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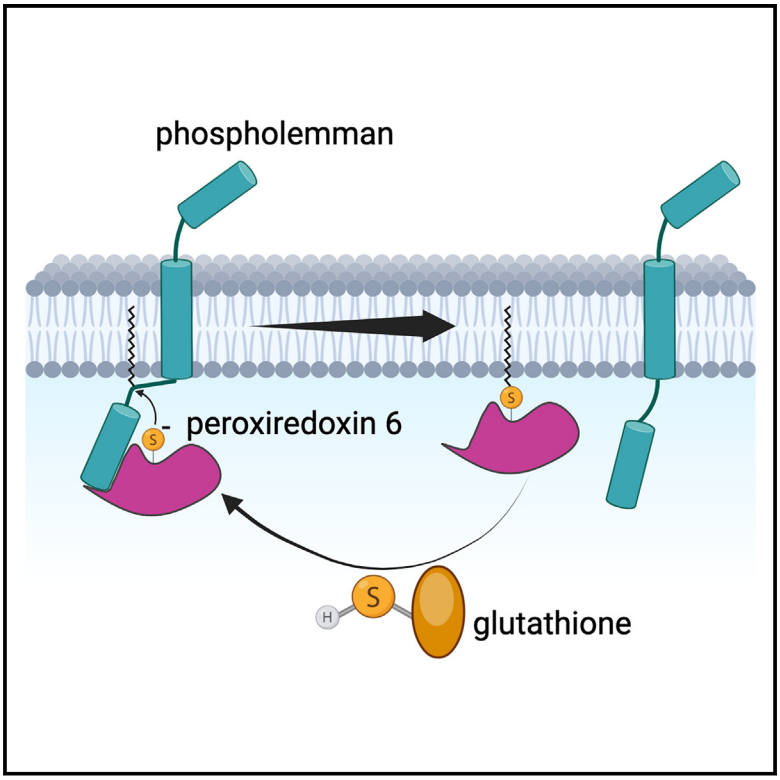
Glutathione-dependent depalmitoylation of phospholemman by peroxiredoxin 6

Graphical abstract



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In brief

The activities and cellular localizations of membrane proteins are frequently controlled by palmitoylation, a reversible covalent post-translational modification. Here, Howie et al. have shown that peroxiredoxin 6 is a novel thioesterase that removes palmitate from the acylated protein phospholemman in a glutathione-dependent mechanism, conferring redox sensitivity on Na pump activity.

Highlights

- Identify peroxiredoxin 6 (Prdx6) as a thioesterase
- Protein depalmitoylation by Prdx6 occurs via its catalytic cysteine
- Phospholemman depalmitoylation by Prdx6 is glutathione dependent
- Prdx6 thioesterase activity confers redox sensitivity on the Na pump

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Glutathione-dependent depalmitoylation of phospholemman by peroxiredoxin 6

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SUMMARY

Phospholemman (PLM) regulates the cardiac sodium pump: PLM phosphorylation activates the pump whereas PLM palmitoylation inhibits its activity. Here, we show that the anti-oxidant protein peroxiredoxin 6 (Prdx6) interacts with and depalmitoylates PLM in a glutathione-dependent manner. Glutathione loading cells acutely reduce PLM palmitoylation; glutathione depletion significantly increases PLM palmitoylation. Prdx6 silencing abolishes these effects, suggesting that PLM can be depalmitoylated by reduced Prdx6. *In vitro*, only recombinant Prdx6, among several peroxiredoxin isoforms tested, removes palmitic acid from recombinant palmitoylated PLM. The broad-spectrum depalmitoylase inhibitor palmostatin B prevents Prdx6-dependent PLM depalmitoylation in cells and *in vitro*. Our data suggest that Prdx6 is a thioesterase that can depalmitoylate proteins by nucleophilic attack via its reactive thiol, linking PLM palmitoylation and hence sodium pump activity to cellular glutathione status. We show that protein depalmitoylation can occur via a catalytic cysteine in which substrate specificity is determined by a protein-protein interaction.

INTRODUCTION

The reversible acylation of cysteine residues of proteins via a thioester bond between the sulfhydryl side chain and the fatty acid palmitate (palmitoylation) regulates membrane association, subcellular location, trafficking, turnover rate, and enzymatic activity of both peripheral and integral membrane proteins.[1] Palmitoylation is achieved enzymatically via the activity of a family of transferase enzymes that have a zinc-finger-containing Asp-His-His-Cys (zDHHC) motif in their active site.[2,3] To date, relatively few palmitate-removing thioesterases have been identified: known depalmitoylases include acyl-protein thioesterase 1 (APT1),[4,5] APT2,[4,6] palmitoyl-protein thioesterase 1 (PPT1),[7] and certain members of the αβ-hydrolase (ABHD) serine hydrolase family of enzymes.[8,9] The discoveries that ABHD17 depalmitoylates N-ras[10] and post-synaptic density protein 95 (PSD-95)[9] and that ABHD10 depalmitoylates Prdx5[11] suggest that the breadth of depalmitoylating enzymes is much greater than has been appreciated to date. Although the palmitoylation of most proteins turns over slowly, some proteins are rapidly palmitoylated and depalmitoylated.[8]

The type 1 integral membrane protein phospholemman (PLM) regulates the ubiquitous sodium/potassium ATPase (Na pump) in excitable tissues such as cardiac muscle by modifying its substrate affinity and maximum transport rate.[12] Phosphorylation of PLM by protein kinase A (PKA) and PKC activates the pump[13]; palmitoylation of cysteines adjacent to the lipid bilayer by zDHHC5 inhibits the pump.[14–16] Although PLM phosphorylation enhances its palmitoylation,[14] the mechanisms underlying PLM depalmitoylation remain undetermined.

In this study, we have shown that PLM and peroxiredoxin 6 (Prdx6) physically interact in cardiac myocytes. Prdx6 catalyzes the reduction of hydrogen peroxide, fatty acid hydroperoxides, and phospholipid hydroperoxides[17] in a two-step reaction in which the Prdx6 active-site nucleophilic cysteine is first oxidized and then regenerated using glutathione.[18] Furthermore, Prdx6 has phospholipase A2 activity and can bind phospholipids.[19] Here, we report that the Prdx6 active-site cysteine can also remove palmitate from PLM by nucleophilic attack of the thioester bond prior to the sulfhydryl group being regenerated in a glutathione-dependent manner. Hence, Prdx6 links the palmitoylation status of PLM to the redox (glutathione) status of the cell.

RESULTS

PLM and Prdx6 physically associate in ventricular muscle

Previously, using tandem mass spectrometry, we identified numerous proteins that co-immunoprecipitate with the cardiac Na pump complex.[20] Apart from the Na pump α subunit, relatively few of these proteins interacted directly with PLM. We sought to identify PLM-interacting proteins by treating

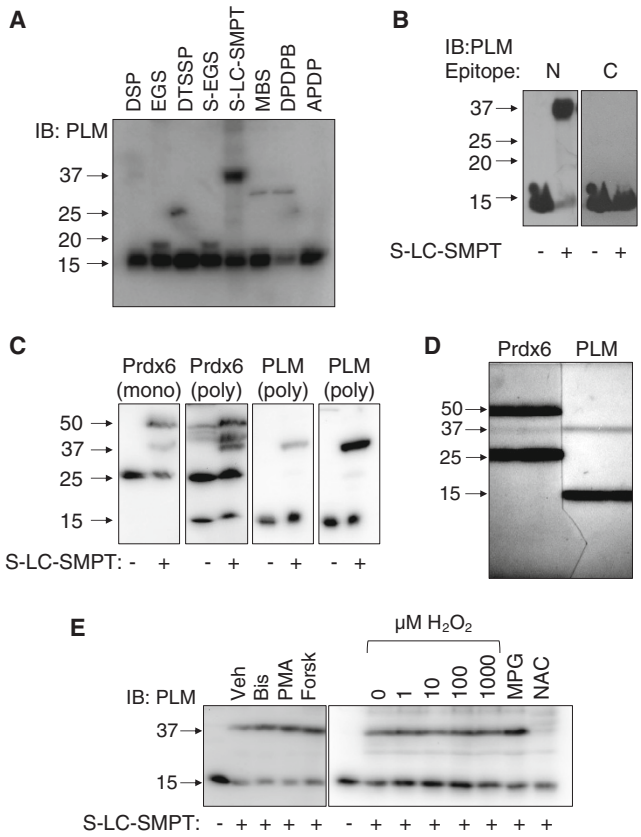

Figure 1. Interaction between PLM and Prdx6 in rat ventricular myocytes

(A) Sulfo-LC-SMPT (sulfosuccinimidyl 6-[α-methyl-α-(2-pyridyldithio)toluamido] hexanoate; 5 mM) quantitatively crosslinked PLM to a 20–25 kDa protein (electrophoretic mobility of PLM 15 kDa, electrophoretic mobility of crosslinked adduct 37 kDa). DSP (dithiobis[succinimidyl propionate]; 2 mM), EGS (ethylene glycol bis[succinimidylsuccinate]; 2 mM), DTSSP (3,3′-dithiobis[sulfosuccinimidylpropionate]; 2 mM), S-EGS (ethylene glycol bis[sulfosuccinimidylsuccinate]; 2 mM), S-LC-SMPT (5 mM), MBS (*m*-maleimidobenzoyl-N-hydroxysuccinimide ester; 1 mM), DPDPB (1,4-Di-[3'-(2′-pyridyldithio)propionamido] butane; 0.8 mM), and APDP (N-[4-(*p*-azidosalicylamido)butyl]-3-(2-pyridyldithio) propionamide; 1 mM).

(B) Crosslinking masks the epitope of an antibody raised against the carboxyl terminus of PLM (C) but not one against the amino terminus (N).

(C) Multiple different Prdx6 and PLM antibodies recognized the 37 kDa crosslinked adduct (mono, monoclonal; poly, polyclonal).

(D) A single crosslinked sample was separated in a single-lane gel. Following transfer, one-half of the membrane was probed for Prdx6 and one-half for PLM. The 37 kDa crosslinked band exactly aligned when the two halves of the membrane were reunited.

(E) Pharmacological treatments that promoted PLM phosphorylation (left) or Prdx6 oxidation (right) had no effect on the PLM-Prdx6 interaction, but glutathione-loading cells with NAC abolished crosslinking between Prdx6 and PLM. Cell treatments: Veh, vehicle (ethanol); Bis, 1 μM bisindolylmaleimide; PMA, 300 nM phorbol-12-myristate-13-acetate; Forsk, 10 μM forskolin, with all applied for 10 min at 35°C; H_2O_2, hydrogen peroxide, applied for 5 min at 35°C; MPG, 1 mM mercaptopropionyl glycine, and NAC, 10 mM N-acetyl cysteine, both applied for 60 min at 35°C. Western blots are representative of at least 5 independent biological replicates.

digitonin-permeabilized rat ventricular myocytes with a series of homo- and hetero-bifunctional crosslinking reagents that had a variety of distances between their reactive groups and then immunoblotting the crosslinking reactions for PLM (Figure 1A). Sulfosuccinimidyl 6-[α-methyl-α-(2-pyridyldithio)toluamido]hexanoate (Sulfo-LC-SMPT; reactive toward sulfhydryls and primary amines; distance between reactive groups: 2 nm) quantitatively crosslinked PLM to a protein whose molecular weight was 20–25 kDa (as the crosslinked adduct had a gel mobility of ca. 37 kDa). The adduct was only recognized in immunoblots with antibodies raised against the extracellular, but not the intracellular, regions of PLM (Figure 1B), suggesting that the interaction between the unidentified protein and PLM occurs in the cytosol and masks the epitope in the PLM intracellular domain that the antibody against the PLM C-tail normally recognizes. We evaluated a variety of detergents (including Triton X-100, Na deoxycholate, and sodium dodecyl sulfate [SDS]) and found that crosslinking between PLM and the unidentified protein was abolished in their presence, suggesting that this protein-protein interaction cannot be studied by co-immunoprecipitation. Furthermore, the crosslinked adduct was resistant to solubilization unless SDS was used, which prevented us from immunoprecipitating after crosslinking.

In separate experiments to identify PLM-interacting proteins, a yeast 2-hybrid screen based on the split-ubiquitin system was conducted in which full-length murine PLM fused to the C-terminal half of ubiquitin was used as bait and a murine adult heart cDNA library fused to the N-terminal half of ubiquitin was used as prey (Dualsystems Biotech). Among the potential PLM interactors identified, Prdx6 was chosen for further analysis and shown to bind directly to PLM in a pairwise interaction test. We therefore investigated whether the 37 kDa crosslinked adduct identified in myocytes was PLM bound to Prdx6. Figure 1C indicates that antibodies against PLM and Prdx6 both recognize the 37 kDa adduct. In myocytes treated with Sulfo-LC-SMPT, Prdx6 migrates at 25 (monomeric Prdx6), 37 (PLM-Prdx6 adduct), and 50 kDa (possibly Prdx6 crosslinked to its glutathione donor GST-π) (Figure 1C). A crosslinked sample was separated on a single-well gel and transferred to a polyvinylidene difluoride (PVDF) membrane, which was subsequently divided and separately probed for PLM and Prdx6 (Figure 1D). When the membrane was joined back together, the crosslinked adduct recognized by both the PLM and Prdx6 antibodies showed identical mobility on SDS-polyacrylamide gel electrophoresis (PAGE). We therefore concluded that the crosslinked adduct consisted of Prdx6 bound to PLM.

The interaction between PLM and Prdx6 is modified by glutathione loading in cells

Next, we investigated whether the interaction between PLM and Prdx6 in ventricular myocytes could be modified by promoting either PLM phosphorylation or Prdx6 oxidation. Figure 1E indicates that the phosphorylation status of PLM does not modify its interaction with Prdx6. Furthermore, the PLM-Prdx6 interaction was not affected by treatment of cells with either exogenous hydrogen peroxide (an oxidizing agent) or the free radical scavenger mercaptopropionyl glycine (an anti-oxidant) (Figure 1E). However, we did find that treating cells with N-acetyl-cysteine

(NAC) abolished the interaction between PLM and Prdx6. NAC is a cell-permeable form of cysteine that increases cellular levels of glutathione, as cysteine availability is the rate-limiting step in glutathione synthesis.[21,22] Unlike the other members of the Prdx family, whose physiological reductant is thioredoxin, Prdx6 utilizes glutathione.[17] Therefore, we hypothesized that the effect of increasing cellular glutathione levels via NAC promoted the activity of PLM-bound Prdx6 toward PLM changing either the structure and/or function of the PLM C-tail, causing Prdx6 to dissociate from it. As Prdx6 is a cellular reducing agent,[17] we first investigated whether Prdx6 bound to PLM protected either the Na pump complex or its locality from oxidative stress. However, we found no evidence that the presence of Prdx6 protected either PLM or the Na pump α subunit from direct oxidation (by assessing reactive cysteine availability with biotinylated iodoacetamide[23]) or modification by oxidized lipids (by measuring protein carbonyl content with 2,4-dinitrophenylhydrazine).

Manipulating cellular glutathione levels modifies the palmitoylation status of PLM in a Prdx6-dependent manner

We investigated palmitoylation of PLM in enzymatically dispersed rat ventricular myocytes by resin-assisted capture of acylated proteins (acyl-RAC) followed by quantitative immunoblotting. Glutathione-loading myocytes (using NAC or cell-permeable glutathione ethyl ester) acutely decreased palmitoylation of PLM but not the caveolar structural protein caveolin 3 (Figure 2A). A similar effect was observed when FT-293 cells engineered to express PLM tagged with yellow fluorescent protein (YFP) were glutathione loaded with NAC (Figure 2B). The effect of glutathione depletion on PLM palmitoylation was assessed by treating the PLM-YFP FT-293 stable cell line with buthionine sulfoximine (BSO; 100 μM, overnight), which inhibits the first enzyme in glutathione biosynthesis γ-glutamylcysteine synthase. Figure 2C shows that depletion of cellular glutathione in cultured cells caused enhanced palmitoylation of PLM.

The thioesterases identified to date (APT1, APT2, PPT1, ABHD17, ABHD10) are all serine hydrolases; other members of this protein family have also been suggested to have thioesterase activity.[8] The nucleophilic active-site serine of these hydrolases attacks the thioester bond linking palmitate to cysteine to facilitate protein depalmitoylation. We considered that the pKa of the nucleophilic cysteine in the active site of Prdx6 (C47, pKa $\sim$6.3[19]) might also be sufficiently low to facilitate the transfer of palmitate from PLM to Prdx6. Since regeneration of the Prdx6 active site is glutathione dependent, we explored the possibility that the effect of manipulating cellular glutathione on PLM palmitoylation is mediated by altering the rate of regeneration of the Prdx6 sulfhydryl group following depalmitoylation of PLM. Figure 2D shows that the glutathione-loading-dependent decrease in PLM palmitoylation in FT-293 cells is abolished following small interfering RNA (siRNA)-mediated silencing of Prdx6, identifying Prdx6 as the glutathione "sensor" responsible for this effect.

Palmitate transfer from PLM to Prdx6

To determine whether transfer of palmitate from PLM to Prdx6 occurs, we reconstituted the recombinant GST-tagged intracellular region of PLM and His-tagged wild-type and C47S Prdx6 *in vitro*. GST-PLM was acylated by incubation with 17-octadecynoic acid (ODYA) co-enzyme A (CoA). The acylated GST-PLM was then biotinylated by reaction of the alkyne-containing 17-ODYA with biotin azide in a copper (I)-catalyzed Huisgen cycloaddition.[24] Biotinylation of GST-PLM was hydroxylamine sensitive (Figure 3A), confirming that the click reaction reported protein acylation.

Incubation of *in-vitro*-acylated GST-PLM with Prdx6 led to transfer of the biotinylated acyl moiety to wild-type, but not C47S, Prdx6 (Figure 3A), indicating that palmitate transfer between these proteins is possible and requires the active-site cysteine of Prdx6. We considered the possibility that either glutathione alone or DTT (the reducing agent used to protect the prdx6 active-site cysteine during protein preparation) participated in the *in vitro* PLM deacylation reaction. *In-vitro*-acylated GST-PLM was reacted with cyanine5.5 azide and incubated with wild-type prdx6, glutathione, or DTT. Only prdx6 deacylated GST-PLM in these experiments (Figure 3B). Furthermore, a small proportion of Prdx6 was found to be palmitoylated in rat ventricular myocytes (Figure 3C). Transient expression of wild-type and C47S Prdx6 in HEK cells confirmed that the active-site cysteine in Prdx6 can be palmitoylated (Figure 3D).

To determine whether the low pKa of the Prdx6 active-site cysteine (C47) was required for it to be palmitoylated, R132, which makes a major contribution to stabilizing the C47 thiolate anion,[25] was mutated to both Ala (aliphatic side chain) and Glu (negative charge). Neutralizing (R132A) or reversing (R132E) the positive charge of R132 in the Prdx6 active site prevented C47 from becoming palmitoylated (Figure 3D), indicating that Prdx6 palmitoylation required the active-site cysteine to have a low pKa.

Palmostatin B inhibits palmitate transfer from PLM to Prdx6

Most depalmitoylating enzymes (thioesterases) that have been identified to date are inhibited by palmostatin B. We considered the possibility that PLM may also be depalmitoylated by other thioesterase enzymes. Although acute application of palmostatin B did not alter basal levels of PLM palmitoylation, it did abolish the impact of glutathione loading on PLM palmitoylation (Figure 3E). We also found that palmostatin B blocked the transfer of palmitate from PLM to Prdx6 *in vitro* (Figure 3F) and therefore concluded that palmostatin B is an inhibitor of Prdx6 depalmitoylation activity.

Quantitative proteomic analysis of the effect of glutathione loading on protein palmitoylation in rat ventricular myocytes

We investigated the impact of glutathione loading on palmitoylation of all proteins in freshly dispersed rat ventricular myocytes using a quantitative proteomic approach employing isobaric tags (iTraq[26]) to compare the relative abundance of proteins purified by acyl-RAC from untreated and NAC-treated cells. As assessed by SDS-PAGE and Coomassie staining, the complement of palmitoylated proteins was not grossly different between control and NAC-treated rat myocytes (Figure 4A) nor mouse hearts that had been perfused with NAC and subjected to fatty acyl biotin

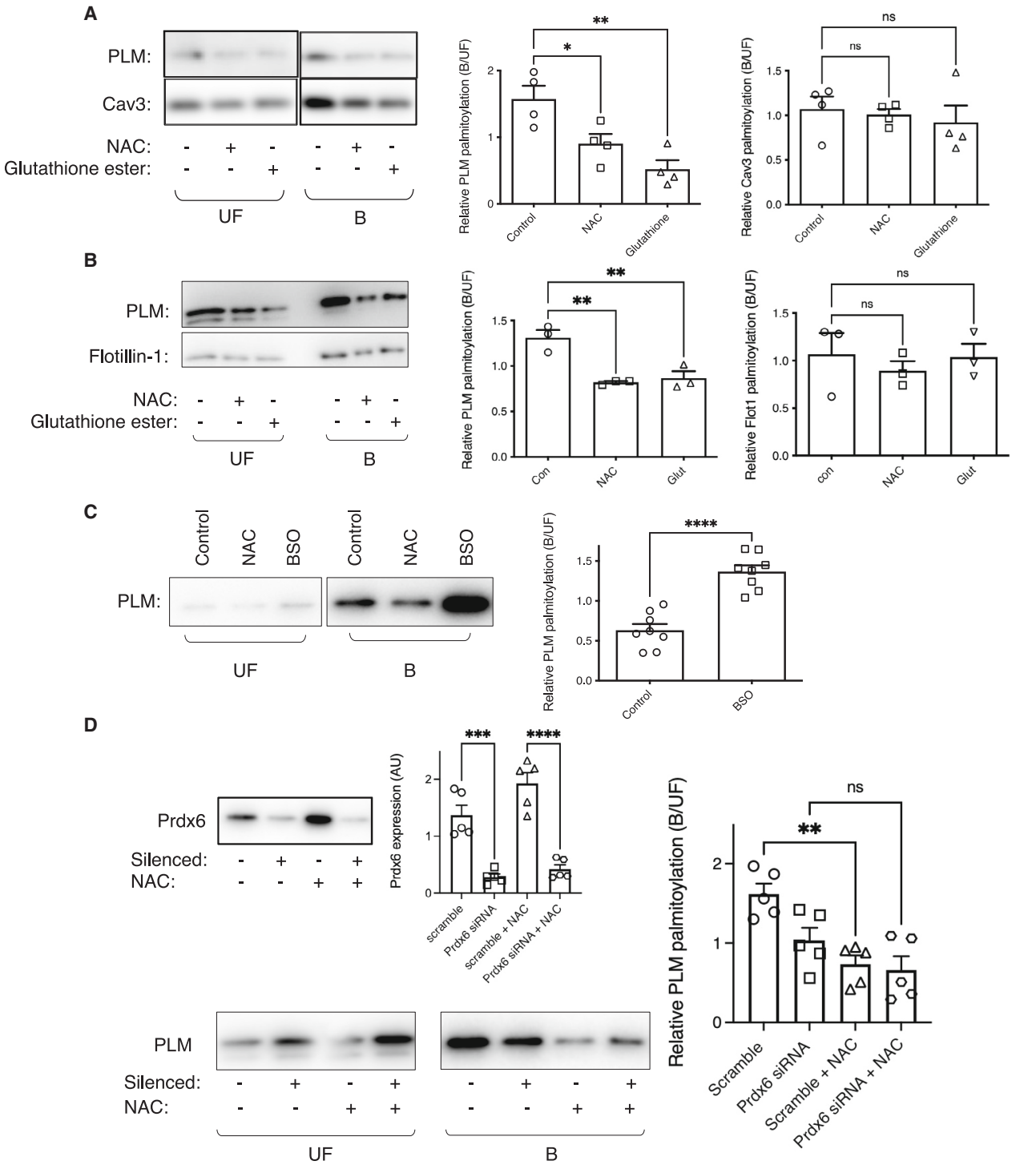

Figure 2. Manipulating cellular glutathione modifies the palmitoylation status of PLM in a Prdx6-dependent manner

(A and B) Glutathione-loading (A) rat ventricular myocytes (n = 4) or (B) FT-293 cells engineered to express PLM-YFP (n = 3) with either NAC or glutathione ethyl ester (60 min) significantly reduced the palmitoylation of PLM.

(C) Glutathione-depleting FT-293 cells engineered to express PLM-YFP (with buthionine sulfoximine [BSO], overnight) significantly enhanced PLM palmitoylation (n = 8).

(D) Prdx6 silencing in FT-293 cells expressing PLY-YFP (left) abolished the effect of glutathione loading on PLM palmitoylation (right) (n = 5).

*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001; (A, B, and D) one-way ANOVA; (C) Student's t test. UF, unfractionated; B, beads. All experimental repeats were biological replicates. A representative blot is provided for each quantitative experiment. Bar chart data are expressed as mean ± SEM.

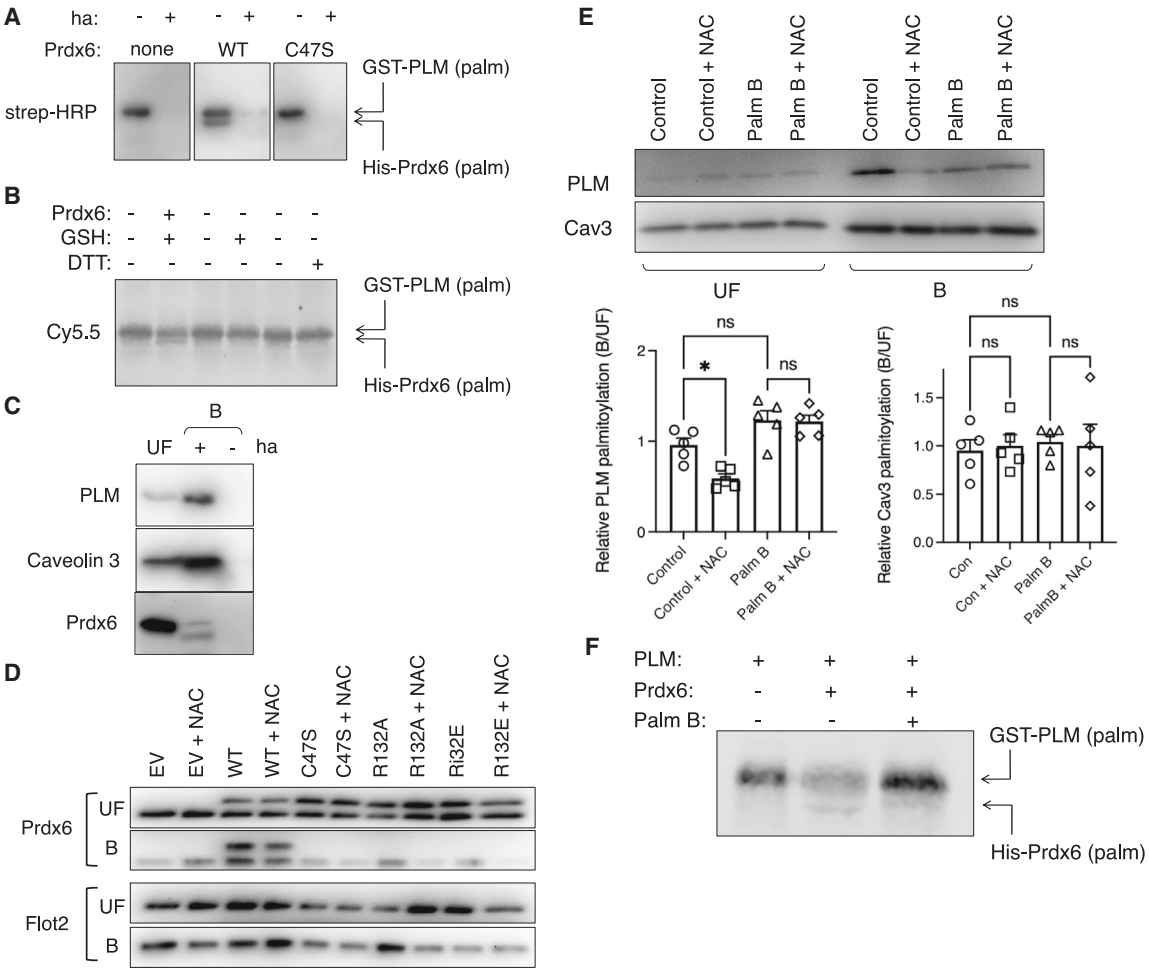

Figure 3. Palmitate transfer from PLM to Prdx6

(A) Palmitate transfer from PLM to Prdx6. Recombinant GST-PLM was acylated *in vitro* by incubation with 17-ODYA CoA and then biotinylated by reaction with biotin azide in the presence of copper (I) and detected using strep-HRP. Labeling specificity was confirmed by HA treatment. 17-ODYA was transferred from PLM to wild-type (WT), but not catalytically inactive (C47S), recombinant Prdx6.

(B) Recombinant GST-PLM was acylated *in vitro* by incubation with 17-ODYA CoA and then biotinylated by reaction with cyanine5.5 azide in the presence of copper (I) and detected using in gel fluorescence. GST-PLM is deacylated by WT Prdx6 but not by either glutathione (GSH; 5 mM) or DTT (1 mM) alone.

(C) A fraction of Prdx6 in rat ventricular myocytes was palmitoylated.

(D) Prdx6 can be palmitoylated at C47 but only when it has a low pKa. HEK cells were transiently transfected with empty vector (EV), WT, C47S, R132A, or R132E mutants in the presence and absence of NAC. Prdx6 palmitoylation at C47 was reduced by glutathione loading. Mutation of R132 (which makes a major contribution to stabilizing the C47 thiolate anion) to either Ala (A) or Glu (E) blocked palmitoylation of Prdx6.

(E) Treatment of ARVM with palmostatin B did not alter basal levels of PLM palmitoylation but did abolish the effect of glutathione loading on PLM palmitoylation (n = 5).

(F) Palmostatin B blocked the transfer of palmitate from PLM to Prdx6 *in vitro*.

*p < 0.05; one-way ANOVA. UF, unfractionated; B, beads. All experimental repeats were biological replicates. A representative blot is provided for each quantitative experiment. Bar chart data are expressed as mean ± SEM.

exchange (FAE; Figure 4B). iTraq analysis showed that the vast majority of the 751 identified palmitoylated proteins in rat cardiac myocytes had the same relative palmitoylation following glutathione loading (representative target proteins are shown in Figure 4C). However, a small number of proteins whose relative palmitoylation was reduced by glutathione loading were identified, most notably cavins 2 (serum deprivation-response protein [SDPR]) and 4 (muscle-related coiled-coil protein [MURC]) as well as myosin heavy chain isoform 6 (uppermost protein band marked in Figure 4A). In contrast, several proteins showed enhanced palmitoylation following glutathione loading (e.g., clathrin heavy chain; Figure 4C), suggesting that glutathione inhibits their depalmitoylation. Full proteomic data are recorded in Table S1.

Prdx6 is the only cytoplasmic peroxiredoxin with thioesterase activity

We evaluated the ability of recombinant peroxiredoxins to acquire palmitate from both palmitoylated proteins in cell lysates

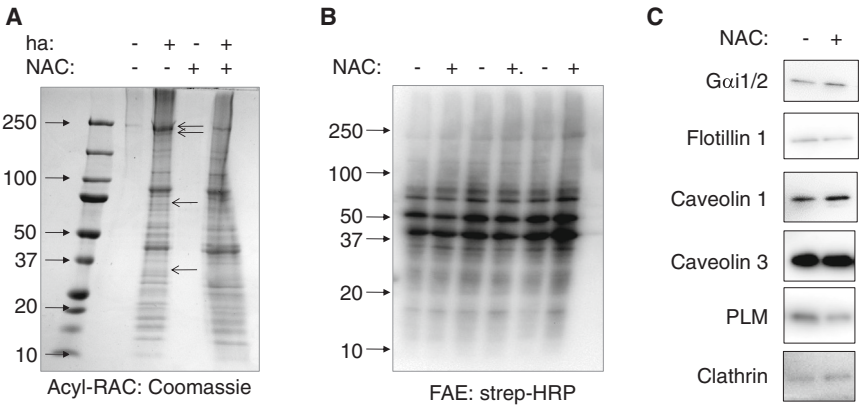

Figure 4. Effect of glutathione loading on protein palmitoylation in cardiac tissue

(A) Palmitoylated proteins were purified by acyl-RAC from control and NAC-loaded RVM, separated by SDS-PAGE, and stained with Coomassie. Few gross differences in the cardiac palmitoyl proteome were evident; notable decreases in abundance are marked with arrows.

(B) Mouse hearts perfused with NAC were subjected to FAE and palmitoylated proteins detected with strep-HRP. Glutathione loading did not have a gross impact on protein palmitoylation.

(C) Mouse hearts perfused with NAC were subjected to acyl-RAC and palmitoylated proteins immunoblotted as shown. Of the targets investigated, only PLM showed reduced palmitoylation following glutathione loading. Western blots are representative of at least 3 biological replicates.

and *in-vitro*-acylated GST-PLM. HEK cells were loaded with 17-ODYA and palmitoylated proteins visualized by reaction with cyanine5.5 azide (Figure 5A). Cell lysates were incubated with different His-tagged peroxiredoxins, which were subsequently purified by Ni^{2+}-affinity chromatography. Transfer of palmitate from acylated proteins in the cell lysates to the recombinant peroxiredoxins was evaluated by SDS-PAGE and in-gel fluorescence (Figure 5B). Wild-type Prdx6 acquired palmitate from the cell lysates but not C47S Prdx6, Prdx1, nor Prdx2, suggesting that the ability to depalmitoylate proteins is unique to Prdx6 among peroxiredoxin isoforms. Similarly, only recombinant wild-type Prdx6 acquired palmitate from *in-vitro*-acylated GST-PLM (Figure 5C).

DISCUSSION

Here, we describe the transfer of palmitate from the cardiac phosphoprotein PLM to the anti-oxidant protein Prdx6 through a nucleophilic transfer reaction involving the active-site cysteine of Prdx6, the regeneration of which is glutathione dependent. We suggest that cellular events in which palmitate is transferred between proteins, including both protein palmitoylation and depalmitoylation reactions, may be favored simply because the pKa of the recipient cysteine is lower than that of the donor.

The ability of a peptide cysteine thiolate anion to cleave the thioester bond in palmitoyl CoA has long been known.[27] This present study demonstrates that thioester bonds linking palmitate groups to cysteine sulfhydryl groups on proteins are also vulnerable to cleavage by thiolate ions in other proteins, suggesting that any nucleophilic active center may depalmitoylate via this mechanism. Although all thioesterases identified to date have active-site nucleophilic serines, our results suggest that depalmitoylating enzymes do not necessarily require such a serine. A reaction scheme suggested by the experimental data is presented in Figure 6.

The transfer of palmitate to a nucleophilic recipient cysteine is highly similar to the transthiolation reactions that occur between E1, E2, and E3 ubiquitin ligases. In that context, the active-site cysteine of an E1 ubiquitin ligase binds ubiquitin via a thioester bond. Transfer of ubiquitin to the E2 ligase is ATP independent and relies on nucleophilic attack of this thioester bond by the active-site cysteine of the E2 ligase.[28] Specificity of ubiquitin transfer is achieved by protein-protein interactions between E1, E2, and E3 ligases akin to that observed here between Prdx6 and PLM.

Among the cellular events regulated by Prdx6, it has previously been shown to interact with both κ opioid receptors and Gαi in a JNK-dependent fashion to regulate opioid receptor sensitivity/tolerance.[29] Prdx6 activity in this context was thought to reduce Gαi palmitoylation via increased free radical production rather than direct depalmitoylation. Nevertheless, the observations of Schattauer and colleagues are consistent with the possibility of Prdx6 functioning as a thioesterase. Interestingly, glutathione supplementation also reduces Gαq palmitoylation in the context of wound healing,[30] suggesting that glutathione-dependent depalmitoylation may also occur in other physiological contexts.

As increasing cellular levels of glutathione increased the rate of PLM depalmitoylation, it suggests that glutathione availability is a rate-determining step in this reaction. Regeneration of the Prdx6 active-site cysteine is dependent first on the glutathione donor GST-π1, which glutathionylates Prdx6 C47, and then the spontaneous reduction of glutathionylated Prdx6 by free cellular glutathione.[18,31,32] Hence, both steps in the regeneration of Prdx6 are glutathione dependent. This, and the relatively low affinity of GST-π1 for glutathione,[18,31] probably explains how supra-physiological concentrations of cellular glutathione increase the rate of both Prdx6 regeneration and PLM depalmitoylation.

Redox regulation of the Na pump

The relationship between Prdx6 and PLM establishes a link between the palmitoylation status of PLM and the redox (glutathione) status of the cell. The redox sensitivity of the cardiac Na pump is well established[33,34]; notably, the cardiac Na pump is rapidly inhibited upon glutathione depletion,[33] but the molecular basis of this effect has remained unclear. As PLM palmitoylation inhibits the Na pump,[14] we propose that glutathione-sensitive depalmitoylation of PLM by Prdx6 acts as a redox sensor for the pump, reducing its activity at times of oxidative stress. Palmitoylation of PLM turns over quickly: we have previously reported phosphorylation-induced palmitoylation within 10 min of agonist application,[14] and in the present study, manipulating cellular glutathione results in changes within 60 min. As

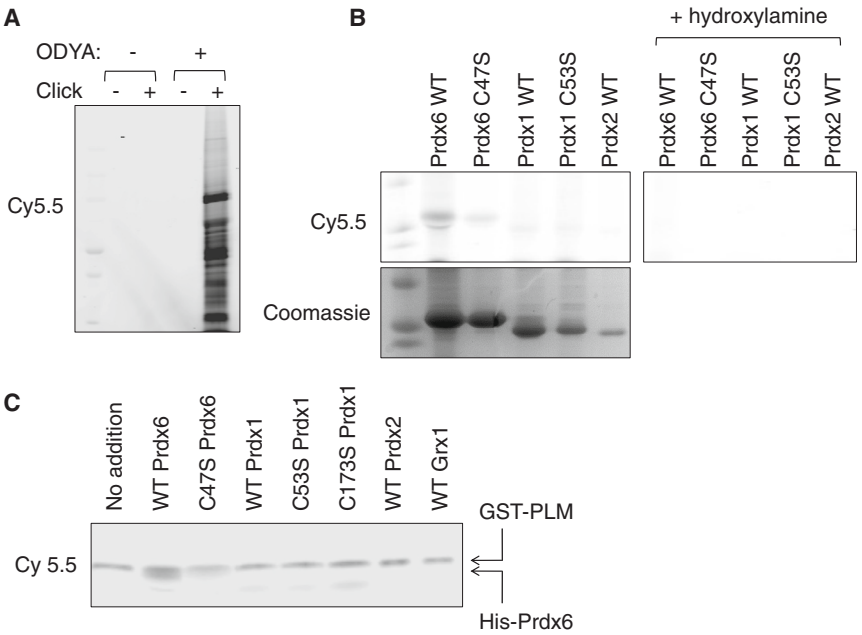

Figure 5. Prdx6 is the only Prdx isoform with depalmitoylase activity

(A) FT-293 cells were loaded with ODYA and palmitoylated proteins visualized by reaction with Cy5.5 azide.

(B) Prdx6, but not Prdx1 or Prdx2, acquired palmitate from cell lysates after labeling palmitoylated proteins with Cy5.5.

(C) Prdx6, but not Prdx1 or Prdx2, acquired palmitate from *in vitro* acylated GST-PLM after labeling with Cy5.5. Experiments are representative of at least 3 biological replicates.

the pump is a major consumer of cellular ATP, its acute inhibition at times of glutathione depletion may protect cardiomyocytes against precipitous ATP depletion and possible cell death. PLM palmitoylation will also prevent terminal cysteine oxidation at times of cellular redox stress.

Palmitate transfer by Prdx6

As well as being an anti-oxidant, Prdx6 has phospholipase A2 activity.[19] The lack of palmitate transfer to C47S Prdx6 indicates that the phospholipase and thioesterases activities of Prdx6 are distinct, as the Prdx6 phospholipase activity is conferred by a catalytic triad of residues consisting of H26, S32, and D140.[35] However, the lipid-binding properties of Prdx6 conferred by this triad are relevant for its targeting to caveolar microdomains[19] (in which PLM resides[20]), and may also assist in targeting Prdx6 to palmitoylated PLM, because the interaction between the two is abolished following depalmitoylation of PLM. Prdx6 has also previously been shown to transfer palmitate to lysophosphatidylcholine (lysoPC).[36] It is, therefore, conceivable that the palmitate that Prdx6 receives from PLM is subsequently transferred to lysoPC.

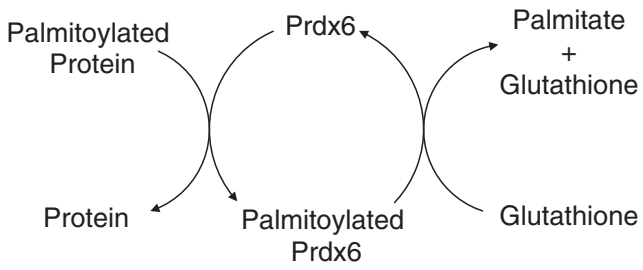

Figure 6. Proposed reaction scheme of Prdx6 depalmitoylase activity

Wider implications

A significant proportion of palmitoylated cysteines are low-pKa nucleophiles.[37,38] Palmitoylation by zDHHC protein acyltransferases (zDHHC-PATs) may therefore be achieved as a result of nucleophilic palmitate transfer from the DHHC motif to the substrate protein, much as we report here from PLM to Prdx6. This is consistent with a paradigm in which the specificity of a palmitoylation/depalmitoylation reaction is achieved by a protein-protein interaction and the movement of palmitate itself by a nucleophilic transfer controlled by the pKa of the recipient thiol. Emerging data support the concept that zDHHC-PAT substrate specificity is determined by protein-protein interactions involving regions of the enzyme far removed from their active site.[15,39–41]

Limitations of the study

Although considerable evidence has been provided demonstrating that Prdx6 depalmitoylates PLM, it is not yet clear whether Prdx6 is a specific thioesterase that only depalmitoylates PLM or if it has activity toward other palmitoylated proteins. When the effect of glutathione loading on protein palmitoylation in rat ventricular myocytes was investigated using quantitative proteomics, several proteins whose relative palmitoylation was reduced by glutathione loading were identified, including cavins 2 (SDPR) and 4 (MURC) as well as myosin heavy chain isoform 6. Further experiments will be required to determine whether the observed reduction in palmitoylation that occurred on glutathione loading was mediated via Prdx6.

Furthermore, in the quantitative proteomics experiment, several proteins were found to have enhanced palmitoylation following glutathione loading such as clathrin heavy chain, suggesting that glutathione may inhibit their depalmitoylation. The molecular basis of this observation currently remains unexplored.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Animals
  - Cell lines
- METHOD DETAILS
  - Isolation of rat ventricular myocytes
  - Crosslinking
  - Cellular palmitoylation assays
  - Glutathione loading and depletion
  - Prdx6 silencing
  - Plasmids and recombinant proteins
  - *In vitro* palmitoylation
  - Quantitative proteomics
  - Palmitate transfer
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2024.113679.

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AUTHOR CONTRIBUTIONS

Conceptualization, W.F. and N.J.F.; methodology, J.H., L.B.T., N.J.F., and W.F.; investigation, J.H., L.B.T., E.B., L.R., F.B.A., J.K., K.J.W., K.L.A., J.K.C.M., and N.J.F.; writing – original draft, W.F. and N.J.F.; writing – review & editing, N.J.F. and M.J.S.; supervision, W.F.; project administration, W.F.; funding acquisition, M.J.S., N.J.F., and W.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Phospholemman	Custom made, amino terminus	Fuller, W.et al.[42]
Phospholemman	Custom made, amino terminus	Fuller, W.et al.,[13] Tulloch, L.B.et al.[14]
Phospholemman	Custom made, carboxyl terminus	Song, J.et al.[43]
Phospholemman	Abcam ab76597	RRID: AB_1952372
Peroxiredoxin 6	Abcam ab133348	RRID: AB_11155931
Peroxiredoxin 6	Thermo Fisher Scientific	LFMA0018 (discontinued)
Flotillin-1	BD Biosciences 610821	RRID: AB_398140
Flotillin-2	BD Biosciences 610384	RRID: AB_397767
Caveolin-1	BD Biosciences 610406	RRID: AB_397788
Caveolin-3	BD Biosciences 610421	RRID: AB_397801
Heterotrimeric G protein α subunit	Thermo Fisher Scientific PA1-1000	RRID: AB_2232440
Clathrin heavy chain	BD Biosciences 610499	RRID: AB_397865
Bacterial and virus strains		
One Shot™ BL21(DE3) Chemically Competent E. coli	Thermo Fisher Scientific	C600003
Chemicals, peptides, and recombinant proteins		
Recombinant prdx6 (wild type)	In house	N/A
Recombinant prdx6 (C47S)	In house	N/A
Recombinant prdx1 (wild type)	In house	N/A
Recombinant prdx1 (C53S)	In house	N/A
Recombinant prdx2 (wild type)	In house	N/A
Collagenase, Type 2	Worthington Biochemical Corp	LS004177
Digitonin	Merck-Millipore	D141
DSP (Dithiobis[succinimidyl propionate])	Thermo Fisher Scientific	22585
DTSSP (3,3′-Dithiobis[sulfosuccinimidylpropionate])	Thermo Fisher Scientific	21578
EGS (Ethylene glycol bis[succinimidylsuccinate])	Thermo Fisher Scientific	21565
S-EGS (Ethylene glycol bis[sulfosuccinimidylsuccinate])	Thermo Fisher Scientific	21566
S-LC-SMPT (sulfosuccinimidyl 6-[α-methyl-α-(2-pyridyldithio)toluamido]hexanoate)	Thermo Fisher Scientific	21568
MBS (*m*-Maleimidobenzoyl-N-hydroxysuccinimide ester)	Thermo Fisher Scientific	22311
DPDPB (1,4-Di-[3'-(2′-pyridyldithio)propionamido]butane)	Thermo Fisher Scientific	21702
APDP (N-[4-(*p*-azidosalicylamido)butyl]-3-(2-pyridyldithio) propionamide)	Thermo Fisher Scientific	27720
Bisindolylmaleimide I	Merck-Millipore	203290
Phorbol 12-myristate 13-acetate	Merck-Millipore	524400
Forskolin	Merck-Millipore	344270
Hydrogen Peroxide solution	Merck-Millipore	H1009
N-Acetyl-L-cysteine	Merck-Millipore	A9165
L-Buthionine-sulfoximine	Merck-Millipore	B2515
N-(2-Mercaptopropionyl)glycine	Merck-Millipore	M6635
Glutathione reduced ethyl ester	Merck-Millipore	G1404
S-Methyl Methanethiosulphonate	Merck-Millipore	64306-1ML
Thiopropyl Sepharose 6B	Cytiva	17042001
Hydroxylamine Sulfate	Thermo Fisher Scientific	10701861

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
N-Ethylmaleimide	Merck-Millipore	E3876-5G
HPDP-Biotin	Thermo Fisher Scientific	21341
Glutathione Sepharose 4B	Cytiva	17075601
Ni Sepharose 6 Fast Flow	Cytiva	17531801
Glutathione	L-Glutathione reduced	G4251-5G
DTT	Melford	D11000–10.0
Streptavidin-Horseradish Peroxidase Conjugate	Cytiva	RPN1231-100UL
Palmostatin B	Merck-Millipore	178501
Lipofectamine 2000	Thermo Fisher Scientific	11668019
Cyanine 5.5 azide	Jena Bioscience	CLK-1059-1
17-Octadecynoic acid	Merck-Millipore	O8382
Coenzyme A sodium salt hydrate	Merck-Millipore	C3144
Acyl-coenzyme A Synthetase	Merck-Millipore	A3352
Palmitoyl Alkyne-Coenzyme A	Cayman Chemical	36470
Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA)	Merck-Millipore	678937
Biotin Azide (PEG4 carboxamide-6-Azidohexanyl Biotin)	Thermo Fisher Scientific	B10184
TCEP (Tris(2-carboxyethyl)phosphine Hydrochloride)	Merck-Millipore	C4706-2G
IPTG (Isopropyl β-D-1-thiogalactopyranoside)	Melford	I56000–1.0
Experimental models: Cell lines		
FT-293 T-REx cells engineered to express PLM-YFP	In house	Tulloch, L.B.et al[14]
FT-293 T-REx cells	Thermo Fisher Scientific	R71007
Experimental models: Organisms/strains		
Sprague-Dawley Rat	Inotiv	002
C5BL/6J mice	Janvier labs	N/A
Oligonucleotides		
ON-TARGETplus Human PRDX6 siRNA SMARTPool	Horizon Discovery	L-019173-00-0005
ON-TARGETplus Non-targeting Control Pool	Horizon Discovery	D-001810-10-05
Prdx6 R132A Forward GACAGCTGCTGT GGTGTTTGTTTTTGGTCC	Eurofins Genomics	N/A
Prdx6 R132A Reverse ACCACAGCAGCT GTCACAGGCATGCC	Eurofins Genomics	N/A
Prdx6 R132E Forward GACAGCTGAA GTGGTGTTTGTTTTTGGTCCTG	Eurofins Genomics	N/A
Prdx6 R132E Reverse ACCACTT CAGCTGTCACAGGCATGCC	Eurofins Genomics	N/A
Recombinant DNA		
Human prdx1 (HIS tag)	N/A	N/A
Human prdx2 (HIS tag)	N/A	N/A
Human prdx6 (HIS tag)	N/A	N/A
Software and algorithms		
The Discovery Series Quantity One 1-D Analysis Software Version 4.6.6, PC	BioRad	LIT-70-9600-Q1-466PC
ImageStudio Lite v5.2.5	LiCOR	N/A
GraphPad v10.0.3	GraphPad Prism	N/A
Deposited data		
Proteomic data	ProteomeXchange	ProteomeXchange: PXD047858

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Will Fuller (will.fuller@glasgow.ac.uk).

Materials availability

All reagents generated in the study are available from the lead contact without restrictions.

Data and code availability

- The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[44] partner repository with the dataset identifier PXD047858.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

This study utilized cardiac tissue from male adult C5BL/6J mice (20-25g, 6–10 weeks age) and male adult Sprague-Dawley Rat rats (200-300g, 7–10 weeks age). Animals were maintained in social groups with free access to food and water under a 12-h light-dark cycle. All protocols involving animals were approved by the University of Glasgow Animal Welfare and Ethics Review Board. Rat and mouse hearts were collected postmortem after sacrificing animals using a method designated Schedule 1 by the Animals (Scientific Procedures) Act 1986 (overdose of pentobarbital administered via intraperitoneal injection followed by cervical dislocation).

Cell lines

FT-293 T-REx cells engineered to express PLM-YFP have been described elsewhere.[14] Cells were maintained in Dulbecco's Modified Eagle's.

Medium (DMEM) medium containing 10% fetal bovine serum, 1% penicillin and streptomycin, 100 μg/mL hygromycin, and 15 μg/mL blasticidin in a 37°C incubator with 5% CO_2.

METHOD DETAILS

Isolation of rat ventricular myocytes

Calcium-tolerant rat ventricular myocytes were isolated by retrograde perfusion of collagenase. Myocytes were allowed to recover from the isolation procedure for 2 h at 35°C before experimentation.

Crosslinking

All crosslinking reagents were applied to isolated ventricular myocytes in phosphate-buffered saline (PBS) supplemented with 0.05% digitonin for 30 min at 4°C.

Cellular palmitoylation assays

Palmitoylated proteins were purified from SDS-solubilized cell or tissues lysates using fatty acyl biotin exchange (FAE[14]) or resin-assisted capture of acylated proteins (acyl-RAC[20,45]). Relative palmitoylation values for PLM, Flotillin-1 (Control, HEK cells) and Caveolin-3 (Control, myocytes) were determined by dividing the band intensities of the bead (B) samples with that of the corresponding unfractionated (UF) samples.

Glutathione loading and depletion

Isolated rat ventricular myocytes, mouse hearts perfused in the Langendorff mode, and cultured cells were glutathione-loaded by incubating with 10 mM N-acetyl cysteine for 60 min at 35°C–37°C. Cultured cells were depleted of glutathione by treatment with the γ-glutamylcysteine synthetase inhibitor buthionine sulfoximine (100 μM) overnight.

Prdx6 silencing

FT-293 expressing PLM-YFP cells were transfected using Lipofectamine 2000 with either SMARTpool siRNA directed against human Prdx6 or a non-targeting control pool (scrambled) and cells treated then lyzed 72 h later.

Plasmids and recombinant proteins

Wild type, C47S, R132A and R132E Prdx6 cDNAs were subcloned into pcDNA 3.1 and expressed in HEK cells by transfection using Lipofectamine 2000. GST-tagged PLM was purified as described previously.[13] His-tagged Prdx6 (wild type and C47S),

Prdx1 and Prdx2 were expressed in BL21 (DE3) cells (induction conditions: 0.5 mM IPTG, 2h at 37°C) and purified using nickel-sepharose.

In vitro palmitoylation

17-ODYA CoA was synthesized from 17-ODYA (1 mM) and CoA (5 mM) by acyl CoA synthetase (10 mUnits/μL) in the presence of 10 mM ATP for 60 min at 37°C. Recombinant GST-PLM was immobilized on glutathione Sepharose beads and acylated by incubation with 17-ODYA CoA or Palmitoyl Alkyne-Coenzyme A for 30 min at 37°C. Unreacted fatty acyl CoA was removed by washing the beads 5 times in 1% Triton X-100 in PBS, and acylated GST-PLM was biotinylated by copper (I) catalyzed Huisgen cycloaddition in the presence of 1 mM TCEP, 1 mM $CuSO_4$, 0.1 mM TBTA and 0.1 mM biotin azide in 50 mM TRIS pH 8.0 and 1% Triton X-100. Beads were washed 5 times with 1% Triton X-100 in PBS. The hydroxylamine-sensitivity of biotin incorporation confirms that it reports acylation of GST-PLM. GST was not acylated by the same protocol. In some experiments biotin azide was replaced with cyanine 5.5 azide, and acylation visualized using in-gel fluorescence.

Prior to reconstitution with acylated biotinylated GST-PLM, His-tagged wild type and C47S Prdx6 were reduced by incubation with 10 mM DTT in 1% Triton X-100 in PBS. The final concentration of dithiothreitol (DTT) in the transfer reaction was 1 mM. Reactions proceeded for 30 min at 37°C, and were quenched by addition of SDS PAGE loading buffer and immediately separated using SDS PAGE, transferred, and probed with streptavidin-horseradish peroxidase (strep-HRP).

Quantitative proteomics

Palmitoylated proteins were purified from control and glutathione-loaded rat ventricular myocytes by acyl-RAC, and eluted in Laemmli buffer supplemented with 100 mM DTT. Proteins were subjected to SDS PAGE for ~1 cm, Coomassie stained, excised as a single band, subjected to in gel digestion, labeled with isobaric tags (iTraq), separated by cation exchange into 10 fractions, and analyzed by mass spectrometry on an LTQ Orbitrap Velos Pro (Thermo) at the FingerPrints Proteomics Unit, School of Life Sciences, University of Dundee. Peptides were matched and assigned using Mascot with a minimum 2 peptides with ion score >34 required for identification. For analysis, rat IPI identifiers supplied by Mascot were mapped to human Uniprot identifiers using a custom-built IPI to Uniprot dictionary. iTraq ratios for all proteins identified from glutathione-loaded cells relative to control cells are reported in Table S1.

Palmitate transfer

FT-293 cells were loaded overnight with 17-ODYA (20 μM) conjugated to fatty acid-free BSA. Cells were lyzed in 1% Triton X-100 in 50 mM TRIS pH 8.0, and palmitoylated proteins visualized by reaction with 20 μM cyanine5.5-azide in the presence of 2 mM $CuSO_4$, 0.2 mM TBTA and 4 mM ascorbate. Lysates were spiked with His-tagged recombinant peroxiredoxins and 10 mM β-mercaptoethanol. Following incubation at 37°C for 60 min, the peroxiredoxins were purified using Ni-sepharose beads. Palmitoylated proteins were visualized after separation by SDS PAGE using in-gel fluorescence with a Li-COR infrared imaging system.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantitative data are presented as mean ± SEM. One-way ANOVA analysis was performed on data that had one independent variable and one quantitative dependent variable (Figures 2A and 2B, 2D; 3E). The Student's T test was used to compare mean values between 2 groups (Figure 2C).

All statistical analysis was carried out using GraphPad Prism. The statistical details for all experiments with quantitative data can be found in the relevant Figure legend. n refers to the number of independent biological experimental replicates that had been carried out.