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A YEAST PAF ACETYLHYDROLASE ORTHOLOG SUPPRESSES OXIDATIVE DEATH

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Abstract

Phospholipids containing *sn*-2 polyunsaturated fatty acyl residues are primary targets of oxidizing radicals, producing pro-apoptotic and membrane perturbing fragmented phospholipids. The only known phospholipases that specifically select these oxidized and/or short-chained phospholipids as substrates are mammalian group VII phospholipases A_2s that were purified and cloned as PAF acetylhydrolases. Platelet-activating factor (PAF) is a short-chained phospholipid, and whether these enzymes actually are PAF hydrolases or evolved as oxidized phospholipid phospholipases is unknown. The fission yeast *S. pombe*, which does not form or use PAF as a signaling molecule, contains an open reading frame potentially homologous to mammalian group VII phospholipase A_2s. We cloned this SPBC106.11c locus and expressed it in distantly related *Saccharomyces cerevisiae* that lack homologous sequences. The *S. pombe* locus encoded a functional phospholipase A_2, now renamed *plg7*$^+$, that hydrolyzed PAF and a synthetic oxidized phospholipid. Expression of human type II PAF acetylhydrolase or *S. pombe* Plg7p enhanced viability of *S. cerevisiae* subjected to oxidative stress. We conclude a single celled organism with an exceedingly spare genome still expresses an unusually discriminating phospholipase A_2, and that selective hydrolysis of phospholipid oxidation products is an early, and critical, way to overcome oxidative membrane damage and oxidant-induced cell death.

Keywords

apoptosis; PAF acetylhydrolase; oxidized phospholipid; phospholipid transport; phospholipase A_2

Introduction

The evolutionary relationship of mammals, the fission yeast *Schizosaccromyces pombe*, and the budding yeast *Saccharomyces cerevisiae* is approximately equidistant, with *S. cerevisiae* and *S. pombe* separating from their common ancestor approximately 420 to 330 million years ago [1]. These yeasts would seem to be protected from the underlying cause of oxidative injury

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and cell death because they do not contain desaturases that form polyunsaturated fatty acids [2] that are the primary targets of chemical oxidation [3]; but in fact, these free living organisms readily accumulate and incorporate the abundant plant polyunsaturated fatty acids they encounter in crushed grapes and malting barley into their membrane phospholipids [4–6]. These yeasts also accumulate whole phosphatidylcholines from their environment [7]. These exogenous phospholipids include short-chained phospholipids [8,9] that are components of red wine and its must [10], or that are formed by chemical oxidation or enzymatic attack on plant phospholipids.

Mammalian cells also accumulate exogenous fragmented phospholipids as intact phospholipids [11]. Some of these disrupt membrane lipid packing [12], while others disrupt mitochondrial integrity and activate the intrinsic apoptotic pathway [11]. To deal with these membrane disruptive phospholipids, mammalian genomes encode a small family of phospholipases A_2 that selectively degrade phospholipids with oxidatively-fragmented [13] or peroxidized [14,15] fatty acyl residues. These enzymes, unlike all other phospholipases A_2, have no activity against intact, long chained phospholipids, and this specific removal of oxidatively damaged phospholipids protects mammalian cells from oxidative death initiated by H_2O_2 exposure [16] or UVB irradiation [17]. These enzymes were originally purified and cloned as PAF acetylhydrolases [18,19], but their role in physiologic and pathologic PAF catabolism is unclear [20] and the presence of a functional ortholog in *C. elegans* [21] involved in epithelial sheet migration potentially indicates a different original purpose for these enzymes.

The *S. pombe* genome, but not that of *S. cerevisiae*, contains a sequence encoding a hypothetical protein that would have 25% identity to human PAF acetylhydrolases [22], and would retain the residues forming the catalytic triad of the mammalian enzyme. Since *S. pombe* does not use PAF as a signaling molecule, a catalytically active enzyme encoded by this SPBC106.11c locus might instead function as an oxidized phospholipid phospholipase positioned to maintain viability in the face of environmental oxidative stress. Here, we show the *S. pombe* genome does encode for a functional PAF acetylhydrolase, and that this enzyme will reduce oxidative cell death. Expression of a functional member of the PAF acetylhydrolase family by a unicellular organism shows these enzymes are ancient responses to environmental stresses.

Material and Methods

Strains and Media

S. pombe strain CHP428 (*h+ ura4-D18 leu1-32 ade6-M210 his7-366*) was purchased from ATCC (201399; Manassas, VA), *S. cerevisiae* wild type diploid expression strain INVSc1 (*MATa his3D1 leu2 trp1-289 ura3-52 MATá his3D1 leu2 trp1-289 ura3-52*) was purchased from Invitrogen (Carlsbad, CA), DY1838 (*MAT α pep4-3 prb1-1122 HIS*Δ*:pGAL10:GAL4 leu2 trp1 ura3-52*) was kindly provided by D. J. Stillman (University of Utah), BY4741*fet3*Δ *(MATa, ura3-52, leu2-3, 2-112, trp1-1, his3-11, 3–15, ade2-1, can1-100, fet3::HIS3*) was a gift from D. R. Winge (University of Utah). *S. cerevisiae* were grown in synthetic complete (SC) minimal media without uracil (2% glucose or 2% galactose and 1% raffinose for expression studies) or YEPD (1% yeast extract, 2% peptone, 2% glucose). *S. pombe* were grown in SC minimal media without histidine or YES media (0.5% yeast extract, 3% glucose, 0.2% ade, his, leu, ura, lys).

Plasmids

pENTR™/D-TOPO® and pYES-DEST52 were purchased from Invitrogen. Cosmid 106 was the kind gift of the Wellcome Trust Sanger Institute (Cambridge, UK). This cosmid was used to clone SPBC106.11c into pYES-DEST52 using the pENTR™/D-TOPO® cloning kit. PCR

of SPBC106.11c (*plg7*$^+$) with Platinum® *Taq* (Invitrogen) to incorporate into pYES-DEST52 with a V5/6xHis C-terminus tag was generated using forward primer 5'-CAC CGA AAT GGG ATT GGG ATT TTC TTC G and reverse primer 5'-GTA CAT AA T TCT TTC CCA CCC AGG. Mutation of SPBC106.11c serine257 to alanine was made with the QuikChange® II site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) using the primers: 5'-AAT TGA TTG TTG CTG GTC ATG CAT TTG GTG CCG CTA CTT GC and 5'-GCA AGT AGC GGC ACC AAA TGC ATG ACC AGC AAC AAT CAA TT. The V5/6xHis tag of *plg7*$^+$ and *plg7*-S257A was removed using the primers 5'-TCC CTC GCA TGG TAC TAA GG and 5'-GGT CGG CGC GCC CAC CCT TTC ACA TAA TTC TTT CCC AC. The PCR product was cleaved with HindIII and BssHII and inserted into *plg7*$^+$-pDEST52 or *plg7*-S257A-pDEST52 cut with the same enzymes. Empty vector pDEST52 was made by cleaving *plg7*$^+$-pDEST52 with BssHII and SacII, blunted using Klenow fragment polymerase (Promega, Madison, WI), and the blunt ends were ligated with T4 Ligase (Promega). A human PAF acetylhydrolase type 2 (HPAFAH2) cDNA clone was purchased from Origene (Rockville, MD, NM_000437, Clone AB3241_B06) and shuttled into pYES-DEST52 using the pENTR™/D-TOPO® cloning kit with the primers 5'-CAC CCT GGG TCG TTT CTC ATT TCC and 5'-GGA AAT GGC CAG TTG TGC GTA C. The resulting plasmid (HPAFAH2-pDEST52) did not have a V5/6xHis tag. The V5/6xHis tag was added onto HPAFAH2-pDEST52 using the primers: 5'-GGA ATG GAT CCC TTT CCG TC and 5'-GGT CGG CGC GCC CAC CCT TGG AAA TGG CCA GTT GTG CCC GCA GGC TGG AC. The PCR product was then cleaved with BamHI and BssHII and inserted into HPAFAH2-pDEST52 cut with the same enzymes. The Δ*plg7* construct was made using the *his7*$^+$ gene from pEA2 (purchased from ATCC) cut with XbaI and EcoRI and inserted into pCI-neo (Promega). The PCR product of the N-terminus of *plg7*$^+$ using the primers 5'-CCT AGC TAG CGG GAT TGG GAT TTT CTT CG and 5'-CGG AAT TCC GAA AAC CTT TCG CAA CTT C was cut with NheI and EcoRI and inserted into pCI-neo-his7. The PCR product of the C-terminus of *plg7*$^+$ using the primers 5'-TGC TCT AGA TTC CCA CGT GTT TGT TTA TGA and 5'-ATA AGA ATG CGG CCG CAT TCT TTC CCA CCC AGG AAT was cut with XbaI and NotI and inserted into pCI-neo-his7-N-terminus *plg7*$^+$ to make the Δ*plg7* construct. *S. cerevisiae* strains were transformed using the *S.c.* EasyComp™ Transformation Kit (Invitrogen); *S. pombe* was transformed with the YEASTMAKER™ yeast transformation system 2 (BD Clontech, San Jose CA).

Sequence analysis

BLAST analysis comparing SPBC106.11c to human plasma (PLA2g7) and type 2 PAF acetylhydrolase (PAFAH2) used the NCBI website http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi. ClustalW alignment of SPBC106.11c, PLA2g7 and PAFAH2 was done using the default settings at website http://align.genome.jp (CLUSTAL output, BLOSUM weight matrix).

Expression studies

A single colony from transformed *S. cerevisiae* was grown in 5 ml SC-ura (glucose) at 30°C overnight, centrifuged at 400xg for 5 min, washed once with sterile water and resuspended in 25 ml SC-ura (galactose and raffinose). Cells were grown overnight at 30°C, centrifuged and resuspended to an optical density (OD) at 550 nm of 100 (GENESYS™ 5, Spectronic) in SDS buffer (1% SDS, 5% glycerol, 125 mM Tris pH 6.8) + Halt™ protease inhibitor cocktail (Pierce, Rockford, IL). Cells were lysed by vortexing with glass beads, boiling for 5 min, then clarified by centrifugation for 2 min at 20,000xg in a microcentrifuge. Twenty µl of clarified lysates were loaded onto a 12% SDS-PAGE gel, transferred to a PVDF membrane (Immobilon™, Millipore, Billerica, MA) and blotted for the V5 epitope (R960, Invitrogen) at 1:2500 and actin (691001, MP Biomedicals, Solon, OH) at 1:1000 dilutions. For activity assays, cells were lysed in 50 mM Tris buffer pH 7.8 with 20 uM E-64, pepstatin A, and 2 mM benzamidine HCl. PAF acetylhydrolase activity assays used 25 µl cell lysates and incubating at 37°C for 15 min with

40 µl 1 nmol [^{3}H]PAF, 5 µl 400 mM DTT and 0.5 µl 0.5 M EDTA. The PAF acetylhydrolase inhibitors Pefabloc SC (Boehringer Mannheim 1429 868, Ingelheim, GE) and methyl arachidonyl fluorophosphonate (MAFP, BIOMOL, Plymouth Meeting, PA, ST 360) were added to lysates at a final concentration of 1 mM and incubated for 30 min at 37°C prior to incubation with ^{3}H-PAF. The reactions were quenched with 50 µl of 10 M glacial acetic acid and excess 0.1 M sodium acetate. Cleaved [^{3}H]acetate was isolated using BAKERBOND™ spe Octadecyl (C_{18}) extraction columns (J.T. Baker, Phillipsburg, NJ). Lysates were normalized by Bradford assay using the Coomassie Protein Assay Kit (Pierce).

Δplg7 generation

S. pombe strain CHP428 was transformed with the Δ*plg7* construct and grown on SC-his media (3% agar) at 30°C. Colonies were replica plated 3 times on SC complete media, then transferred back to SC-his plates to select for stable transformants. Colonies were screened for correct insertion of construct by colony PCR as described previously [23,24]. Briefly, a colony was resuspended in 10 uL zymolyase mixture (2.5 mg/ml zymolyase (Sigma, St. Louis, MO), 1.2 M sorbitol, 0.1 M sodium phosphate, pH 7.4) and incubated for 15 min at 37°C. Two µl of the spheroplasts were used in a 25 µL PCR reaction with *Taq* DNA polymerase (Promega) using primers: d1 5' CCT TAA TCA TCG CGG TCC TA with d2 5' AGG CTT TTT CCA TCT CCT GA, or d3 5' TG CAA ACG AAA GAT TCA CA with d4 5' AAA ACG AAC CGG CTA AAA GG to detect successful integration. The d4 primer with the Y5 primer (5' TCT CGC GAT ACT GAA CAA CG) was used to detect the presence of wild type *plg7*$^+$.

Supplementation and oxidation assays

S. cerevisiae were grown for 2 days in 25 ml SC-ura (galactose and raffinose) + 1% Igepal at 25°C with shaking. Cells were recovered by centrifugation at 400xg, washed once with sterile water and a portion of the cells resuspended to an OD (550 nm) of approximately 0.2 in 19 ml of SC-ura (galactose and raffinose) + 1 ml filter sterilized 5% Igepal or 1 ml of 20 mM linolenic acid (Sigma) in 5% Igepal. The cells were grown overnight at 25°C shaking, and 1 ml was removed for oxidation detection by fluorescence. Five µl of 2 mM Bodipy® 581/591 C11 (Invitrogen) was added to 1 ml of cells, and incubated rocking at room temperature for 30 min. Cells were collected by centrifugation at 400xg for 5 min and resuspended in 2-(4-morpholino)-ethane sulfonic acid (MES) buffer pH 5.5, 1% glucose to obtain an OD (550 nm) of 1. Two hundred fifty µl were removed as nontreated samples, and the remaining amount treated with a final concentration of 50 µM $CuSO_4$. One hundred twenty five µl of cells were aliquoted in duplicate into a flat-bottomed black 96-well plate and fluorescence was measured in 5 min increments with a 485 nm, 20 nm bandwidth excitation and a 528 nm, 20 nm bandwidth emission filter at a sensitivity of 50 in a Synergy HT fluorimeter (BIO-TEK®). For microscopy, cells were allowed to adhere to 8-well chamber coverslips coated with 1 mg/ml poly-L-lysine (Sigma). Thirty µl of cells were incubated on coverslips for 1–2 min, and washed once with MES buffer. Cells were treated, or not, with a final concentration of 50 µM $CuSO_4$ and visualized after 60 min by confocal microscopy using a 488 nm Argon laser, 520/10 nm emission filter (60X 1.42NA oil objective on an FV300 Olympus IX81 microscope).

Phospholipid mass spectrometry

Hexadecyl azelaoyl choline phosphoglyceride (AzPC; Cayman Chemical Co., Ann Arbor, MI) was quantified in *S. cerevisiae* expressing Plg7p or its S257 mutant by supplementing the cells with linolenate and then exposing the cells to Cu^+ for 0, 30 or 60 min as above. The cell wall was digested with zymolase, the spheroplasts recovered by centrifugation, lysed by dounce homogenizer and their lipids extracted [25]. Polar phospholipids were resolved from free fatty acids with aminopropyl extraction columns and the recovered PAF-like lipids were

reconstituted in methanol for analysis by LC/ESI/MS/MS [26] in comparison to [^{2}H]PAF as described [11].

Viability assays

S. cerevisiae were grown in mock or linolenic acid supplemented media as with the oxidation detection assay and collected the next day at 400xg for 5 min. Pellets were washed twice with sterile water before the cell pellets were resuspended in water. Suspensions were added to 20 ml of sterile MES buffer to an OD (550 nm) of 0.6–0.8 (culture ODs were matched within 1%). After 10 min equilibration, a portion was removed for nontreated control, and the rest of the cells were treated with 50 μM $CuSO_4$. Aliquots were removed after 15 and 60 min, diluted 10-fold serially and 7 μl of each dilution was spot plated on YEPD (3% agar). Cells were grown for 4 days at room temperature. For cell counting, 100 μl of 10^{-3} and 10^{-4} cell dilutions were plated on YEPD media and grown for 4 days at room temperature. Viability was determined as the fraction of cells that formed colonies after treatment with $CuSO_4$ compared to untreated cells.

RESULTS

S. pombe expresses an enzymatically active group VII PAF acetylhydrolase homolog

BLAST analysis against the human plasma PAF acetylhydrolase (PLA2g7) revealed a putative PAF acetylhydrolase open reading frame in the *S. pombe* genome. This open reading frame, SPBC106.11c, has 25% identity and 44% similarity to PLA2g7 (Fig. 1) and 24% identity and 41% similarity to human type 2 PAF acetylhydrolase (gene name PAFAH2). SPBC106.11c does not share homology with the N-terminus of PLA2g7, including the 17-amino acid sequence predicted to be a secretion signal [27]. ClustalW analysis reveals the consensus lipase sequence GXSXG is conserved in the yeast and human isoforms (Fig. 1). Also conserved are the amino acids Ser257, Asp291, and His368, which were previously shown to be critical for enzyme activity (Ser273/236, Asp296/259, His351/315 for PLA2g7 and PAFAH2 respectively) [28,29]. These residues form a catalytic triad characteristic of esterases and lipases, and retention of all the essential residues suggest SPBC106.11c might encode a functional enzyme. Based on the information below, we have renamed the open reading frame SPBC106.11c as *plg7*, and hence the encoded protein as Plg7p, to emphasize the similarity of this gene to mammalian group VII phospholipases A_2 [30].

Δplg7 S. pombe have decreased, but residual, PAF acetylhydrolase activity

We targeted the single homolog of PAF acetylhydrolase in *S. pombe* by sequence analysis to generate a knockout of *plg7*$^+$. A plasmid construct was generated with approximately 200 bp of the 5' and 3' ends of *plg7*$^+$ flanking the *his7*$^+$ gene from pEA2 (Fig. 2A). Transformation of strain CHP428 (ATCC# 201399) with the knockout construct yielded colonies that were selected for stable incorporation of the plasmid. Successful integration was analyzed in 85 colonies using PCR primers internal to the his7$^+$ gene and external to *plg7*$^+$ for detection of Δ*plg7* (Fig. 2A). Generation of Δ*plg7* was successful in 5 out of 85 colonies, illustrated in the PCR results from one of the colonies (Fig. 2B). Appropriate sized bands were present in Δ*plg7* but not in wild type cells; the opposite was true for detection of intact *plg7*$^+$ (Fig. 2B) and confirmed by sequence analysis (data not shown). These results show the previous inability to recover an insertional mutant at this locus by the *S. pombe* genome project was not due to a lethal defect caused by an absence of Plg7p.

We found that Δ*plg7* displayed reduced PAF hydrolytic activity compared to wild type *S. pombe*, but that half of this activity remained after *plg7*$^+$ deletion (Fig. 2C). Accordingly, the selective PAF acetylhydrolase inhibitor Pefabloc SC [31] reduced wild type PAF hydrolysis by 50%, and this residual hydrolytic activity in Δ*plg7* was not sensitive to Pefabloc inhibition.

Similar results were obtained in experiments using the PAF acetylhydrolase inhibitor [32] methyl arachidonoyl fluorophosphonate (MAFP; data not shown). A significant change in phenotype of Δ*plg7* versus wild type was not observed using various challenges (heavy metal, oxidative stress, and temperature). *S. pombe* therefore constitutively express at least two enzymes with phospholipase activity against short chain phospholipids, with only Plg7p containing a catalytically essential serine residue.

plg7^{+} expressed in *Saccharomyces cerevisiae* encodes a functional PAF acetylhydrolase

S. cerevisiae are as distantly related to *S. pombe* as humans are to either yeast, and a search of the *S. cerevisiae* genome revealed no homologous sequence to plg7 or mammalian PAF acetylhydrolase genes (not shown). Accordingly, the lysate of wild type *S. cerevisiae* INVSc1 hydrolyzed PAF significantly less well (16±6 vs. 196±54 dpm/h/μg) than a lysate of a wild type *S. pombe* (CHP 428), suggesting this lower background would reveal whether plg7p is active and has a role in protecting cells against oxidative stress. We cloned *plg7* and a mutant (Ser257Ala), which replaces what is predicted to be the active site serine, into the *S. cerevisiae* pYES-DEST52 vector that introduced V5 and 6xHis tags at the C-terminus. We transformed these clones into *S. cerevisiae* strain DY 1838 (Fig. 3A) and found both clones expressed proteins at the predicted size of 55 kDa. The locus SPBC106.11c therefore is a functional gene, now *plg7*$^{+}$, encoding the protein Plg7p.

We determined whether Plg7p is a catalytically active enzyme, but found that the chimera Plg7p/V5/6xHis in crude lysates or after purification on a chelation affinity column did not hydrolyze PAF (data not shown). Similarly, expression constructs with the V5 and 6xHis tags moved to the amino terminus were without activity, as were constructs containing Protein A tags. However, expression of native Plg7p without modified termini in the *S. cerevisiae* strain INVSc resulted in increased PAF hydrolytic activity compared to empty vector (Fig. 3B). The activity levels of Plg7p were similar to the human intracellular type 2 PAF acetylhydrolase expressed under the same conditions. We also found that the mutant Plg7p-S257A had little or no activity suggesting the conserved serine residue is essential for catalysis by Plg7p. We tested whether targeted covalent modification of the active site serine by Pefabloc SC [31], a modified sulfonyl fluoride that inhibits PLA2g7 and PAFAH2 encoded enzymes, or methyl arachidonyl fluorophosphonate [32] would inhibit Plg7p. We found that each reagent greatly reduced, but did not abolish, Plg7p and HPAFAH2 activity in crude lysates (Fig. 2C). We attribute the residual activity to endogenous serine- and Ca^{++}- independent esterolytic activity of unknown origin.

We tested the ability of Plg7p to hydrolyze the synthetic oxidized phospholipid azelaoyl phosphatidylcholine. This cytotoxic phospholipid arises from oxidative fragmentation of the 9,10 bond of *sn*-2 oleoyl, linoleoyl and linolenoyl residues, and is the prominent phospholipid fragmentation product of phospholipids containing these residues [33]. We found that a crude *S. cerevisiae* Plg7p lysate hydrolyzed azelaoyl phosphatidylcholine (0.6 ± 0.1 nmol/mg/h), while the S257 mutant had little activity (0.03 ± 0.02 nmol/mg/h) in this assay.

S. cerevisiae expressing Plg7p are protected against oxidative death

Over-expression of type II PAF acetylhydrolase protects mammalian cells against oxidative stress and death [16,17], potentially because its short-chained phospholipid substrates [13, 34,35] either initiate apoptosis via the mitochondria-dependent apoptotic caspase cascade [11] or from Ca^{++} overload subsequent to PAF receptor activation [36]. To avoid the complication of receptor signaling in mammalian cells, we used the yeast transition metal-dependent oxidation model developed by Avery et al. [37] to test whether Plg7p expressed in *S. cerevisiae* conferred protection from oxidant injury. We found (Fig. 4A) that supplementation of the wild type *S. cerevisiae* strain INVSc1 with linolenic acid ($C_{18:3}$) caused

a time dependent increase in fluorescence from Bodipy® 581/591 C11, a membrane-intercalated indicator lipid that shifts its fluorescence in response to oxidation. The addition of the transition metal Cu^+ alone to INVSc1 did not initiate membrane oxidation, but a combination of $C_{18:3}$ supplementation followed by exposure to Cu^+ increased the fluorescence over that produced by $C_{18:3}$ supplementation alone.

We used confocal microscopy to image the oxidative environment detected by the Bodipy® 581/591 C11 dye, and found punctate intracellular staining with a diffuse halo closely associated with the plasma membrane (Fig. 4B). Remarkably, there was great variation among these genetically identical cells in the level of membrane oxidative stress that individual cells experienced, with some cells apparently being completely unaffected by Cu^+ exposure. *S. cerevisiae* peroxiredoxins metabolize hydroperoxides [38] and lipid hydroperoxides [39] to reduce oxidative stress that can vary with the age of the culture [40], and previous work [41] shows there is significant cell cycle variation in oxidant sensitivity in this system with a 5-fold variation in a critical Cu^+ resistance protein with cell cycle and cell replicative age [42,43]. Alternatively, we considered that intracellular Cu^+ might be limiting, and hence limit oxidant stress in some of the cells, because Cu^+ limits its own uptake [44]. We therefore used a *fet3*Δ strain that displays extreme sensitivity to Cu^+ due to abnormalities in Fe/Cu homeostasis [41,45]. The *fet3*Δ cells displayed increased levels of Bodipy® 581/591 C11 fluorescence in an oxidized lipid environment when supplemented with $C_{18:3}$ and treated with Cu^+ (Fig. 4C), but still these cells were heterogeneous in their response to oxidative stress.

We reduced the effect of the variation of oxidative stress among individual cells [41,43] by plating and immobilizing INVSc1 to assess colony number, an approach where the background arising from cells not subjected to oxidative stress is quantifiable. We treated INVSc1 with Cu^+ for 0, 10 or 60 min in culture and then plated serial dilutions to immobilize the cells. Exposure to Cu^+ reduced the number of viable cells in a time-dependent way (Fig. 5A), and cells supplemented with $C_{18:3}$ prior to this exposure displayed enhanced sensitivity to the transition metal. Introduction of a *plg7*$^+$ expression plasmid into copper challenged, $C_{18:3}$-supplemented INVSc1 suppressed Cu^+ toxicity (Fig. 5B). Quantitation showed the presence of *plg7*$^+$ resulted in an average 6-fold increase in viability compared to no plasmid ($n=3$; $p < 0.05$).

We challenged $C_{18:3}$-supplemented *fet3*Δ cells expressing Plg7p with Cu^+ and found that cells also had increased viability compared to cells expressing the inactive Plg7p-S257A (Fig. 5C). Similar levels of oxidation were detected by Bodipy® 581/591 C11 fluorimetry in both Plg7p and Plg7p-S257A expressing cells (not shown), so each strain encountered the same level of oxidative stress. We observed similar results in *fet3*Δ expressing HPAFAH2 compared to cells with empty vector (Fig. 5D). These results show that *S. cerevisiae* cells are sensitive to oxidative death when supplemented with a polyunsaturated fatty acid, and that viability can be enhanced by over-expression of active Plg7p.

Discussion

The genome of the yeast *S. pombe*, a fission yeast distantly related to both animals and the budding yeast *S. cerevisiae*, has been fully sequenced. This revealed it to be the smallest sequenced eukaryotic genome, yet it contains genes with introns, genes homologous to human disease genes, and clusters of genes regulating cell function related to those found in higher eukaryotes [22]. The Gene Ontology Project (http:www.geneontology.org) infers that SPBC106.11c should be a member of the phospholipase family, but the function of this locus remained undefined because it is among the ~10% of the potential genes that could not be ablated in a high throughput insertional mutagenesis screen [46]. Expression tagging shows

the SPBC106.11c locus is among the several hundred genes induced in response to H_2O_2 and Cd^{++} exposure [47], and so may play a cytoprotective role.

We cloned the *S. pombe* open reading frame SPBC106.11c and expressed it in *S. cerevisiae*, which lacks confounding homologous sequences, to find that this locus is a functional gene encoding an enzymatically active member of the group VII phospholipase A_2 family [30]. We found that, like the mammalian group VII enzymes, the *S. pombe* member Plg7p was Ca^{++}-independent and it required the Ser residue in the midst of the GXSXG lipase motif for catalysis. Additional evidence that Plg7p contains essential features of the mammalian homolog is that the selective serine-directed inhibitors Pefabloc SC and methyl arachidonyl fluorophosphonate that irreversibly inhibit human plasma PAF acetylhydrolase also inhibited Plg7p.

We ectopically expressed Plg7p in *S. cerevisiae* to test its function in a system where lipid peroxidation is lethal, but yet also does not employ PAF as a signaling entity. Polyunsaturated fatty acids can be incorporated into *S. cerevisiae* lipid by supplementing their growth media with linoleate, rendering the cells more susceptible to Cu^{+}-induced lipid peroxidation and cell death [48]. We found by serial plating that *S. cerevisiae* expressing Plg7p were protected against this oxidative stress, and that this protection required the deduced active site serine. We found a similar level of protection when we expressed the mammalian phospholipase A_2 group VII enzyme PAFAH2. The results from this reduced system indicate that membrane lipid peroxidation and death can be suppressed after an oxidative attack has been initiated, and that both the human and *S. pombe* enzyme act in the same way to hydrolyze structurally damaged phospholipids and maintain viability.

The group VII family of phospholipases A_2 was originally described in studies of PAF catabolism [49], but their role in inflammation or homeostasis in an oxidizing environment has not yet been fully defined. One family member, the liver type II gene (PAFAH2), recently has been genetically ablated to discover that the mice develop and behave normally, but that repair of liver after CCl_4 damage is delayed [50]. In contrast, genetic ablation of a *C. elegans* group VII phospholipase A_2 ortholog interfered with normal epithelial morphogenesis [21]. Over-expression studies show the mammalian enzymes protect against complex disease and apoptosis [51–56], protect skin epidermis from UVB irradiation [17], and protect transfected CHO cells from exogenous peroxides [16]. However, whether the process(es) affected by the PAF acetylhydrolases in these studies derive from the signaling role of PAF or from the deleterious effect of oxidized phospholipids, and hence whether the relevant substrate(s) of the enzymes are PAF or oxidized phospholipids, cannot be easily be distinguished in mammalian systems that use PAF as a signaling molecule.

The natural habitat of the budding and fission yeasts *S. cerevisiae* and *S. pombe*, respectively, are largely undefined. *S. cerevisiae* inhabits the skin of grapes, likely a consequence of cultivation, but also exists as a free-living organism in soil beneath broad-leafed trees [57]. These free-living cells encounter unknown and uncontrollable environments far different from that established in laboratories. The lipid composition of *S. cerevisiae* grown in culture is simple because these cells lack a dehydrogenase other than the Δ9 desaturase that generates pamitoleate and oleate [2]. Defined culture media contains no polyunsaturated fatty acids, and so neither do laboratory-grown yeast [58]. In contrast, *S. cerevisiae* [4,5] and *S. pombe* [6] grown outside the laboratory do contain polyunsaturated fatty acids because polyunsaturated fatty acids are abundant lipids in plants and their seeds [59]. The must of crushed grapes contains micromolar levels of free linoleate ($C_{18:2}$)—the primary polyunsaturated fatty acid of plants—and an equivalent amount esterified as linoleoyl-phosphatidylcholine [60]. Depending on the variety [61], crushed grapes contain a third to half as much linolenate ($C_{18:3}$) as linoleate. Ethanol generated by the fermentation of grape must, malt wort, and cereal flour reduces yeast viability [62]. This ethanol-induced loss of viability is ameliorated by

increasing membrane fluidity by capturing environmental polyunsaturated fatty acids [62], so free living yeast will contain oxidizable polyunsaturated fatty acids.

Yeast also encounter oxidized polyunsaturated fatty acids, oxidized polyunsaturated phospholipids, and phospholipid cleavage products with short and functionalized *sn*-2 residues in their environment. Plants contain 9-lipoxygenase (Lox1) and 13-lipoxygenase (Lox2) that form free hydroperoxy fatty acids, but the preferred substrates of both enzymes are esterified fatty acyl esters, neutral lipids and phosphatidylcholine [63]. Accordingly, the vast majority of these enzymatic lipid hydroperoxide products in plants are esterified [64], and esterified fatty acyl hydroperoxides can account for up to 6% of the total polyunsaturated fatty acyl pool [65,66].

Plants also contain a fatty acid hydroperoxide lyase that cleaves the carbon backbone of free and esterified [67] hydroperoxy lipids adjacent to the hydroperoxy function to form two aldehyde-bearing fragments [65]. Cleavage of free or esterified 9-hydroperoxy fatty acids by this enzyme generates precursors, derived from the ω-end of the fatty acid, used in the synthesis of oxilipins and volatile organic molecules that signal the plant, its neighbors, and beneficial insects [68–70]. This cleavage simultaneously generates phospholipids with a 9-carbon aldehyde esterified at the *sn*-2 position that can then be oxidized to the acid, which are the azelaoyl phosphatidylcholines. *S. pombe* and *S. cerevisiae* internalize the oxidatively- and enzymatically-generated short-chained phospholipids they encounter in their environment in a process requiring ATP cassette proteins and the interacting gene product lem3p/ROS3p [9, 71]. This provides a ready-made source of ethanolamine and choline headgroups [72,73], but also means they must be prepared to appropriately metabolize cytotoxic short-chained phospholipids. This, we now show, is accomplished by Plg7p.

Even though *S. pombe* has one of the smallest eukaryote genomes—encoding just 4,824 proteins [22]—among this handful of genes is one that codes for an enzyme that specifically hydrolyzed oxidized phospholipids and protects against oxidative cell death. Actually, our data suggest that at least one other activity is also present that degrades or remodels incoming phospholipid oxidation products. This metabolic function is retained by the homologous group VII mammalian phospholipases A^2, suggesting the original, and potentially remaining, purpose of PAF acetylhydrolases is catabolism of phospholipid oxidation products.

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Abbreviations

HAzPC, hexadecyl azelaoyl phosphatidylcholine; MAFP, methyl arachidonoyl fluorophosphonate; PAF, Platelet-activating Factor.

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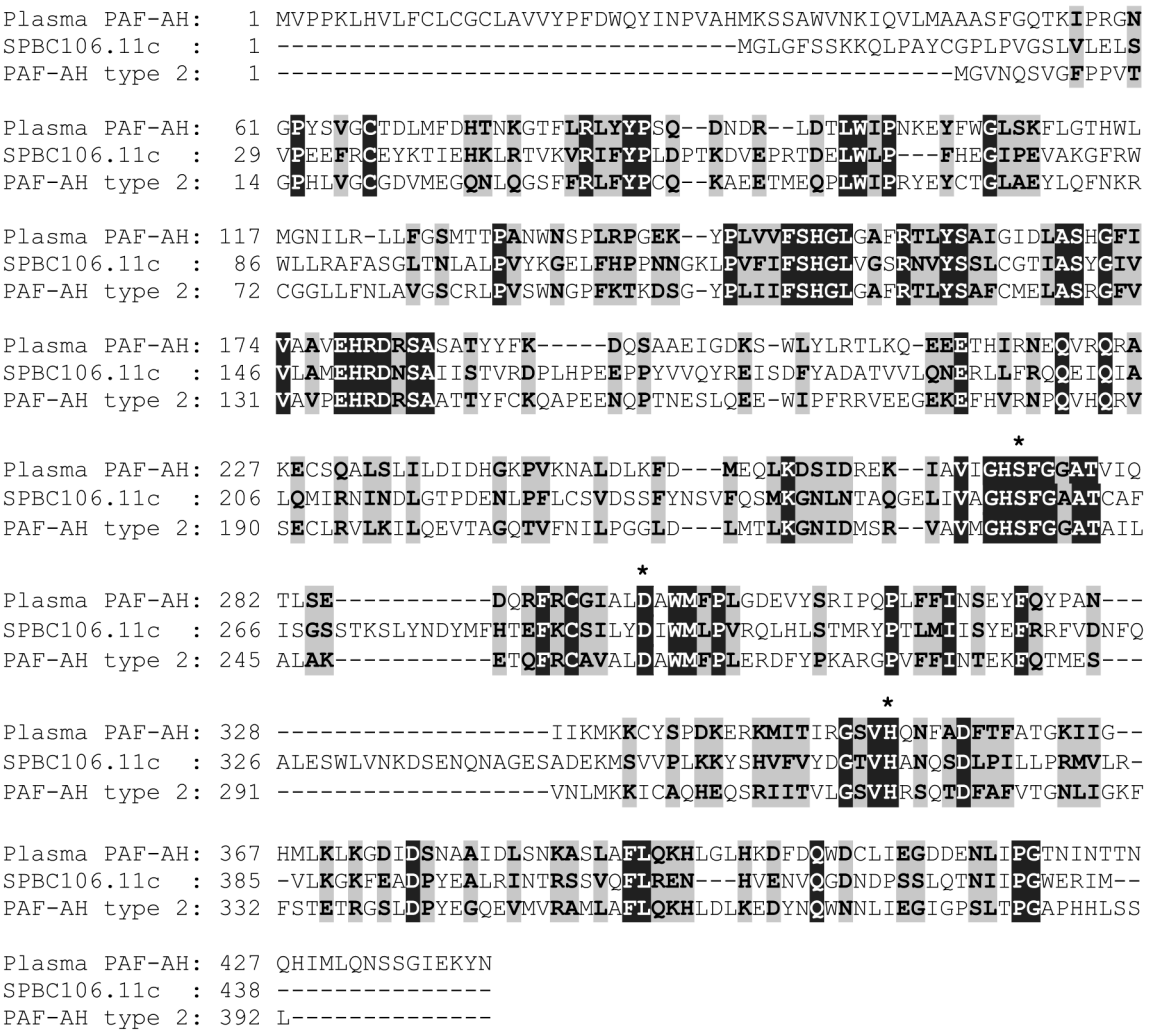

Figure 1. The *S. pombe* genome contains a sequence homologous to the human plasma and type 2 PAF-acetylhydrolases

Sequence alignment of the *S. pombe* locus SPBC106.11c, human plasma PAF acetylhydrolase (PLA2g7) and type 2 PAF-acetylhydrolase (HPAFAH2) using ClustalW, BLOSUM program. Characters highlighted in black are exact matches, characters highlighted in gray are similar in identity. Amino acids marked (*) are essential for enzyme activity.

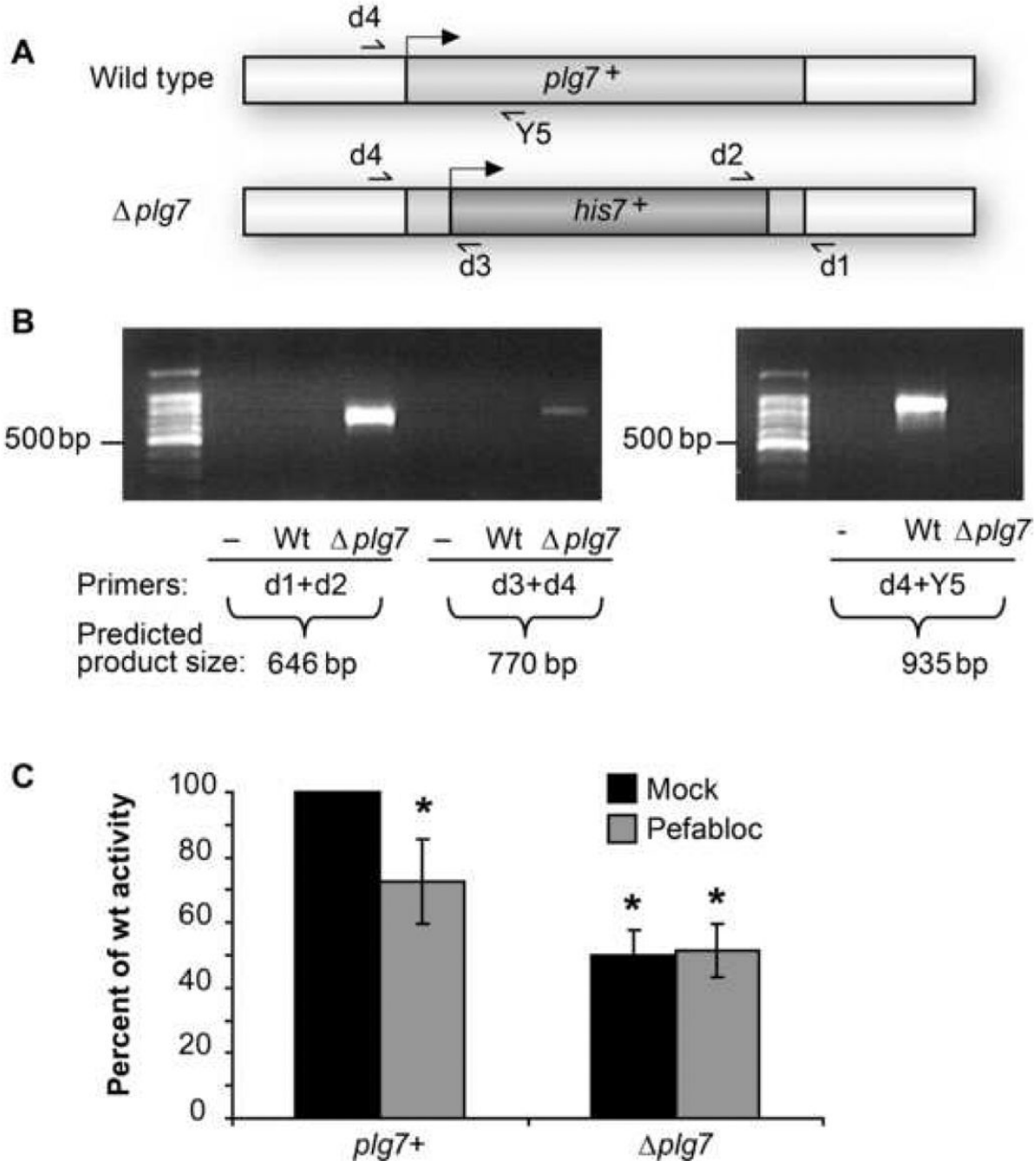

Figure 2. Δ*plg7* cells have decreased but residual PAF-AH activity

(A) Gene deletion scheme to replace *plg7*$^+$ with *his7*$^+$ in *S. pombe* strain CHP428. Approximately 200 base pairs of both ends of *plg7*$^+$ were added to a construct with the *his7*$^+$ gene. 5' ends of the genes are indicated with black arrows. (B) PCR analysis of *plg7*$^+$::*his7*$^+$ gene replacement in wild type (Wt) and Δ*plg7* cells using genomic and *his7*$^+$ specific primers; (−) = negative PCR reaction. PCR detecting intact genomic *plg7*$^+$ in wild type and Δ*plg7* cells. (C) PAF-AH activity assay of OD (550 nm) normalized wild type and Δ*plg7* cells, lysed and pre-treated with mock or 100 μM Pefabloc SC. Values are expressed relative to mock treated wild type lysates, n=2 in duplicate * = P<0.001 vs *plg7*$^+$ (One Way Anova, Student t-test).

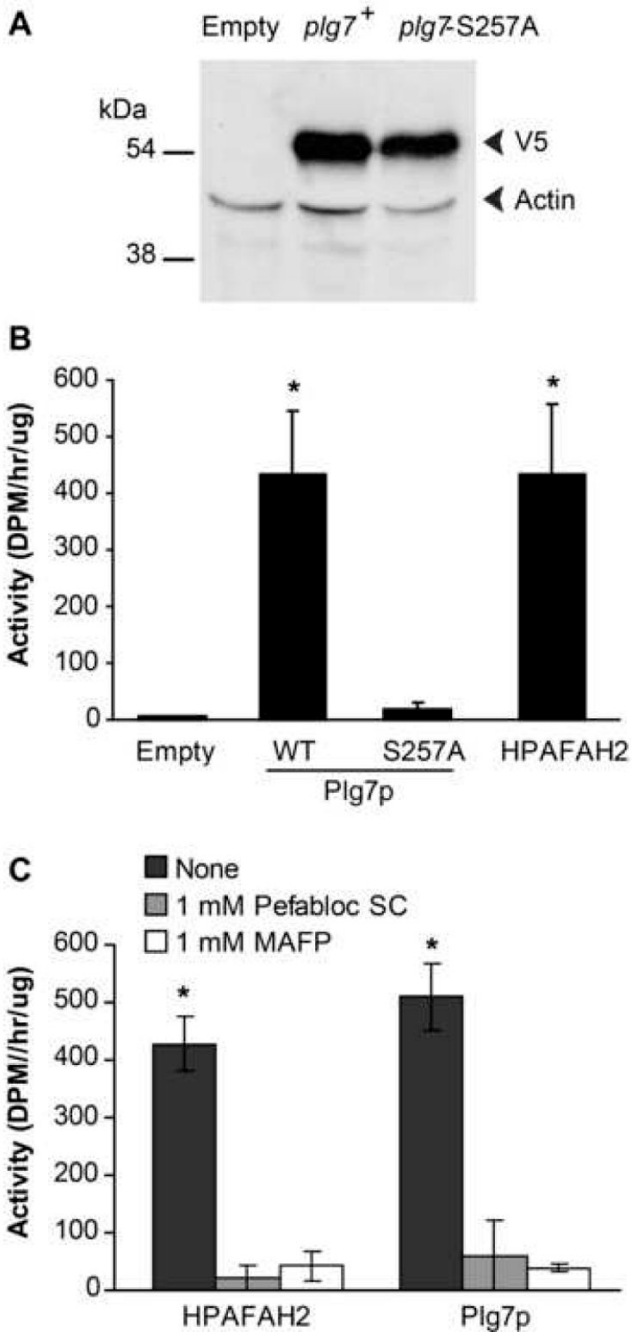

Figure 3. *plg7*$^{+}$ encodes a protein with Ser257 dependent PLA_2 activity

(A) Western blot of lysates from empty vector, *plg7*$^{+}$, or *plg7*-S257A-pDEST52 transformed DY 1838 cells using a combination of antibodies against the V5 epitope tag (upper band) and a diluted antibody to actin (lower band). (B) PAF-AH activity assay of cell lysates from empty vector, non-tagged *plg7*$^{+}$, *plg7*-S257A, or human PAFAH2-pDEST52 transformed INVSc1 cells, normalized by total protein. (C) PAF-AH activity assay of cell lysates from non-tagged *plg7*$^{+}$ or human PAFAH2-pDEST52 DY 1838 transformed cells, treated with 1 mM Pefabloc SC or MAFP. Results for B: n=4, in duplicate * = P<0.05 vs empty, Plg7p-S257A; results for C: n=3, in duplicate * = P<0.05 vs Pefabloc SC, MAFP (One Way ANOVA, Tukey Test).

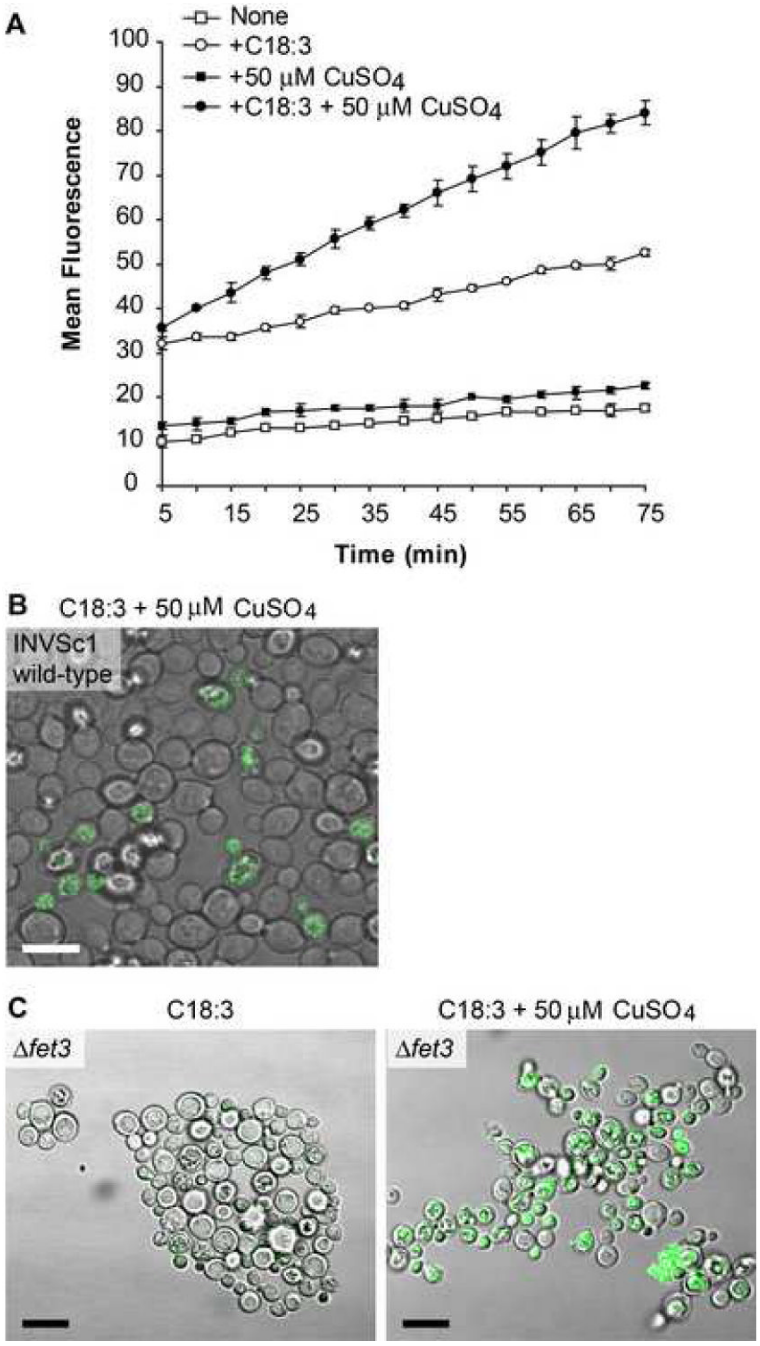

Figure 4. *S. cerevisiae* supplemented with linolenic acid is sensitive to oxidation

(A) INVSc1 wildtype cells loaded with the lipophilic dye Bodipy® 581/591 C11 that fluoresces after oxidation were examined by fluorimetry using 488/20 nm bandwidth excitation, 528/20 nm bandwidth emission in a representative experiment done in duplicate. Key: Non treated (open square), copper treated (filled square), $C_{18:3}$ supplemented (open circle), and $C_{18:3}$ supplemented and copper treated (filled circle). (B) INVSc1 strain was supplemented with $C_{18:3}$, loaded with Bodipy® 581/591 C11, and treated with 50 µM $CuSO_4$ for 60 min. Cells were visualized by confocal microscopy using a 488 nm excitation laser. (C) *fet3*Δ cells supplemented with $C_{18:3}$ and loaded with Bodipy® 581/591 C11 were visualized after 60 min of mock or 50 µM $CuSO_4$ treatment. Scale bar = 10 µm.

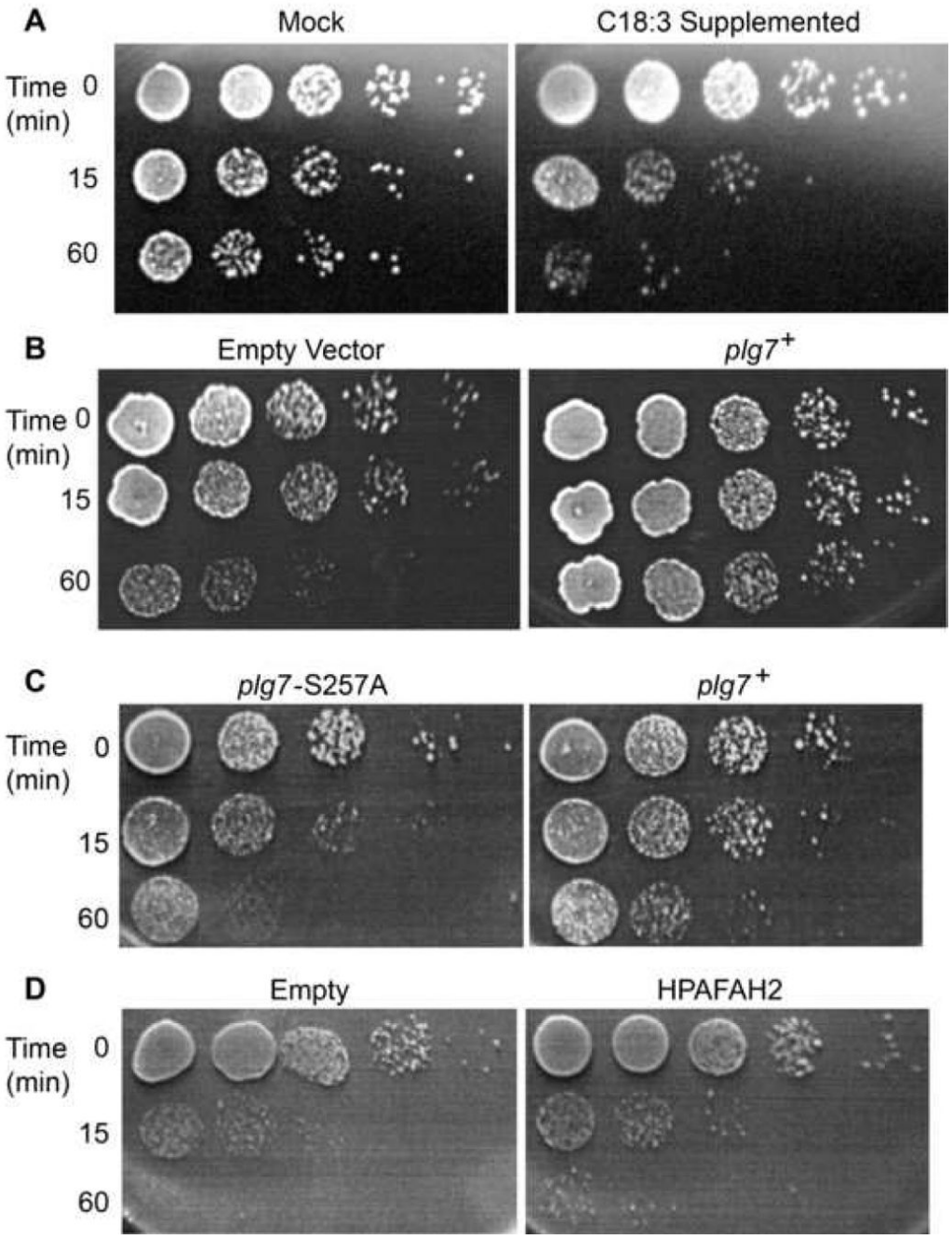

Figure 5. *plg7*$^{+}$ protects *S. cerevisiae* against oxidative stress

(A) Viability assay of INVSc1 cells −/+ $C_{18:3}$ supplementation, treated with 50 μM $CuSO_4$ for 0, 10 or 60 min, diluted 10-fold serially and plated on YEPD plates. (B) $C_{18:3}$ supplemented INVSc1 cells were transformed with empty vector or *plg7*$^{+}$pDEST52 and challenged with 50 μM $CuSO_4$ for the stated times, then serially diluted and plated to test viability. (C) Viability assay using *fet3*Δ cells transformed with *plg7*$^{+}$, *plg7*-S257A. (D) HPAFAH2-pDEST52, or empty vector, supplemented with 1 mM $C_{18:3}$, treated with 50 μM $CuSO_4$, serially diluted and grown on YEPD plates for 4 days at room temperature.