Acetylome Profiling Reveals Extensive Lysine Acetylation of the Fatty Acid Metabolism Pathway in the Diatom *Phaeodactylum tricornutum*

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The abbreviations used are: ACCase, acetyl-CoA carboxylase; AcP, acetyl-phosphate; CoA, acetyl Coenzyme A; E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase; FabI, enoyl-ACP reductase; FabD, malonyl-CoA: ACP transacylase; FabZ, 3R-hydroxyacyl-ACP dehydrase; FabF, 3-oxoacyl-ACP synthase; FabG, 3-oxoacyl-ACP reductase; FDR, false discovery rate; GO, Gene Ontology; KATs, lysine acetyltransferases; KDACs, lysine deacetylases; LACS, long chain acyl-CoA synthase; Lys, $N^\epsilon$-lysine; MS, mass spectrometry; PTM, post-translational modification; SIR2, Silent Information Regulator 2; SRT, sirtuin-type deactylases; Ub, ubiquitin; WT, wild-type.

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**Summary**

N\(^e\)-lysine acetylation represents a highly dynamic and reversibly regulated post-translational modification widespread in almost all organisms, and plays important roles for regulation of protein function in diverse metabolic pathways. However, little is known about the role of lysine acetylation in photosynthetic eukaryotic microalgae. We integrated proteomic approaches to comprehensively characterize the lysine acetylome in the model diatom *Phaeodactylum tricornutum*. In total, 2324 acetylation sites from 1220 acetylated proteins were identified, representing the largest dataset of the lysine acetylome in plants to date. Almost all enzymes involved in fatty acid synthesis were found to be lysine acetylated. Six putative lysine acetylation sites were identified in a plastid-localized long-chain acyl-CoA synthetase. Site-directed mutagenesis and site-specific incorporation of N-acetyllysine in acyl-CoA synthetase show that acetylation at K407 and K425 increases its enzyme activity. Moreover, the nonenzymatically catalyzed overall hyperacetylation of acyl-CoA synthetase by acetyl-phosphate can be effectively deacetylated and reversed by a sirtuin-type NAD\(^+\)-dependent deacetylase with subcellular localization of both the plastid and nucleus in *Phaeodactylum*. This work indicates the regulation of acyl-CoA synthetase activity by site-specific lysine acetylation and highlights the potential regulation of fatty acid metabolism by lysine acetylation in the plastid of the diatom *Phaeodactylum*. 
Introduction

Diatoms are a highly diverse group of eukaryotic unicellular microalgae living in marine and freshwater environments, and are considered to be responsible for one-fourth of global primary productivity (1, 2). Photosynthetic diatoms are of ecological significance in aquatic ecosystems and the global carbon cycle, and some diatoms are considered as promising sources for the production of renewable and sustainable biodiesel (3) as they can accumulate large amounts of triacylglycerols when their growth is limited by nutrients. The pennate diatom *Phaeodactylum*, a model diatom species with a sequenced genome (4), has evolved a set of sophisticated cellular and molecular mechanisms to cope with a wide variety of nutrient stresses. Over the past five years, metabolic change triggered by nutrient stress has been examined using omics-based approaches, which show that *Phaeodactylum* has the ability to globally regulate metabolic pathways at both transcriptional and translational levels, enabling their survival under nutrient stress (5-9). However, post-translational control of gene regulation and potentially protein stability and activity in *Phaeodactylum* is only beginning to be understood. A recent study reported the identification of multiple post-translational modifications (PTMs) on histones of *Phaeodactylum*, revealing the dynamic feature of chromatin modifications in regulating target genes in response to nitrogen limitation (10). These histone modifications demonstrate the importance of PTMs in defining chromatin states and regulating gene expression in response to nutrient stress.

Of more than 200 reported PTMs, N°-lysine (Lys/K) acetylation is one of the most abundant and extensively studied PTMs with an evolutionary conservation from prokaryotes to eukaryotes (11). The best-known function of Lys acetylation is the regulation of histone proteins by affecting chromatin structure and gene expression (12). In contrast to irreversible Nα-acetylation, the reversible Lys acetylation is catalyzed by Lys acetyltransferases (KATs) and reversed by Lys deacetylases (KDACs) (13), making it an important mechanism to reversibly regulate protein function. In enzymatically catalyzed Lys acetylation reactions, KATs catalyze the transfer of an acetyl group from acetyl Coenzyme A (CoA) to the ε-amino group of protein lysine residues; whereas the KDAC activity removes the acetyl group from the
modified lysine residue. In addition to enzymatic catalysis, Lys acetylation can also occur by chemical catalysis (14). Over the past decade, the development of new approaches to identify acetylated proteins, especially mass spectrometry-based proteomics, has advanced the research of Lys acetylation beyond histone protein modification. Substrates of Lys acetylation have been extended from histone proteins to a wide variety of non-histone proteins/enzymes involved in diverse biological processes, coupling Lys acetylation to cellular metabolism beyond epigenetic control of gene expression and chromatin dynamics that is associated with histones. Analyses of the overall picture of Lys acetylomes in yeast and humans greatly broaden its regulatory scope, and demonstrate that it contributes not only to the regulation of nuclear function but also to the control of cytoplasmic and mitochondrial functions (15-17).

While proteome-wide analyses of acetylated proteins have been reported in several organisms, including bacteria (18-20), yeast (15), plants (21, 22) and human (16, 17, 23, 24), little is known about the acetylomes in photosynthetic microalgae. In this study, we integrated anti-acetyllysine-based enrichments, high accuracy MS techniques and bioinformatic analyses to profile the Lys acetylome in the model diatom Phaeodactylum, and we identified 2324 unique acetylation sites from the peptides of 1220 proteins. We performed proteome-wide analysis of Lys acetylated proteins in Phaeodactylum, and characterized the effect of Lys acetylation on enzyme activity of a plastid-localized long-chain acyl-CoA synthetase, which shows that acetylation enhances its enzyme activity in vitro. Our results provide a comprehensive view of the acetylome in Phaeodactylum and pave the way to understand the functional importance of Lys acetylation for cellular metabolic enzymes in this model diatom.

**Experimental Procedures**

**Cell Culture and Protein Extraction**—Axenic cells of Phaeodactylum tricornutum Bohlin (CCMP 2561) from the culture collection of the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Bigelow Laboratory for Ocean Sciences, USA) were grown as previously described (8). For proteomic analysis, cells \((4 \times 10^5 \text{ cells mL}^{-1})\) from mid-logarithmic phase cultures were inoculated in artificial seawater enriched with f/2 (25) at 22 °C, bubbled with filtered air and continuously irradiated with 100
μmol photons m$^{-2}$ s$^{-1}$.

To obtain a large amount of the Lys acetylation proteome in this model diatom, cells at mid-logarithmic phase were exposed to different stresses for 12 h, including nitrogen deficiency (−N), iron deficiency (−Fe), and phosphate deficiency (−P). To inhibit endogenous protein deacetylase activity, nicotinamide was added to the cell cultures at a final concentration of 10 mM and incubated for an additional 30 min. The cells were harvested by centrifugation (6,000 g for 5 min at 10 °C), washed with f/2 medium twice and frozen by liquid nitrogen. Then, the sample was grinded with liquid nitrogen, transferred to 15 mL centrifuge tube and sonicated three times with an output of 135 W (JY92-IIN; Ningbo Scientz Bio-technology Co., Ltd., Ningbo, Hangzhou, Zhejiang, China) on ice-water (2 s on/2 s off) for about 30 min in lysis buffer (8 M urea, 10 mM DTT, 3 μM trichostatin A, 50 mM nicotinamide, 2 mM EDTA and 1 % protease inhibitor cocktail set VI [Calbiochem, Darmstadt, Germany]). The whole cell lysate was then centrifuged (20,000 g at 4 °C for 10 min) to remove the remaining debris, after which the protein was precipitated with cold 15 % trichloroacetic acid at −20 °C for 2 h. Following another round of centrifugation at 4 °C for 10 min, the supernatant was discarded, leaving the precipitate which was washed with cold acetone three times. The precipitated protein was redissolved in buffer (8 M urea, 100 mM NH₄CO₃, pH 8.0) and the protein concentration was determined using the BCA Protein Assay Kit (TIANGEN, Beijing, China).

Trypsin Digestion, HPLC Fractionation, and Affinity Enrichment of Acetylated Peptides—The protein in solution was reduced with 10 mM DTT for 1 h at 37 °C and alkylated with 20 mM iodoacetamide for 45 min at room temperature in the dark. Then, the protein sample was diluted by 100 mM NH₄CO₃ to urea concentration less than 2 M. Finally, sequencing grade Trypsin (Promega, Madison, WI, USA) was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1: 100 trypsin-to-protein mass ratio for a second 4 h-digestion.

The digested peptides were fractionated by high pH reverse-phase HPLC using an Agilent 300Extend C₁₈ column (5 μm particles, 4.6 mm ID, 250 mm length). Briefly, peptides were first separated into 80 fractions with a gradient of 2 % to 60 % acetonitrile in 10 mM NH₄CO₃ at pH 10 for over 80 min. Then,
the peptides were combined into 8 fractions and then collected and dried by vacuum centrifuging.

The resulting peptides were enriched by agarose-conjugated the anti-acetyllysine antibody (PTM Biolabs, Chicago, IL, USA). Briefly, tryptic peptides dissolved in NETN buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 % NP-40) were incubated with anti-acetyllysine antibody conjugated protein A agarose beads at 4 °C overnight with gentle rotation. The beads were washed four times with NETN buffer and then with ddH2O two times. The bound peptides were eluted from the beads by 0.1 % TFA, combined and vacuum-dried. The resulting peptides were cleaned with C18 ZipTips from Millipore (Billerica, MA, USA) according to the manufacturer’s instructions.

**LC-MS/MS Analysis**—The enriched peptides were dissolved in 0.1 % FA, directly loaded onto a reversed-phase pre-column (Acclaim PepMap 100; Thermo Fisher Scientific, Waltham, MA, USA), and separated using a reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Fisher Scientific). The gradient was comprised of an increase from 5 % to 20 % solvent B (0.1 % FA in 98 % ACN) for 20 min, 20 % to 35 % for 8 min and climbing to 80 % in 2 min then holding at 80 % for the last 5 min. The resulting peptides were eluted at a constant flow rate of 300 nl/min on an EASY-nLC 1000 UPLC system and analyzed by Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific).

The peptides were subjected to a nanospray ionization source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTM Plus (Thermo Fisher Scientific) coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were selected for MS/MS using a NCE setting of 30 and ion fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 1E4 in the MS survey scan with 10.0 s dynamic exclusion. The electrospray voltage applied was 2.0 kV. Automatic gain control was used to prevent overfilling of the ion trap; 5E4 ions were accumulated for the generation of the MS/MS spectra. For MS scans, the m/z scan range was 350 to 1800. MS2 fixed first mass was set as 100.

**Data Search**—The MS/MS data was processed using MaxQuant with an integrated Andromeda search
engine (v.1.3.0.5). Tandem mass spectra were searched against the *P. tricornutum* (10, 402 sequences) database concatenated with reverse decoy database. Trypsin/P was specified as a cleavage enzyme allowing up to 4 missing cleavages. Mass error of precursor ions was set to 20 ppm for the first search, 5 ppm for the second search and 0.02 Da for fragment ions. Carbamidomethylation (Cys) was set as fixed modification and oxidation (Met), and deamidation (Asn/Gln), acetylation (Lys) and protein N-terminal were specified as variable modifications. Minimum peptide length was set at 6. Search results were filtered out by eliminating all putative hits with an Andromeda score < 40. The estimated false discovery rate (FDR) thresholds for proteins, peptides and modification sites were specified at a maximum 1%. The site localization probability was set as $\geq 0.75$. All the other parameters in MaxQuant were set to default values. All the raw data were deposited in a publicly accessible database, PeptideAtlas (www.peptideatlas.org) (26). All the MS/MS spectra were manually inspected to improve the reliability of the results, and the spectra were kept only if a series of at least three successive b- or y-ions were present (27, 28). Finally, those MS/MS spectra with high-quality were used for further analyses in this study.

**Bioinformatics Analysis**—Annotation of acetylproteins based on the Gene Ontology (GO) terms including biological process and molecular function was performed using Blast2GO software (29-31). Protein subcellular localization was predicted by ngLOC (32). Because nuclear-encoded plastid-localized proteins in *Phaeodactylum* generally possess bipartite N-terminal pre-sequences that contain a conserved sequence motif (‘ASAFAP’ motif), we additionally used a customized prediction tool (ASAFind) to predict plastid localization of identified acetylated proteins as previously described (33). Functional enrichment analysis of the identified acetylated proteins was performed by BinGO 2.44 plugin (34) in the Cytoscape platform (35). Lys-acetylated motifs were analyzed by soft motif-x (36, 37). All the database protein sequences of *Phaeodactylum* were used as a background database parameter and other parameters as default. To analyze Lys acetylation sites, the ratios of 6 amino acids surrounding the acetylation sites upstream and downstream were calculated, and the position-specific heat map was generated by plotting the $\log_{10}$ of the ratio (38-40). Predictions of secondary structures were performed using NetSurfP (41). The mean secondary structure probabilities of modified lysine residues were compared with those of control
residues for all identified acetylated proteins, and p values were calculated as previously described (42). Further, orthologs of acetylproteins that were identified were searched using BLASTP to evaluate the conservation across species (43). KEGG mapper was used to analyze the KEGG pathway to evaluate the metabolic pathway involving acetylproteins (44, 45). The structural models of FABZ (bd1143), FABFa (37367), FABI (10068), FABG (13073) and FABD (37652) were prepared from the previously determined crystal structure by using the modeling server SWISS-MODEL and the three-dimensional structures of the identified acetylproteins were modeled using The PyMOL Molecular Graphics System (version 1.7.2, http://www.pymol.org).

*Site-directed Mutagenesis*—Site directed mutagenesis of K407 (K407R, AAA>AGA, K407Q, AAA>CAA, K407Ac, AAA>TAG) and K425 (K425R, AAG>AGA, K425Q, AAG>CAA, K425Ac, AAG>TAG) of the ptACSL1 gene within the pMBP-C plasmid was performed using the Transformer™ Site-Directed Mutagenesis Kit (Takara, Dalian, Liaoning, China) and the procedure specified by the manufacturer. Colonies were selected by PCR, and positive mutants were verified by DNA sequencing.

*Genetically Encoding N*-Acetyllysine in Recombinant ptACSL Proteins*—To generate a homogenously K407- or K425-acetylated ptACSL1 construct, we used a three-plasmid based system as previously described (46, 47). The site-specific incorporation of N-acetyllysine was directed by a *Methanosarcina barkeri* acetyl-lysyl-tRNA synthetase/tRNACUA pair in response to the amber codon. We cloned the wild-type (WT) ptACSL1 into the pET21b vector producing a C-terminal 6×His-tagged construct, and incorporated an amber codon at K407 (AAA>TAG) or K425 (AAG>TAG) using site-directed mutagenesis as described above. The amber constructs (K407- and K425-acetylated ptACSL1 construct) were overexpressed in LB media with spectinomycin (50 μg/ml), kanamycin (50 μg/ml), and ampicillin (100 μg/ml) and with supplementation of 2 mM N-acetyllysine (Sigma-Aldrich) and 20 mM nicotinamide at the time of induction. WT ptACSL1, K407- and K425-acetylated ptACSL1 recombinant proteins were purified and used for enzyme activity measurement.

*In Vitro Protein Acetylation and Deacetylation Assay*—In vitro protein acetylation and deacetylation
assays were performed as described by You et al. (48) with minor modifications. For the acetylation assay, 2 µg of purified ptACSL1 was incubated with 20 mM AcP in 150 mM Tris-HCl (pH 7.3), 10% glycerol, 10 mM MgCl₂, and 150 mM NaCl. After incubation for 4 h at 37°C, the samples were mixed with 5×SDS loading buffer in a boiling water bath for 5 min and then separated by 12% SDS-PAGE. The acetylation level was analyzed by Western blotting using HRP-conjugated rabbit anti-acetyllysine antibody (1:1500).

For the deacetylation assay, 2 µg of purified acetylated ptACSL1 was incubated with 0.5 µg recombinant ptSTR5 in 50 mM HEPES (pH 8.5) buffer containing 1 mM MgCl₂ and 1mM NAD⁺. After 4 hours of incubation at 37 °C, the samples were mixed with 5×SDS loading buffer in a boiling water bath for 5 min and then separated by 12% SDS-PAGE. The acetylation level was analyzed by western blot using HRP-conjugated rabbit anti-acetyllysine antibody (1:1500).

**Acyl-CoA Synthetase Activity Assay**—Long-chain acyl-CoA synthetase activity was measured using an NADH consumption assay (49). Reactions contained HEPES buffer (50 mM, pH 7.5), TCEP (1 mM), ATP (2.5 mM), CoASH (0.5 mM), MgCl₂ (5 mM), phosphoenolpyruvate (3 mM), NADH (0.1 mM), pyruvate kinase (5 units), myokinase (5 units), lactate dehydrogenase (10 units), and sodium oleate as the acyl substrate (0.2 mM). Reactions were started by the addition of WT and mutant ptACSL1 protein (30 nM), and changes in the absorbance at 340 nm were monitored for 10 min in a 96-well plate format using a Microplate Spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA). Specific activities were calculated from the extinction coefficient of 12440 M⁻¹ cm⁻¹ for the oxidation of two molecules of NADH for each AMP released and reported as mmol min⁻¹ (mg of protein)⁻¹.

Experimental details for plasmid construction, recombinant ptACSL1 enzyme expression and purification, antibody production, western blotting, immunoelectron and fluorescence microscope, and DAPI staining are described in supplemental material.

**Experimental Design and Statistical Rationale**—This first large-scale analysis of the Lys acetylome in a photosynthetic microalga was performed from a single biological preparation and analysis of acetylated peptides from whole cell lysates of axenic cultures of *P. tricornutum* (CCMP 2561), a WT strain which
was grown on nutrient-replete or nutrient-deprived f/2 medium. Acetylated peptides from whole cell lysates were fractionated and enriched using high efficiency immune-affinity enrichment strategies, and analyzed with high-accuracy nanoflow LC-MS/MS technology (Fig. 1A). FDR thresholds for proteins, peptides, and modification sites were specified at maximum 1%. Data source of the Phaeodactylum proteome was from the Phaeodactylum tricornutum Annotated Standard Draft (Project ID: 1091374). Release version of 10, 402 entries was actually searched using the Filtered Models ("best") combined with Proteins (chromosomes): Phatr2_chromosomes_geneModels_FilteredModels2_aa.fasta.gz and Proteins (unmapped): Phatr2_bd_unmapped_GeneModels_FilteredModels1_aa.fasta.gz data (release date 2008/10/06). GO analysis using Blast2GO (29-31) and GO enrichment using Hypergeometric test (34) were performed. Benjamini-Hochberg FDR correction was carried out at a significance level of 0.05. Lys-acetylated motifs were analyzed by motif-x (36, 37) at a significance level of 0.000001. Secondary structures were predicted using NetSurfP (41) with *p*-values calculated by Wilcoxon test.

In the functional study, sequence- and Lys-acetylation-specific antibodies were prepared to confirm acetylated K407 (α-ptACSL1-acK407), K425 (α-ptACSL1-acK425), K321 (α-ptACSL4-acK321) and K325 (α-ptACSL4-acK325). To establish the functional consequence of Lys acetylation on Phaeodactylum LACS enzymes, we investigated the effect on LACS activity of acetylation of two Lys sites (K407 and K425). Three replicates were prepared and analyzed for each of the biological samples: WT, site directed mutagenesis of K407 and K425 of ptACSL1. The mutagenesis included a nonacetylated state K407R, K425R, and a mimic-acetylated state K407Q and K425Q. These recombinant proteins were purified and used for enzyme activity measurement. To generate a homogenously K407- or K425-acetylated ptACSL1 construct, we used a three-plasmid based system according to previous findings (46, 47). The amber codon at K407 (AAA>TAG) and K425 (AAG>TAG) was incorporated using site-directed mutagenesis. WT ptACSL1, K407-, and K425-acetylated ptACSL1 recombinant proteins were purified and used for enzyme activity assays. Mean and standard deviation of the three independent experiments are presented and the statistical tests (paired *t* test) are used to analyze the data in the respective figures and/or manuscript sections.
Results

Enrichment of Acetylation Sites in Phaeodactylum—Previous studies have described the global survey of Lys acetylation in plants, but none to our knowledge have focused on this PTM in diatoms. To facilitate a systems-level understanding of Lys acetylation-dependent events in diatoms, we utilized acetylome analysis to reveal the regulatory role of Lys acetylation in cellular metabolism in Phaeodactylum. First, we performed western-blot analysis to identify the distribution of Lys acetylation on Phaeodactylum proteins using anti-acetyllysine antibody. Figure 1A showed that multiple Lys acetylated proteins with distinct patterns of acetylation were detected under different growth conditions, representing the presence of diverse acetylated events in this organism. Then, the total proteins from exponentially growing cells under these various stress conditions were extracted and digested to explore the global picture of Lys acetylated events in this model diatom. Finally, acetylated peptides were enriched using acetyllysine antibody and the modified sites were analyzed by 2D-LC/MS/MS (Figure 1B). Our data cover 2324 unique acetylation sites identified, belonging to 1220 acetylated proteins (supplemental Table S1), implying that protein acetylation may play an important role in a wide variety of physiological processes. The high mass accuracy of the modified peptide data obtained from MS was estimated and the average absolute mass error was 0.5803 ppm in our data (Figure 1C). Among these proteins, approximately 58.11% of these acetylated proteins contained one single acetylated site, while 21.72% contained two acetylated sites. Notably, more than 6.15% proteins carried at least five identified sites, including two long chain acyl-CoA synthetases (ptACSL1 and ptACSL4) (Figure 1D). This dataset provides a systems-wide view of Lys acetylation in Phaeodactylum.

Functional Characterization and Enrichment of Acetylated Proteins - To gain a better understanding of the role and the distribution of the identified acetylproteins in Phaeodactylum, GO function classification analysis of these proteins were performed according to their biological process, and molecular function using Blast2GO, and subcellular localization using ngLOC and ASAFind (supplemental Fig. S1, supplemental Table S2 and Table S3). Analysis of the biological process showed that multiple proteins were acetylated in cellular nitrogen compound metabolic process (252), small molecule metabolic process
13

(224), macromolecule metabolic process (160), organonitrogen compound metabolic process (150) and other processes. Molecular function analysis indicated that acetylated proteins associated with catalytic activity and binding accounted for over half of all the identified acetylproteins. Based on subcellular localization prediction, 347 (28.4%) were assigned to the nucleus, 156 to the cytoplasm, 114 to the plasma membrane, 95 to the mitochondria, 67 to the chloroplast, 16 to the endoplasmic reticulum, 425 to other subcellular compartments. A multiple testing correction was also applied and the statistical test was performed as shown in supplemental Table S4. The subcellular localization of Lys-acetylated proteins was further investigated by immunoelectron microscopy. Lys-acetylated proteins were distributed in the whole cell including the plastid, nucleus and vacuole (supplemental Fig. S2A-D). As a negative control, almost no anti-acetylsine signal was observed when the chemically acetylated BSA was used to block the anti-acetylsine antibody (supplemental Fig. S2E).

GO enrichment analyses (biological process, molecular function, and cellular component) were conducted for further determination of proteins targeted for Lys acetylation (supplemental Table S5). In the biological process ontology, acetylproteins in biological process (p-value, 2.73E-14), metabolic processes (p-value, 1.44E-12), cellular biosynthetic processes (p-value, 4.28E-12) and so on were significantly enriched. GO enrichment analysis on the category of molecular functions demonstrated that many functions were highly enriched in the structural constituent of ribosome (p = 2.68E-06) and in structural molecule activity (p = 3.39E-06). These findings suggest that acetylated proteins in *Phaeodactylum* may play essential roles in various cellular processes.

*Analysis of Acetylated Sites*—To evaluate the conservation of the sequences of the neighboring regions of these acetylated lysines in *Phaeodactylum*, the occurrences of six flanking amino acids frequencies upstream and downstream surrounding the acetylation site were compared with those of all lysine residues that occur in this model diatom proteome using motif-x. Recent studies have found amino acid residue over- or under represented at particular positions surrounding the acetylated lysine site in plants (50, 51) and bacteria (18, 20, 52-54). In this study, we defined 17 significantly enriched acetylation site motifs from 1894 unique sites accounting for 81.5% of sites identified, namely LKacY, KacY, LKac,
Kac*F, I*Kac*L, YKac, K****Kac, FKac, KacH, KacF, K***Kac, I*Kac, Kac*L, Kac*Y, KacW, K*****Kac, F**Kac (Figure 2A and supplemental Table S6), and they display distinct abundance (supplemental Fig. S3) (Kac indicates the acetylated lysine, and * represents a random amino acid residue). Three of these motifs, enrichment of Y, H or F on the +1 position toward the C terminus appeared frequently as previously described (50, 51). The motifs KacH, KacY, Kac*F and KacF were also reported in strawberry (50) and KacH, KacY and KacF in bacteria (18, 52, 53), showing that plants and bacteria shared some conserved motifs with the diatom. Interestingly, considerable nonpolar hydrophobic amino acids such as F on the -1/+1/+2 positions, I on the -1 position, L on the -1/+2 positions and W on the +1 position were observed among these motifs. Several motifs, including Y on the +1 accompanied by L on the -1 position or L on the +2 / I on the -1 position were first identified in plants. Additionally, heatmap analysis was conducted to show the frequency of different amino acids around the acetylated lysine (Figure 2B). Colors were plotted by intensity map and represented the log10 of the ratio of frequencies within acetyl-13-mers versus non-acetyl-13-mers (red for enrichment and blue for depletion). It could be observed that the residue preferences for acetylated peptides are Y at the −1 and +1 positions, F at the -1/+1/+2 positions, and L on the -1/+2 positions, most of which are in accordance with the previous findings by motif-x analysis. These data show the preference of several amino acid residues for acetylation in *Phaeodactylum*.

Secondary structure analysis was performed to reveal the relationship between the Lys acetylation with local secondary structures of acetylated proteins. It demonstrates that the acetylation sites distribution was about 52.5% in α-helix, 11.3% in β-sheet, and 36.2% in coil (Figure 2C), which is similar to the distribution in the cyanobacterium *Synechocystis* (20) but differs from secondary structural regions of acetylated lysines identified in rice (55) and most of the other bacterial acetylomes (52, 54). This finding suggests that Lys acetylation might prefer to be located in α-helix structures in photosynthetic organisms.

**Comparative Analysis with Other Acetylomes**—Lys acetylation is an ancient and conserved PTM across both prokaryotes and eukaryotes. To reveal the metabolic pathway regulated by conserved acetylated proteins, we performed a systematic analysis of the orthologs of acetylproteins by comparing the
Phaeodactylum acetylproteome with several previous reported acetylproteomes of cyanobacteria, yeast, and plant. Orthologs of 749 (61.4%) acetylated proteins can be detected in the other acetylproteomes. of 1220 acetylated proteins from Phaeodactylum, 227, 14, 63, 35, 183, 481, 563 proteins share sequence similarity with orthologs from Arabidopsis thaliana (21, 22), Vitis vinifera (51), Solanum tuberosum (56), Oryza sativa (55), Glycine max (57), Fragaria ananassa (50), Saccharomyces cerevisiae (15, 58), and Synechocystis sp. PCC 6803 (20), respectively (supplemental Fig. S4 and supplemental Table S7). Comparative analysis of these acetylproteomes revealed that Lys acetylation might regulate a number of functionally conserved proteins among these organisms. For example, our data found that three out of six Lys-acetylated sites in the manganese stabilizing protein (PsbO), including K131, K184 and K219, were located in KL, GGER and DPKGR highly conserved regions (59) from Phaeodactylum (protein ID 20331). The site of K184 in GGER region was also detected to be acetylated in Arabidopsis and Fragaria (versus K216 in Arabidopsis and K215 in Fragaria), and K219 in DPKGR region detected in Synechococcus sp. PCC 7002 (versus K190 in Synechococcus sp. PCC 7002) (60), implying the modifications events may occur in other photosynthetic organisms.

Lysine Acetylated Enzymes in the Fatty Acid Synthesis Pathway—Similar to higher plants, diatoms synthesize fatty acids in plastids through the type II fatty acid synthesis pathway, in which each enzymatic activity is encoded by a discrete protein. At least seven enzymes of the fatty acid synthase system, plastidial acetyl-CoA carboxylase (ACCase1), cytosolic acetyl-CoA carboxylase (ACCase2), enoyl-ACP reductase (FabI), malonyl-ACP: ACP transacylase (FabD), 3R-hydroxyacyl-ACP dehydrase (FabZ), 3-oxoacyl-ACP synthase (FabF) and 3-oxoacyl-ACP reductase (FabG) were found to be Lys-acetylated, indicating a possible role of acetylation in regulating fatty acid synthesis in Phaeodactylum (supplemental Fig. S5). These Lys-acetylated enzymes catalyze the major reactions of carbon fixation and carbon chain elongation in the fatty acid synthesis pathway. For example, ACCase, the first rate-limiting enzyme of fatty acid synthesis pathway, catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA. The Phaeodactylum genome encodes two ACCases, ptACC1 located in the plastid and ptACC2 in the cytosol (61). We identified 11 Lys acetylation sites at ptACC1, among which 7 sites are located at the conserved
functional domains. By contrast, we detected only 3 Lys acetylation sites at the cytosolic ptACC2. Prediction of the secondary structure of ptACC1 shows that approximately 72.7% (8/11 sites) of the acetylation sites are located in the region of ordered secondary structures, and 7 acetylated lysines are located in α-helix and 1 site was in β-sheet (supplemental Fig. S6). In addition to ACCases, 5 proteins (FabD, FabF, FabG, FabZ and FabI) were identified to be acetylated at 9 Lys sites, of which 7 acetylated sites were located in α-helix and 1 site was in β-sheet (supplemental Fig. S5). This observation suggests a structural preference of acetylation sites for ordered secondary structure to disordered structure in these enzymes.

*Functional Characterization of Lysine Acetylation of Acyl-CoA Synthetase ptACSL1*—Long-chain acyl-CoA synthetase (LACS, EC 6.2.1.3), belonging to the AMP-forming acyl-CoA synthetase superfamily, catalyzes the esterification of free fatty acids to their activated form of acyl-CoAs, a key activation step necessary for the utilization of fatty acids by many lipid metabolic pathways such as phospholipid and TAG biosynthesis, and fatty acid β-oxidation (62). In the LACS-catalyzed two-step reaction, free fatty acid is converted to an acyl-AMP intermediate through the pyrophosphorolysis of ATP, followed by the coupling of the activated carbonyl carbon to the thiol group of CoA, and then releases AMP and fatty acyl-CoA (63). LACS enzyme appears to play a fundamental role in providing activated acyl group for nearly all fatty acid-related metabolic pathways. The diatom Phaeodactylum, which contains high concentration of eicosapentaenoic acid (20: 5) in its fatty acid profile, has at least 5 genes annotated to encode putative long-chain acyl-CoA synthetases. In our previous study, of the 5 putative LACS proteins, ptACSL1 and ptACSL4 were found to have the ability to complement growth deficiency of the yeast Saccharomyces cerevisiae double mutant FAA1ΔFAA4Δ, and to facilitate exogenous fatty acid uptake (64). Here both ptACSL1 and ptACSL4 were identified to be abundantly Lys acetylated. As many as six different Lys acetylation sites (K407, K425, K683, K648, K662, and K710) were identified in ptACSL1. Similarly, ptACSL4 was also found to be acetylated at six different lysine sites (K86, K153, K305, K321, K325, and K601). To confirm Lys acetylation of ptACSL1 and ptACSL4, we developed sequence- and Lys-acetylation-specific antibodies to recognize acetylated K407 (α-ptACSL-acK407),
K425 (α-ptACSL1-acK425), K321 (α-ptACSL4-acK321) and K325 (α-ptACSL4-acK325). We confirmed that these four Lys residues were acetylated in vivo in *Phaeodactylum* cells and Lys-acetylated ptACSL1 and ptACSL4 exhibited an expression pattern which was different from those recognized by sequence-specific antibodies under nutrient stress (Figure 3A and B). As the negative control, no signal was detected when Lys-acetylation-specific antibodies were pre-incubated with the respective acetylated peptides prior to further immunoblot analysis. To establish the functional consequence of Lys acetylation on *Phaeodactylum* LACS enzymes, we investigated the effects of site-specific acetylation (K407 and K425 located at the AMP-binding domain of ptACSL1, Figure 4A) on LACS activity.

To evaluate the effect of acetylation of individual Lys sites on ptACSL1 activity, we first mutated individually K407 and K425 located AMP-binding domain of ptACSL1 to glutamine (K407Q, K425Q), which is noncharged and commonly used to mimic acetylated lysine, or to arginine (K407R, K425R) which is similarly charged and cannot be acetylated. The results of enzyme activity assay showed that all point mutations led to a decrease in LACS activity (Figure 4B). These two lysines therefore appear to be important for the catalytic activity of ptACSL1. Furthermore, ptACSL1 with the mutation of K407Q had an enzyme activity slightly higher than that of K407R, and a similar result was observed for K425. Site-directed mutagenesis suggests that acetylation on K407 and K425 might be a positive regulatory modification on ptACSL1 activity.

To further demonstrate the effect of Lys acetylation on LACS activity of ptACSL1, we used the three-plasmid-based system of genetically encoding N⁵-acetyllysine to synthesize recombinant proteins in *Escherichia coli* (46, 47). This expression system allows the overexpression and purification of recombinant ptACSL1 proteins with 100% acetylation at K407 or K425 due to the suppression of the K407-TAG and K425-TAG stop codon by the N⁵-acetyllysine-conjugated amber suppressor tRNA. We purified K407-, K425-acetylated, and unacetylated ptACSL1 and compared their enzyme activity. As shown in Figure 4C, acetylation of K407 and K425 led to a significant increase in LACS activity of ptACSL1 when compared to that of unacetylated ptACSL1. These results demonstrate that acetylation at
K407 and K425 enhances ptACSL1 activity.

Because sirtuin-catalyzed deacetylation is a common mechanism for the regulation of acyl-CoA synthetases in bacteria, we attempted to identify putative sirtuins having the ability to deacetylate ptACSL1 in *Phaeodactylum*. To examine whether sirtuin-type deactylases (SRT) are capable of deacetylating ptACSL1 in vitro, we overexpressed four *Phaeodactylum* sirtuin homologs in *E. coli* with full-length coding sequences (ptSRT2, protein id: 21543; ptSRT3, protein id: 39523; ptSRT4, protein id: 45850; and ptSRT5, protein id: 52135). Among these four sirtuin homologs, only two recombinant sirtuin proteins (ptSRT3 and ptSRT5) could be induced and purified to be soluble proteins. We prepared the acetylated ptACSL1 using nonenzymatic catalysis by acetyl-phosphate (AcP). When the acetylated ptACSL1 was incubated with purified ptSRT5, ptACSL1 could be effectively deacetylated in the presence of NAD⁺, whereas ptSRT3 was unable to induce the deacetylation of ptACSL1 in the same reaction system (Figure 4D). Furthermore, addition of nicotinamide, a specific inhibitor of the SIRT family of deacetylases, completely inhibits ptSRT5 activity for deacetylation of acetylated ptACSL1 (Figure 4E). These results suggest that ptACSL1 acetylated by AcP can be reversed by ptSRT5, a sirtuin protein of the SIRT family of deacetylases in *Phaeodactylum*.

To determine the subcellular localization of *Phaeodactylum* ptACSL1 and ptSRT5, we constructed GFP fusions of the full-length open reading frames of ptACSL1 and ptSRT5 which are C-terminally fused with eGFP, respectively. We transformed these fusion constructs into *Phaeodactylum* and used to define the localization of them in *Phaeodactylum*. Fluorescent observation of positive transformants showed that full-length ptACSL1-eGFP fusion protein directed GFP in the plastid of *Phaeodactylum* (Figure 5A). For *Phaeodactylum* transformants expressing ptSRT5-eGFP construct, a GFP signal was detected in the complex plastid as well as in the nucleus (Figure 5B). Because both ptACSL1 and ptSRT5 can be localized in the plastid, we reasoned that ptACSL1 may be regulated by reversible Lys acetylation and deacetylation by ptSRT5 in the plastid of *Phaeodactylum*.

**Discussion**
Regulation of Acyl-CoA Synthetase Activity by Lysine Acetylation in Prokaryotes and Eukaryotes—Over the past decades, acetylome profiling has identified a large number of Lys acetylated proteins. Most acetylated proteins are metabolic enzymes that are implicated in numerous cellular functions beyond the transcriptional regulation of histones (65, 66). Regulation of bacterial acyl-CoA synthetase activity by Lys acetylation has been well studied in Rhodopseudomonas palustris. In this photosynthetic α-proteobacterium, the protein acetyltransferase RpPat can recognize and acetylate at least 10 AMP-forming acyl-CoA synthetases involved in the activation of short, medium, and long chain fatty acids and aromatic acids. All these acyl-CoA synthetase substrates acetylated by RpPat contain the conserved PXGK(ac) motif at the acetylation site (67), and the presence of such conserved motif is necessary but not sufficient for substrate recognition by RpPat (68). The alignment of protein sequences of acyl-CoA synthetases that have been reported to be acetylated in prokaryotes and eukaryotes shows that the conserved lysine that is acetylated in all these prokaryotic acyl-CoA synthetases is substituted to Ala in Phaeodactylum ptACSL1 and Val in the yeast scFaa1p, at an equivalent position to K598 in RpPrpE (supplemental Fig. S7). This substitution reveals a positional conservation of acetylated lysine of acyl-CoA synthetases among bacteria but not eukaryotes. In this study, site-directed mutagenesis and site-specific acetylation demonstrate that acyl-CoA synthetase activity of ptACSL1 is positively regulated by K407 and K425 acetylation (Figure 4), which contrasts to the negative regulation of Lys acetylation at the conserved K609 on acyl-CoA synthetase from the gram-negative enterobacterium Salmonella enterica (69). There is probably a distinct Lys acetylase/deacetylase catalytic mechanism for Phaeodactylum LACs from that in bacteria. Because the number of acetylated eukaryotic acyl-CoA synthetases is very limited, it is now difficult to determine whether or not a bacteria-like conserved motif in which Lys is acetylated is also present in eukaryotic acyl-CoA synthetases. Notably, unlike the single acetylation event that occurs at Lys residue in bacterial acyl-CoA synthetases, eukaryotic acyl-CoA synthetases appear to be acetylated at multiple Lys sites, which may suggest the regulatory complexity of acyl-CoA synthetases by Lys acetylation in eukaryotes.
Cross-regulation between Acetylation and Ubiquitylation—In addition to acetylation, reversible protein phosphorylation and ubiquitylation are also two prominent and ubiquitous PTMs regulating protein function. Ubiquitylation is an important and frequently studied PTM, through which a Lys residue of a substrate protein is modified by the small protein ubiquitin (Ub) or a Ub chain consisting of multiple or poly-Ub molecules. Typically, the activities of three enzymes, namely E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligase), are required for ubiquitylation (70). The *Phaeodactylum* genome is predicted to encode approximately 5 E1s, 12 E2s, and 25 E3s, of which 2 E1s, 2 E2s, and 4 E3s were identified to be acetylated, indicating that the ubiquitylation machinery might be regulated by protein acetylation in *Phaeodactylum*. The effect of acetylation of a conserved Lys residue in the N-terminal helix (‘KRX<sub>2</sub>KEX<sub>3</sub>L’) in *Drosophila* E2 enzyme on its activity has been characterized by genetic and biochemical analysis. The results show that acetylation of this conserved Lys residue impaired the function of ubiquitin conjugating enzyme E2 (71). It was found that acetylation of this Lys residue is conserved from yeast to human. In our acetylome study, we identified Lys acetylation of two E2 enzymes in *Phaeodactylum*, Pt_E2_22525 (K90) and Pt_E2_36198 (K95 and K97). Sequence alignment of Pt_E2_22525 and Pt_E2_36198 with yeast, fly, and human E2 enzymes revealed the presence of the conserved N-terminal motif ‘KRX<sub>2</sub>KEX<sub>3</sub>L’ in these two E2 enzymes from *Phaeodactylum* (supplemental Fig. S8). However, we found that acetylation did not occur at the corresponding conserved Lys site in the N-terminal motif but occurred at the lysine in the C-terminal region of *Phaeodactylum* E2s. The difference in the acetylation site of E2 enzymes suggests that acetylation might regulate protein function of *Phaeodactylum* E2s in a manner distinct from that in other eukaryotic organisms. Nevertheless, Lys acetylation of multiple components of the *Phaeodactylum* ubiquitylation machinery may represent an example of cross-regulatory events and one form of cross-talk between acetylation and ubiquitylation in *Phaeodactylum*.

Acetylation of Metabolic Enzymes is Controlled by a Limited Number of Acetyltransferases and Deacetylases—The acetylation level of histones and metabolic enzymes within the cell may be predominantly determined by the activity of both KATs and KDACs. In *Phaeodactylum*, 24 genes were
annotated to encode KATs, of which 6 KATs are putative histone modifiers. In this study, we identified at least 39 acetylated Lys sites in *Phaeodactylum* histones, including the highly conserved H4 isoform 1b, H5, three variants of histone H2 (H2A isoform 1, 3b and H2B isoform 1a) and two variants of histone H3 (H3 isoform 1a and 2). Given the presence of 6 nucleus-localized KATs that mediate the dynamic modification of histones, Lys acetylation of a large number of non-histone proteins and metabolic enzymes might be catalyzed by the other 18 KATs with the predicted subcellular localization of other compartments.

The *Phaeodactylum* KDACs are encoded by 15 genes and can be grouped into two large classes: the classic Histone Deacetylase and Silent Information Regulator 2 (SIR2). The SIR2 class of KDAC, also called sirtuins, catalyzes the deacetylation reaction in an NAD⁺-dependent manner. The *Phaeodactylum* genome encodes four sirtuins, of which ptSRT5 is localized in the plastid (Figure 5B). The unrooted phylogenetic tree, which was constructed with putative protein sequences of four *Phaeodactylum* sirtuins and other eukaryotic and prokaryotic sirtuin core domain sequences, shows that two *Phaeodactylum* sirtuins are class II and class IV sirtuins while the other two could not be classified into these five subgroups (Figure 6A). Among the four *Phaeodactylum* sirtuins, ptSRT5 belongs to the class IV sirtuins that are not present in prokaryotes, and it shares 44%–53% sequence identity with other homologous sirtuins. Further sequence alignment of class IV sirtuins led to the identification of several short motifs of conserved amino acids (Figure 6B). These motifs include GAGISTXXGIPXXR, GPXXXWT, QNXDGLH, and HG that are strictly conserved in all known sirtuins, and CXXC that may be a zinc finger domain. In our study, the deacetylation activity of ptSRT5 was confirmed by incubation with ptACSL1 that was acetylated by AcP (Figure 4 D and E). In vitro, the nonenzymatic acetylation of ptACSL1 using AcP led to overall hyperacetylation of ptACSL1, and identification of the acetylated sites of ptACSL1 by LC-MS/MS revealed the acetylation of 25 Lys sites (supplemental Fig. S9), including those 6 acetylation sites in our acetylome analysis (K407, K425, K683, K648, K662, and K710). Although hyperacetylation of ptACSL1 could be deacetylated by ptSRT5, deacetylase activity was not
observed when ptACSL1 with specific acetylation on K407 or K425 was used as substrate in our in vitro assay (data not shown). This result is consistent with the observation that there was still residual unacetylated ptACSL1 when AcP-acetylated ptACSL1 was deacetylated by ptSRT5 (Figure 4 D and E), and might suggest Lys site selectivity of ptSRT5 on ptACSL1. Because ptSRT5 contains a typical SIR2 domain and has a subcellular location of the plastid and nucleus (Figure 5 B), we postulated that ptSRT5 might catalyze Lys deacetylation in both the plastid and nucleus in *Phaeodactylum*. In addition to regulation of the activity of metabolic enzymes, sirtuins modulate gene silencing, aging, energy metabolism and also determine the cellular life span in eukaryotes (72, 73). Biological significance of the SIR2 family deacetylases in diatom remains to be determined.

Together, our data presented here demonstrate that extensive Lys acetylation occurs in cellular enzymes involved in fatty acid metabolism in *Phaeodactylum*. Further functional studies by site-directed mutagenesis (on K407 and K425) and site-specific incorporation of N°-acetyllysine in recombinant ptACSL1 (at K407 and K425) show that site-specific acetylation at these two Lys sites increases ptACSL1 activity. Western blot analysis with Lys-acetylation-specific antibodies (against K407 and K425) reveals the dynamically acetylated state of K407 and K425 sites of ptACSL1 under different nutrient stress conditions. In addition, we identified ptSRT5 as a plastid- and nuclear-localized sirtuin-type deacetylase and found that it possessed deacetylase activity for ptACSL1, indicating the potential role of ptSRT5 in regulating fatty acid metabolism. Our work provides a large-scale dataset of the Lys-acetylated proteome for elucidating the functional significance of Lys acetylation modification in fatty acid metabolism in the model diatom *Phaeodactylum* in the future.
Footnotes

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DATA AVAILABILITY
The raw mass spectrometry data, annotated spectra of acetylated peptides, and the whole of the results folder created by MaxQuant as well as the version of MaxQuant software have been deposited in the PeptideAtlas database that can be accessed with the identifier PASS01080 (http://www.peptideatlas.org/PASS/PASS01080).
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Figure Legends

Figure 1. Overview of the lysine acetylome in *Phaeodactylum tricornutum*. *A*, Anti-acetyllysine western blotting analysis in response to nutrient stress. Equal amounts of protein samples were extracted under normal cultural condition (C), nitrogen deficiency (−N), phosphate deficiency (−P) and iron deficiency (−Fe). The gel stained with Coomassie Brilliant Blue acts as a loading control. *B*, The workflow of integrated strategy for global mapping of the lysine acetylome analysis. *C*, Mass error distribution of the lysine-acetylated peptides. *D*, Pie chart illustration of the number of lysine acetylation sites per protein.

Figure 2. Bioinformational analysis of lysine acetylation sites. *A*, Over-represented Lys-acetylation motif. Amino acid frequency plots of 13-residue regions centered on identified acetylation sites using motif-x. Seventeen statistically significantly enriched acetylation site motifs were identified. *B*, The intensity map shows the relative abundance of amino acids in positions from -6 to +6 flanking the acetylated lysine site based on all identified acetylation sites compared to the overall proteome amino acid frequency distribution. The colors in the intensity map represent the log10 ratios of frequencies within acetyl-13-mers versus the frequencies within non-acetyl-13-mers (red for enrichment and blue for depletion). *C*, Distribution of acetylated and nonacetylated lysine in the protein secondary structures. The probabilities of acetylated lysines in different secondary structures (α helix, β strand and coil) were compared with the secondary structure probabilities of all lysines on all the proteins identified in this study.

Figure 3. Western blot analysis of levels of acetylation on ptACS1 (*A*) and ptACS4 (*B*) proteins under nutrient stress (normal cultural condition (C), nitrogen deficiency (−N), phosphate deficiency (−P) and iron deficiency (−Fe)) using acetyl-ptACS1 (K407 and K425) and acetyl-ptACS4 (K321 and K325) antibodies, respectively.

Figure 4. Site-specific acetylation of ptACSL1 increases the acyl-CoA synthetase activity and the hyperacetylation of ptACSL1 by acetyl phosphate can be effectively reversed by the sirtuin family deacylase ptSRT5. *A*, Domain architecture of the long-chain acyl-CoA synthetase ptACSL1 and the
sirtuin-type deacetylase ptSRT5. The domain analysis was performed with InterProScan implemented in the EMBL-EBI Workplace. The protein lengths are displayed on the right. Different domains are represented by different colors and lengths at their relative position in the protein sequence from the N-terminus to the C-terminus. TM, transmembrane region. B, Effect of site-directed mutagenesis on ptACSL1 activity. The p value was calculated by paired t test. C, Site-specific acetylation of K407 and K425 increases enzyme activity of ptACSL1. Acetylation levels of the purified ptACSL1 protein without or with site-specific acetylation were determined by Western blotting using the anti-acetylated lysine antibody and the acyl-CoA synthetase activity was measured. D, The sirtuin family deacetylase ptSRT5 deacetylates ptACSL1. E, ptSRT5-catalyzed deacetylation of ptACSL1 which was acetylated by acetyl phosphate is inhibited by nicotinamide (NAM).

Figure 5. Subcellular localization of the long-chain acyl-CoA synthetase ptACSL1 and the sirtuin-type deacetylase ptSRT5 in *Phaeodactylum tricornutum*. A, Expression of eGFP fusion protein indicated the localization of ptACSL1 in complex plastid. GFP, enhanced green fluorescent protein; Chl/GFP, overlay of plastid and GFP fluorescence. B, *Phaeodactylum* cells expressing the ptSRT5:eGFP construct showed a green fluorescence inside both the plastid and nucleus. Chl/DAPI, overlay of plastid and nucleic region stained with DAPI (4',6-diamidino-2-phenylindole); BF/Chl/DAPI, overlay of bright field cells, plastid and nucleic region stained with DAPI; scale bar represents 5.0 μm.

Figure 6. Phylogenetic tree of prokaryotic and eukaryotic sirtuins and alignment of sirtuins from IV class. A, An alignment of the conserved domains of sirtuins was analyzed using ClustalW, and NJ phylogram was constructed using Mega 6. *Phaeodactylum* sirtuins were shown in blue. Scale bar indicates 0.05 substitutions per amino acid position. B, Sirtuins from IV class was aligned and motifs of partial conserved amino acid residues were shown. SIRT: sirtuins from *Homo sapiens*; *B. sub*: *Bacillus subtilis*; *C. ace*: *Clostridium acetobutylicum*; *C. dif*: *Clostridioides difficile*; *S. aur*: *Staphylococcus aureus*; *T. mar*: *Thermotoga maritima*; *M. tub*: *Mycobacterium tuberculosis*; *E. coli*: *Escherichia coli*; *A. aeo*: *Aquifex aeolicus*; *A. ful*: *Archaeoglobus fulgidus*; *P. aby*: *Pyrococcus abyssi*; *A. per*: *Aeropyrum pernix*; *A. tha*: *Arabidopsis thaliana*; *O. sat*: *Oryza sativa* L.; *D. mel*: *Drosophila melanogaster*; *C. ele*: *Caenorhabditis*
elegans; S.cer: Saccharomyces cerevisiae; L.maj: Leishmania major; S.coe: Streptomyces coelicolor; M.avi: Mycobacterium avium.
**Figure 1**

A. SDS-PAGE

B. Cell culture under stress conditions

1. Cell harvesting and protein extraction
2. Proteolysis and acetylated peptides enrichment
3. LC-MS/MS analysis
4. Functional analysis

C. Average absolute mass error = 0.5803 (ppm)
   STDEV = 0.5706 (ppm)

D. Functional analysis:
   - ptACSL:
     - antiAc
     - antiAc
     - Acetylated
     - NAD
     - Sirtuin
   - anti-AcK
   - NAD
   - Sirtuin

Legend:
- 1
- 2
- 3
- 4
- >=5

Mass error [ppm]

m/z

-4 -2 0 2 4 6

0 400 800 1200 1600

Average absolute mass error = 0.5803 (ppm)
STDEV = 0.5706 (ppm)
Figure 2

A

B

C

Log₁₀ (amino acid frequency)

0.525 0.400 0.113 0.132 0.362 0.468

Mean Second Structure Probability

0.0402 0.756 0.00455

p value

0.00455

Acetylated-Lysines

Nonacetylated-Lysines

Alpha-helix 0.0402

Beta-strand 0.756

Coil 0.00455
**Figure 3**

A

| Protein          | C  | N  | P  | Fe  |
|------------------|----|----|----|-----|
| \(\alpha\)-ptACSL1-acK407 |    | 70 KDa |    |     |
| \(\alpha\)-ptACSL1-acK407-peptide blocking |    |       |    |     |
| \(\alpha\)-ptACSL1-acK425 |    | 70 KDa |    |     |
| \(\alpha\)-ptACSL1-acK425-peptide blocking |    |       |    |     |
| Loading control  | 15 KDa |    |    |     |

B

| Protein          | C  | N  | P  | Fe  |
|------------------|----|----|----|-----|
| \(\alpha\)-ptACSL4-acK321 |    | 70 KDa |    |     |
| \(\alpha\)-ptACSL4-acK321-peptide blocking |    |       |    |     |
| \(\alpha\)-ptACSL4-acK325 |    | 70 KDa |    |     |
| \(\alpha\)-ptACSL4-acK325-peptide blocking |    |       |    |     |
| Loading control  | 15 KDa |    |    |     |
Figure 4

A

ptACSL1

N

AMP-binding

KK

C (721 aa)

ptSRT5

N

SIR2 family

C (307 aa)

B

LACS activity (nmol/mg/min)

0

50

100

150

anti-His

anti-AcK

UnAc-ptACSL1

K407Ac-ptACSL1

K425Ac-ptACSL1

WT

K407Q

K407R

K425Q

K425R

p<0.05*

p<0.05*

p<0.01**

C

LACS activity (nmol/mg/min)

anti-His

anti-AcK

UnAc-ptACSL1

K407Ac-ptACSL1

K425Ac-ptACSL1

D

ptACSL1WT

anti-AcK

ptSRT5

anti-AcK

ptSRT3

Acetylated

-  

-  

-  

-  

-  

NAD+

-  

-  

+  

-  

-  

Sirtuin

-  

-  

-  

+  

+  

E

anti-His

anti-AcK

Acetylated

NAD+

NAM

-  

-  

-  

-  

-  

-  

-  

-  

-  

+  

+  

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Figure 5

**Bright field Chlorophyll GFP Chl/GFP**

(A) nucleus

(B) plastid

**Bright field Chlorophyll GFP Chl/DAPI BF/Chl/DAPI**

(BF/Chl/DAPI)

5 μm

5 μm

plastid

nucleus
Figure 6