Sterol and Diacylglycerol Acyltransferase Deficiency Triggers Fatty Acid-mediated Cell Death*

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Deletion of the acyltransferases responsible for triglyceride and steryl ester synthesis in Saccharomyces cerevisiae serves as a genetic model of diseases where lipid overload is a component. The yeast mutants lack detectable neutral lipids and cytoplasmic lipid droplets and are strikingly sensitive to unsaturated fatty acids. Expression of human diacylglycerol acyltransferase 2 in the yeast mutants was sufficient to reverse these phenotypes. Similar to mammalian cells, fatty acid-mediated death in yeast is apoptotic and presaged by transcriptional induction of stress-response pathways, elevated oxidative stress, and activation of the unfolded protein response. To identify pathways that protect cells from lipid excess, we performed genetic interaction and transcriptional profiling screens with the yeast acyltransferase mutants. We thus identified diacylglycerol kinase-mediated and transcriptional profiling screens with the yeast acyltransferase mutants. We thus identified diacylglycerol kinase-mediated phosphatidic acid biosynthesis and production of phosphatidycholine via methylation of phosphatidylethanolamine as modifiers of lipotoxicity. Accordingly, the combined ablation of phospholipid and triglyceride biosynthesis increased sensitivity to saturated fatty acids. Similarly, normal sphingolipid function, diabetes, and the metabolic syndrome.

Lipotoxicity, the phenomenon of cell death due to lipid imbalance, is a common consequence of obesity and likely influences many of the morbidities associated with this syndrome, including diabetes and atherosclerosis. The mechanisms by which an imbalance in lipid metabolism culminates in cell death differ for each offending metabolite and surprisingly are poorly understood. For example, saturated and unsaturated fatty acids are pro-apoptotic in most cell types (1) but achieve this through different pathways (for review see Ref. 2). The cytotoxicity of saturated fatty acids such as palmitate likely arises from elevated ceramide levels and/or reactive oxygen species (3, 4). By contrast, the accumulation of oleate, an unsaturated fatty acid, promotes apoptosis through activation of serine/threonine protein phosphatases such as PP2Cα/β (5). Similarly, subcellular cholesterol accumulation induces cellular stress responses such as the unfolded protein response pathway, apoptosis, and necrosis (6).

Any process that limits the accumulation of lipid alcohols (such as diacylglycerol and sterols) or fatty acids is likely to be cytoprotective. One such reaction is their mutual co-esterification to produce neutral lipids (e.g. triglyceride (TG) or steryl ester (SE)). Subsequently, these lipids are sequestered in cytoplasmic lipid droplets (CLDs), the preponderance of which correlates with lipidotoxic states such as obesity and fatty liver disease (7). To date, three acyltransferase gene families independently direct the terminal and committed steps of neutral lipid synthesis in eukaryotes (8). In mammals, the esterification of sterol is accomplished by members of the acyl-CoA: cholesterol acyltransferase (ACAT) family or in serum by lecithin-cholesterol acyltransferase. ACAT members carry out the reaction in an acyl-CoA-dependent fashion, whereas lecithin-cholesterol acyltransferase uses phospholipids as an acyl donor. Diacylglycerol (DAG) esterification is primarily the activity of DGAT2 (a member of the diacylglycerol acyltransferase (DGAT) 2 gene family) or DGAT1 (a member of the ACAT family).

In Saccharomyces cerevisiae, the ACAT-related enzymes Are1p and Are2p are responsible for the esterification of sterols (9–12). DAG esterification during the logarithmic phase of growth is critical for optimal growth on oleate and also results in a decreased resting DAG level. This suggests that DAG esterification could be a general mechanism for the regulation of neutral lipid synthesis in Saccharomyces cerevisiae.
growth is mediated by Lro1p (a lecithin-cholesterol acyltransferase ortholog that uses DAG and phospholipids as substrates (13, 14)), whereas DGA1, the sole representative of the DGAT2 gene family in this organism, directs synthesis of TG during stationary phase (11, 15, 16). Yeast strains that lack all four acyltransferase genes are viable but completely lack neutral lipids and thus present a genetically malleable system to investigate fatty acid-induced cytotoxicity (17).

We show here that elimination of neutral lipid biosynthesis results in contrasting effects of saturated and unsaturated fatty acids on cellular metabolism and viability and that this primarily reflects differential channeling of fatty acids into phospholipids. We describe the use of genetic interaction screens and transcriptional profiling to identify pathways that interact with the neutral lipid acyltransferases (see also Ref. 18). Defects in several components of endoplasmic reticulum homeostasis impose limitations on growth of ARE1, ARE2, LRO1, or DGA1 mutants, implicating them as evolutionarily conserved components of lipid homeostasis that protect a cell from lipotoxicity.

EXPERIMENTAL PROCEDURES

General—Media preparations (yeast extract, peptone, dextrose; YPD, synthetic; SCD), molecular and yeast genetic procedures, DNA modification, and oligonucleotide purifications (QIAquick gel extraction kit, Qiagen) were according to conventional (19) or the manufacturers’ protocols.

Yeast Strains and Growth, Plasmids, and Transformation—The yeast strains used in this study (supplemental Table S1) were derived from W303 (20) or s288C (Open Biosystems). Strains with deletions in ORP1 or CHS1 and CYB5 or OLE1 were obtained from Susan Henry (21) and Charles Martin (22, 23), respectively. Synthetic genetic interactions with neutral lipid acyltransferase genes were determined from an ER-Golgi epi-

DNA Microarray Analysis—RNA was extracted from yeast cells grown at 30 °C to ~0.650 (A600 nm) in YPD or in YPD plus 0.01 mM oleate and used to prepare cDNA for hybridization to Affymetrix S98 Yeast GeneChip arrays (28). Results are in triplicate, and each set was normalized using Robust Multichip Average (29, 30) followed by comparison analysis using the LIMMA module in conjunction with affyInGUI (31–33) found in the R software package (Linear Models for Microarray Data; version 2.2.0).

Fluorescence Microscopy—Yeast cells were grown in appropriate media supplemented with 20 μM adenine to minimize autofluorescence of the vacuole associated with ade2 mutants. For CLD analysis, cells were stained with Nile Red (1 μg/ml) or envisaged for GFP reporters using a long pass GFP filter (excitation 440 nm). Cell viability using the FUN1 stain was determined after overnight exposure using a phosphorimager (GE Healthcare).

Lipid Analysis and Metabolic Labeling—[9,10-3H]Oleic acid and [9,10-3H]palmitic acid (PerkinElmer Life Sciences) pulse labeling in exponential phase was performed with 0.1 μCi/ml [3H]oleate or 0.5 μCi/ml [3H]palmitate in YPD ± fatty acids at 30 °C for 1 h (11). Strains transformed with hDGAT2 were grown to mid-log phase in 2% galactose, 1% raffinose media. Lipids were isolated by hexane/isopropyl alcohol (2:1) organic extraction of dried cell pellets and resolved by TLC in petroleum ether/diethyl ether/acetone (84:15:1). Phospholipids were further resolved with a second solvent of chloroform/methane/acetone/water (60:50:1:4) (26). Lipids were detected using iodine staining and quantified via scintillation counting. Capillary gas chromatography analysis of fatty acid composition following esterification was performed on a Hewlett-Packard 5890A equipped with a Supelco™ 10 fused silica capillary column (30 m, 0.25 mm internal diameter), essentially as described (27).

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Verification of Gene Expression Changes—RNA was prepared from mutant cells as above and used to generate first-strand cDNA (SuperScript First-strand Synthesis System, Invitrogen). Real time PCRs were performed with the MyiQ single-color real time PCR detection system (Bio-Rad), SYBR Green 2X Supermix (Bio–Rad), and the indicated primers. Expression levels were calculated relative to ACT1 using the MyiQ real time detection software (34). Northern blots were performed using 15 μg of RNA (28), hybridized with PCR-generated gene-specific probes, and analyzed after overnight exposure using a phosphorimager (GE Healthcare).

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Lipotoxicity in Budding Yeast

RESULTS

Phenotypes of CLD-deficient Strains—Neutral lipids represent a safe harbor for cytotoxic lipids enabling cells to efficiently store lipid precursors in a biologically inert form. We and others have described viable yeast strains with combined deletions of the ARE1, ARE2, LRO1, and DGA1 genes that lack neutral lipids and CLDs (11, 15, 16) (confirmed in Fig. 1A). Surprisingly, the sterol content of membranes, fluid phase endocytosis, and pH and the proteolytic capability of the vacuole in the mutants were equivalent to control strains as determined by staining with lipophilic dyes (Filipin, MDY-64, LysoSensor Green DND-189, carboxy-DCFDA, and CMAC-Arg, respectively; data not shown). By contrast, we discovered that the protein composition of the CLD organelle is responsive to changes in neutral lipids. 3-Keto sterol reductase (ERG27) fused to GFP (37) was coincident with Nile red staining of lipid droplets in control cells and unaffected by loss of sterol esterification (Fig. 1A). However, loss of TG synthesis in the dga1Δ lro1Δ strains or complete loss of neutral lipids in the quadruple mutant resulted in Erg27-GFP retention at the ER (Fig. 1A).

Another critical role of neutral lipid biosynthesis is to provide important membrane and energy reserves, most likely in response to nutrient limitation to the cell. Under such circumstances, diploidy yeast differentiate (sporulate) to the haploid state and accumulate neutral lipids, both within and between daughter cells, the latter presumably of maternal origin (Fig. 1B) (38–40). We generated homozygous quadruple acyltransferase mutants (2n) that were completely deficient in neutral lipid accumulation and observed a severe block in spore formation (Fig. 1B). After 8 days in nitrogen-deficient media, 4.3% of quadruple mutant cells had sporulated versus 62.5% of control diploid cells (averaged over multiple fields of view). This suggests a requirement for neutral lipids and/or CLDs or alternatively an inhibitory effect of lipid substrate accumulation during early sporulation, perhaps at entry into meiosis.

Compensatory Pathways for Neutral Lipid Deficiency—The viability of mutants in the neutral lipid biosynthetic pathway was surprising given the key role these enzymes play in metabolite storage and detoxification. We reasoned this is likely due to regulatory responses to an overabundance of the precursors to these reactions, i.e., alcohols (sterols or diacylglycerols) and fatty acids, as well as the utilization of alternate systems that bypass the toxic effects of free fatty acids. In mammalian systems this homeostasis initiates at the transcriptional level (41, 42). We therefore profiled the transcriptional status of are1Δ are2Δ (SE-deficient), lro1Δ dga1Δ (TG-deficient), and are1Δ are2Δ lro1Δ dga1Δ (lacking neutral lipids) strains using Affymetrix oligonucleotide microarrays (supplemental Tables S2–S5). The data, generated in triplicate, were analyzed using linear modeling (see “Experimental Procedures”).

Global Gene Expression Changes as a Result of Steryl Ester or Triglyceride Deficiency—Yeast cells deficient in sterol esterification have reduced ergosterol biosynthetic rates and maintain free ergosterol at levels equivalent to control cells (9, 43). Surprisingly, there was no evidence of statistically significant transcriptional regulation of the sterol biosynthetic pathway in sterol esterification-defective strains. The major pathways influenced by loss of sterol esterification determine or respond to membrane status (e.g. down-regulation of vesicular transporters such as Uso1p, Cog7p, and Dsl1p). We also observed a statistically significant decrease in the expression of several components of iron metabolism such as the iron-responsive transcriptional regulator AFT1 and Aft1p target genes such as FET3. These results (supplemental Tables S2 and S3) were confirmed via Northern blotting (Fig. 2A). Many steps in ergosterol biosynthesis incorporate iron into their structure in various forms as follows: as a mono- or binuclear species, as a part of an Fe-S cluster, or as heme groups (44). These observations indicate an unexpected feedback interaction between iron and sterol homeostasis that may confer an alternate avenue by which sterol biosynthesis is regulated.

Triglyceride deficient dga1Δ lro1Δ mutants exhibited a statistically significant reduction in multiple key components of phospholipid homeostasis relative to control strains. The positive transcriptional regulator INO2 and its targets CHO1, CHO2, PSD1, OPI3, and EPT1 were down-regulated in the mutant cells (supplemental Tables S2 and S4 and Fig. 2B). There is an intimate relationship between triglycerides and phospholipids; both biosynthetic pathways converge through common intermediates (diacylglycerol and fatty acids); thus it is not surprising that they are coordinately regulated.

Global Gene Expression Changes Due to Neutral Lipid Deficiency—Total ablation of neutral lipid biosynthesis led to many changes in gene expression (supplemental Tables S2 and
Lipotoxicity in Budding Yeast

Expression of Human DGAT2 Prevents Lipotoxicity—The mechanism that underlies the death of UFA-treated neutral lipid-deficient yeast cells is likely multifactorial. To confirm that restoration of TG biosynthesis was sufficient to restore cell viability, we expressed a human DGAT2 cDNA in these mutants (Fig. 3, D and E), an approach used previously to characterize mammalian neutral lipid biosynthesis (25). Expression of human DGAT2 in these yeast strains resulted in a marked elevation of TG synthesis (supplemental Fig. S1), complete rescue of the oleate-induced inviability of the quadruple deletion strain (Fig. 3D), and deposition of NL in cytoplasmic lipid droplets as observed by Nile red staining (Fig. 3E). This is a critical observation that suggests it is the accumulation of TG precursors or loss of the CLD compartment that limits the growth of the TG-deficient strains.

Transcriptional Response to UFA Challenge—To assess the primary cellular response to fatty acids in the context of neutral lipid deficiency, we assessed global gene expression changes between control and are1Δ are2Δ lro1Δ dga1Δ strains grown for 6 h in sub-lethal concentrations of oleate (0.01 mm, see supplemental Table S6). Differential gene expression between control and mutant cells was determined by p value (p ≤ 0.05) or by using the T-profiler algorithm, which correlates microarray expression data with DNA sequences immediately upstream of the ORFs (45) (supplemental Fig. S2). Despite the absence of a growth phenotype at this concentration of UFA, expression of all genes classified under the GO category “stress response” were increased relative to control strains (supplemental Fig. S2A). Four consensus motifs associated with differential gene expression summarize the data (Table 1). This includes an overall higher expression of genes containing the stress response-related RLM1 (366 ORFs) and STRE (960 ORFs) consensus motifs compared with that of all other genes on the microarray (positive T-value). Concomitant with the increase in stress-responsive genes was a general decrease of genes involved in transcription, RNA, ribosome, and protein metabolism (supplemental Fig. S2B), including RNA polymerase III subunits RPC17 and RPC34. The rRPE and PAC consensus motifs found on genes associated with rRNA and ribosome biosynthesis (1297 and 248 ORFs, respectively) displayed a negative T-profiler value indicating an overall decrease in expression of genes within this category (Table 1). Thus, even at sub-lethal concentrations, it is clear that neutral lipid-deficient cells initiate a general stress response to accommodate toxic fatty acids such as oleate.

Lipoapoptosis in Budding Yeast—Programmed cell death as a consequence of surplus free fatty acids has been described in

S5), many of which were confirmed by Northern hybridizations or quantitative real time PCR (Fig. 2). Consistent with the are1Δ are2Δ or dga1Δ lro1Δ double mutants, the quadruple mutant strain also displayed decreased expression of genes involved in iron and phospholipid metabolism. Additionally, we observed statistically significant decreases in several genes responsible for ergosterol biosynthesis, namely ERG3, ERG4, and ERG5 (supplemental Table S6 and Fig. 2A). Thus it appears that loss of the CLD compartment, as arises when neutral lipids are absent, has a more significant impact on the lipid transcriptome than does loss of sterol ester or TG alone.

Exogenous Fatty Acids Are Lipotoxic—To provoke a viability response to neutral lipid deficiency, we incubated yeast cells with increasing concentrations of saturated or unsaturated free fatty acids, reasoning that these were common substrates to all of the enzymes under study and would reach toxic levels in the deletion strains. All cells, regardless of genotype, absorbed equal volumes of UFA, reasoning that these were common substrates to all strains lacking sterol esterification were unaffected by exogenous fatty acids.

Expression of human DGAT2 cDNA in these mutants (Fig. 3, D and E), an approach used previously to characterize mammalian neutral lipid biosynthesis (25). Expression of human DGAT2 in these yeast strains resulted in a marked elevation of TG synthesis (supplemental Fig. S1), complete rescue of the oleate-induced inviability of the quadruple deletion strain (Fig. 3D), and deposition of NL in cytoplasmic lipid droplets as observed by Nile red staining (Fig. 3E). This is a critical observation that suggests it is the accumulation of TG precursors or loss of the CLD compartment that limits the growth of the TG-deficient strains.

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several organisms (3, 46) and is likely to play a major role in the metabolic syndrome of obesity, diabetes, and atherosclerosis. We therefore investigated the mechanism of cell death in yeast acyltransferase mutants treated with oleate or palmitoleate (Fig. 4). Senescence as opposed to quiescence (i.e. toxicity versus starvation) is the predominant outcome of UFA treatment; colony formation on fatty acid-free media following oleate pre-incubation (0.5 mM) was markedly impaired in cells lacking neutral lipids (Fig. 4A).

The accumulation of reactive oxygen species (ROS) is an early indicator and possible cause of cell death in most organisms. To assess the impact of such molecules in lipotoxicity in yeast, we stained cells with DHR 123 (Fig. 4B). DHR 123-positive staining in the quadruple acyltransferase mutants treated with sub-lethal levels of oleate (0.01 mM) was absent from control strains or any cell incubated in the presence of saturated fatty acids suggesting that ROS accumulation is an early component of UFA-mediated cell death in this yeast mutant.

The integrity of the ER in mammalian cells is a major component of cell homeostasis and often responds to stressors, such as ROS accumulation, by activation of the unfolded protein response (UPR) transcriptional cascade. In turn this can trigger apoptosis (47). In yeast and humans a key initiating

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**TABLE 1**

Consensus motifs associated with statistically significant (t-test) transcriptional changes in are1Δ are2Δ dga1Δ lro1Δ strains after growth in 0.01 M oleate

The T-value represents the change in the average activity of sets of genes based on Gene Ontology, chromatin immunoprecipitation-chip experiments, and known transcription factor consensus motifs (45).

| Motif       | Transcription factor | T-value | ORFs |
|-------------|----------------------|---------|------|
| TAWWWW/TAGM | RLM1                 | 4.61    | 366  |
| CCCCT       | STRE                 | 3.61    | 960  |
| AAAAATTT    | rRE                 | -5.54   | 1297 |
| CGATGAG      | PAC                  | -6.81   | 248  |

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**FIGURE 3. Exogenous free fatty acids are cytotoxic to strains lacking triglycerides.**

A, growth curve analysis of control and mutant strains in YPD + 0.5 mM palmitate. B, control and mutant strains were plated in serial dilutions on YPD + 0.5 mM palmitoleate. Results are representative of at least three experiments performed in triplicate. C, growth curve analysis of control and mutant strains in YPD + 0.5 mM palmitoleate. D, growth curve analysis of control and are1Δ are2Δ dga1Δ lro1Δ (4Δ) strains harboring hDGAT2 in media supplemented with 0.5 mM oleate. E, Nile red fluorescence detecting cytoplasmic neutral lipid deposition in control or are1Δ are2Δ dga1Δ lro1Δ (4Δ) strains harboring hDGAT2. VC = vector control.

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event in this response is the Ire1p-mediated mRNA splicing of a transcription factor (Hac1p in yeast and XBP1 in metazoans) that activates numerous metabolic events, including phospholipid biosynthesis and sterol esterification (for example, human ACAT2 and yeast ARE1 are both induced by the UPR). We assessed splicing of HAC1 as a reporter of the UPR in strains lacking neutral lipid biosynthesis, by Northern hybridization (Fig. 4C). The presence of sub-toxic levels of oleate (0.05 mM) clearly induced the splicing of HAC1 and thus the UPR; however, SFA at a 10-fold higher concentration had no effect on this aspect of ER quality control.

To further investigate the mechanism of cell death, we assessed the impact of fatty acids using the yeast cell viability stain FUN1 (Fig. 4D). In control cells, FUN1 is transported to the vacuole and converted into a cylindrical intravacuolar structure. This process has been shown to require mitochondrial ATP as addition of uncoupling agents prevents cylindrical intravacuolar structure formation (48). By contrast, triglyceride- and neutral lipid-deficient strains exhibited diffuse FUN1 staining after growth in 0.05 mM UFA, suggesting metabolic debilitation due to mitochondrial dysfunction. Finally, we assessed the role of programmed cell death in this phenomenon by propidium iodide (PI) staining of cell nuclei and phosphatidylserine exposure at the outer leaflet of the plasma membrane as judged by annexin V binding (Fig. 4E). PI staining alone is indicative of necrosis; however, the combination of PI staining and phosphatidylserine exposure indicates a late stage of apoptosis (35). TG-deficient strains clearly underwent lipoapoptosis after treatment with UFAs (0.1 mM). Treatment of any strain with saturated fatty acids was without phenotype by these assays.
Fate of Fatty Acids in the Absence of Neutral Lipid Synthesis

Saturated or unsaturated fatty acid cytotoxicity is cell type-dependent in mammalian systems and arises by different mechanisms (2, 49–51). To investigate the mechanism of lipotoxicity in NL-deficient yeast, we assessed the metabolism of oleate or palmitate in control and mutant cells treated with unlabeled fatty acids for 6 h (Fig. 5). This time course had no detectable impact on growth rates. Using capillary gas chromatography (Fig. 5A), we observed a remodeling of endogenous fatty acids in the NL-deficient strains such that oleate accumulated at the expense of palmitate and palmitoleate, consistent with elevated fatty acid elongation and desaturation. The short term inclusion of exogenous UFA (0.5 mM oleate) markedly and specifically elevated the UFA composition of all cells, with greater impact when TG synthesis was genetically ablated. A similar bolus application of 0.5 mM palmitate to cells specifically and significantly elevated cellular palmitate with little impact on UFA suggesting that elongation and desaturation of exogenous fatty acids did not occur in this time period with this quantity of SFA. In control cells, 70–80% of fatty acids were incorporated into neutral lipids (TG and SE), regardless of saturation (Fig. 5, B and C). However, in cells lacking the ability to synthesize neutral lipids, the metabolism of UFA versus SFA was markedly divergent. In the mutant cells, ~40% of palmitate was preferentially incorporated into phospholipids (Fig. 5B), mainly phosphatidylcholine and phosphatidylserine (a 2–3-fold elevation relative to control cells, see Fig. 5D). Phosphatidylcholine mass in the NL-deficient strain was also elevated ~2-fold (supplemental Fig. S3). By contrast, oleate incorporation into phospholipids in the quadruple mutant strain was similar to wild-type cells (~6%, see Fig. 5C). As a consequence, ~90% of total cell-associated counts accumulated as free oleate in the quadruple mutant.

Epistatic Interactions with NL Biosynthetic Genes—We present here a genetic model of lipotoxicity in which cells lacking

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**FIGURE 5. Phospholipid synthesis is protective against lipotoxicity.** A, fatty acid species as measured by capillary gas chromatography. The percentage of major long chain fatty acid species was measured at mid-log in control and mutant (4Δ) strains after growth in YPD, YPD + 0.5 mM of oleate (YPD0), or palmitate (YPDP) for 6 h. The data are the average of two experiments, in duplicate. Lipid synthesis (expressed as a percentage of total) in control and dga1Δ lro1Δ are1Δ are2Δ strains grown in 0.5 mM palmitate (YPDP; B) or oleate (YPD0; C) until mid-log followed by a 1 h [3H]palmitic acid or [3H]oleic acid pulse, respectively. D, phospholipid synthesis (expressed as a percentage of total lipid) after growth of control and dga1Δ lro1Δ are1Δ are2Δ strains in 0.5 mM oleate or palmitate until mid-log followed by a 1 h [3H]oleic acid or [3H]palmitic acid pulse, respectively. Asterisks indicate significant difference (p < 0.05). PL, phospholipid; FFA, free fatty acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine.
TABLE 2
Epistatic interactions with acyltransferase genes

| Acyltransferase | Interacting gene (ORF) | Interacting gene function | e-MAP score | Growth rate of deletion strain |
|-----------------|------------------------|---------------------------|-------------|-------------------------------|
| ARE1           | CHO2 (YGR157W)         | Phosphatidylcholine biosynthesis | −4.47       | 1.13 ± 0.15 1.40 ± 0.04*     |
| ARE2           | UBX2 (YML013W)         | Protein catabolism (ubiquitin-mediated) | −3.06       | 1.16 ± 0.01 0.91 ± 0.01      |
| LRO1           | OP13 (YJR073C)         | Phosphatidylcholine biosynthesis | −3.84       | 1.05 ± 0.12 1.30 ± 0.03*     |
|                 | SFB2 (YNL049C)         | COP1 vesicle-mediated transport (SEC24 family) | −3.09       | 1.10 ± 0.11 1.14 ± 0.04*     |
| DGAl           | BFR1 (YOR198C)         | mRNA metabolism/vesicle-mediated transport | −8.16       | 1.01 ± 0.10 1.20 ± 0.07*     |
| DGAl           | DOP1 (YDR141C) (essential) | Membrane organization | −6.82       | 0.98 ± 0.01 0.95 ± 0.01      |
|                 | GET2 (YER083C)         | Golgi-ER vesicle-mediated transport | −6.17       | 0.97 ± 0.08 0.55 ± 0.01*     |
|                 | DGK1 (YOR311C)         | Diacylglycerol kinase | −5.76       | 1.15 ± 0.18 0.99 ± 0.03      |
|                 | TSC3 (YBR058C-A)       | Sphingolipid biosynthesis | −4.04       | 1.16 ± 0.09 0.49 ± 0.04*     |
|                 | YOR285W                | Unknown                   | −3.94       | 1.04 ± 0.11 1.03 ± 0.08      |
|                 | OLE1 (YGL055W) (essential) | Stearoyl-CoA desaturase | −3.32       | 0.96 ± 0.02 0.93 ± 0.05      |
|                 | ICE2 (YIL090W)         | ER organization           | −3.15       | 1.03 ± 0.02 0.92 ± 0.04      |

a Significant change is indicated (p value < 0.05; Student’s t test).

The genetic ablation of the yeast neutral lipid biosynthetic genes for neutral lipid biosynthesis succumbs to the toxic effects of substrate accumulation. The successful exploitation of this paradigm rests on the application of genetic screens to identify interacting pathways and thus new facets of lipid homeostasis. Numerous interactions between genes encoding early secretory pathway proteins have been observed in synthetic lethality screens (e.g. Epistatic Mini Array Profiling (eMAP) (24)). We focused on mutations that were growth-impaired when combined with lesions in any one of the neutral lipid biosynthetic pathways (eMAP score less than −3, see Table 2 and Fig. 6A). The majority of interactions were with DGA1, likely because it encodes the predominant pathway for TG synthesis during the late logarithmic/stationary phase. Strains deleted at these loci were then assessed for alterations in TG synthesis during the late logarithmic/stationary phase.

Other gene deletions were epistatic with mutations in the acyltransferases (Table 2 and Fig. 6A) and also impacted lipid metabolism. Strains lacking the COPII-vesicle coat protein encoded by SFB2 or bearing lesions in the UBX2 gene (involved in ER-associated degradation) synthesize less triglyceride and were growth-compromised when combined with LRO1 or ARE2 deletions, respectively (Fig. 6). Strains deleted for mediators of vesicular transport (BFR1 and GET2) displayed altered incorporation of fatty acids into neutral lipids (Fig. 6B) and altered growth in response to exogenous UFA (Table 2). Get2p along with the GET1 and GET3 gene products form the GET complex required for protein transit between ER and Golgi. Given our observations with get2Δ strains, we also assessed GET1 and GET3 deletion mutants. Loss of any component of this complex compromised oleate incorporation into TG or SE (Fig. 6B); however, only GET3 deletions resembled get2Δ strains with respect to palmitoleate sensitivity (Fig. 6C).

DISCUSSION

The model described here facilitates the manipulation of genetic and environmental pressures that lead to cellular dysfunction due to lipid excess. This phenomenon is among a pallet of events associated with syndromes such as obesity, type 2 diabetes, and atherosclerosis. Disease onset reflects the metabolic saturation or genetic deficiency of cellular homeostatic mechanisms that normally protect the body from a lipid surplus. Oleate-induced lipotoxicity arises due to interruption of a protein phosphorylation/dephosphorylation cycle (5), whereas saturated fatty acids, such as palmitate, induce toxicity by elevating ceramide and/or reactive oxygen species (3, 55). In Chinese hamster ovary cells, saturated fatty acid toxicity is overcome in the presence of oleate by channeling into TG biosynthetic pathways (56). Diminution of TG biosynthesis renders saturated and unsaturated fatty acids equally toxic (56).

The genetic ablation of the yeast neutral lipid biosynthetic pathway and consequential loss of the CLD compartment com-
Lipotoxicity in Budding Yeast

Combined with the addition of exogenous UFAs mimic the acute lipid overload (lipotoxicity) that often besets nonadipose tissues. By assessing the response of these yeast strains to increasing concentrations of UFA, we have characterized the events that lead to cell death. The cascade initiates with a generalized transcriptional stress response followed by elevated reactive oxygen species, induction of the unfolded protein response, metabolic dysfunction, and apoptosis (Fig. 7). These results suggest that lipotoxicity studies in yeast are relevant to higher eukaryotes and represent a suitable model to study this process in greater detail.

It is unclear if the transcriptional and biochemical effects associated with neutral lipid deficiency result from the loss of the lipid molecules or are by-products of the loss of CLDs. This cellular compartment, consisting of a neutral lipid (commonly TG or SE) core encased in a polar lipid monolayer embedded with proteins, exists in the cytosol of all eukaryotes (57, 58) and in the ER-lumen of specialized cells such as hepatocytes. In the budding yeast, S. cerevisiae, we have investigated the impact of losing this depot by ablating neutral lipid biosynthesis. We demonstrate that the produc-

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**TABLE 3**

| Strain               | YPD | YPD + 0.5 mM palmitate |
|----------------------|-----|------------------------|
| Control              | 1.0 ± 0.0 | 1.0 ± 0.0            |
| Dga1Δ lro1Δ          | 1.0 ± 0.02 | 0.9 ± 0.10           |
| Dga1Δ lro1Δ opi3Δ    | 0.95 ± 0.10 | 0.66 ± 0.11*         |
| Dga1Δ lro1Δ cho2Δ    | 1.0 ± 0.03 | 0.64 ± 0.15*         |

* Significant change is indicated (p value < 0.05; Student’s t test).
Lipotoxicity in Budding Yeast

One of the most striking epistatic relationships is the synthetic interaction of the pathways defined by DGK1 and DGA1. Both enzymes modify diacylglycerol by phosphorylation or esterification, respectively, and thus control the flux of fatty acids into phospholipids or triacylglycerol. The loss of both pathways would thereby expose a cell to distortions in DAG that could lead to inviability. Unexpectedly, DGK1 is an interaction hub for this subset of genes; the great majority of genes that interact with the sterol and DAG acyltransferases also interact with DGK1 (Fig. 6A). Similarly, Tsc3p, a positive regulator of serine-palmitoyltransferase activity (61), the first reaction in sphingolipid biosynthesis, was required for optimal growth in the context of impaired neutral lipid production or exposure to palmitoleate. This phenomenon is further supported by transcriptional changes to many genes involved in phospholipid formation in the TG-deficient strains. We conclude that the synthesis of neutral lipids acts as a buffering mechanism allowing cells to maintain viability in the face of excess lipid exposure and that phospholipid synthesis, particularly for saturated fatty acids, represents an alternate salvage point.

The ability of neutral lipid-deficient yeast to utilize the phospholipid biosynthetic machinery to maintain cellular homeostasis in the presence of toxic fatty acids prompted us to search for other compensatory pathways. Deletion of GET2, encoding a subunit of the GET complex, a multicomponent pathway for Golgi-ER (i.e. retrograde) transport, genetically interacts with a DGA1 deletion to compromise growth. Moreover, loss of GET2 or GET3 confers sensitivity to exogenous UFA and reduced steryl ester or TG biosynthesis. GET mutations are exceptionally pleiotropic, with phenotypes as diverse as metal sensitivity, mitochondrial fragmentation, temperature sensitivity, and defective sporulation. This has been explained based on a role of the GET proteins and their metazoan counterparts in the insertion of "tail-anchored" proteins into ER membranes (62). Our results link this process to lipid metabolism in terms of how a eukaryotic cell deals with toxic levels of fatty acids.

We reasoned that the identification of pathways that respond or compensate for defects in neutral lipid synthesis could lead to a better comprehension of CLD formation in all cells and the pathogenesis and treatment of human illnesses that have lipotoxicity as a component. A high number of the yeast genes identified here have human orthologs, suggesting the pathways are conserved through evolution. This includes diacylglycerol kinase, the phosphatidylethanolamine N-methyltransferases, and GET3, which is orthologous to human ASNA1. Intriguingly, PEMT activity is elevated in diabetic states (63) and the ASNA1 gene product is...
Lipotoxicity in Budding Yeast

required for insulin secretion in murine and nematode models (64). Our findings thus provide a mechanistic link between lipotoxicity and insulin secretion that was not previously apparent. We hypothesize that our approach to developing *S. cerevisiae* as a model of lipid-mediated cell death has identified new components of human diseases such as type II diabetes where lipid overload is implicated as causative.

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