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The Putative *Saccharomyces cerevisiae* Hydrolase Ldh1p Is Localized to Lipid Droplets[▽]

Sven Thoms,[1]† Mykhaylo O. Debelyy,[1] Melanie Connerth,[2] Günther Daum,[2] and Ralf Erdmann[1]*

Abteilung für Systembiochemie, Institut für Physiologische Chemie, Medizinische Fakultät der Ruhr-Universität Bochum, D-44780 Bochum, Germany,[1] and Institute of Biochemistry, Graz University of Technology, A-8010 Graz, Austria[2]

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Here, we report the identification of a novel hydrolase in *Saccharomyces cerevisiae*. Ldh1p (systematic name, Ybr204cp) comprises the typical GXSXG-type lipase motif of members of the α/β-hydrolase family and shares some features with the peroxisomal lipase Lpx1p. Both proteins carry a putative peroxisomal targeting signal type1 (PTS1) and can be aligned with two regions of homology. While Lpx1p is known as a peroxisomal enzyme, subcellular localization studies revealed that Ldh1p is predominantly localized to lipid droplets, the storage compartment of nonpolar lipids. Ldh1p is not required for the function and biogenesis of peroxisomes, and targeting of Ldh1p to lipid droplets occurs independently of the PTS1 receptor Pex5p.

Peroxisomes and lipid droplets (LDs) are ubiquitous eukaryotic organelles involved in lipid metabolism. LDs appear as oleosomes in plants, as adiposomes in mammals, or as lipid particles/bodies/droplets in yeasts and constitute a family of morphologically and biogenetically similar organelles (19). LDs are bound by a phospholipid monolayer and serve as the main storage sites for nonpolar lipids, mainly triacylglycerols (TAG) and cholesteryl ester (CE) (6, 7). LDs derive from the endoplasmic reticulum (ER), possibly by inclusion of nonpolar lipids between the two ER leaflets, eventually leading to the budding of nascent LDs (1, 6, 24, 27, 36). A large number of LD proteins have been identified by proteomic studies (12). In recent years, it has become evident that LDs, rather than being solely lipid storage sites, play a dynamic role in lipid biosynthesis, metabolism, degradation, and trafficking (6). Peroxisomes are particularly engaged in the β-oxidation of long- and very long-chain fatty acids (16). Notably, in yeast, peroxisomes are the only site of fatty acid β-oxidation (37). In mammals, peroxisomes are also involved in bile acid and plasmalogen synthesis, as well as amino acid metabolism (37, 38). Defective peroxisome biogenesis can lead to severe heritable diseases in humans (32). Such biogenesis defects are caused by mutations in PEX genes coding for proteins required for peroxisome biogenesis, collectively called peroxins (25, 34). The majority of peroxisomal matrix proteins are directed to peroxisomes by a peroxisomal targeting signal type1 (PTS1). The three amino acids SKL (serine-lysine-leucine) at the very C terminus of a protein represent the first PTS1 discovered. Generally, PTS1 comprises tripeptides with the consensus sequence [SAC][KRH][LM]. The PTS1 is recognized in the cytosol by the cycling import receptor Pex5p (8). Masking of the PTS1 by the addition of protein tags interrupts PTS1-Pex5p association and prevents peroxisomal localization (40). A peroxisomal targeting signal type 2 (PTS2) is located within the first 20 amino acids of the N terminus of some peroxisomal proteins. Peroxisomal proteins with a PTS2 are recognized by the import receptor Pex7p (20, 21, 42).

Here, we report the identification of a novel hydrolase in *S. cerevisiae*. The gene sequence of *LDH1* predicts a GXSXG-type motif that is typical of α/β-hydrolases and/or lipases (31). Bioinformatics analysis suggests that *LDH1* (*YBR204C*) encodes a novel peroxisomal protein, due to its putative PTS1 (17). In the present study, however, we show that Ldh1p is not required for the function and biogenesis of peroxisomes and that Ldh1p primarily localizes to LDs, independently of the peroxisomal protein import machinery.

MATERIALS AND METHODS

Strains and plasmids. *S. cerevisiae* strains BY4742, BY4742 Δ*yor084w*, BY4742 Δ*ybr204c*, BY4742 Δ*pex5*, and BY4742 Δ*pex1* were obtained from EUROSCARF (Frankfurt). BY4742 ERG6-RFP was obtained from W. K. Huh (San Francisco, CA). BY4742 ERG6-RFP Δ*ybr204c* was constructed by gene replacement using kanMX6 from pUG6 and primers 5′-CTAGAAGAGATTG TTCAAAATGCAGAAAATGCAGCTGATTTGGTCGTACGCTGCAGGTC GAC-3′ and 5′-GCACGAAAATCTAGTTACGCAATGTGAAATCTAGAAA ACCTTCTAATCGATGAATTCGAGCTCG-3′. BY4742 Δ*pex5*Δ*ldh1* and BY4742 Δ*pex1*Δ*ldh1* were constructed from BY4742 Δ*pex5* and BY4742 Δ*pex1* by gene replacement using a pUG6 vector and primers 5′-GCTAGAAGAGATTG TTCAAAATGCAGAAAATGCAGCTGATTTGGTCGTACGCTGCAGGTC GAC-3′ and 5′-GCACGAAAATCTAGTTACGCAATGTGAAATCTAGAAA ACCTTCTAATCGATGAATTCGAGCTCG-3′ after removal of loxP-kanMX6-loxP marker cassettes (13, 14). The yeast media have been described previously (9, 10). For construction of pUG35-LDH1 (Ldh1p-GFP), PCR-amplified YBR204c (primers RE2444 [5′-GCGCGGATCCATGAATATGGCAG AACGTGCA-3′] and RE2445 [5′-GCGCAAGCTTCAATTTGGAATTATCA ATCAC-3′]) was introduced into BamH I and HindIII sites of pUG35. For construction of pUG36-LDH1 (GFP-Ldh1p), PCR-amplified YBR204C (primers RE2444 [5′-GCGCGGATCCATGAATATGGCAGAACGTGCA-3′] and RE2446 [5′-GCGCAAGCTTCTACAATTTGGAATTATCAATCAC-3′]) was introduced into BamHI and HindIII sites of pUG36. All constructs were confirmed by DNA sequencing. The GFP-SKL plasmid has been described previously (29).

Nile Red and Oil Red O staining. For Nile Red staining (39), yeast cells in stationary phase were washed and resuspended in phosphate-buffered saline (PBS) (150 mM NaCl, 1.7 mM KH_2PO_4, 5.2 mM Na_2HPO_4). The cells were stained with Nile Red solution (0.0005% in PBS, diluted from a 0.01% stock solution in acetone) for 15 min at room temperature in the dark. The cells were

* Corresponding author. Mailing address: Institut für Physiologische Chemie, Ruhr-Universität Bochum, Universitätsstr. 150, D-44780 Bochum, Germany. Phone: 49 234 322 4943. Fax: 49 234 321 4266. E-mail: Ralf.Erdmann@rub.de.

† Present address: Universitätsmedizin Göttingen, Abteilung für Pädiatrie und pädiatrische Neurologie, Georg-August-Universität Göttingen, D-37099 Göttingen, Germany.

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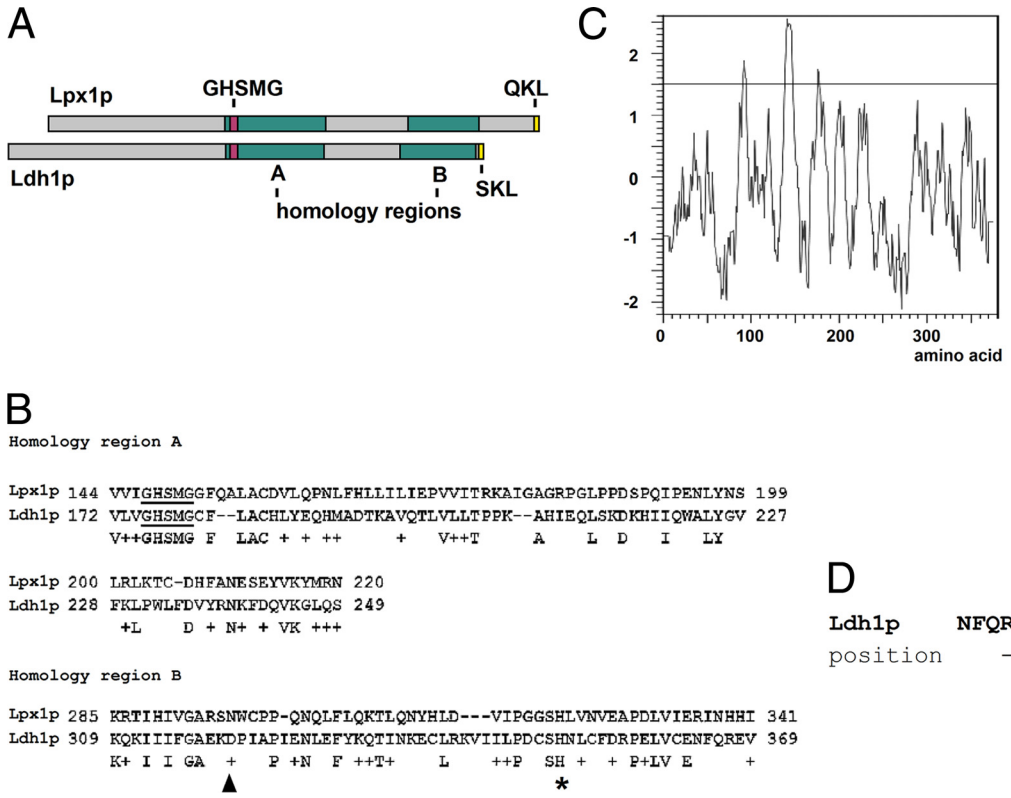

FIG. 1. Ldh1p and Lpx1p from *S. cerevisiae* are similar proteins with a hydrolase/lipase motif. (A) Similarities between Lpx1p (predicted mass, 43.7 kDa; 387 amino acids; theoretical pI, 8.16) and Ldh1p (predicted mass, 43.3 kDa; 375 amino acids; theoretical pI, 6.36) are indicated: two regions of homology, the first of which contains the GHSMG hydrolase/lipase motif of the GXSXG consensus. Both proteins carry a (putative) PTS1, QKL, or SKL. (B) Alignment of the two regions of homology of Lpx1p and Ldh1p exhibiting 28% (region A) and 27% (region B) amino acid identities. Asterisk, histidine of the probable catalytic triad; arrowhead, aspartate of the probable catalytic triad in Ldh1p. The GXSXG hydrolase/lipase motif is underlined; similar amino acids are indicated by a plus symbol. (C) Hydropathy plots of Ldh1p. The Kyte-Doolittle plot was calculated with a window size of 11. Values greater than 1.8 indicate very hydrophobic regions. (D) C terminus of Ldh1p. The amino acids in positions −2 and −5 are likely to interfere with peroxisomal targeting.

then washed six times with PBS to remove surplus dye. For Oil Red O staining (26, 39), yeast cells in stationary phase were washed twice, fixed by 4% formaldehyde in PBS for 20 min, and washed twice again. The cells were then stained with Oil Red O (0.2% in a water-isopopanol [1:1] mixture) for 15 min at room temperature in the dark and washed six times before microscopic analysis.

Image acquisition. Samples were fixed with 0.5% (wt/vol) agarose on microscope slides. Fluorescence microscopic images were recorded on an AxioPlan 2 microscope (Zeiss) equipped with a αPlan-FLUAR 100×/1.45 oil objective and an AxioCam MRm camera (Zeiss) at room temperature. If necessary, contrast was linearly adjusted using the image acquisition software AxioVision 4.8 (Zeiss).

Subcellular fractionation and organelle isolation. Subcellular fractionation and gradient centrifugation for the analysis of peroxisomes and mitochondria of Δ*ldh1* were carried out as described previously (29, 33). Cell fractionation and LD isolation for the subcellular localization of Ldh1p have been described previously (5, 11, 28).

RESULTS

Ldh1p and Lpx1p: two similar hydrolases. Ldh1p shares some features with the peroxisomal lipase Lpx1p (33) (Fig. 1). Both proteins have almost the same predicted molecular mass, namely, 43 kDa for Ldh1p and 44 kDa for Lpx1p. Both proteins carry a putative PTS1, the prototypical SKL in Ldh1p, and glutamine-lysine-leucine (QKL) in Lpx1p (Fig. 1A). Furthermore, both proteins can be aligned with two regions of homology (Fig. 1A and B), with one in the central domain, comprising the lipase motif GHSMG (4, 35), indicative of members of the α/β-hydrolase family. In the case of Ldh1p, the amino acids adjacent to the active-site serine are identical in the two proteins, namely, histidine (H) and methionine (M). Hydropathy plots indicated a pronounced hydrophobic region in the centers of both proteins. Amino acids 130 to 154 of Ldh1p comprise a hydrophobic core region, 138VVELIFVLV 146, and amino acids 154 to 177 of Lpx1p comprise the core region, 164LLILIEPVVI173 (Fig. 1C).

Absence of a synthetic phenotype of Δ*ldh1* and Δ*lpx1* in peroxisome biogenesis. Ldh1p carries the prototypical yet putative PTS1 and has been speculated to be a peroxisomal matrix protein (17). Therefore, we first tested the effect of an *LDH1* deletion on peroxisome biogenesis. Postnuclear supernatants (PNS) were prepared from wild-type and Δ*ldh1* strains and analyzed by density gradient centrifugation. The gradient fractions were assayed for peroxisomal catalase and mitochondrial cytochrome *c* oxidase activity (Fig. 2A). The distribution of neither of these proteins indicated a significant change in the abundance or density of peroxisomes or mitochondria, suggesting that peroxisomal and mitochondrial biogenesis remain functional after deletion of *LDH1*. As a defect in peroxisome biogenesis would affect peroxisome presence or density, we conclude that Ldh1p is not a peroxin. Altogether, the avail-

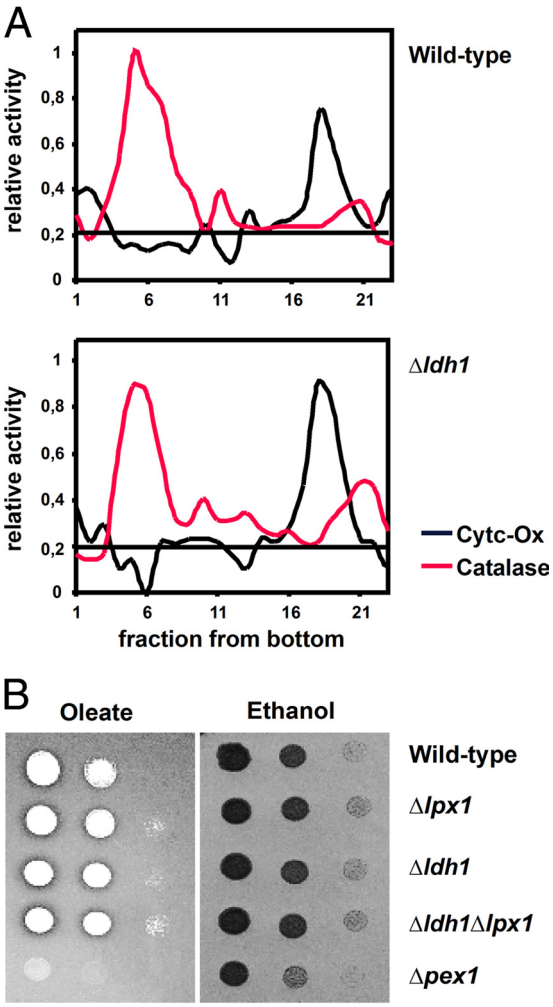

FIG. 2. Ldh1p is dispensable for peroxisome biogenesis and function. (A) Postnuclear supernatants prepared from oleate-induced wild-type and Δ*ldh1* strains were fractionated by density gradient centrifugation, and each fraction was analyzed for catalase (peroxisome) and cytochrome *c* oxidase (mitochondria) activities. The absence of Ldh1p has no influence on the apparent densities of peroxisomes and mitochondria. (B) Growth on oleate is not affected by deletion of the lipase gene *LDH1* or *LPX1* or both. Single or double deletions of *LDH1* and *LPX1* were spotted on oleate and ethanol plates with equal cell numbers in a series of 10-fold dilutions and grown for 3 days at 30°C.

able evidence suggested that Lpx1p and Ldh1p might be proteins exerting similar or redundant functions. Most mutants whose peroxisome biogenesis or functions are affected are characterized by a growth defect on oleic acid (9). We therefore tested the single and double knockouts of *LPX1* and *LDH1* for growth on oleate as the only carbon source (Fig. 2B). Neither of these knockouts had its growth on oleic acid affected, suggesting that Lpx1p and Ldh1p do not form a redundant pair in peroxisome function.

Ldh1p localizes to the lipid droplet membrane. Next, we investigated the subcellular distribution of Ldh1p. Ldh1p was expressed from a plasmid as N-terminally or C-terminally tagged green fluorescent protein (GFP) fusion proteins that localized to a particular organelle about 1 to 2 μm in diameter with several copies in a cell (Fig. 3A). Ldh1p specifically localized to the surface membranes of these organelles. We reasoned that the organelles were fragmented vacuoles, endosomes, or LDs. Thus, we coexpressed marker proteins for the organelles together with the Ldh1p fusion proteins and found that Ldh1p perfectly colocalized with Erg6p, the δ(24)-sterol methyl transferase (Fig. 3A, top), which is a major and prominent LD protein (18). Both proteins localize to the surface membrane of LDs. Ldh1p colocalized with Erg6p when GFP was localized at the N terminus or the C terminus of the protein (Fig. 3A). Localization of Ldh1p in LDs was also confirmed by Oil Red O staining (Fig. 3B). Ldh1p contains a perfect consensus for a PTS1 at its extreme C terminus. The fact that some LD proteins contain a C-terminal localization signal (22) and the possibility of a common origin of peroxisomes and LD encouraged us to test whether the PTS1 of Ldh1p is required for LD targeting. We found that neither masking of the SKL by expression of the GFP at the C terminus of Ldh1p nor deletion of the PTS1 receptor protein Pex5p interfered with targeting of Ldh1p (Fig. 3C). Thus, the PTS1-like C terminus of Ldh1p does not function as a classical peroxisomal targeting signal, nor does it interfere with targeting of the polypeptide to LD.

To verify the localization of Ldh1p, we performed cell fractionation analysis with a yeast strain that expressed plasmid-encoded Ldh1p-GFP. LDs were isolated by flotation on a density gradient (5, 28). Subcellular fractions of the gradient were analyzed by immunoblotting with polyclonal antibodies against GFP and organelle-specific marker enzymes (Fig. 4). These data revealed that Ldh1p-GFP was highly enriched in LD, as represented by the LD marker proteins Erg1p (squalene epoxidase) and Erg6p, but Ldh1p-GFP also cofractionated to some extent with the peroxisomal marker protein Fox1p (fatty-acyl coenzyme A oxidase) and the mitochondrial marker protein Por1p (mitochondrial porin) (Fig. 4). It has been shown that some LD proteins are not exclusively found in this compartment but also localize to the ER; in contrast, Ldh1p appears to localize to LD and, possibly to a lesser extent, to mitochondria and peroxisomes.

The biogenesis of peroxisomes and lipid droplets does not require *LDH1*. To test whether deletion of *LDH1* influences the intracellular distribution or morphology of peroxisomes, we analyzed wild-type and Δ*ldh1* strains expressing the peroxisomal marker protein GFP-SKL by fluorescence microscopy. Microscopic inspection of the LD was performed by Oil Red O staining (Fig. 5). These results showed that the morphological appearance of peroxisomes, as well as the frequently observed proximity to LD, was not affected by deletion of *LDH1*. Having shown that Ldh1p is targeted to LD independently of the soluble PTS1 receptor, we investigated whether Ldh1p is required for the biogenesis of LDs. After introducing a Δ*ldh1* knockout into the genomically tagged ERG6-red fluorescent protein (RFP) marker strain for LD, we found that LD could still be formed in the absence of Ldh1p (Fig. 6A). We confirmed these findings by LD staining with Nile Red (Fig. 6B) and Oil Red O (Fig. 6C). Taking these data together, it appears that Ldh1p is not required for the formation of LD.

DISCUSSION

Ldh1p is a lipid droplet hydrolase with an SKL terminus. Ldh1p contains the consensus sequence for a classical peroxi-

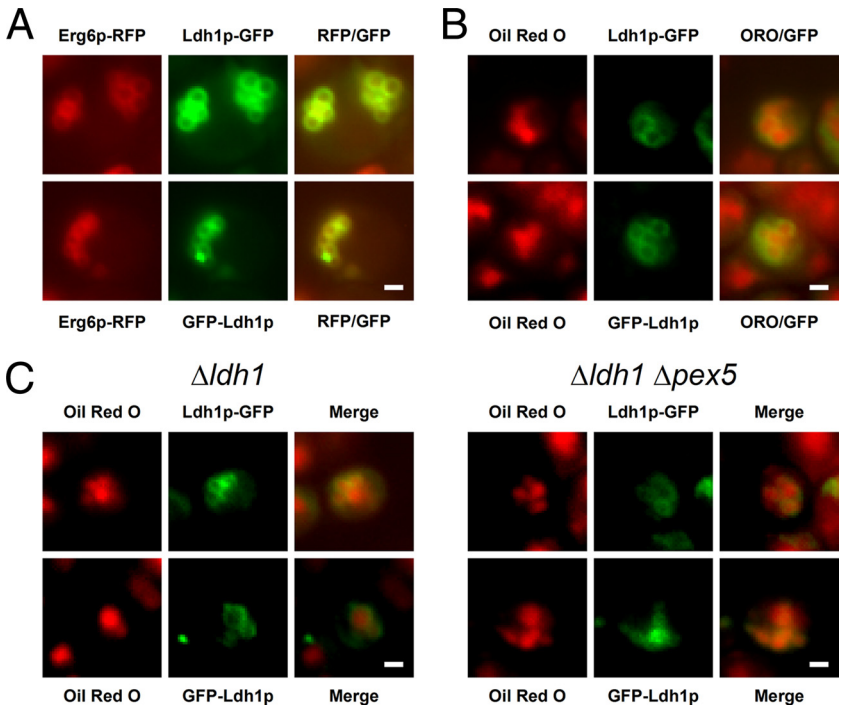

FIG. 3. Ldh1p primarily localizes to lipid droplets, and its localization is independent of the peroxisomal import receptor Pex5p. (A) Ldh1p colocalizes with the LD marker protein Erg6p [δ(24)-sterol methyl transferase]. GFP-Ldh1p and Ldh1p-GFP were coexpressed in a yeast strain with genomically tagged Erg6p-RFP. Bar, 1 μm. (B) Ldh1p colocalizes with the LD marker dye Oil Red O. GFP-Ldh1p and Ldh1p-GFP were coexpressed in a wild-type yeast strain. Bar, 1 μm. (C) Ldh1p localization is independent of the peroxisomal PTS1 pathway. GFP was fused to either the C terminus (top images) or the N terminus (bottom images) of Ldh1p. Also, in a Δ*pex5* deletion mutant, Ldh1p localization to LD was not compromised (right). In both cases Ldh1p colocalizes with the LD marker dye Oil Red O.

somal targeting signal, but the protein is primarily targeted to LD and not to peroxisomes. Peroxisomal exclusion of Ldh1p is likely due to the upstream sequences with charged amino acids in positions −2 and −5 (Fig. 1D). These positions are adverse to Pex5p binding and peroxisomal localization, for which polar/hydrophilic or positively charged amino acids in position −2 are preferred. In our case, the negatively charged amino acid is not even counteracted by neighboring amino acids, giving the likely explanation for dominating peroxisomal exclusion. The classical PTS1, SKL, is not completely sufficient to target protein to peroxisomes if the upstream sequences are not supportive. We show that the majority of Ldh1p is an LD protein that is targeted independently of the PTS1-binding Pex5p. This view is confirmed by applying a PTS1 prediction algorithm

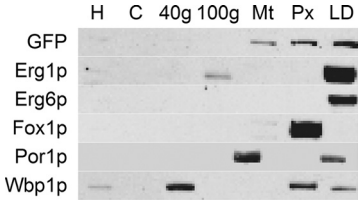

FIG. 4. Subcellular localization of Ldh1p. (A) Organelles from the wild-type strain carrying Ldh1p-GFP were isolated from cells grown to stationary phase in oleic acid-containing medium. Proteins from the subcellular fractions were precipitated, and the same amounts were separated by SDS-PAGE and analyzed by Western blotting using primary antibodies against marker enzymes, as indicated. The same amounts of proteins were loaded; therefore, the intensity of the GFP band does not represent the relative distribution of Ldh1p between LDs, mitochondria, and peroxisomes. The presence of organelles was detected with primary antibody against marker enzymes, as indicated. Erg1p, squalene epoxidase; Erg6p, δ(24)-sterol methyl transferase (lipid droplets); Fox1p, fatty-acyl coenzyme A oxidase (peroxisomes); Por1p, porin (mitochondria); Wbp1p (endoplasmic reticulum); H, homogenate; C, cytosol; 40g, 40,000 × *g* microsomes (endoplasmic reticulum); 100g, 100,000 × *g* microsomes (endoplasmic reticulum); Mt, mitochondria; Px, peroxisomes.

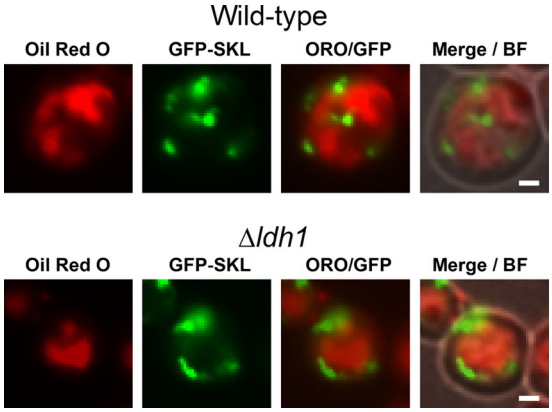

FIG. 5. The association of lipid droplets and peroxisomes is not affected by deletion of *LDH1*. Shown is fluorescence microscopy of wild-type yeast and the Δ*ldh1* strain transformed with pGFP-SKL. LDs were stained with Oil Red O (ORO). BF, bright field. Bar, 1 μm.

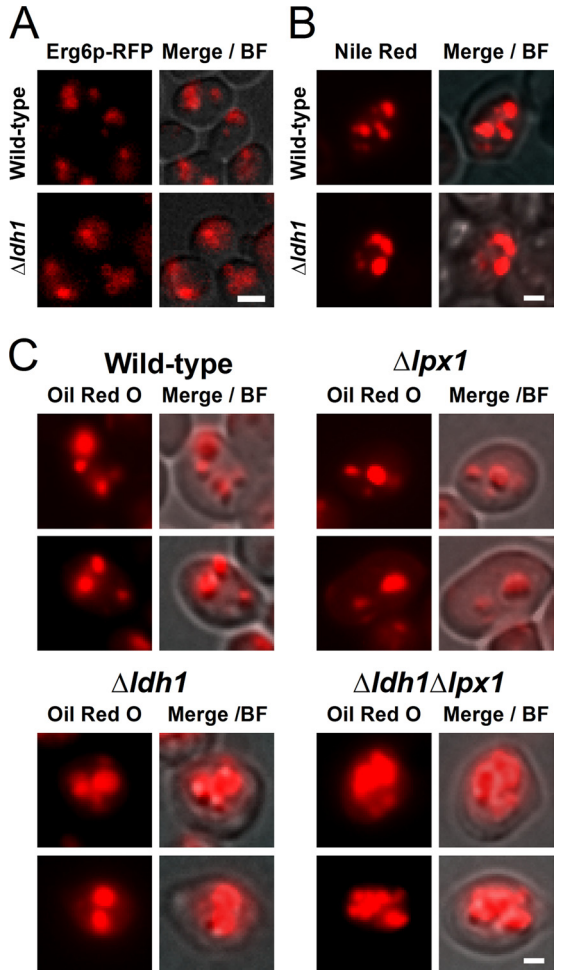

FIG. 6. Lipid droplet biogenesis is independent of Ldh1p. (A) Comparison of Erg6p-RFP localization in a wild-type strain and a Δ*ldh1* deletion strain. Bar, 2 μm. (B) Localization and morphology of Nile Red-stained LDs from the wild type and a Δ*ldh1* deletion strain. Bar, 1 μm. (C) LD morphology in the wild type and Δ*lpx1*, Δ*ldh1*, and Δ*ldh1*Δ*lpx1* deletion strains. LDs were stained with Oil Red O. BF, bright field. Bar, 1 μm.

(http://mendel.imp.ac.at/pts1/) (23) that does not predict peroxisomal localization for Ldh1p.

LD localization signals are only poorly characterized. It has been suggested that LD localization signals are constituted of hydrophobic residues at the C terminus of a protein (22, 41). A Kyte-Doolittle plot of Ldh1p indicated a region with particularly high hydrophobicity from amino acids 130 to 154 (Fig. 1C). This stretch might be required to target and/or to attach Ldh1p to LDs. Indeed, our data show that LD targeting is not abrogated when GFP is added to the C terminus or the N terminus of Ldh1p. Thus, targeting information within central parts of Ldh1p, rather than at its termini, is sufficient for the LD localization. Interestingly, the Lpx1p stretch of high hydrophobicity is in a similar location in the primary sequence, namely, at amino acids 154 to 177. The hydrophobic stretches in Ldh1p are likely not classical transmembrane domains (TMD), because LDs are bound by a single monolayer membrane of phospholipids.

Extended localization studies of Ldh1p-GFP showed that at least a portion of the polypeptide is targeted to peroxisomes and mitochondria. While this triple localization may reflect the true cellular scenario, we also have to take into account that partial targeting of Ldh1p to peroxisomes and mitochondria may be due to the overexpression of Ldh1p-GFP.

We were able to show that Ldh1p and the lipase Lpx1p are not redundant, provided that other enzymes, probably with somewhat lower homology, cannot compensate for a defect in the two enzymes. Both peroxisomes and LD function in concert in lipid metabolism. LDs require the action of triacylglycerol lipases to metabolize nonpolar lipids, while peroxisomes represent the sole cellular site for fatty acid oxidation. It is thus possible that the peroxisomal Lpx1p and the LD Ldh1p play a physiological role in lipid metabolism by mobilizing fatty acids and channeling them to their site of degradation. LDs, as fatty acid depot organelles, can be the storage sites for nonpolar lipids that are further metabolized in peroxisomes. For this reason, and not surprisingly, LDs have been found in proximity to peroxisomes in different organisms (2, 15, 30). It was also shown that *S. cerevisiae* peroxisomes attach to LDs or even project into LDs, which was interpreted as an intimate interaction between the two compartments (3). Our work on Ldh1p and Lpx1p shows that, beyond a metabolic collaboration, peroxisomes and LDs may be equipped with similar hydrolases.

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