Regulation of membrane lipid composition is crucial for many aspects of cell growth and development. Lipins, a novel family of phosphatidate (PA) phosphatases that generate diacylglycerol (DAG) from PA, are emerging as essential regulators of fat metabolism, adipogenesis, and organelle biogenesis. The mechanisms that govern lipid translocation onto membranes are largely unknown. Here we show that recruitment of the yeast lipin (Pah1p) is regulated by PA levels onto the nuclear/endoplasmic reticulum (ER) membrane. Recruitment requires the transmembrane protein phosphatase complex Nem1p-Spo7p. Once dephosphorylated, Pah1p can bind to the nuclear/ER membrane independently of Nem1p-Spo7p via a short amino-terminal amphipathic helix. Dephosphorylation enhances the activity of Pah1p, both in vitro and in vivo, but only in the presence of a functional helix. The helix is required for both phospholipid and triacylglycerol biosynthesis. Our data suggest that dephosphorylation of Pah1p by the Nem1p-Spo7p complex enables the amphipathic helix to anchor Pah1p onto the nuclear/ER membrane allowing the production of DAG for lipid biosynthesis.

Lipids play multiple key roles in membrane biogenesis, in signaling cascades, or in energy metabolism. These pathways depend largely on the regulated activation and recruitment of enzymes that respond to changes in membrane lipid composition. Lipins define a unique family of phosphatidate phosphatase (PAP) enzymes, conserved from yeasts to mammals, that catalyze a fundamental reaction in lipid and membrane biogenesis: the dephosphorylation of phosphatidate (PA) to diacylglycerol (DAG) (1), which is then acylated to produce triacylglycerol (TAG), the major form of fat stored in lipid droplets. In addition, both PA and DAG are intermediates for the biosynthesis of membrane phospholipids (Fig. 1A) (2, 3).

Consistent with these key functions, recent studies have implicated lipins in a variety of processes in different systems. In budding yeast, the single lipin orthologue Pah1p regulates phospholipid and TAG content (1) as well as the transcription of many genes encoding lipid biosynthetic enzymes (4). Mammals express three paralogues called lipin 1, 2, and 3 that exhibit distinct genotypes, lipin 1, 2, and 3 that exhibit distinct patterns of stacked ER membranes to the outer nuclear envelope (15) and found that in both cases there was no Pah1p-GFP recruitment onto membranes of cells expressing GAL-PAH1 mutant (11, 12). Moreover, the levels of PAP activity suggests that Pah1p comes from the fact that DGK1 overexpression rescues the toxicity caused by the overexpression of Pah1p activity (11). In media containing raffinose, where GAL-DGK1 is not induced, Pah1p-GFP expressed at endogenous levels shows a diffused soluble localization and cells display normal, round nuclei as visualized by the inner nuclear membrane reporter HEH2-CHERRY (Fig. 1B). As shown previously, in galactose-containing medium the nuclear membrane expanded in GAL-DGK1 cells (11) and similar to the nuclei of spo7Δ cells (13), expansion took place at the membrane associated with the nucleolus (Fig. 1C). Interestingly, Pah1p-GFP colocalized with the expanding nuclear envelope, although it was excluded from the rest of the nuclear envelope and ER (Fig. 1B). Time course analysis showed that in most cells recruitment was initiated and maintained onto the nucleolar-associated membrane (Fig. 1C).

Results and Discussion

To investigate the regulation of lipin membrane translocation, we applied a genetic approach in yeast. Specifically, we asked whether increasing the levels of PA, the substrate of Pah1p, could enhance its recruitment onto membranes. To do this, we used a GAL-DGK1 construct to overexpress the transmembrane DAG kinase Dgk1p that localizes to the nuclear/ER membrane. Dgk1p uses CTP as a phosphate donor to phosphorylate DAG and generate PA (11, 12) (Fig. 1A), and therefore its overexpression causes phenotypes similar to those described in pah1Δ (4, 11, 12). Further genetic evidence that Dgk1p activity antagonizes that of Pah1p from the fact that DGK1 overexpression rescues the toxicity caused by the overexpression of Pah1p activity (11).

In media containing raffinose, where GAL-DGK1 is not induced, Pah1p-GFP expressed at endogenous levels shows a diffused soluble localization and cells display normal, round nuclei as visualized by the inner nuclear membrane reporter HEH2-CHERRY (Fig. 1B). As shown previously, in galactose-containing medium the nuclear membrane expanded in GAL-DGK1 cells (11) and similar to the nuclei of spo7Δ cells (13), expansion took place at the membrane associated with the nucleolus (Fig. 1C). Interestingly, Pah1p-GFP colocalized with the expanding nuclear envelope, although it was excluded from the rest of the nuclear envelope and ER (Fig. 1B). Time course analysis showed that in most cells recruitment was initiated and maintained onto the nucleolar-associated membrane (Fig. 1C). In contrast, Pah1p-GFP was not recruited onto membranes of cells overexpressing the catalytically dead DGK1[D177A] mutant (11, 12). Moreover, co-over-expression of GAL-PAH1 in cells expressing GAL-DGK1 resulted in complete loss of Pah1p-GFP membrane labeling and concomitantly restored normal nuclear shape (Fig. 1B). The fact that the recruitment of Pah1p-GFP depends on (i) a catalytically active Dgk1p and (ii) the levels of PA activity suggests that PA is required for this translocation step onto membranes. To test whether structural changes to the nuclear/ER membrane, rather than the elevated PA levels, could be responsible for Pah1p-GFP recruitment, we overexpressed the nuclear envelope protein Esclp1, which causes similar structural defects to the nucleus as Dgk1p (14), or Hmg1p, which causes accumulation of stacked ER membranes to the outer nuclear envelope (15) and found that in both cases there was no Pah1p-GFP recruit-

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membrane (Fig. S1). Taken together, these data indicate that Pah1p-GFP can be recruited in a PA-dependent manner onto a nuclear/ER membrane domain.

We first asked whether the membrane translocation of Pah1p-GFP could be driven only by the interaction of its catalytic site with its substrate PA. Pah1p belongs to a superfamily of Mg$^{2+}$-dependent phosphatases that use a DXDX(T/V) catalytic motif to dephosphorylate their substrates. A Pah1p-[D398A D400A]-GFP mutant at this motif, which is catalytically dead (16), still associates with membranes, both in GAL-DGK1 or in pah1Δ cells (Fig. 1D), indicating that recruitment is independent of the catalytic activity of Pah1p and suggesting the presence of other Pah1p domains that are responsible for membrane binding.

We next examined the role of the Nem1p-Spo7p, a protein phosphatase complex that localizes to the nuclear/ER membrane and dephosphorylates Pah1p (4, 17). Nem1p is the catalytic subunit and Spo7p is the regulatory subunit that binds stably to the catalytic domain of Nem1p and is required for the activity of the holoenzyme in vivo and in vitro (4, 18). Because Nem1p and Spo7p are integral membrane proteins, we asked whether binding of Pah1p to this complex is required for its membrane recruitment. Indeed, although Spo7p-Cherry showed an ER localization in raffinose media, it colocalized with membrane-bound Pah1p-GFP in GAL-DGK1 cells (Fig. 2A). Importantly, PA-dependent translocation of Pah1p-GFP was blocked in nem1Δ spo7Δ cells (Fig. 2B), indicating that the phosphatase complex is required for this step. However, membrane translocation was restored in nem1Δ spo7Δ cells expressing PAH1-7A (Fig. 2B), a phosphorylation deficient mutant where seven phosphoserides have been replaced by alanines (17). Therefore, once Pah1p is dephosphorylated, its membrane binding no longer requires the Nem1p-Spo7p complex. Thus, there must be other factor(s) responsible for maintaining Pah1p at its membrane-bound state.

Bioinformatic analysis suggests that Pah1p contains a predicted amphipathic helix in its extreme amino-terminal end (residues 1 to 18, Fig. 3A) (19). Because amphipathic helices are known to mediate reversible binding of proteins with membranes, we assayed recruitment of two Pah1p mutants, one lacking the predicted helix (Pah1p-Δ[3-16]-GFP) and a second, where three bulky residues within the hydrophobic phase of the helix (L8, V11, W15) were changed to alanines (Pah1p-AH3A-7A-GFP, Fig. 3A). Although in both cases the helix mutations did not affect the stability of Pah1p (Fig. 3C), recruitment was completely abolished in GAL-DGK1 cells (Fig. 3B). Therefore the hydrophobic phase of the helix is required for membrane recruitment. We next asked whether dephosphorylation of Pah1p would bypass the requirement of the amphipathic helix for membrane association. As seen in Fig. 3B, a Pah1p-AH3A-7A-GFP fusion was not recruited in GAL-DGK1 Δ spo7Δ cells (Fig. 2B), indicating that recruitment is independent of its catalytic domain required for this step. However, membrane translocation was restored in nem1Δ spo7Δ cells expressing PAH1-7A (Fig. 2B), a phosphorylation deficient mutant where seven phosphoserides have been replaced by alanines (17). Therefore, once Pah1p is dephosphorylated, its membrane binding no longer requires the Nem1p-Spo7p complex. Thus, there must be other factor(s) responsible for maintaining Pah1p at its membrane-bound state.

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overexpression of NEM1-SPO7 mixed micelles (20). Two assays, which monitor distinct types of interactions of Pah1p with PA, were performed: In a first set of experiments, the enzyme activities were measured as a function of the molar concentration of PA (e.g., number of micelles containing PA) by maintaining the ratio of Triton X-100 to PA in the micelle at 10:1 (Fig. 3B). In a second set of assays, activities were measured as a function of the surface concentration of PA (mole percent, e.g., number of PA molecules on a micelle surface) while keeping the molar concentration of PA constant at 0.2 mM (Fig. 4C). 

The helix mediates the binding of Pah1p onto PA/Triton X-100 to PA was maintained at 0.2 mM, and the Triton X-100 concentration was held constant at 0.2 mM, and the Triton X-100 concentration was varied to obtain the indicated surface concentrations for the first assay reflect the interactions of the enzyme with the micelle surface, whereas for the second assay reflect its interactions with PA during catalysis within the surface. Although in both assays the Pah1p-AH3A mutant exhibited a decrease in the V_{max} its K_{m} value showed an increase only for the first (molar PA) but not the second (surface PA) assay. These data indicate that the affinity of Pah1p-AH3A for the initial binding step to the Triton X-100/PA surface is lower. Once Pah1p binds to the surface, however, its interaction with PA is no longer affected by the amphipathic helix.

To examine further the role of the helix in membrane recruitment, we used a fluorescence-based assay that measures

**Fig. 3.** An amino-terminal amphipathic helix mediates the membrane recruitment of Pah1p-GFP in vivo. (A) Schematic of the primary structure of Pah1p, with the seven phosphorylation sites indicated (P). The position of the predicted amphipathic helix (dark gray diamond, residues 1 to 18) and its helical wheel plot with the hydrophobic phase (dark gray residues) are shown. Asterisks indicate the three hydrophobic residues mutated in the PAH1-ΔAH3A mutant. (B) Wild-type RS453 cells expressing GAL-DGK1 and the indicated PAH1-GFP fusions were grown and visualized as in Fig. 1B. Bar, 5 μm. (C) Cell extracts from the strains in B were analyzed by Western blotting using the indicated antibodies. (D) Serial dilutions of wild-type cells expressing the indicated constructs were spotted onto synthetic plates lacking inositol and supplemented with glucose (Left) or galactose (Right). Cells were grown for 2 or 4 d, respectively. (E) Serial dilutions of pah1Δ cells expressing the indicated constructs were spotted onto synthetic plates supplemented with galactose and grown for 4 d.

**Fig. 4.** Helix-dependent recruitment of Pah1p onto liposomes. (A) SDS-PAGE of the purified Pah1p and Pah1p-AH3A from yeast, stained with Coomassie blue. (B and C) The helix mediates the binding of Pah1p onto PA/Triton X-100 micelles and is required for its PAP activity. The PAP activities of Pah1p or Pah1p-AH3A were measured as a function of the indicated molar (B) or surface (C) concentrations of PA. For the experiment shown in B, the molar ratio of Triton X-100 to PA was maintained at 10:1 (9.1 mol % PA). For the experiment shown in C, the molar concentration of PA was held constant at 0.2 mM, and the Triton X-100 concentration was varied to obtain the indicated surface concentrations. The kinetic parameters for Pah1p or Pah1p-AH3A for each of the two assays are shown. Kinetic data were analyzed according to the Michaelis–Menten and Hill equations using the enzyme kinetics module of SigmaPlot software. The data shown are means ± SD from triplicate enzyme determinations. (D) Effect of dephosphorylation on the liposome-dependent increase in fluorescence of Pah1p or Pah1p-AH3A. Native and dephosphorylated forms of Pah1p were incubated with increasing concentrations of liposomes that were made of phosphatidylcholine and PA at a molar ratio of 10:1. Following 10 min incubation, the increase in fluorescence was measured. The data are plotted with respect to total phospholipid concentration in the liposomes and are means ± SD from triplicate determinations. Open symbols: native proteins, closed symbols: dephosphorylated proteins. The dissociation constants (K_{d}) shown were determined as previously described (32).
Pah1p-liposome interactions. The interaction of Pah1p with liposomes can be monitored by an increase of tryptophan fluorescence intensity with a shift from 350 to 343 nm in the wavelength of the maximum emission. This increase indicates a change from a hydrophilic to a more hydrophobic environment around the tryptophan residues. The native Pah1p-AH3A mutant purified from yeast showed little difference in liposome binding when compared to the wild-type Pah1p (Fig. 4D, open symbols). In vitro dephosphorylation of the wild-type Pah1p resulted in a threelfold decrease in the Kd, reflecting an increase in liposome binding (Fig. 4D). The dephosphorylation of the Pah1p-AH3A mutant, however, did not result in an increase in binding (Fig. 4D). Thus, as seen with the Pah1p-GFP fusions in vivo, dephosphorylation of Pah1p promotes membrane binding only in the presence of the amphipathic helix.

We then tested the functional significance of the Pah1p helix in lipid production. Lack of PAH1 affects the cellular levels of the major phospholipid classes: phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylglycerol (PG). PHA1 cells exhibited increased levels of PE, PI, and PG, and PA levels decreased in the presence of [2-3H]acetate. The phospholipids were extracted, separated by two-dimensional thin-layer chromatography. Each data point represents the mean SD of three experiments. (Fig. 5A). Expression of PAH1-AH3A in the pah1Δ mutant also led to an increase in PE, PI, and PA and decrease in PC (Fig. 5A). In addition to these changes in phospholipids, neutral lipid analysis of the PAH1-AH3A mutant showed that both DAG, generated by Pah1p, and TAG generated by acylation of DAG, decreased to levels comparable to those seen in the pah1Δ mutant (Fig. 5B). Therefore, the helix is required for the lipid biosynthetic functions of Pah1p. Consistent with these effects on lipid metabolism, PAH1-AH3A cells displayed nuclear/ER membrane expansion also seen in pah1Δ cells (Fig. 5C).

In summary, we find that (i) the levels of PA control the localization of the lipin Pah1p, (ii) the Nem1p-Spo7p phosphatase complex and the amphipathic helix cooperate to tether Pah1p onto the nuclear/ER membrane, and (iii) the helix is essential for the biosynthetic functions of Pah1p. Although PA is important for Pah1p recruitment, additional factors (such as PA effector proteins or other phospholipids that are metabolically linked to PA) may work together to recruit Pah1p. The Nem1p-Spo7p relocation in Dgk1p-overexpressing cells and its requirement for Pah1p recruitment suggest it may be part of a machinery that determines localized membrane translocation of Pah1p in response to PA. Because dephosphorylated Pah1p no longer depends on Nem1p-Spo7p but still requires the helix for recruitment in vivo, we support the idea that a prime function of dephosphorylation is to enable helix membrane binding. Indeed, dephosphorylation of Pah1p enhances its liposome binding in vitro, but only in the presence of a functional amphipathic helix. How dephosphorylation could control helix function is unclear, but structural changes within Pah1p or interaction with another factor could both expose the helix and stabilize recruitment. It should be noted that because PA and DAG play essential functions onto the membranes of other organelles such as mitochondria, Golgi complex, or lipid droplets, it is possible that additional and organelle-specific interactions of Pah1p may operate on these membranes. Nevertheless, the effects of Pah1p-AH3A on lipids levels suggest that the helix mediates the recruitment of Pah1p to the nuclear/ER membrane where the biosynthesis of the bulk cellular pools of phospholipids and TAG takes place.

Sequence analysis suggests that the Pah1p helix is an ArfGAP1 lipid packing sensor, an amphipathic helical motif that adsorbs preferentially on curved membranes and is proposed to act as a membrane curvature sensor. The ER is made of flat membrane sheets that include the nuclear membrane, and tubules with high cross-sectional curvature that are stabilized by reticulons. This raises the intriguing possibility that Pah1p converts PA into DAG preferentially on ER tubules. However, PA-dependent recruitment of Pah1p-GFP takes place on a nuclear membrane domain (Fig. 1B) that, as expected, lacks reticulons (Fig. S2), suggesting that Pah1p is able to bind membrane sheets. An alternative role of helix in the well-known deformations of PA and DAG bilayers: Changes in the curvature of PA-enriched membranes may be detected by the helix that would then mediate the binding of Pah1p.

Lipins act at a key branch point of lipid biosynthesis that commits the glycerol backbone to membrane phospholipids and triglyceride. Therefore, membrane recruitment of lipins could be a major regulatory step during membrane biogenesis or energy storage in lipid droplets. Mammalian lipins respond to several stimuli: Fatty acids cause translocation of a soluble PAP activity on microsomal membranes, whereas insulin causes phosphatidate and increase of the soluble pool of lipin 1 in 3T3-L1 adipocytes. Whether, as in yeast, these changes require a membrane-bound receptor, such as the mammalian Nem1p orthologue Dullard (28), is not known. Mammalian lipins 1 and 2 can functionally replace Pah1p in yeast (29) and contain predicted amphipathic helices at their amino-terminal ends although they lack the distinctive serine residues of the polar phase of the Pah1p helix. Future studies should address whether similar mechanisms operate during the membrane translocation of lipins in other systems.
Materials and Methods

Yeast Strains, Media, and Growth Conditions and Plasids. Yeast strains and plasmids used in this study are listed in Tables S1 and S2, respectively. Yeast cells were grown in synthetic medium (SC) containing 2% glucose, lacking the appropriate amino acids for plasmid selection. To assay growth of yeast cells in media lacking inositol, synthetic medium was prepared using yeast nitrogen base lacking inositol (Bio101). GAL1/10-dependent overexpression was performed by changing the carbon source of early log phase cells from 2% raffinose to 2% galactose.

For growth assays on plates, yeast cells were grown in the corresponding SC liquid medium to early logarithmic phase. Ten microliters of serial tenfold dilutions were spotted onto the appropriate SC plates and incubated at 30 °C for 2–4 d.

Yeast Cell Extracts and Antibodies. Yeast cells (20 OD600) from a logarithmically grown culture were centrifuged, washed once with water, and lysed in SDS-sample buffer with glass beads and three rounds of boiling (2 min)/vortexing (30 s). Extracts were spun for 10 min (14,000 × g, 10 min) and 15 μL of each supernatant was analyzed by Western blot analysis. The anti-GFP antibody is a gift from Fulvio Reggiori (Utrecht). Western blot signals were developed using ECL (GE Healthcare).

Light Microscopy. Cultures for experiments involving galactose induction were set as follows: Yeast strains were grown at 30 °C in minimal selective medium. Two microtubules were transferred onto a glass slide, covered with a 22-μm cover glass, and immediately imaged live at room temperature. Cultures for experiments not involving galactose induction were set as described above with the following differences: Media contained glucose as carbon source and cells from 10 mL were pelleted when they reached mid log phase (OD600 = 0.4 to 0.6) and resuspended in glucose containing medium. Images were acquired on an epifluorescence microscope consisting of an inverted CCD type, and a 100 X plan-apochromatic 1.4NA objective lens (Carl Zeiss Ltd). The microscope was controlled by the Imaris OpenLab software (VS). GFP was recorded with Carl Zeiss Filter set 52 (488052-0000-000) (excitation band pass 488/10 nm, emission band pass 525/50). mCherry was recorded with Carl Zeiss Filter set 00 (488000-0000-000) (excitation band pass 530-585 nm, emission long pass 615 nm). The brightness and contrast of the resulting images were adjusted using Photoshop (Adobe).

Purifications of Native Pah1p and Pah1p-AH3A from Yeast. RS453 cells overexpressing the PAH1-PTA or PAH1-AH3A-PTA fusions from the GAL1/10 promoter were grown in galactose-containing medium to an OD600 of 0.7. PTA fusion affinity purification and tobacco etch virus protease-dependent elution of the native proteins was performed as previously described (17). Purified proteins eluted in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.05% TX-100, 20% glycerol were snap frozen into liquid nitrogen and kept at −80 °C.

D Dephosphorylation of Pah1p and Pah1p-AH3A. Pah1p proteins were incubated for 1 h at room temperature with 50 units of lambda protein phosphatase (New England Biolabs) in 50 mM Tris-HCl (pH 7.5) buffer containing 2 mM MnCl2, 2 mM dithiothreitol, 0.01% Brij 35, 0.1 mM EGTA, and 100 mM NaCl.

Preparation of Large Unilamellar Phospholipid Vesicles. Large unilamellar phospholipid vesicles (liposomes) were prepared with diethyl phosphatidylcholine and dioleoyl phosphatidate at a molar ratio of 10:1 by lipid extrusion (30). Briefly, phospholipids in chloroform were evaporated under nitrogen to form a thin lipid film. The phospholipids were then resuspended in 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 1 mM EDTA. After five freeze-thaw cycles in dry ice and warm water, the phospholipid suspension was extruded 11 times through a polycarbonate filter (100-nm pore size).

Fluorescence Measurements. Fluorescence measurements were carried out in a Fluoromax-3 fluorimeter (HORIBA Jobin Yvon Inc.) at room temperature in 200 μL of 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 1 mM EDTA. After five freeze-thaw cycles in dry ice and warm water, the phospholipid suspension was extruded 11 times through a polycarbonate filter (100-nm pore size).

Preparation of Triton X-100/PA-Mixed Micelles. PA in chloroform was transferred to a glass test tube, and the solvent was eliminated in vacuo for 1 h. Triton X-100 was added to PA to prepare Triton X-100/PA-mixed micelles. The mole percent of a lipid in a Triton X-100/lipid-mixed micelle was calculated using the following formula: mol % lipid = 100 × [lipid/molar] / ([lipid/molar] + [Triton X-100/molar]). The total lipid concentration in the Triton X-100/lipid-mixed micelles was kept below 15 mol % to ensure that the structure of the lipid-mixed micelles was similar to that of pure Triton X-100 micelles.

PA Phosphatase Assays and Kinetic Analysis. Mg2+-dependent PA phosphatase activity was measured by following the release of water-soluble 32P from chloroform-soluble 32PPA as described previously (31). Enzyme reactions and kinetic analysis with purified Pah1p proteins from yeast were performed as previously described (1). Kinetic and statistical analyses were performed using the enzyme kinetics module of SigmaPlot software.

Labeling and Analysis of Lipids. Steady-state labeling of phospholipids and neutral lipids with 32P and [2-3H]acetate, respectively, was performed as described previously (1). Phospholipids were separated by two-dimensional thin-layer chromatography on silica gel plates using the described previously (1). Phospholipids were separated by two-dimensional thin-layer chromatography on silica gel using chloroform/methanol/water (65:36:3, v/v/v) as the developing solvent. Radiolabeled lipids were visualized by phosphorimaging and subjected to ImageQuant analysis.

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