN_3, resuspended in zymolyase buffer (10 mM NaN_3, 1.4 M sorbitol, 50 mM potassium phosphate, pH 7.5, 40 mM β-mercaptoethanol, 1 mg ml^{-1} zymolyase 20T) and incubated for 1-2 h at 30 °C without shaking. Spheroplasts were collected by centrifugation at 800 × *g* for 5 min at 4 °C and washed with one volume of zymolyase buffer lacking β-mercaptoethanol and zymolyase. For experiments using PEG-mal or UBI-mal, NaN_3 and β-mercaptoethanol were omitted. Spheroplasts were lysed in buffer A (0.2 M sorbitol, 5 mM $MgCl_2$, 0.1 M potassium phosphate, pH 7.4) supplemented with 2 mM EDTA, 10 μg ml^{-1}

leupeptin, 2 μg ml^{-1} aprotinin, 5 μg ml^{-1} pepstatin A, 2 mM PMSF, 2 mM AEBSF, 1 x EDTA-free Roche protease inhibitor cocktail, and 50 μg ml^{-1} DNAse by vortexing for 1 min and forcing them 10 times through a 0.4 mm gauge needle. After centrifugation at 800 × *g* for 5 min at 4 °C, the supernatant was filtered through Millipore Millex AP-20, then centrifuged at 16,000 × *g* for 30 min at 4 °C to pellet the microsomes. The supernatant was discarded; microsomes were resuspended in small volumes of buffer A.

Synthesis of Ubiquitin-EMCS and Ubiquitin-GMBS (UBI-mal). Ubiquitin is a 76 amino acids long (8.5-kDa) protein, which does not contain any cysteine but 7 lysine residues. It was conjugated with the heterobifunctional crosslinkers sulfo-EMCS or sulfo-GMBS, each carrying two different groups, a maleimide and a N-hydroxysuccinimide (NHS) ester reacting with sulfhydryl and primary amino groups, respectively. This yielded a highly water-soluble, entirely membrane-impermeable cysteine labeling reagent, ubiquitin-maleimide (UBI-mal). Sulfo-EMCS and sulfo-GMBS were stored desiccated at -20°C. Vivaspin 500 (MWCO 3 kDa) columns and were washed with buffer E (50 mM NaH_2PO_4/Na_2HPO_4, pH 7.2) prior to use. 25 mg (= 2.92 μmol) of ubiquitin were dissolved in a final volume of 760 μl buffer E and transferred to the reaction tube containing 2.4 mg (= 5.85 μmol) Sulfo-EMCS dissolved in 870 μl buffer E. The reaction mixture was incubated for 30 min at room temperature on a wheel. Excess (non-reacted) cross-linker was removed from the mixture by concentrating the solution in a Vivaspin 500 column, washing twice with buffer E, then concentrating to a volume of 849 μl. 131 μl 1.5 M sorbitol (0.2 M final) were added, and the resulting 3 mM ubiquitin-EMCS solution was either used immediately for the cysteine labeling experiments or stored as frozen aliquots at -20°C. GMBS was coupled in the same way but using buffer F (250 mM KCl, 10 mM NaCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) and uncoupled GMBS was removed at the end by adding a 10 fold molar excess of lysine in the form of prewashed Agarose-poly-L-lysine and further incubation for 15' at room temperature. Agarose-poly-L-lysine was removed by centrifugation. All experiments were performed with ubiquitin-EMCS except for Fig. S1B, where ubiquitin-GMBS was used.

Cysteine accessibility assay using UBI-mal. To probe the accessibility of the cysteines of Gpt2p, several technical problems had to be solved. The traditional cysteine accessibility methods rely on the use of small impermeant alkylating reagents (Bogdanov et al., 2005). It however has been reported that the ER membranes of mammalian cells are permeable for such small reagents, unlike lysosomal, Golgi or plasma membranes. Yet, ER membranes were not permeable to polyethyleneglycol-5000-maleimide (PEG-mal)(Le Gall et al., 2004). As judged by the derivatization of Kar2p, an ER lumenal soluble protein possessing a single cysteine residue, our yeast ER microsomes are permeable for PEG-mal at room temperature, are usually tight at 0 °C

or 4°C (Fig. S1A), but not in all experiments. Ubiquitin maleimide (UBI-mal) on the other hand was found to be impermeant even at room temperature (Fig. S1B). From a given cell line, 4 aliquots of 50 μg microsomal proteins were resuspended in 100 μl buffer, 2 aliquots in buffer G (0.2 M sorbitol, 5 mM $MgCl_2$, 100 mM NaH_2PO_4/Na_2HPO_4, pH 7.2), one in buffer H (= Buffer G + 1% w/v DDM), one in buffer I (= Buffer G + 1% w/v SDS). Samples were preincubated for 30 min on ice-cold water (0°C), except microsomes in buffer I, which were incubated at room temperature. 50 μl (150 nmol) of UBI-mal (ubiquitin-EMCS) or, for control, 50 μl of buffer G were added. After a further 30 min at the same temperature, the reactions were stopped by addition of 25 mM DTT and further incubation for 15 min. Samples were heated for 20 min at 65°C in Laemmli sample buffer and separated by SDS-PAGE. Proteins were transferred on a PVDF membrane using 25 mM Tris, 192 mM glycine, pH 8.3 as buffer; Western blotting was performed using anti-V5, anti-Kar2p, and anti-FLAG antibodies.

Phosphopeptide analysis. For the preparation of large amounts of Gpt2p we adapted a published protocol (Drew et al., 2008) as follows: BY4742 cells harboring pBF733 (*GAL1*$_{UAS}$-*GPT2*-V5-His_6 in pYES2NT/B with C-terminally tagged WT *GPT2*) were pre-cultured in 20 ml of YNB 2% galactose, then in 200 ml of YNB 2% glucose. 3x 2L of YNB 0.1% glucose were inoculated to give an A_{600} of 0.12 and grown overnight at 30°C to an A_{600} of about 1. Galactose (2% final) was added to induce protein expression for 2h at 30°C, during which the A_{600} rose to between 2 and 2.5). All media lacked uracil, to maintain the plasmid. The cells were harvested at 4°C, then washed twice in buffer B (50 mM KH_2PO_4/K_2HPO_4 pH 7.5, 1 mM EDTA, 0.6M sorbitol, 1x Roche Complete EDTA-free protease inhibitors cocktail) and the cell pellets (about 2 x 6'000 A_{600} units of cells (equivalent to 6'000 ml of culture having an A_{600} of 1)) were frozen in liquid N_2 and stored at -20°C.

Cell pellets were thawed on ice and resuspended to 200 A_{600} units ml^{-1} of fresh buffer C (0.2M sorbitol, 50 mM Na_2HPO_4/NaH_2PO_4 pH 6.0, 5 mM $MgCl_2$, 20 μg ml^{-1} DNase, 2 mM PMSF, 1x Roche Complete EDTA-free protease inhibitors cocktail, pepstatin A 5 μg ml^{-1}, 5 mM iodoacetamide). A volume of glass beads was added to two volumes of cell suspension, and the cells were broken in a planetary mono mill (Fritsch Pulverisette 6, Idar-Oberstein, Germany) at 4°C. The beads were filtered and the lysate centrifuged at 1000 g, 4°C, for 5 min to remove cell debris and nuclei. The membranes were pelleted by ultracentrifugation (Sorvall Discovery M120) at 150'000 g, 4°C, for 2h, then resuspended in 4 ml of buffer D (0.2M sorbitol, 50 mM Na_2HPO_4/NaH_2PO_4 pH 6.0, 5 mM $MgCl_2$, 100 mM $CaCl_2$, 1 mM PMSF, 1x Roche Complete EDTA-free protease inhibitors cocktail, pepstatin A 5 μg ml^{-1}). The membranes were diluted to 3.5 mg protein ml^{-1} of buffer E (8 mM Na_2HPO_4 1.4 mM KH_2PO_4, 10 mM imidazole pH 8.0, 436 mM NaCl, 2.6 mM KCl, 10% glycerol, 0.9 mM Triton X-100 (0.0562 % (w/v)), 1 x Roche Complete EDTA-free protease inhibitors cocktail,

5 μg ml^{-1} pepstatin) and more Triton X-100 was added for complete solubilization (20 x the amount of microsomal proteins). The volume was adjusted with buffer E to reduce the Triton X-100 concentration to 2%, and the membranes were solubilized for 2 h on a rotating wheel, at 4°C. Insoluble material was sedimented by ultracentrifugation (45 min at 100'000 g, 4°C). One ml of QIAGEN Ni-NTA superflow resin, pre-equilibrated with binding buffer, was added to the solubilized membranes and the binding reaction was performed overnight at 4°C, on a wheel. The lysate and resin were poured into 5 ml gravity-flow columns with a filter of 30 μm pore diameter (Pierce, Thermo Scientific, 89897). The flow-through was reloaded a second time; non-binding proteins were washed away by first adding 5 column volumes of buffer E and then 4 column volumes of buffer E with 20 mM imidazole. The bound material was eluted by adding 2 x 2 ml of buffer E containing 250 mM imidazole. Part of the first elution (2 μg protein μl^{-1}) was immediately heated for 15 min at 65°C in sample buffer, the rest was frozen as aliquots in liquid nitrogen and stored at -20°C. Purification was monitored by Bradford assay of protein content, by Western blotting, and by colloidal Coomassie blue (Sigma) staining of 7% SDS gels. 40 μl of the first elution in sample buffer was separated on a 7% SDS gel (1.5 mm). The gel was stained during 15 min with Coomassie G250 (Serva) without fixing. Both bands corresponding to hyper- or hypo- phosphorylated Gpt2p were cut, frozen in liquid nitrogen and used for mass spectrometric analysis.

Bands were excised from SDS-PAGE gel and digested, as described (Shevchenko et al., 1996; Wilm et al., 1996), with sequencing-grade trypsin (Promega).

Extracted peptides were analyzed on a hybrid LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) interfaced to an Ultimate 3000 RSLC nano HPLC system (Dionex, Olten, Switzerland). Solvents used for the mobile phase were 97:3 H_2O:acetonitrile (v/v) with 0.1 % formic acid (**A**) and 20:80 H_2O:acetonitrile (v/v) with 0.1 % formic acid (**B**). Each sample was injected twice on the Orbitrap mass spectrometer, using either CID or HCD for peptide fragmentation.

Peptides were loaded onto a trapping microcolumn Acclaim PepMap100 C18 (20 mm x 100 µm ID, 5 µm, Dionex) in H_2O:acetonitrile 97:3 (v/v) + 0.1 % formic acid at a flow rate of 3.5 µl min^{-1}. After 12 min, they were back-flush eluted and separated on a reversed-phase nanocolumn Acclaim PepMap RSLC 100A (75 µm ID x 25 cm, 2 µm, Dionex) at a flow rate of 300 nl min^{-1} with a gradient from 6 to 72 % acetonitrile in 0.1% formic acid (total run time: 95 min).

For spraying, an uncoated needle (New Objective silicatips^{TM}, 20 µm ID, 10 µm tip ID) was used with a voltage of 2.9 kV, and the mass spectrometer capillary transfer temperature was set at 300°C. Lock mass option was used for full MS scan recalibration with a polydimethylcyclosiloxane ion from ambient air (*m/z* 445.12003). Data-dependent acquisition

was controlled by Xcalibur 2.1 software (Thermo Fisher Scientific). In CID mode, the 20 most intense precursor ions detected in the full MS survey performed in the Orbitrap (range 350-1500 *m/z*, resolution 60'000 at *m/z* 400) were selected and fragmented. MS/MS was triggered by a minimum signal threshold of 3'000 counts, carried out at relative collision energy of 35 %, with isolation width of 4.0 amu and wideband activation, and fragment ions were analyzed in the ion trap. In HCD mode, the 15 most intense precursor ions detected in the full MS survey performed in the Orbitrap (range 300-1700 *m/z*, resolution 30'000 at *m/z* 400) were selected and fragmented. MS/MS was triggered by a minimum signal threshold of 3'000 counts, carried out at relative collision energy of 40 %, with isolation width of 1.7 amu, and fragment ions were analyzed in the Orbitrap at a resolution of 7'500. Only precursors with a charge higher than one were selected for fragmentation and the *m/z* of fragmented precursors was then dynamically excluded from any selection during 25 s. From *raw* files, MS/MS spectra were exported as *mgf* files (Mascot Generic File, text format) using extract_msn script (Thermo Fisher Scientific).

All MS/MS samples were analyzed using Mascot 2.3 (Matrix Science, London, UK). Mascot was set up to search the SwissProt database (release 2011_03, **www.uniprot.org**,) restricted to *Saccharomyces cerevisiae* taxonomy (7'320 sequences after taxonomy filter), and a custom database containing usual contaminant proteins (digestion enzymes, keratins, etc.). Trypsin (cleavage at K, R) was used as the enzyme definition. Mascot was searched with a fragment ion mass tolerance of 0.5 Da for CID data and 0.02 Da for HCD data, a parent ion tolerance of 10 ppm, allowing 2 missed cleavages. Iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Acetylation at the proteins' N-terminal end, deamidation of asparagine and glutamine, oxidation of methionine, and phosphorylation of serine, threonine and tyrosine were specified as variable modifications. Phosphosite localization was based on Mascot Delta Score described before (Savitski et al., 2011).

GPAT assay. *Sct1Δ* cells were grown in YPD to stationary phase. Microsomes were prepared by breaking cells with glass beads in buffer A supplemented with 2 mM EDTA. Cell walls were removed by centrifugation for 5 min at 800 g, 4 °C, the supernatant was filtered through a 0.8 μm glass fiber filter (Millipore, Millex-AP, AP20) and the microsomes were sedimented by centrifugation at 16'000 x g for 30 min, 4 °C. 100 μg microsomal proteins were diluted into buffer A and preincubated for 15 min at 0 °C with acyl-CoA (22.5 μM C16:0-CoA and 22.5 μM C18:1-CoA final conc.), DTT (1 mM final conc.) and with or without Triton X-100 (0.1% v/v) and further incubated for 45 min after addition of TNBS. GPAT activity was measured in a 200 μl assay volume by adding 0.2 μCi of [^{14}C]glycerol-3-phosphate diluted with cold G3P to a specific activity of 5 mCi mmol^{-1} at room temperature for 20 min. Reactions were stopped by adding 600 μl $CHCl_3$/MeOH (1/1), 200 μl $CHCl_3$ and 320 μl 1% $HClO_4$. The lower organic

phase was washed 3 times with 400 μl 1% $HClO_4$, and samples were analyzed by TLC in $CHCl_3$/MeOH/acetic acid/5% aqueous sodium bisulfite (67/26/4.4/2.6). Plates were analyzed by phosphorimaging and radioscanning (Berthold Services GmbH, Switzerland). Control experiments showed a) that GPAT activity was linear for at least 10 min over a range of microsomal protein concentrations, b) that the product is stable (Fig. S7A, B), and c) that even at the end of a 20 min incubation, most of radioactivity still is in the form of G3P (Fig. S7C).

Supplemental Figure legends

Fig. S1. Permeability of yeast microsomes for PEG-maleimide. A, microsomes from BY4742 WT cells were incubated in the absence or presence of DDM (D) or SDS (S) with PEG-mal (5 mM) for indicated time spans (min) either at 0 °C (upper panel) or at room temperature (lower panel). Untagged and mass-tagged Kar2p was observed after SDS/PAGE and Western blotting using anti-Kar2p antibody. Note that Kar2p has a single cysteine residue, derivatization of which induces a mass shift of about 10 kDa. **B**, microsomal membranes derived from WT BY4742 cells were prepared as above. In this experiment the membrane pellet was resuspended and incubated for 15 minutes with 200 μM β-mercaptoethanol. Re-centrifuged microsomes were then incubated in the presence or absence of detergent with freshly prepared ubiquitin-GMBS (0.9 mM) for 30, 60 and 90 minutes at room temperature.

Fig. S2. Functionality of *GPT2* alleles containing only one cysteine. Sporulation of diploid *gpt2::TRP1/GPT2 sct1::HIS3/SCT1* (FBY2250) strains harboring *URA3* plasmids containing either WT *GPT2* or mutated *GPT2* alleles yielded viable *gpt2::TRP1 sct1::HIS3* (2Δ) cells. **A**, 2Δ cells harboring either WT *GPT2* or *GPT2* single-cysteine mutant alleles (all cysteines deleted except for a single cysteine at positions 77, 254 or 297) were plated on selective media supplemented with 1 M sorbitol, 5'-fluoroorotic acid (FOA) and containing galactose or raffinose as the carbon source as indicated. **B**, yeast cells containing the *GAL1* promoter driven Gpt2p-V5-His_6 construct were grown in raffinose at 30 °C, at time 0 galactose was added from a concentrated solution to induce Gpt2p-V5-His_6. After a further culture for 0 to 2 hours, lysates were prepared and probed by Western blotting using anti-V5 antibody.

Fig. S3. Functionality of cysteine mutated *GPT2* alleles used in Fig. 3. A, model of Gpt2p topology with natural cysteines in orange, cysteine substitutions in loop L1-2 in turquoise. **B-E**, we constructed new *gpt2* alleles containing only the cysteines indicated on top of each double panel in 2μ vectors behind the *GAL1* promoter and followed by a C-terminal V5-His_6 tag. Non-natural cysteines are in bold. To test the functionality of the cysteine-modified alleles, the

vectors were introduced into a *gpt2::TRP1/GPT2 sct1::HIS3/SCT1* diploid (FBY2250). After sporulation and tetrad dissection, we screened for growth of viable *gpt2Δ sct1Δ* progeny on galactose and glucose-containing medium. **B**, progeny from 3 complete tetratype tetrads having the indicated genotypes and harboring Gpt2p-5CS-C77-254-297-V5-His_6 was streaked on YNB with galactose as a carbon source without and with 5'-fluoroorotic acid (FOA) as indicated. Plates were photographed after 3 days at 30°C. Genotypes were deduced by PCR and by growth on medium lacking His or Trp (YNBGal-His, YNBGal-Trp)(not shown). The critical *gpt2Δ sct1Δ* (2Δ) strains rescued by Gpt2p-5CS-C77-254-297-V5-His_6 are highlighted in red. **C – E**, progeny from one complete or incomplete tetrad carrying a plasmid born *gpt2* allele was precultured either in galactose or glucose for 16 h. Ten fold dilutions were then spotted onto YNB plates containing the same carbon source as the preculture. Plates were photographed after 3 days at 30°C. Genotypes of the strains analyzed are on the left. A *gpt2Δ sct1Δ* strain rescued by WT *GPT2* in the same vector was added for comparison to each tetrad progeny.

Fig. S4. Functionality of *GPT2* alleles used for cysteine accessibility studies in Fig. 4. Functionality was tested as described in Fig. S3C-E.

Fig. S5. Viability of *gpt2Δ sct1Δ* cells containing epitope-tagged *GPT2* alleles. A, sporulation of diploid *gpt2::TRP1/GPT2 sct1::HIS3/SCT1* (FBY2250) strains harboring *URA3* plasmids containing either WT *GPT2* or mutated *GPT2* alleles yielded viable *gpt2::TRP1 sct1::HIS3* (2Δ) cells. Ten fold dilutions of 2Δ cells containing *GAL1* promoter driven *GPT2* constructs with HA- or VSVG-epitopes inserted at positions 235 or 412 were plated on selective (-His, -Trp) or non-selective media containing amino acids, uracil, adenine, 5'-fluoroorotic acid (FOA) and galactose (YNBGal) or glucose (YPD) as indicated. Note that the low expression of Gpt2p-412-VSVG and Gpt2p-412-HA on glucose is sufficient to rescue the lethality of the *gpt2::TRP1 sct1::HIS3* double mutation. **B**, samples denoted with 1 to 3 stars in Fig. 5A - C were run side by side in a single SDS-PAGE gel and different parts of the Western blot were probed with the appropriate antibodies. It appears that the larger fragments of Gpt2 proteins having tags at amino acids 235 or 412 are the same, suggesting that they encompass both tag insertion sites, 235 and 412. The Gpt2p-412-VSVG produced a unique intermediate of about 50 kDa whereas the Gpt2p-235-VSVG and Gpt2p-235-HA produced a unique intermediate of about 44 kDa. Thus, the smaller fragments generated are different, as might be expected. Note that the unique 25-kDa fragment of Gpt2p-235-HA and Gpt2p-235- VSVG (Fig. 5A, B) was allowed to run out of the gel.

Fig. S6. Phosphosites detected by mass spectrometry in purified Gpt2p. The two Coomassie blue stained bands corresponding to the upper and lower band of Gpt2p were

excised from an SDS-PAGE gel and processed for mass spectrometry as described in supplemental experimental procedures. This allowed to identify with high confidence the phosphosites that are underlain black; peptides identified are underlain in yellow, acyltransferase motifs I (79-86) and III (258-262) are in red, motifs II (137) and IV (271 or 296) in green. Transmembrane helices predicted by TOPCONS global prediction are underlined.

Fig. S7. GPAT activity in *sct1Δ* microsomes. A, 200 μl aliquots of microsomes (100 μg of protein) were preincubated for 1 h on ice without or with 0.1% TX-100, [^{14}C]-G3P was added and samples were incubated for 2, 5, 10 and 20 min. Products were analyzed by TLC and lipids quantified by radioscanning. **B**, 0, 2.5, 6.4, 16, 40 and 100 μg of microsomal proteins from *sct1Δ* cells were completed to total amount of 100 μg protein with boiled microsomes, preincubated for 1 hour on ice and GPAT activity was measured during 10 min as in panel A. **C**, aliquots of the aqueous phase of Folch separations performed on lipid extracts in the experiment of panel A were taken and analyzed by TLC on plastic supported cellulose (0.1 mm Cellulose Polygram CEL300, Macherey Nagel) using as a solvent MeOH/1 M NH_4Ac pH8.5/0.2M EDTA (70/20/0.5). Quantification by radioscanning allowed to assess the percentage of [^{14}C]G3P present at all moments either as a percentage of the sum of [^{14}C]G3P + [^{14}C]glycerol, or as percentage of the total radioactivity present in the lane.

Supplemental Tables

Table SI. Yeast strains and plasmids

Name	Genetic markers	Source/reference
Strains		
STY50	*MAT***a** *his4-401 leu2-3,112 trp1-1 ura3-52 hol1-1 SUC2::LEU2*	(Strahl-Bolsinger and Scheinost, 1999)
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	EUROSCARF
W303-1B	*MATα*, *ade2-1 can1-100 ura3-1 leu2-3 112 trp1-1 his3-11,15*	Paul Kirchmann
gpt2Δ/GPT2 sct1Δ/SCT1 (FBY2250)	*MAT***a**/α *ura3/ura3 his3/his3 leu2/leu2 trp1/trp1 ade2/ade2 can1/can1 gpt2::TRP1/GPT2 sct1::HIS3/SCT1*	This study
gpt2Δ (CMY204)	*MAT***a** *ura3-1 his3-1115 leu2-3112 trp1-1 ade2-1 can1-100 gpt2::TRP1*	Christopher R. Mc Master
sct1Δ (CMY201)	*MATα ura3-1 his3-1115 leu2-3112 trp1-1 ade2-1 can1-100 sct1::HIS3*	Christopher R. Mc Master
2Δ.GPT2 (FBY2252)	*ura3 his3 leu2 trp1 ade2 can1 gpt2::TRP1 sct1::HIS3* containing plasmid pBF733	This study
2Δ.GPT2-C77	*ura3 his3 leu2 trp1 ade2 can1 gpt2::TRP1 sct1::HIS3*	This study

(FBY7407)	containing plasmid pBF736	
2Δ.GPT2-C254 (FBY7410)	*ura3 his3 leu2 trp1 ade2 can1 gpt2::TRP1 sct1::HIS3* containing plasmid pBF730	This study
2Δ.GPT2-C297 (FBY7431)	*ura3 his3 leu2 trp1 ade2 can1 gpt2::TRP1 sct1::HIS3* containing plasmid pBF737	This study
2Δ.GPT2-235-VSVG (FBY2259)	*ura3 his3 leu2 trp1 ade2 can1 gpt2::TRP1 sct1::HIS3* containing plasmid pBF225	This study
2Δ.GPT2-235-HA (FBY7404)	*ura3 his3 leu2 trp1 ade2 can1 gpt2::TRP1 sct1::HIS3* containing plasmid pBF223	This study
2Δ.GPT2-412-VSVG (FBY2255)	*ura3 his3 leu2 trp1 ade2 can1 gpt2::TRP1 sct1::HIS3* containing plasmid pBF228	This study
2Δ.GPT2-412-HA (FBY2253)	*ura3 his3 leu2 trp1 ade2 can1 gpt2::TRP1 sct1::HIS3* containing plasmid pBF227	This study
sct1Δ	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ybl011w::KanMX*	EUROSCARF
FBY2272	STY50 with pJK90 harboring *GPT2*-P109-HA-*SUC2-HIS4C*	(Kim et al., 2003)
FBY2274	STY50 with pJK90 harboring *GPT2*-K120-HA-*SUC2-HIS4C*	(Kim et al., 2003)
FBY7378	STY50 with pJK90 harboring *GPT2*-P259-HA-*SUC2-HIS4C*	(Kim et al., 2003)
FBY7379	STY50 with pJK90 harboring *GPT2*-G262-HA-*SUC2-HIS4C*	(Kim et al., 2003)
FBY7380	STY50 with pJK90 harboring *GPT2*-P333-HA-*SUC2-HIS4C*	(Kim et al., 2003)
FBY7381	STY50 with pJK90 harboring *GPT2*-P467-HA-*SUC2-HIS4C*	(Kim et al., 2003)
FBY2280	his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, trp1::KanMX4 containing plasmid pBF649 (2x FLAG-*GPI8)*	This study
FBY2344	his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, trp1::KanMX4 containing plasmids pBF649 and pBF733 (WT *GPT2*)	This study
FBY2295	his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, trp1::KanMX4 containing plasmids pBF649 and pBF237 (*GPT2-C77-254-297*)	This study
FBY2289	his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, trp1::KanMX4 containing plasmids pBF649 and pBF233 (*GPT2-C77-110-254-530*)	This study
FBY2305	his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, trp1::KanMX4 containing plasmids pBF649 and pBF239 (*GPT2-C77-254-530*)	This study
FBY2306	his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, trp1::KanMX4 containing plasmids pBF649 and pBF240 (*GPT2-C77-110-254*)	This study
FBY2314	his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, trp1::KanMX4 containing plasmids pBF649 and pBF241 (*GPT2-C77-99-254-297*)	This study
FBY2315	his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, trp1::KanMX4 containing plasmids pBF649 and pBF242 (*GPT2-C77-243-254-297*)	This study
FBY2316	his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, trp1::KanMX4 containing plasmids pBF649 and pBF243 (*GPT2-C77-250-254-297*)	This study
FBY2317	his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, trp1::KanMX4 containing plasmids pBF649 and pBF244 (*GPT2-C77-251-254-297*)	This study
FBY2349	his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, trp1::KanMX4 containing plasmids pBF649 and pBF246 (*GPT2-C77-254-297-349*)	This study
FBY2351	his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, trp1::KanMX4 containing plasmids pBF649 and pBF248 (*GPT2-C77-254-297-330-331-332*)	This study
FBY2352	his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, trp1::KanMX4 containing plasmids pBF649 and pBF249 (*GPT2-C77-254-297-345-346-347*)	This study

Plasmids		
pJK90	2μ, *URA3*, TPI_{UAS}-HA-*SUC2-HIS4C*	(Kim et al., 2003)
pYES2NT/B	2μm, *URA3*	INVITROGEN
pBF649	2x FLAG-*GPI8* in pBF617, CEN/ARS, *TRP1*	I. Imhof
pBF720	$GAL1_{UAS}$-*GPT2*-V5-His_6 in pYES2NT/B containing an uncorrected N719D mutation	This study
pBF733	$GAL1_{UAS}$-*GPT2*-V5-His_6 in pYES2NT/B	This study
pBF736	$GAL1_{UAS}$-*GPT2-C77*-V5-His_6 in pYES2NT/B*	This study
pBF730	$GAL1_{UAS}$-*GPT2-C254*-V5-His_6 in pYES2NT/B*	This study
pBF737	$GAL1_{UAS}$-*GPT2-C297*-V5-His_6 in pYES2NT/B*	This study
pBF223	$GAL1_{UAS}$-*GPT2*-235-HA-V5-His_6 in pYES2NT/B	This study
pBF225	$GAL1_{UAS}$-*GPT2*-235-VSVG-V5-His_6 in pYES2NT/B	This study
pBF227	$GAL1_{UAS}$-*GPT2*-412-HA-V5-His_6 in pYES2NT/B	This study
pBF228	$GAL1_{UAS}$-*GPT2*-412-VSVG-V5-His_6 in pYES2NT/B	This study
pBF237	$GAL1_{UAS}$-*GPT2-C77-254-297*-V5-His_6 in pYES2NT/B*	This study
pBF233	$GAL1_{UAS}$-*GPT2-C77-110-254-C530*-V5-His_6 in pYES2NT/B*	This study
pBF239	$GAL1_{UAS}$-*GPT2-C77-254-530*-V5-His_6 in pYES2NT/B*	This study
pBF240	$GAL1_{UAS}$-*GPT2-C77-110-254*-V5-His_6 in pYES2NT/B*	This study
pBF241	$GAL1_{UAS}$-*GPT2-C77-99-254-297*-V5-His_6 in pYES2NT/B*	This study
pBF242	$GAL1_{UAS}$-*GPT2-C77-243-254-297*-V5-His_6 in pYES2NT/B*	This study
pBF243	$GAL1_{UAS}$-*GPT2-C77-250-254-297*-V5-His_6 in pYES2NT/B*	This study
pBF244	$GAL1_{UAS}$-*GPT2-C77-251-254-297*-V5-His_6 in pYES2NT/B*	This study
pBF246	$GAL1_{UAS}$-*GPT2-C77-254-297-349*-V5-His_6 in pYES2NT/B*	This study
pBF248	$GAL1_{UAS}$-*GPT2-C77-254-297-330-331-332*-V5-His_6 in pYES2NT/B*	This study
pBF249	$GAL1_{UAS}$-*GPT2-C77-254-297-345-346-347*-V5-His_6 in pYES2NT/B*	This study

* numbers indicate positions of all remaining cysteines (natural or substituted) present in the *gpt2* allele carried by the vector.

Table SII. Primers used for construction of different plasmids

Number	**Purpose/name**	**Sequence (5'-3')**
918	Gpt2 forward	aggtggtttgttacgcatgcaagcttgatatcgaaatgtctgctcccgctgccgatc
1387	Gpt2-P109-DTR	gatggtctagaggtgtaaccacttgagttcttaggaggcattctggatcggg
1386	Gpt2-K120-DTR	gatggtctagaggtgtaaccacttgagttcttaggtttcttaaaactcgactcagc
919	Gpt2-P259-DTR	gatggtctagaggtgtaaccacttgagttcttagggggaaaatacctacacagcccttc
920	Gpt2-G262-DTR	gatggtctagaggtgtaaccacttgagttcttaggaccaccctcggggaaaatacctac
921	Gpt2-P333-DTR	gatggtctagaggtgtaaccacttgagttcttaggtggggagtccttatacatttcg
922	Gpt2-P467-DTR	gatggtctagaggtgtaaccacttgagttcttaggtggagtgaagagaatagaacc
966	*GPT2* amplification	cagctaagcttataatgtctgctcccgctgcc
967	*GPT2* amplification	cagctgcggccgctcgagttctttcttttcgtgttctcttttctgtcttac
968	235 epitope insert.	gcagagaaaggatccatcgacaatacggaaactttcca
969	235 epitope insert.	gtcgatggatcctttctctgcatatttaaaattagtaccattagtc
970	412 epitope insert.	ggtatatgacggatcctacaacaggaaattagattcagtggg
971	412 epitope insert.	cctgttgtaggatccgtcatataccagtttttttaagtgaataattct
1003	HA epitope insert.	gctgggatcctatccttacgatgttccagactacgcaggatccactt
1004	HA epitope insert.	aagtggatcctgcgtagtctggaacatcgtaaggataggatcccagc
1005	VSVG epitope insert.	acttggatcctatactgatattgaaatgaataggctaggtaaaggatccagtg
1006	VSVG epitope insert.	cactggatcctttacctagcctattcatttcaatatcagtataggatccaagt
1338	*GPT2* SCAM-C77S	tgccaaccatccttgtgtctgcccctcatgcaaatcagttc
1339	*GPT2* SCAM-C110S	tcccgatccagaatgccttcttttgttactgctgagtcgag

1340	*GPT2* SCAM-C254S	atcacttgcatacgaagggctctgtaggtattttccccgaggg
1341	*GPT2* SCAM-C297S	tgaaagttgctgttgtaccctctggtttgcattatttccacag
1343	*GPT2* SCAM-C440A	aaattagaagcattgagggcttttgtaactttgatcgttcg
1344	*GPT2* SCAM-C472A	ttcactccaattttcattattgctcgcgtatactcagaaaagaagg
1346	*GPT2* SCAM-C530S	tggcaagaaaacaacactattctcgcatctgggttccttcc
1348	*GPT2* SCAM-C603S	tctagagttgactgctgtttctaacgatttaggacctttgg
1520	*GPT2* SCAM-S254C	atcacttgcatacgaagggctgtgtaggtattttccccgaggg
1342	*GPT2* SCAM-S297C	tgaaagttgctgttgtaccctgtggtttgcattatttccacag
1538	*GPT2* SCAM-T99C	tcgggactttcccgctgagcacttcagcaaacgggtttg
1540	*GPT2* SCAM-Q243C	gagaaaatcgacaatacggaaactttctgcagtgtttttgatcacttgcatacgaag
1541	*GPT2* SCAM-H250C	atacctacacagcccttcgtacacaagtgatcaaaaacactctg
1542	*GPT2* SCAM-T251C	tttccagagtgtttttgatcacttgcattgcaagggctgtgtaggt
1546	*GPT2 SCAM-F349C*	ctggagcattttcggtaacagaacacaaagaattggtgatctttttag
1544	*GPT2 SCAM-KDS330CCC*	gtggtggatgggaaatatggcgaaatgtattgttgctgcccacgtgagaccgtttccaaactac
1543	*GPT2 SCAM-TNS345CCC*	tctggagcattttcggtaacagaaaacaaacaacagcagatctttttagtagtttggaaacggtctc

Table SIII. Surface exposure predictions for native and substituted cysteines of Gpt2p

Residue / Substitution	Prediction	Relative Surface Accessibility	Absolute Surface Accessibility (ASA)	ASA-mut/ ASA-nat
Natural cysteines				
C77	B	0.019	2.597	-
C110	B	0.089	12.552	-
C254	B	0.076	10.642	-
C297	B	0.015	2.148	-
C440	B	0.190	26.620	-
C472	B	0.039	5.448	-
C530	B	0.209	29.316	-
C603	B	0.020	2.752	-
Cysteine substitutions				
T99C	E / E	0.466 / 0.398	64.690 / 55.907	0.864
Q243C	E / E	0.461 / 0.467	82.317 / 65.581	0.797
H250C	E / E	0.576 / 0.517	104.811 / 72.643	0.693
T251C	E / E	0.700 / 0.697	97.062 / 97.831	1.008
K330C	E / E	0.583 / 0.604	119.861 / 84.858	0.708
D331C	E / E	0.651 / 0.640	93.795 / 89.926	0.959
S332C	E / E	0.451 / 0.486	52.822 / 68.262	1.293
T345C	E / E	0.272 / 0.241	37.754 / 33.879	0.897
N346C	E / E	0.558 / 0.547	81.706 / 76.757	0.939
S347C	B / B	0.176 / 0.190	20.639 / 26.606	1.289
F349C	E / E	0.424 / 0.411	85.037 / 57.747	0.679

Surface exposure of natural cysteines of Gpt2p and of amino acids that were replaced by cysteine were predicted using the predictor available at http://www.cbs.dtu.dk/services/NetSurfP. For substitutions, the relative and absolute values were obtained for the native and the mutated sequence and separated by a slash. Absolute surface accessibility (ASA) values (Petersen et al., 2009) are slightly diminished after the substitution of natural residues by cysteines, as indicated by the ratio “ASA-mutated/ASA-natural” ratio given in the last column. This ratio on average is 0.92.

Supplemental references

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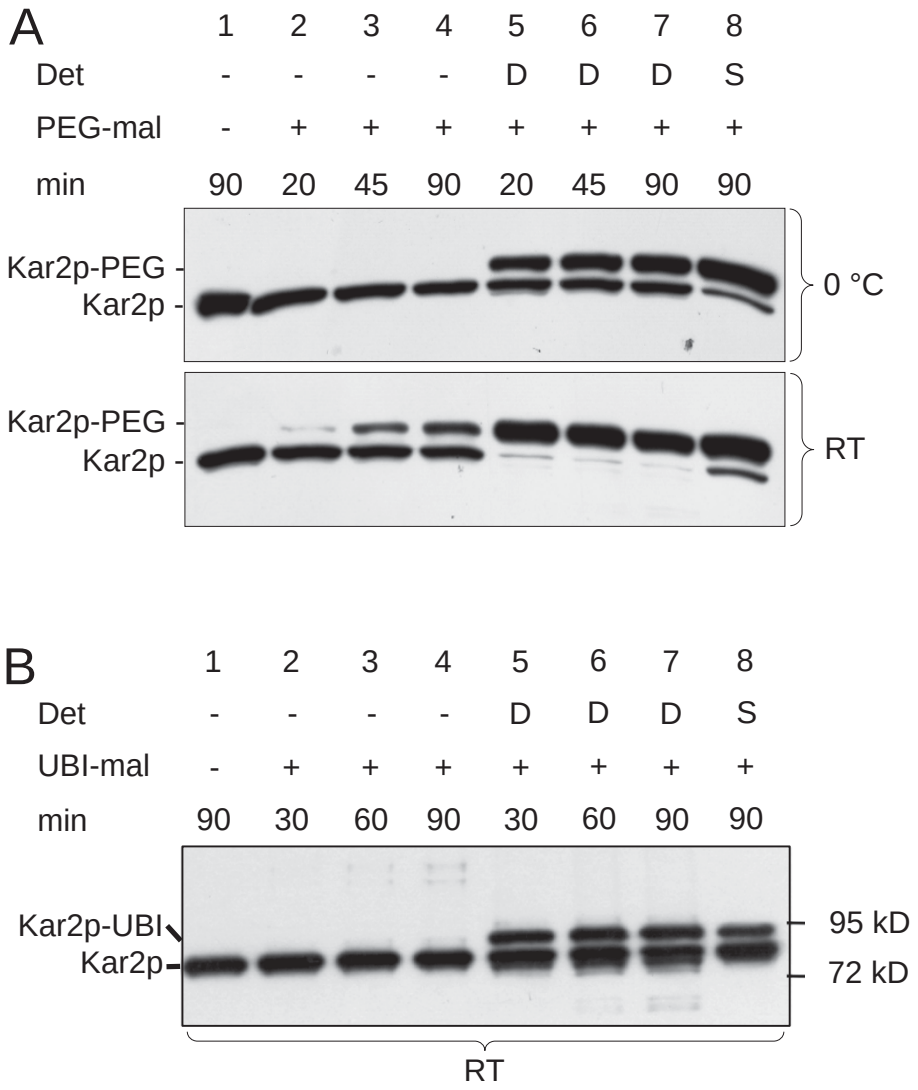
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Kar2p
0 °C
Kar2p-PEG
Kar2p
RT
B
1 2 3 4 5 6 7 8
Det - - - - D D D S
UBI-mal - + + + + + + +
min 90 30 60 90 30 60 90 90
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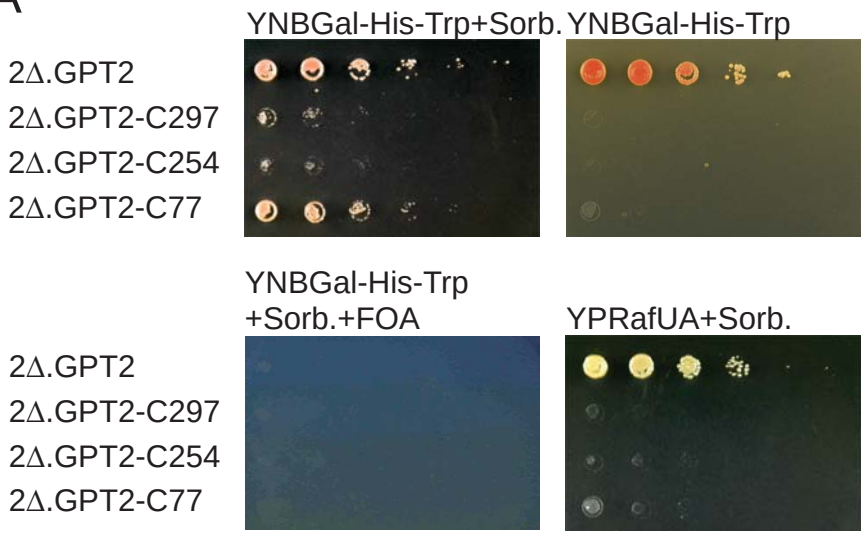
Kar2p
95 kD
72 kD
RT

Fig. S1A, B

A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2Δ.GPT2-C254
2Δ.GPT2-C77
YNBGal-His-Trp
+Sorb.+FOA
YPRafUA+Sorb.
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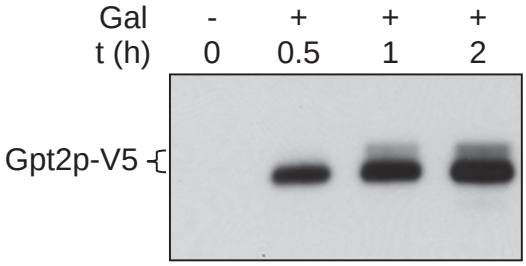
2Δ.GPT2-C254
2Δ.GPT2-C77

B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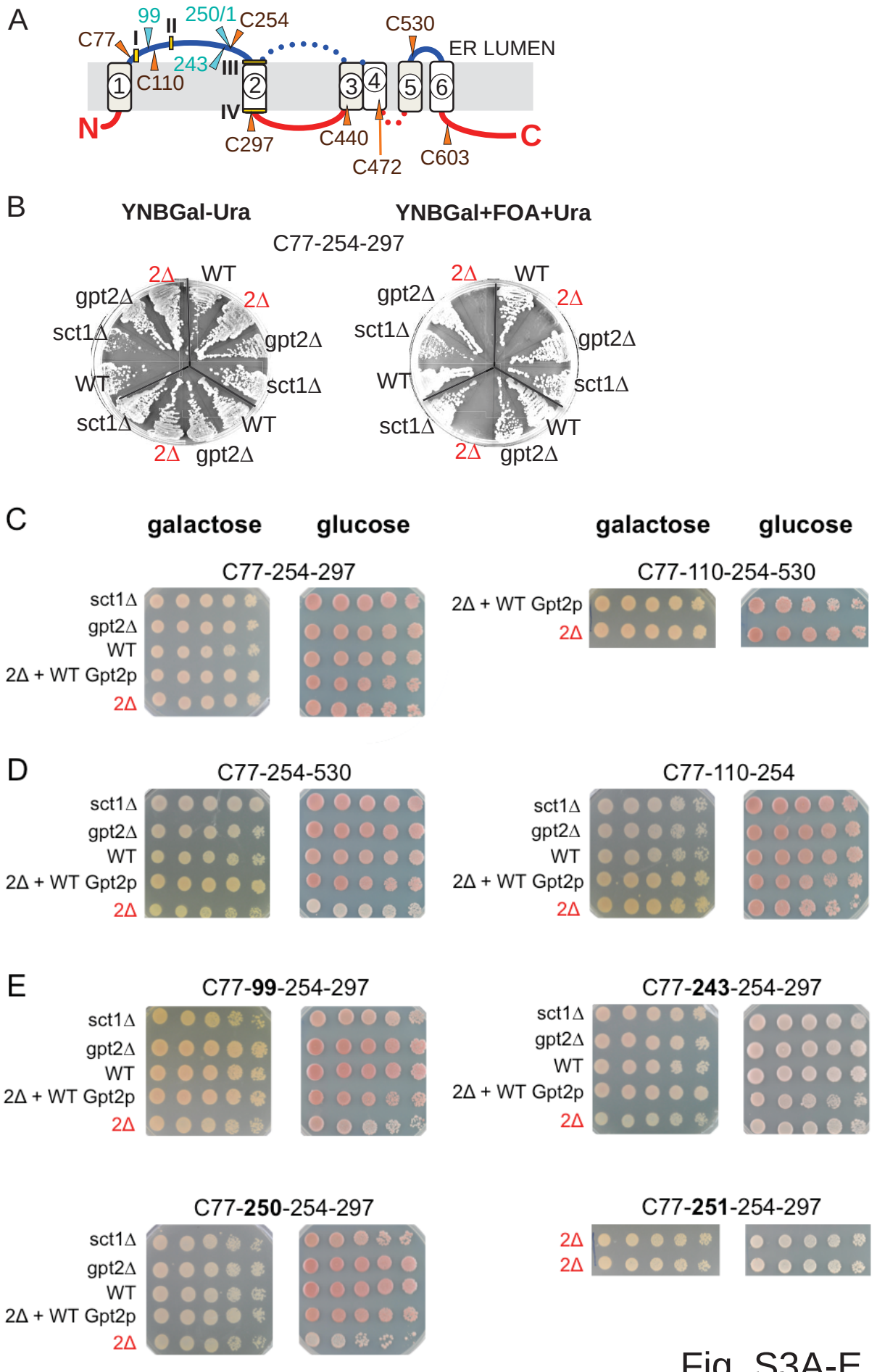
Gpt2p-V5

Fig. S2A, B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C254
C77
I
II
243
III
C110
C530
ER LUMEN
1
2
3
4
5
6
IV
N
C
C297
C440
C472
C603
B
YNBGal-Ura
YNBGal+FOA+Ura
C77-254-297
2Δ
WT
gpt2Δ
sct1Δ
C
galactose
glucose
C77-254-297
C77-110-254-530
2Δ + WT Gpt2p
D
C77-254-530
C77-110-254
E
C77-99-254-297
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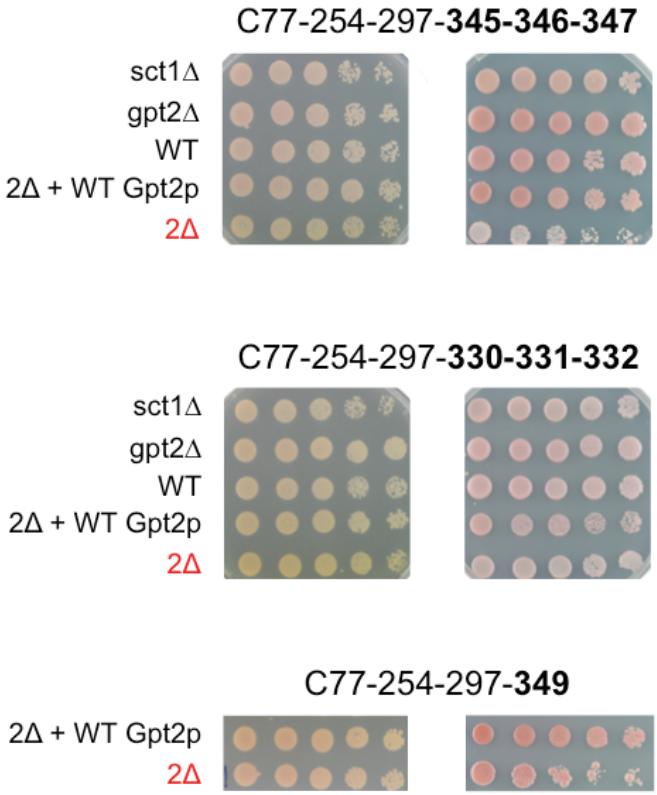
C77-250-254-297
C77-251-254-297

Fig. S3A-E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WT
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2Δ
C77-254-297-**330-331-332**
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WT
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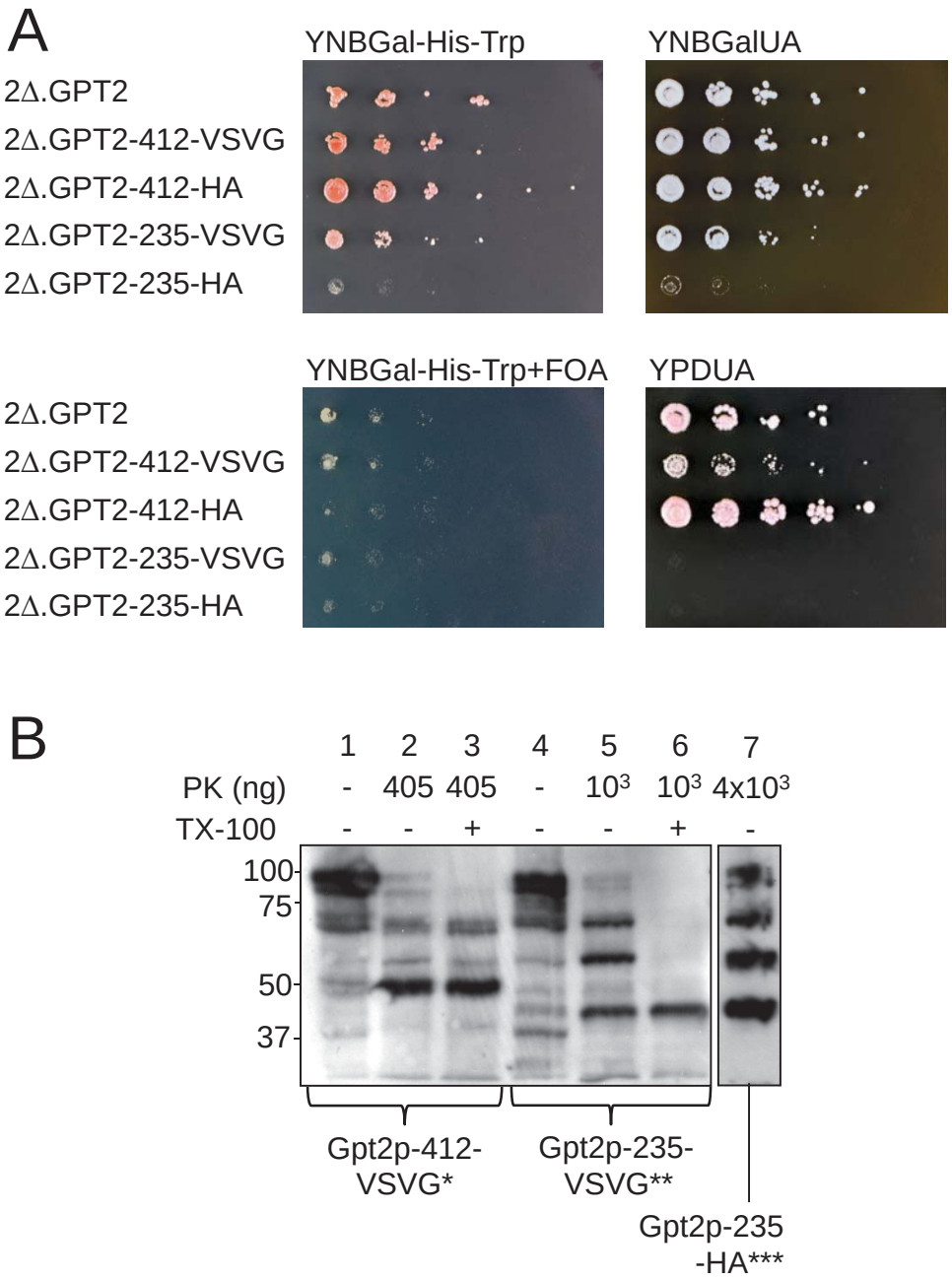
2Δ
C77-254-297-**349**
2Δ + WT Gpt2p
2Δ

Fig. S4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2Δ.GPT2-412-HA
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2Δ.GPT2-235-HA
YNBGal-His-Trp+FOA
YPDUA
2Δ.GPT2
2Δ.GPT2-412-VSVG
2Δ.GPT2-412-HA
2Δ.GPT2-235-VSVG
2Δ.GPT2-235-HA
B
1 2 3 4 5 6 7
PK (ng) - 405 405 - 10³ 10³ 4x10³
TX-100 - - + - - + -
100
75
50
37
Gpt2p-412-VSVG*
Gpt2p-235-VSVG**
Gpt2p-235-HA***

Fig. S5

Gpt2p, upper band, 65% coverage

```
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  1  MSAPAADHNA AKPIPHVPQA SRRYKNSYNG FVYNIHTWLY DVSVFLFNIL
 51  FTIFFREIKV RGAYNVPEVG VPTILVCAPH ANQFIDPALV MSQTRLLKTS
101  AGKSRSRMPC FVTAESSFKK RFISFFGHAM GGIPVPRIQD NLKPVDENLE
151  IYAPDLKNHP EIIKGRSKNP QTTPVNFTKR FSAKSLLGLP DYLSNAQIKE
201  IPDDETIILS SPFRTSKSKV VELLTNGTNF KYAEKIDNTE TFQSVFDHLH
251  TKGCVGIFPE GGSHDRPSLL PIKAGVAIMA LGAVAADPTM KVAVVPCGLH
301  YFHRNKFRSR AVLEYGEPIV VDGKYGEMYK DSPRETVSKL LKKITNSLFS
351  VTENAPDYDT LMVIQAARRL YQPVKVRLPL PAIVEINRRL LFGYSKFKDD
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451  FSVFAILSLP GSILFTPIFI ICRVYSEKKA KEGLKKSLVK IKGTDLLATW
501  KLIVALILAP ILYVTYSILL IILARKQHYC RIWVPSNNAF IQFVYFYALL
551  VFTTYSSLKT GEIGVDLFKS LRPLFVSIVY PGKKIEEIQT TRKNLSLELT
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651  SIHSIGSLAS NALSRVNSRG SLTDIPIFSD AKQGQWKSEG ETSEDEDEFD
701  EKNPAIVQTA RSSDLNKENS RNTNISSKIA SLVRQKREHE KKE
```

Gpt2p, lower band, 63% coverage

```
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 51  FTIFFREIKV RGAYNVPEVG VPTILVCAPH ANQFIDPALV MSQTRLLKTS
101  AGKSRSRMPC FVTAESSFKK RFISFFGHAM GGIPVPRIQD NLKPVDENLE
151  IYAPDLKNHP EIIKGRSKNP QTTPVNFTKR FSAKSLLGLP DYLSNAQIKE
201  IPDDETIILS SPFRTSKSKV VELLTNGTNF KYAEKIDNTE TFQSVFDHLH
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651  SIHSIGSLAS NALSRVNSRG SLTDIPIFSD AKQGQWKSEG ETSEDEDEFD
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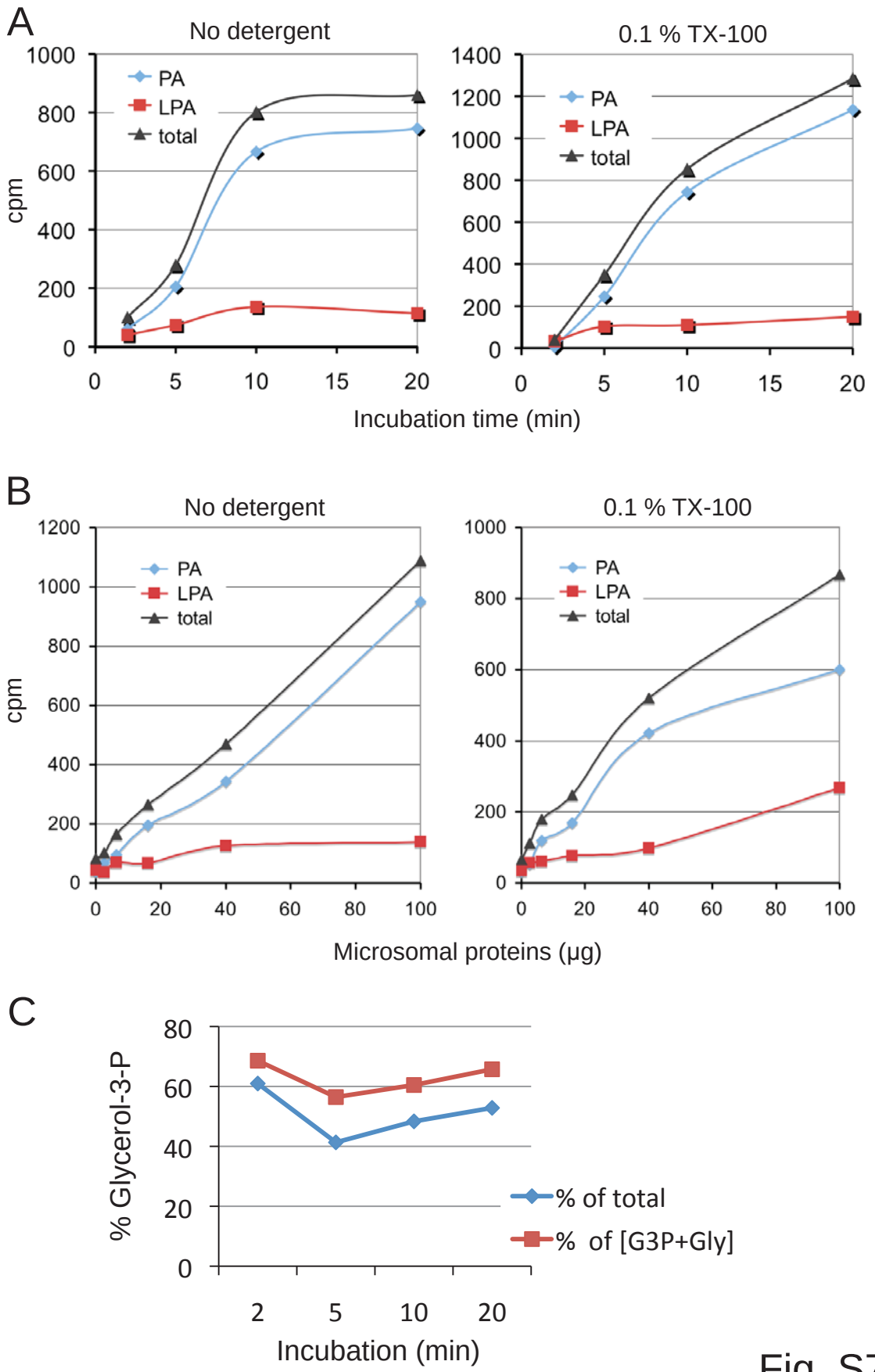
Fig. S6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total
cpm
Microsomal proteins (µg)
C
% Glycerol-3-P
% of total
% of [G3P+Gly]
Incubation (min)

Fig. S7A-C