NuA4 Lysine Acetyltransferase Complex Contributes to Phospholipid Homeostasis in Saccharomyces cerevisiae

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ABSTRACT Actively proliferating cells constantly monitor and readjust their metabolic pathways to ensure the replenishment of phospholipids necessary for membrane biogenesis and intracellular trafficking. In Saccharomyces cerevisiae, multiple studies have suggested that the lysine acetyltransferase complex NuA4 plays a role in phospholipid homeostasis. For one, NuA4 mutants induce the expression of the inositol-3-phosphate synthase gene, INO1, which leads to excessive accumulation of inositol, a key metabolite used for phospholipid biosynthesis. Additionally, NuA4 mutants also display negative genetic interactions with sec14-1ts, a mutant of a lipid-binding gene responsible for phospholipid remodeling of the Golgi. Here, using a combination of genetics and transcriptional profiling, we explore the connections between NuA4, inositol, and Sec14. Surprisingly, we found that NuA4 mutants did not suppress but rather exacerbated the growth defects of sec14-1ts under inositol-depleted conditions. Transcriptome studies reveal that while loss of the NuA4 subunit EAF1 in sec14-1ts does derepress INO1 expression, it does not derepress all inositol/choline-responsive phospholipid genes, suggesting that the impact of Eaf1 on phospholipid homeostasis extends beyond inositol biosynthesis. In fact, we find that NuA4 mutants have impaired lipid droplet levels and through genetic and chemical approaches, we determine that the genetic interaction between sec14-1ts and NuA4 mutants potentially reflects a role for NuA4 in fatty acid biosynthesis. Altogether, our work identifies a new role for NuA4 in phospholipid homeostasis.

KEYWORDS triacylglycerols steryl esters inositol/choline responsive elements (ICREs) FAS1/FAS2 cerulenin

Cellular processes, such as proliferation and intracellular trafficking, depend on a vast network of proteins involved in regulating phospholipid metabolism (Figure 1). Membrane phospholipid composition is, in part, established by highly conserved families of lipid-binding proteins that, as their name suggests, bind cellular lipids to facilitate lipid transport, modulate enzymatic activity, and even regulate the transcription of metabolic genes (reviewed in Henry et al. 2012; Olkkonen 2013; Jackson and Bouvet 2014; Tripathi et al. 2014). Phospholipid metabolism can also be fine-tuned by signaling pathways such as post-translational modifications. Indeed, it has recently emerged that lysine acetylation contributes to lipid metabolism by regulating both gene expression and the activity of metabolic enzymes (reviewed in Drazic et al. 2016; Menzies et al. 2016).

Several genome-wide genetic screens performed in Saccharomyces cerevisiae have revealed interactions between the highly conserved lysine acetyltransferase (KAT) complex NuA4 and phospholipid metabolic genes, suggesting a role for NuA4 in phospholipid homeostasis. NuA4 is a 13-subunit KAT complex containing the essential catalytic domain Esa1 (reviewed in Doyon and Cote 2004) and held together by the scaffolding protein Eaf1 (Auger et al. 2008; Mitchell et al. 2008). One role for NuA4 is the regulation of chromatin remodeling and gene transcription through the acetylation of histones H4 and H2A-Z (reviewed in Lu et al. 2009), and growing evidence indicates that NuA4 also targets nonhistone proteins (reviewed in Downey and Baetz 2016).
Interestingly, several functional genomic screens have determined that NuA4 subunit mutants display an excessive accumulation of inositol, also called an Opi- phenotype (Hancock et al. 2006; Salas-Santiago and Lopes 2014). Opi1 is a lipid-binding protein that represses transcription of target genes including the inositol-3-phosphate synthase, INO1, as well as other phospholipid metabolic genes containing inositol/choline-responsive elements (ICREs) within their promoters (Greenberg et al. 1982; Hirsch and Henry 1986; White et al. 1991). These genes are derepressed under inositol-depleted conditions through a mechanism involving the relocation of Opi1 from the nucleus to the endoplasmic reticulum, where it binds to phosphatidic acid (Loewen et al. 2003, 2004). Deleting OPI1 leads to the overexpression of INO1, which in turn causes an excessive accumulation of inositol, referred to as the Opi- phenotype (Greenberg et al. 1982). High-throughput genomic screens have identified many other mutants that derepress INO1 transcription and cause excessive accumulation of inositol, including mutants of the NuA4 complex (EAF1, EAF3, EAF5, EAF7, YAF9, and ESA1) (Hancock et al. 2006; Salas-Santiago and Lopes 2014). Interestingly, despite binding to its promoter, NuA4 is not required for the transcriptional activation of INO1 (Suka et al. 2001; Konarzewska et al. 2012), suggesting that the Opi- phenotype of NuA4 mutants is due to an uncharacterized role for NuA4 in the regulation of ICRE-containing genes as well as phospholipid homeostasis. NuA4 subunit mutants are unique among Opi- mutants as they also negatively interact with a mutant of the phospholipid-remodeling protein, Sec14, providing an opportunity to dissect the particular role of NuA4 in phospholipid metabolism.

Sec14 is an essential phospholipid-binding protein that coordinates the metabolism of phosphatidylinositol-4-phosphate (PI-4-P) with phosphatidylethanolamine (PC) at the Golgi to create a lipid environment necessary for trafficking events (reviewed in Ghosh and Bankaitis 2011). Sec14-deficient mutants cause multiple effects related to phospholipid metabolism, including increased intracellular PC (McGee et al. 1994; Xie et al. 2001) and decreased PI-4-P (Hama et al. 1999; Li et al. 2002; Schaaf et al. 2008). However, Sec14-deficient mutants also exhibit growth defects under inositol-depleted conditions (Patton-Vogt et al. 1997), which is associated with an inability to derepress INO1 expression leading to decreased inositol biosynthesis under conditions lacking an exogenous source of inositol (Calbertson and Henry 1975; Villa-Garcia et al. 2011). Synthetic genetic screens identified a mutant of the NuA4 complex, eaf7Δ, as one of several mutants that display a growth defect in combination with the temperature-sensitive mutant sec14-G266N (sec14-1P) (Curwin et al. 2009). A separate high-throughput synthetic dosage lethal screen found that overexpression of OSH4, a gene whose function directly antagonizes Sec14 by depleting PI-4-P (Fang et al. 1996; LeBlanc and McMaster 2010; Alfaro et al. 2011), resulted in decreased viability in the NuA4 mutants eaf5Δ and eaf7Δ, and lethality in yng2Δ (Mitchell et al. 2011).

The negative genetic interaction between sec14-1P and NuA4 mutants are unexpected as one might anticipate that overproduction of inositol (Opi- phenotype) of the NuA4 mutants would suppress the growth defect in sec14-1P cells. This dichotomy provides an elegant means to start dissecting the function of NuA4 within this pathway. Here, we report that deletion or mutation of NuA4 subunits increased the growth defect in sec14-1P cells under inositol-depleted conditions. Further investigation through the use of RNA sequencing (RNA-seq) of sec14-1Peaf1Δ cells revealed that although transcription of INO1 was significantly upregulated, the transcription of other ICRE-containing genes was not. This suggests that the role of NuA4 under inositol-depleted conditions extends beyond inositol biosynthesis, most likely through other aspects of phospholipid homeostasis. In fact, we find that NuA4 mutants have impaired lipid droplet levels, which implies defects in the biosynthesis of triacylglycerol (TAG) and sterol esters (SEs). Through genetic and chemical approaches, we determine that the genetic interaction between sec14-1P and NuA4 mutants potentially reflects a role for NuA4 in fatty acid biosynthesis. Altogether, our work identifies a role for NuA4 in phospholipid homeostasis through regulation of fatty acid synthesis and lipid droplets.

MATERIALS AND METHODS
Yeasts, plasmids, and media
All strains used in this study are in the BY4741 (S288C) background and are listed in Table 1. Strains were generated either by standard mating methods or a PCR-mediated gene insertion/deletion technique (Longtine et al. 1998). Strains from the Deletion Mutant Array (DMA) collection (GE, catalog no. YSC1053) were confirmed by PCR. A tetracycline/doxycycline inducible promoter (ptet) was used for CDS1 overexpression, as

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**Figure 1** Overview of phospholipid metabolism. Key lipid metabolic proteins mentioned in the text are indicated, transcription factors/repressors are in circles, and lipids are indicated in black rectangles. CDP-DAG, cytidine diphosphate diacylglycerol; PA, phosphatidic acid; PI, phosphatidylinositol; PI-4-P, phosphatidylinositol-4-phosphate; TAG, triacylglycerol; SE, steryl ester.
Previously described (Roney et al. 2016). All cells were grown in either standard YPD or SC media, as indicated. SC-inositol media was made by mixing 6.7 g/liter of YNB with ammonium sulfate, without inositol (Sigma, catalog no. D3447), choline chloride (Sigma, catalog no. C7017), or dH$_2$O was added to media after autoclaving to the concentration desired. Plates containing cerulenin (Sigma, catalog no. C2389) also contained 1% Biir 58 detergent (Sigma, catalog no. P5884-100G) to help dissolve the drug.

**Spot assays**

Cultures were grown in YPD, SD, or SC–inositol (+75 μM myo-inositol) as indicated at 30°C prior to being diluted to an OD$_{600}$ of 0.1, and four times 10-fold serial dilutions (OD$_{600}$ = 0.1, 0.01, 0.001, and 0.0001) were plated and incubated for 2 d at the indicated temperatures. Images of spot assays were taken with the Bio-Rad Chemidoc XRS system under EPI-white light illumination and on the autoexposure setting.

**RNA-seq**

The strains sec14-1ts (CMY505) and sec14-1ts::ptet-CDS1-TAP-LEU2,HIS3 sec14-1ts-NATMX eaf1Δ::KANMX (YKB3935) were grown in triplicate in 50 ml of YPD at 30°C to midlog growth phase (OD$_{600}$ = 0.5), then shifted to prewarmed YPD media at 33.5°C for 2 hr before harvesting, washing with dH$_2$O, and flash-freezing on dry ice. Cells were subsequently lysed by resuspending pellets in 100 μl of lyticase reaction solution [1.2 M sorbitol (Sigma, catalog no. S1876); 1 mg/ml lyticase (Sigma, catalog no. SRE0018)] and incubated at 30°C for 30 min. RNA was purified using the RNA mini-kit (Ambion, catalog no. 12183018A) following the manufacturer’s yeast extraction protocol. Purified RNA extracts were then twice treated with DNaseI (Promega, catalog no. M6101) for 1 h at 37°C each time followed by a phenol/chloroform extraction. After two DNaseI treatments, purified RNA was resuspended in 30 μl of RNase-free water. Three micrograms of purified RNA spiked with 1.5 μg of ERCC RNA control (Ambion, catalog no. G456740) was depleted of ribosomal RNA (rRNA) using the Ribo-Zero Gold rRNA depletion kit for yeast (Illumina, catalog no. MRZY1306) and resuspended in 10 μl of RNase-free water. Successful depletion of the 18S and 26S rRNA was confirmed using the Agilent Bioanalyzer RNA 6000 Pico quantification kit (Agilent Technologies, catalog no. 5067-4626). All six RNA-seq libraries were multiplexed and sequenced on the Life Technologies Ion Torrent Proton platform using a single Ion Proton Chip.

| Strain ID               | Genotype            | Source               |
|-------------------------|---------------------|----------------------|
| YKB1079/BY4741          | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Brachmann et al. (1998) |
| YKB3333                 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 eaf1Δ::KANMX | DMA collection (GE) |
| YKB3144/CMY505          | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sec14-1ts-NATMX | Fain et al. (2007) |
| YKB3935                 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sec14-1ts-NATMX eaf1Δ::KANMX | This study |
| YKB3292                 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 eaf7Δ::KANMX | DMA collection (GE) |
| YKB4068                 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sec14-1ts-NATMX eaf7Δ::KANMX | This study |
| YKB4236                 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 esa1Δ::HIS3 esa1::L254pm-URA3 | This study |
| YKB4242                 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sec14-1ts-NATMX esa1Δ::HIS3 esa1::L254pm-URA3 | This study |
| YKB4325                 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ade2Δ::ptet-CDS1-TAP-LEU2,HIS3 | This study |
| YKB4326                 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ade2Δ::ptet-CDS1-TAP-LEU2,HIS3 sec14-1ts-NATMX | This study |
| YKB4327                 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ade2Δ::ptet-CDS1-TAP-LEU2,HIS3 sec14-1ts-NATMX | This study |
| YKB4328                 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ade2Δ::ptet-CDS1-TAP-LEU2,HIS3 eaf1Δ::KANMX | This study |
| YJP1078                 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dga1Δ::KANMX iro1Δ::KANMX are1Δ::KANMX are2Δ::KANMX | Gaspar et al. (2011) |
| YKB4337                 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sec14-1ts-NATMX dga1Δ::KANMX iro1Δ::KANMX are1Δ::KANMX are2Δ::KANMX | This study |
| YKB4338                 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 eaf1Δ::URA3 dga1Δ::KANMX iro1Δ::KANMX are1Δ::KANMX are2Δ::KANMX | This study |
| YKB4339                 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sec14-1ts-NATMX eaf1Δ::URA3 dga1Δ::KANMX iro1Δ::KANMX are1Δ::KANMX are2Δ::KANMX | This study |
Resulting reads were aligned against the \textit{S. cerevisiae} S288c genome (version GCF\_000146045.2\_R64) using the MAPQ aligner provided with the Torrent Suite (v5.0.4). Raw sequencing reads have been deposited at the NCBI SRA archive under accession number PRJNA350552. Variation in sample processing during RNA depletions and library construction was assessed using the ERCC analysis plugin (v5.0.0.0). The number of reads aligning to each annotated transcript was determined using bedtools (v2.17.0) (Quinlan and Hall 2010). Differential expression of transcripts between sec14-1F and sec14-1P/Δ was determined using DESeq2 (v1.10.1) (Love et al. 2014). Transcripts with a fold change of two or more and an adjusted p value \leq 0.05 were considered to be differentially expressed. Gene ontology term analysis of enriched biological processes was conducted by using the DAVID bioinformatic online database (https://david.ncifcrf.gov) (Huang da et al. 2009). Significantly downregulated or upregulated genes were clustered into functionally related groups using the Gene Functional Classification Tool. The highest ranking Biological Process term was subsequently assigned for each group.

\section*{qRT-PCR}

RNA extracts were prepared as above but without RNA depletion. Purified RNA (2 μg) was reverse transcribed using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosciences, catalog no. 4368814) and resulting cDNA was diluted 1:10 for qRT-PCR. Transcript levels were measured using the SoFast EvaGreen Supermix for qPCR (Bio-Rad, catalog no. 172-5201) with the Bio-Rad MiniOpticon Real-Time PCR system and CFX manager software. Primers used for qRT-PCR are as follows: (1) \textit{INO1} forward (Forw) primer: 5′-TCTCTGTTGGCCATGGTTAG-3′ and reverse (Rev) primer: 5′-CTTTCAAGGTTGTTGCAAGAT-3′; (2) \textit{FAS1} Forw: 5′-TGGAAGG TGGTCCATCTCAGA-3′ and Rev: 5′-GCACTAGATTCTTCGCA-3′; (3) \textit{ACT1} Forw: 5′-GCCAGAAAGATGGGAAAT-3′ and Rev: 5′-TGATATGGCAACGACCTTCAA-3′; (4) \textit{TDH3} Forw: 5′-GGCATGGTGTGATCAAGAC-3′ and Rev: 5′-TCTGTGTTGCAGATCAGAC-3′; (5) \textit{CDS1} Forw: 5′-GCCATGGTTGATGACACAG-3′ and Rev: 5′-TCTCTGTTGGCCATGGTTAG-3′; (6) \textit{YEH1} Forw: 5′-TTCTCAGGGCACTACACAGG-3′ and Rev: 5′-AAGGGACCCAGGATACTGC-3′; (7) \textit{BCP1} Forw: 5′-TGATTAATATGCCACCGGAA-3′ and Rev: 5′-ATGGTTGGTCA CGCCAAAGTG-3′; (8) \textit{TDH3} Forw: 5′-CTGTCAGGTTGAACAGG AAACAC-3′ and Rev: 5′-CAACGGTGTTCAACACGGACGCA-3′; (9) \textit{ACT1} Forw: 5′-GCCCTTCTAGTTCCTACCCA-3′ and Rev: 5′-GTAAATGGAAAGCAGGT-3′. A standard curve was constructed using a five times fivefold serial dilution series of pooled cDNA sample from the original individual reverse transcription reactions starting at a 1:2 dilution. The geometric averaging of two internal reference genes, \textit{TDH3} and \textit{ACT1}, was used for normalization of relative expression levels (Pfafli 2001) using the Bio-Rad CFX Manager software. Three biological and two technical replicates were used for each sample. Statistical analyses were completed using a two-tailed unpaired t-test (p \leq 0.05) with the GraphPad Prism software.

\section*{Immunoblotting}

Samples were grown in SC media at 30° until midlog growth phase (OD\textsubscript{600} = 0.5–0.6), then shifted to SC media with indicated amounts of doxycycline for 4 hr, before being harvested by centrifugation, washed in water, aliquoted in 1.5-ml Eppendorf tubes, and flash frozen in liquid nitrogen. Pellets were resuspended in 30% TCA lysis buffer and lysed mechanically by vortex with glass beads. Cell debris and glass beads were removed by centrifugation. Whole cell extract (WCE) was diluted in Laemmli loading dye and stored at −80°. The prepared samples were boiled at 95° for 10 min prior to separation by SDS-PAGE on a linear SDS-polyacrylamide gel (7.5%). Western blotting was performed using a semidry transfer apparatus from Bio-Rad (Trans-Blot SD Semi Dry Electrophoretic Transfer Cell; catalog no. 170-3940). Blocking, primary, and secondary incubations were performed with 5% milk in TBS-T (0.1% Tween 20; VWR, catalog no. CA95017-122L). Primary incubations were carried out at 4° overnight with rabbit α-TAP antibodies (1:10,000 dilution; Thermo Fisher, catalog no. P1CAB1001) and secondary incubations at room temperature for 1 hr with peroxidase-conjugated goat α-rabbit lgG (1:10,000 dilution; Chemicon, catalog no. AP307P). Chemiluminescence was detected using Immobilon Western Chemiluminescent HRP substrate (Millipore, catalog no. WBKLS0500) and developed on a ChemiDoc XRS system (Bio-Rad, catalog no. 170-8070).

\section*{Lipid droplet}

Cultures were grown in SC-inositol (+75 μM myo-inositol) media at 30° to midlog growth phase (OD\textsubscript{600} = 0.5), washed in dH\textsubscript{2}O, and transferred to SC-inositol media supplemented with or without 75 μM of myo-inositol and grown for 2 hr at 30°. Cells were subsequently incubated with 10 μM BODIPY 493/503 (Life Technologies, catalog no. D3922) for 10 min at room temperature in their residing media and washed with dH\textsubscript{2}O. Cells were pelleted, resuspended in SC-inositol media (± or − inositol), and loaded onto prefreshed microscope slide with coverslips. Images were taken using the CellVoyager CV1000 disk confocal microscope (Yokogawa Electric Corporation, Musashino Tokyo, Japan). Brightfield (20% intensity, 100-ms exposure time, 100% gain) and BODIPY fluorescence, using FITC filters (25% intensity, 50-ms exposure time, 20% gain), were taken across multiple fields of view to capture at least 100 cells on the 100x oil immersion objective. Images for each field of view were taken at 0.2-μm steps for a total of 30 images. Image analysis was done using a custom MATLAB script. Bright field images were first thresholded using an adaptive thresholding algorithm. The resulting thresholded images were segmented using our MATLAB script that incorporated previously published code (Ricciova et al. 2013), as well as our own code that makes use of functions found in the MATLAB Image Processing Toolbox. The cell outlines obtained through segmentation were used to quantify the area (in pixels) and total fluorescence from the fluorescence images. Fluorescence was quantified using the middle z-stack for each cell. Fluorescence density was calculated by dividing total fluorescent area by total area. A total of three biological replicates with >100 cells were analyzed for each sample. Mean fluorescence for each sample are converted relatively to wild-type samples in inositol-supplemented conditions. Statistical analyses were performed by one-way ANOVA with the Tukey’s multiple comparisons test, using the GraphPad Prism software.

\section*{Data availability}

Strains and code are available upon request. Supplemental Material, File S1 contains the complete RNA-seq data set and gene functional classification analysis. Raw sequencing reads have been deposited at the NCBI SRA archive under accession number PRJNA350552.

\section*{RESULTS}

NuA4 mutants exacerbate the growth defects of \textit{sec14-1F} under inositol-depleted conditions

The growth defects of \textit{sec14-1F} on inositol-depleted media can be suppressed by mutants which exhibit an overproduction of inositol phenotype (Opi-) due to the derepression and transcription of the rate-limiting biosynthetic enzyme within this pathway, \textit{INO1} (Patton-Vogt et al. 1997; Chang et al. 2002). As NuA4 mutants exhibit a derepression of \textit{INO1} and excessive inositol production, or Opi- phenotype...
(H Hancock et al. 2006; Salas-Santiago and Lopes 2014), we anticipated that NuA4 mutants would suppress the growth defect in sec14-1ts under inositol-depleted conditions. To test this hypothesis, NuA4 mutants eaf1Δ and eaf7Δ, and the temperature sensitive esa1 mutant, esa1-l254Pts (Clarke et al. 1999), were crossed with sec14-1ts and growth was assessed under both inositol-supplemented and -depleted conditions (Figure 2). As expected, sec14-1ts eaf1Δ and sec14-1ts eaf7Δ single mutants grew similarly between both the conditions (Figure 2, A and B), we were surprised to see that esa1-l254Pts cells displayed growth defects in inositol-depleted media even at 30°C (Figure 2C). In combination, the double mutants sec14-1ts eaf1Δ, sec14-1ts eaf7Δ, and sec14-1ts esa1-l254Pts all showed a synthetic growth defect at 30°C in inositol-depleted media compared to sec14-1ts (Figure 2). These results suggest that, although eaf1Δ and eaf7Δ do not display detectable growth defects under inositol-depleted conditions like esa1-l254Pts, these mutants do in fact exacerbate the inositol-depleted growth defects for sec14-1ts under normally permissive temperatures. As NuA4 mutants derepress INO1 transcription and secrete excessive amounts of inositol (H Hancock et al. 2006; Salas-Santiago and Lopes 2014), the root cause of the growth defect under inositol-depleted conditions of sec14-1ts eaf1Δ, sec14-1ts eaf7Δ, and sec14-1ts esa1-l254Pts cells appears independent of inositol production.

ICRE-regulated phospholipid genes are not fully derepressed in an EAF1 mutant background

We next sought to determine if ICRE-regulated or phospholipid genes were derepressed on a NuA4 mutant under conditions where Sec14 is inactivated, and if so, would dysregulation of these genes explain the genetic interactions between sec14-1ts and NuA4 mutants. Using next-generation RNA-seq, we compared mRNA gene expression between sec14-1ts and sec14-1ts eaf1Δ. RNA samples from three biological replicates were extracted from midlog phase cells that were shifted to semi-permissive temperatures (33.5°C) for 2 hr to briefly inactivate sec14-1ts. In summary, >8 million reads were obtained for each sample, identifying at least 89% of the annotated open reading frames in the Saccharomyces genome database (Cherry et al. 2012; Engel et al. 2014). Analysis of the ERCC spike-in standard revealed that all samples had an R² correlation ranging from 0.92 to 0.95 (Figure S1A in File S2). These results indicate our rRNA depletion and library construction was consistent and unbiased across samples. Principal component analysis demonstrated that the majority of the variance between samples is attributable to the differences between the two different strains and not their biological replicates (Figure S1B in File S2). Only a subset of genes was found to be significantly downregulated (103 genes, 1.84%) or upregulated (183 genes, 3.11%) in sec14-1ts eaf1Δ compared to sec14-1ts by a fold change of two or more and FDR adjusted p-value ≤ 0.05 (Figure 3A and File S1).

Deletion of EAF1 in the sec14-1ts background resulted in the decreased expression of the purine biosynthesis genes (ADE1, ADE12, ADE17) phosphate metabolic genes (PHO3, PHO5, PHO11, PHO12) and a large cluster of genes associated with ribosomal biogenesis (File S1), which is in agreement with previously published transcriptome studies on NuA4 mutants (Reid et al. 2000; Krogan et al. 2004; Nourani et al. 2004; Lindstrom et al. 2006; Cheng et al. 2015). Upregulated genes common to previous eaf1Δ transcriptome studies and our sec14-1ts eaf1Δ transcriptome included the stress response genes (DDR2, HSP12, HSP26) (Lindstrom et al. 2006; Cheng et al. 2015).

Together, these results indicate that deletion of EAF1 in the sec14-1ts background impacts the transcriptome similar to what has been previously reported for NuA4 mutants in the wild-type background. However, our main purpose was to assess if ICRE-containing genes or genes implicated in phospholipid metabolism were differentially regulated by NuA4 in the sec14-1ts background.

If NuA4 is regulating transcription of ICRE-containing genes, one would anticipate that other ICRE-containing phospholipid metabolic genes would be similarly derepressed as INO1 (Figure 3C), however, this was not the case. While the expression of the fatty acid synthases FAS1 and FAS2 (Kuziora et al. 1983) were significantly upregulated in sec14-1ts eaf1Δ cells, the expression of phosphatidate cytidylyltransferase (CDP-diglyceride synthetase) CDS1 (Shen et al. 1996; Shen and Downham 1997) was downregulated (Figure 3C). Indeed, the expression of the majority of ICRE-containing phospholipid genes did not display significant changes in gene expression in our transcriptome profile,
including the transcription factor \textit{INO2}, whose expression remained close to basal level (Figure 3C). Derepression of \textit{INO1} expression is normally preceded by increased levels of \textit{INO2} which dimerizes with \textit{INO4} and binds to ICRE gene promoters to promote transcription (Ambroziak and Henry 1994; Ashburner and Lopes 1995). Our transcriptome analysis provides evidence of \textit{INO1} derepression occurring independently of \textit{INO2} derepression, suggesting that the transcriptional derepression of \textit{INO1} in \textit{sec14-1tseaf1Δ} is not characteristic of the typical Opi- transcriptional response.

Extending our search beyond just ICRE-regulated genes, we were surprised to find that only eight genes involved in phospholipid metabolism displayed significant transcriptional change between \textit{sec14-1tseaf1Δ} and \textit{sec14-1ts} (Figure 3B). In addition to \textit{CDS1}, downregulated genes included the SE hydrolase \textit{YEH1} (Koffel et al. 2005), the phospholipase B \textit{PLB2} (Fyrst et al. 1999), and \textit{BCP1}, which encodes a protein implicated in phosphatidylinositol-4,5-bisphosphate synthesis (Audhya and Emr 2003). Upregulated genes included the inositol-3-phosphate synthase \textit{INO1}, the phospholipase B \textit{PLB3} (Merkel et al. 1999), and \textit{FAS1} and \textit{FAS2}. As recent evidence suggests that the contribution of phospholipase B enzymes toward phospholipid turnover in vivo is negligible (Mora et al. 2012), we focused our attention on the remaining six genes. For each of the lipid metabolic genes, the changes in expression detected by next-generation sequencing analysis were confirmed through qRT-PCR (Figure S2 in File S2).

The misregulation of a handful of phospholipid metabolic genes, most notably that of \textit{CDS1}, suggests that perturbation of phospholipid homeostasis may contribute to the growth defects displayed in \textit{sec14-1tseaf1Δ} under inositol-depleted conditions. Given the essential role of \textit{CDS1}, we predicted that its downregulation in \textit{sec14-1tseaf1Δ} could not rescue the growth defects under inositol-depleted conditions (Figure S3 in File S2). Therefore, downregulation of \textit{CDS1} by itself is not the cause of the growth defects in \textit{sec14-1tseaf1Δ}.

\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Transcriptional profiling of \textit{sec14-1tseaf1Δ} reveals that most ICRE-regulated phospholipid genes are not significantly derepressed. (A) Volcano plot for differentially expressed genes, \textit{sec14-1tseaf1Δ} (YKB3935) vs. \textit{sec14-1ts} (YKB3144), from RNA sequencing experiments. Differential expression levels of aligned sequences were calculated using significant thresholds set at log2 fold change over two and FDR adjusted p-value $\leq 0.05$. Significant differentially expressed genes are in red. Blue shows the significantly changed lipid metabolic genes described in (B). (B) Table listing significantly downregulated and upregulated lipid metabolic genes. (C) Relative expression levels of ICRE-containing phospholipid genes between \textit{sec14-1tseaf1Δ} and \textit{sec14-1ts}. Genes are ordered from lowest to highest fold-change expression. Genes in red are discussed further in the text.}
\end{figure}
Lipid droplet dynamics is impaired in eaf1Δ cells

We next wanted to determine which, if any, lipid metabolic pathways were affected in eaf1Δ cells that might explain the genetic interaction between sec14-1ts and eaf1Δ. We opted to explore if the growth defect in sec14-1tsfl eaf1Δ under inositol-depleted conditions is potentially due to defects in TAG and SE biosynthesis for two reasons. First, we found altered expression of CDS1, FAS1, and FAS2, which all encode proteins implicated in neutral lipid synthesis (reviewed in Eisenberg and Buttner 2014; Fernandez-Murray and McMaster 2016) (Figure 1). Second, under inositol-depleted conditions, cells require inositol as well as the ability to synthesize both TAG and SE (both neutral lipids) for survival (Gaspar et al. 2011). TAG and SE biosynthesis can be completely abolished in yeast by deleting DGA1, LRO1, ARE1, and ARE2, genes encoding the proteins responsible for all neutral lipid biosynthesis in yeast (Oelkers et al. 2002; Sandager et al. 2002). Intriguingly, like sec14-1tsfl eaf1Δ strains, a yeast strain in which all TAG and SE biosynthesis is abolished (dga1Δlro1Δare1Δare2Δ) is not able to grow in the absence of inositol at higher temperatures despite INO1 expression (Gaspar et al. 2011). The formation of lipid droplets is an established proxy for TAG and SE biosynthesis (Wang 2015); therefore, we examined lipid droplet formation under inositol-supplemented and -depleted conditions in the wild-type, sec14-1ts, eaf1Δ, and sec14-1tsfl eaf1Δ strains at 30°C using a fluorescent lipid droplet marker. As expected, upon inositol depletion, wild-type cells display a nearly threefold increase in fluorescence of the lipid droplet marker, whereas there is no increase in dga1Δlro1Δare1Δare2Δ cells where TAG and SE biosynthesis is abolished (Figure 4 and Figure S4 in File S2) (Gaspar et al. 2011). Much like wild-type cells, upon inositol depletion, sec14-1tsΔ cells emitted a nearly threefold increase in fluorescence density compared to inositol-supplemented conditions (Figure 4, A and B). In contrast, deletion of EAF1 in either the wild-type or sec14-1tsΔ background resulted in a reduction in the fluorescence intensity of lipid droplets, which suggests that some aspect of lipid droplet dynamics is significantly impaired in eaf1Δ cells. Furthermore, although the reduction of fluorescence intensity of lipid droplets was not as striking in eaf1Δ and sec14-1tsfl eaf1Δ cells, it was clearly reduced in esa1-1L254PΔ and sec14-1tsesa1-1L254PΔ cells. As both eaf1Δ and esa1-1L254PΔ cells display reduced KAT activity, this suggests that the catalytic activity of the NuA4 complex is involved in the regulation of lipid droplet and potentially neutral lipid biosynthesis (Figure S5 in File S2). If the defect in lipid droplets seen in NuA4 mutant cells is a reflection of neutral lipid biogenesis, this potentially could explain the increased growth defects of sec14-1tsfl eaf1Δ, sec14-1tsfl eaf7Δ, and sec14-1tsesa1-1L254PΔ cells under inositol-depleted conditions.

Inhibition of de novo fatty acid biosynthesis has a detrimental effect on sec14-1ts growth

To investigate if the genetic interaction between sec14-1ts and eaf1Δ was due to a role of Eaf1 (and presumably NuA4) in neutral lipid biosynthesis, we examined the growth of sec14-1tsΔ strains when both TAG and SE biosynthesis are eliminated (Figure 5A). As previously shown, dga1Δlro1Δare1Δare2Δ (d.l.a.aΔ in Figure 5) cells do not display defects on inositol-supplemented media, but become inositol auxotrophs at 37°C (Gaspar et al. 2011) (Figure 5A). If the genetic interaction between sec14-1ts and eaf1Δ was due to decreased neutral lipid synthesis caused by deletion of Eaf1, we would predict that sec14-1ts dga1Δlro1Δare1Δare2Δ cells would display similar growth defects on inositol-depleted media as sec14-1tsfl eaf1Δ. However, in inositol-supplemented or -depleted conditions at 30°C, dga1Δlro1Δare1Δare2Δ combined with sec14-1ts does not impair growth. Further, the addition of dga1Δlro1Δare1Δare2Δ does not increase the growth defects of sec14-1tsfl eaf1Δ cells at 30°C. Indeed, we detect a negative genetic interaction between eaf1Δ and dga1Δlro1Δare1Δare2Δ on inositol-supplemented media at 37°C, suggesting that Eaf1, and presumably NuA4, function in separate pathways. Altogether, this suggests the growth defect in sec14-1tsfl eaf1Δ under inositol-depleted conditions or at higher
enzymatic activity of fatty acid synthases. For this purpose, we used cerulenin, a fatty acid analog that inhibits the sec14-1ts synthesis.

- Inositol-supplemented (75 mM myo-inositol) SC media or inositol-depleted conditions (Patton-Vogt et al. 1982), to determine the effect of inhibiting fatty acid biosynthesis pathway.

**DISCUSSION**

Here we set out to begin to dissect the biological role for NuA4 in phospholipid homeostasis. Surprisingly, we found that NuA4's role in inositol auxotrophy is not simply a reflection of regulation of ICRE-regulated phospholipid genes. Rather, through dissection of the genetic interaction between sec14-1 and eaf1Δ, our work provides evidence for a role of NuA4 in contributing to the regulation of neutral lipid and fatty acid biosynthesis pathway.

**NuA4 mutants increase the inositol auxotrophy of sec14-1Δ cells despite INO1 derepression**

NuA4 mutants are known to exhibit the Opi- phenotype, which is the overproduction of inositol caused by the derepression and transcription of INO1 (Hancock et al. 2006; Salas-Santiago and Lopes 2014). As deletion mutants of the CDP-choline pathway that exhibit an Opi- phenotype suppress the growth defect in the sec14-1Δ mutant under inositol-depleted conditions (Patton-Vogt et al. 1997; Chang et al. 2002), we initially hypothesized that the introduction of NuA4 mutants into a sec14-1Δ background would rescue growth on inositol-depleted media. Instead, we found that the combination of sec14-1Δ and NuA4 mutants resulted in an increased growth defect under inositol-depleted conditions despite INO1 derepression (Figure 2, Figure 3, and Figure S2A in File S2). However, comparative to op1Δ cells that are considered to have a strong Opi- phenotype, NuA4 mutants display only an intermediate Opi- phenotype, as determined by the extent of the derepression of an INO1-CYC-lacZ reporter gene (Hancock et al. 2006).

Similarly, our transcriptome analysis indicated only a twofold derepression of INO1 expression (Figure 3), which differs considerably from the 56.7-fold increase in an opi1Δ background (Santiago and Mamoun 2003). Despite this fact, we did not expect the growth defect in sec14-1Δ under inositol-depleted conditions to worsen in combination with NuA4 mutants. This suggests that survival under inositol-depleted conditions requires more than just INO1 induction and inositol production. Our transcriptional data showed that partial derepression and transcription of INO1 was occurring in sec14-1Δeaf1Δ, but derepression was not extended to all ICRE-regulated phospholipid genes. The main transcriptional activator of ICRE-regulated genes, INO2, which is normally induced under similar conditions as INO1, remained at basal levels of transcription in sec14-1Δeaf1Δ cells (Figure 3). This suggests that the induction of INO1 in sec14-1Δeaf1Δ cells and other NuA4 mutants (Hancock et al. 2006; Salas-Santiago and Lopes...
2014) may not be dependent on INO2, potentially explaining why most ICRE-regulated genes are not induced in sec14-1tscaiΔ cells.

One possible mechanism for the induction of INO1 expression in sec14-1tscaiΔ cells could be through the downregulation of CDS1 expression (Figure 3 and Figure S2D in File S2). It has been shown that lowering expression of the essential gene CDS1 causes an Opp phenotype and induction of INO1 expression independent of INO2 derepression, much like NuA4 mutants (Shirra et al. 2001; Feddersen et al. 2007) and increases fatty acid biosynthesis, causing a dramatic accumulation of lipid droplets (Hob人員er et al. 2014).

Is NuA4 regulating lipid droplet dynamics through the fatty acid biosynthesis pathway? A decrease in lipid droplet staining despite the derepression of INO1 expression is also a characteristic of the quadruple mutant dga1Δ/sfl1Δ/are1Δ/are2Δ, which is unable to synthesize neutral lipids (Figure S4 in File S2) (Mul1ner and Daum 2004; Gaspar et al. 2011). Further, like our esa1-L254P mutant cells (Figure 2C), dga1Δ/sfl1Δ/are1Δ/are2Δ cells are sensitive to inositol-depleted conditions at higher temperatures (Figure 5A) (Gaspar et al. 2011). However, the loss of neutral lipid biosynthesis did not account for the negative genetic interaction between NuA4 and Sec14 mutants, nor did it account for the increased growth of sec14-1tscai Δ on inositol-depleted media at 30°C (Figure 5A).

An alternative explanation could be that defective lipid droplet formation in caiΔ and other NuA4 mutants is caused by a lack of fatty acid substrate. We demonstrated that the inhibition of the fatty acid biosynthesis pathway by cerulenin dramatically increased the fatty acid substrate. We demonstrated that the inhibition of the fatty acid biosynthesis pathway negatively affects neutral lipid biosynthesis and lipid droplet formation as they are the principal substrates in these pathways (Henry et al. 2012; Wang 2015). Similar to NuA4 mutants, downregulation of the fatty acid biosynthesis pathway leads to the derepression and transcription of INO1 (Shirra et al. 2001). Whether the inhibition of the fatty acid biosynthesis pathway is the primary cause behind INO1 transcriptional activation in NuA4 mutants remains to be confirmed. Conversely, the opposite effect is observed when this pathway is activated. Overexpression of genes responsible for fatty acid biosynthesis represses the transcription of INO1 (Shirra et al. 2001; Feddersen et al. 2007) and increases fatty acid biosynthesis, causing a dramatic accumulation of lipid droplets (Hob入mer et al. 2014).

How is NuA4 regulating the fatty acid biosynthesis pathway? Our work suggests that NuA4 is a positive regulator of the fatty acid biosynthesis pathway. Based on this theory, one potential mechanism is through NuA4-dependent transcriptional regulation of fatty acid biosynthesis genes. However, transcriptome analysis on NuA4 mutant subunits have not identified significant decreases in fatty acid biosynthesis genes (Krogan et al. 2004; Lundstrom et al. 2006; Chen et al. 2015). Indeed, our work identified an increase in mRNA expression of FAS1 and FAS2 in sec14-1tscaiΔ cells, which may reflect the cells’ attempts at compensating for decreased lipogenesis flux upon deletion of EAF1 (Figure 3). NuA4 has also been implicated in the inhibition of AMPK1/Snl1, and NuA4 mutants display hyperactivated Snl1 (Lu et al. 2011). As Snf1 phosphorylates and inhibits the activity of acetyl-CoA carboxylase (Acc1), the first step of fatty acid de novo synthesis (Hob入mer et al. 2014), NuA4 might regulate fatty acid biosynthesis pathway by reducing Snf1 activity and maintaining Acc1 activity. Indeed, activity of Acc1 is reduced in WCEs from NuA4 mutants (Rollins et al. 2017). Alternatively, and perhaps not mutually exclusively, NuA4 may also have potential targets within the fatty acid biosynthesis pathway. In fact, lysine acetylation of proteins in this pathway, including Fas1 and Fas2, have been detected in both yeast and higher organisms (Choudhary et al. 2009; Henriksen et al. 2012; Weinert et al. 2014; Downey et al. 2015; Madsen et al. 2015). While the mechanism(s) through which NuA4 is contributing to cellular lipid homeostasis remain unclear, our work shows that NuA4 has underappreciated roles in lipid homeostasis.

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