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Architecture and function of yeast phosphatidate phosphatase Pah1 domains/regions

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ABSTRACT

Phosphatidate (PA) phosphatase, which catalyzes the Mg^{2+}-dependent dephosphorylation of PA to produce diacylglycerol, provides a direct precursor for the synthesis of the storage lipid triacylglycerol and the membrane phospholipids phosphatidylcholine and phosphatidylethanolamine. The enzyme controlling the key phospholipid PA also plays a crucial role in diverse aspects of lipid metabolism and cell physiology. PA phosphatase is a peripheral membrane enzyme that is composed of multiple domains/regions required for its catalytic function and subcellular localization. In this review, we discuss the domains/regions of PA phosphatase from the yeast *Saccharomyces cerevisiae* with reference to the homologous enzyme from mammalian cells.

1. Introduction

In the model eukaryote yeast *Saccharomyces cerevisiae*, the *PAH1*-encoded phosphatidate (PA) phosphatase (3-*sn*-phosphatidate phosphohydrolase, EC 3.1.3.4) catalyzes the Mg^{2+}-dependent dephosphorylation of PA to produce diacylglycerol (DAG) [1] (Fig. 1). Pah1 is a highly regulated enzyme that largely controls whether the key lipid intermediate PA is utilized for the synthesis of membrane phospholipids or the neutral lipid triacylglycerol (TAG) [2–8] (Fig. 1). There are promiscuous lipid phosphate phosphatase enzymes (i.e., App1 [9], Dpp1 [10], and Lpp1 [11]) that utilize PA, as well as lysoPA and DAG pyrophosphate, as a substrate, but they are not involved in de novo lipid synthesis [5,12]. Instead, these enzymes presumably serve other cellular functions that include lipid signaling and endocytosis [2,9,12].

The importance of Pah1 in maintaining the phospholipid/TAG balance is exemplified by a plethora of deleterious phenotypes exhibited by yeast cells that lack the enzyme [7,12]. One of the notable phenotypes is the massive expansion of the nuclear/endoplasmic reticulum (ER) membrane [13], which is ascribed to increases in PA content and phospholipid synthesis that occur at the expense of TAG synthesis [1,14,15]. The increase in phospholipid synthesis correlates with the derepression of phospholipid synthesis genes via the Henry regulatory circuit [13,16], whereas the reduction in TAG synthesis correlates with a decrease in lipid droplet numbers [17–19]. The imbalance of lipid homeostasis in cells bearing the *pah1*Δ mutation correlates with additional phenotypes that include a loss in cell wall strength [20,21], defects in nucleotide metabolism [22], abnormal chromosomal insertions [23], hypersensitivity to oxidative stress [24], fatty acid-induced toxicity [17], an inability to fuse vacuoles [25] or degrade cellular components [26], and the inability to grow on non-fermentable carbon sources [1,27] or at elevated temperatures [1,13,27]. Ultimately, the *pah1*Δ mutation causes a reduction in chronological life span with apoptotic cell death in the stationary phase [24]. Some of the *pah1*Δ mutant phenotypes (e.g., derepression of phospholipid synthesis genes and massive nuclear/ER membrane expansion) are dependent on the synthesis of PA via the CTP-dependent DAG kinase Dgk1 that utilizes a DAG pool that is not derived from the Pah1-catalyzed reaction [7,17,18,28] (Fig. 1). The source of this specific pool of DAG is currently unknown.

Pah1 is conserved throughout eukaryotic evolution with counterpart enzymes in plants [29,30], worms [31], flies [32,33], mice [34,35], and humans [1,36]. In fact, the discovery that *PAH1* encodes the Mg^{2+}-dependent PA phosphatase in *S. cerevisiae* [1,37] made it possible to

Abbreviations: DAG, diacylglycerol; ER, endoplasmic reticulum; HAD, haloacid dehalogenase; IDR, intrinsically disordered region; PA, phosphatidate; RP, regulation of phosphorylation; TAG, triacylglycerol.

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study the enzyme function of the orthologous proteins in higher eukaryotic organisms [2,12]. In mammals, the Mg^{2+}-dependent PA phosphatase is encoded by the *LPIN1–3* genes and their protein products are known as lipins [34,35]. Lipin 1 deficiency in humans and mice causes rhabdomyolysis [38,39], and deficiency in mice is also characterized by hepatic steatosis during the neonatal period, lipodystrophy, insulin resistance and peripheral neuropathy [34,40]. In mice, lipin 1 overexpression results in increased lipogenesis and obesity [41]. Polymorphisms in the human *LPIN1* gene are associated with insulin resistance and metabolic syndrome [42]. Human lipin 2 deficiency causes chronic recurrent multifocal osteomyelitis and congenital dyserythropoietic anemia [43,44], whereas genetic variations in the human *LPIN2* gene are associated with type 2 diabetes [45]. These observations further underscore the importance of PA phosphatase function in lipid synthesis and cell physiology. The enzyme discovery, identifications of its encoding genes, its enzymological and kinetic properties, and the genetic and biochemical regulations of the enzyme in *S. cerevisiae* and other eukaryotic systems have been reviewed elsewhere [2,5,6,8,12,46–55]. The purpose of this review is to summarize advances in our understanding of how the *S. cerevisiae* Pah1 architecture governs its PA phosphatase function and regulation. While *S. cerevisiae* Pah1 and mammalian lipins are conserved with respect to their mode of action and catalytic cores, they are different with respect to their structural and regulatory determinants that are be discussed in this review.

2. Phosphorylation/dephosphorylation regulates the location, function, and stability of Pah1

Pah1 is a peripheral membrane protein, and its PA phosphatase activity occurs at the nuclear/ER membrane surface [1,56,57] (Fig. 2). Yet, the enzyme is largely associated with the cytosol [1,57–60]. The subcellular location of Pah1 and thus its catalytic function is primarily controlled by posttranslational modifications in the form of phosphorylation and dephosphorylation [8] (Fig. 2). Pah1 is phosphorylated by multiple protein kinases [61–67] and, in general, the phosphorylated enzyme is localized in the cytosol of the cell [61,68] (Fig. 2). The phosphorylation not only serves to sequester Pah1 to the cytosol, but it also protects the enzyme against degradation by the 20S proteasome [69,70]. Additionally, some phosphorylations of Pah1 stimulate PA phosphatase activity, whereas other phosphorylations inhibit the activity [61,66,67].

In contrast to the phosphorylation of Pah1 by multiple protein kinases, its dephosphorylation is catalyzed by a single protein phosphatase complex, namely Nem1 (catalytic subunit)-Spo7 (regulatory subunit), in the nuclear/ER membrane [13,68,71,72] (Fig. 2). The function of Nem1-Spo7 is to recruit and dephosphorylate Pah1 at the nuclear/ER membrane [8,13,57,58,68,72]. The dephosphorylation permits Pah1 to associate with its membrane-imbedded substrate PA [73], and in addition, it derepresses PA phosphatase activity [72]. Whereas the dephosphorylation of Pah1 is required for its function at the nuclear/ER membrane, it also renders the enzyme susceptible to proteasomal degradation [69,70]. The Nem1-Spo7 complex itself is regulated for its protein phosphatase activity. The complex is stimulated by the Pah1 substrate PA [74], but inhibited by the ER-associated protein Ice2 [75]. Like Pah1, the Nem1 and Spo7 subunits are both subject to phosphorylation by protein kinases A [76] and C [77]. Given the requirement of Nem1-Spo7 in the membrane localization and catalytic activity of Pah1, it is not surprising that cells bearing the *nem1*Δ or *spo7*Δ mutation exhibit the same phenotypes shown by the *pah1*Δ mutant [71,78,79]. Where the broad strokes of this phosphorylation/dephosphorylation-mediated regulation mechanism are conserved, the specifics differ between the yeast and mammalian PA phosphatases.

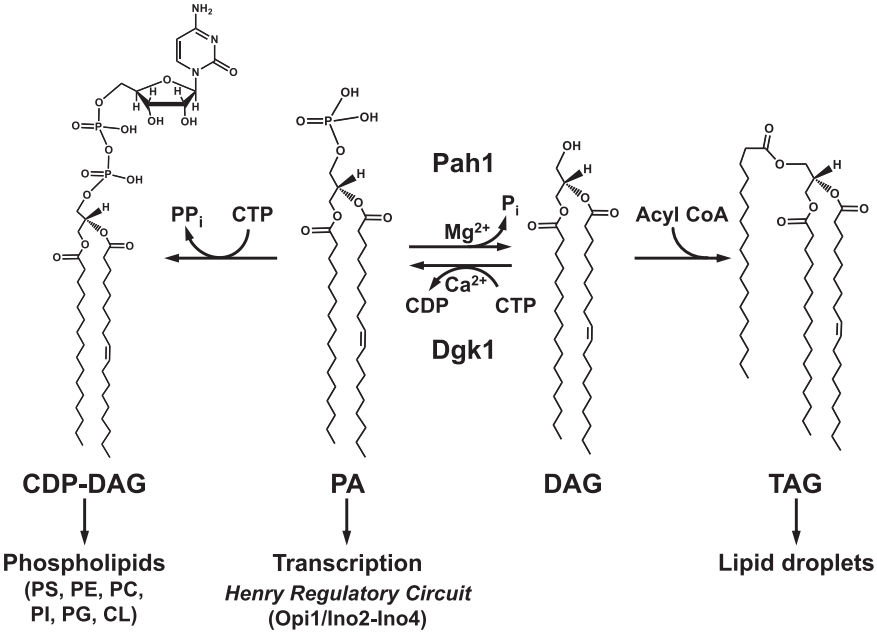


Fig. 1. Roles of PA phosphatase Pah1 in lipid synthesis. The structures of CDP-DAG, PA, DAG, and TAG are shown with fatty acyl groups of 16 and 18 carbons with and without a single double bond where indicated. Pah1 plays a key role in the production of DAG for TAG synthesis and thereby controls the use of PA for the synthesis of membrane phospholipids via CDP-DAG. The PA phosphatase reaction is counterbalanced by the CTP-dependent conversion of DAG to PA by Dgk1. In addition to its role as a precursor in lipid synthesis, PA signals the transcriptional regulation of phospholipid synthesis genes via the Henry (Opi1/Ino2-Ino4) regulatory circuit. Under certain conditions (i.e., choline and/or ethanolamine supplementation), the DAG generated by the PA phosphatase reaction is utilized for the synthesis of phosphatidylcholine and/or phosphatidylethanolamine via the Kennedy pathway (not shown). More comprehensive pathways of lipid synthesis, along with details of the Henry regulatory circuit may be found in Refs. [3,4]. *PS*, phosphatidylserine; *PE*, phosphatidylethanolamine; *PC*, phosphatidylcholine; *PI*, phosphatidylinositol, *PG*, phosphatidylglycerol; *CL*, cardiolipin.

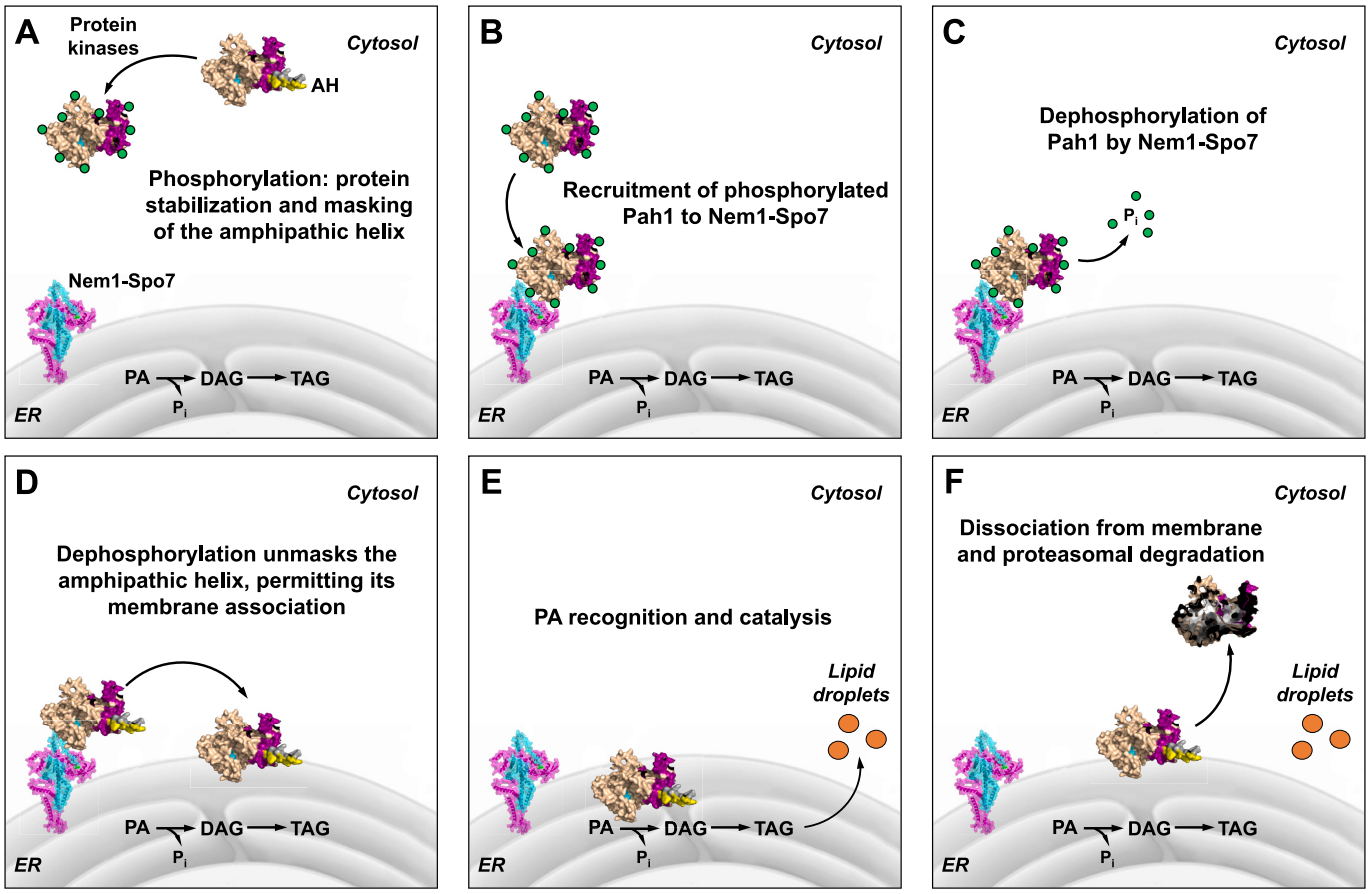


Fig. 2. Model for the phosphorylation/dephosphorylation-mediated regulation of Pah1 function. The PA phosphatase activity of Pah1 is primarily controlled by the localization of the enzyme. Following expression, Pah1 is present in the cytosol where it is phosphorylated by many protein kinases (*A*). The multiple phosphorylations mask the amphipathic helix and protect the enzyme against degradation by the 20S proteasome. Phosphorylated Pah1 is recruited (*B*) and dephosphorylated (*C*) by Nem1-Spo7 present at the nuclear/ER membrane. The dephosphorylation exposes the amphipathic helix to permit Pah1 to associate with the membrane surface (*D*). The membrane-associated Pah1 recognizes PA for its catalytic reaction to generate DAG, which is acylated to form TAG that is stored in lipid droplets (*E*). Following rounds of reaction, Pah1 dissociates from the nuclear/ER membrane for proteasomal degradation (indicated by *dark shading*) (*F*). AlphaFold2 structures of Pah1 and Nem1-Spo7 are depicted. For simplicity, some domains/regions (e.g., acidic tail and IDRs) of Pah1 are not shown. Nem1 is colored pink and Spo7 is colored blue. Green dots on Pah1 represent a phosphate group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Domains and regions that govern catalytic function and regulate the localization of Pah1

In this section, we provide an overview of the mode of action and regulation of Pah1 as mediated by its domains and regions. The overview is followed by specific/mechanistic details of the domains and regions. Different domains and regions of Pah1 are associated with its catalytic activity or translocation to and interaction with the nuclear/ER membrane (Figs. 3 and 4). The N-LIP and the haloacid dehalogenase (HAD)-like domains are required for PA phosphatase activity [1,80]. The HAD-like domain contains the canonical DXDX(T/V) catalytic motif that is essential to catalysis [80] (Fig. 4). The crystal structure of *Tetrahymena thermophila* Pah2, a minimal Pah1 ortholog consisting of only amphipathic helix, N-LIP, and HAD-like domains [81], shows that the conserved domains interact to form the catalytic core [82] (Fig. 4C). This interaction is also depicted in the AlphaFold2 predicted structures of *S. cerevisiae* Pah1 and human lipin 1α (Fig. 4A and B). The N-terminal amphipathic helix of Pah1 is required for interaction with the

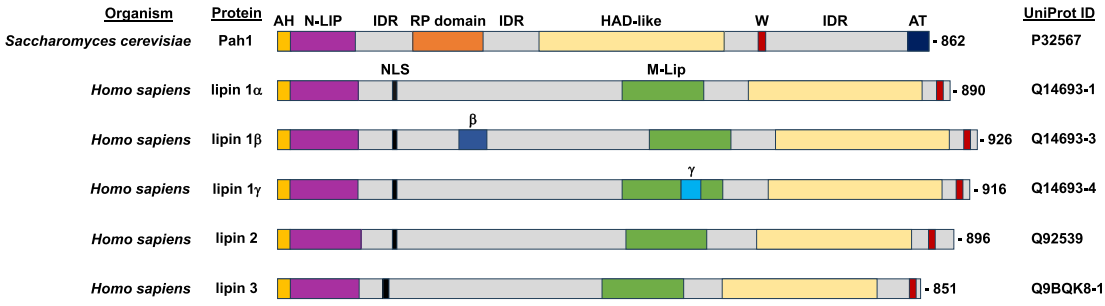


Fig. 3. Linear schematics of yeast Pah1 and human lipins. The domains and regions of Pah1 and human lipin isoforms. *AH*, amphipathic helix; *W*, conserved tryptophan residue; *AT*, acidic tail; *NLS*, nuclear localization signal; *M-Lip*, middle lipin domain; *β*, lipin 1β specific sequence; *γ*, lipin 1γ specific sequence.

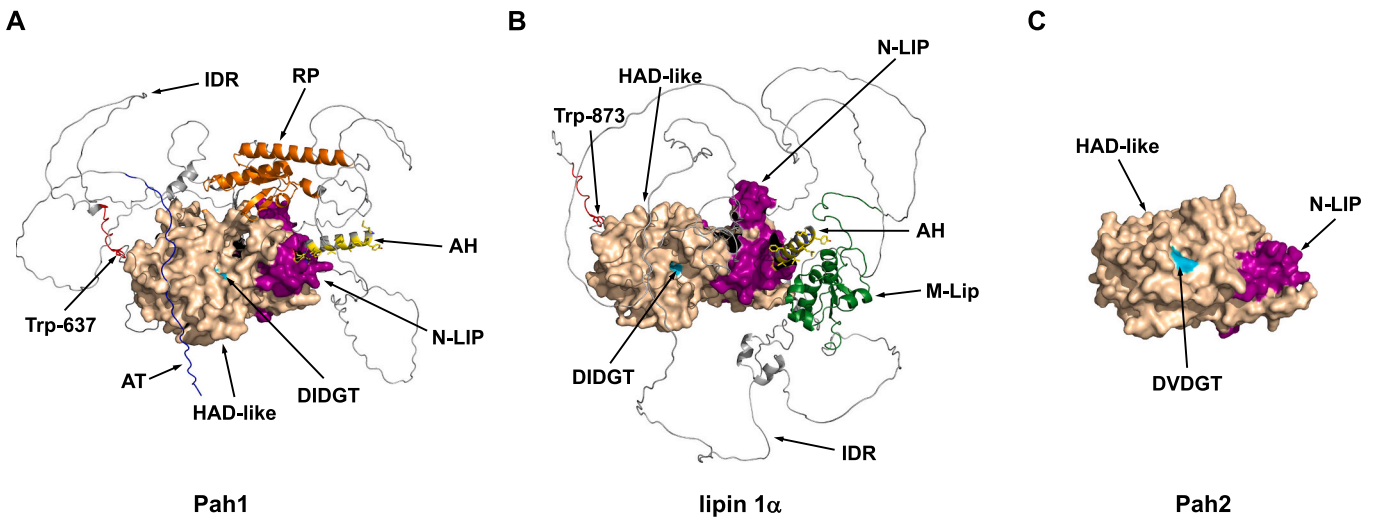


Fig. 4. Predicted structures of *S. cerevisiae* Pah1 and human lipin 1α. The structures of Pah1 (*A*) and lipin 1α (*B*) are predicted by AlphaFold2 and visualized using the PyMol program. The crystal structure of *T. thermophila* Pah2 (*C*) is shown for comparison.

membrane, facilitating the active site to recognize PA in the membrane [57]. The WRDPLVDID domain, which is C-terminal to the HAD-like domain [83], is not required for catalysis per se, but is required for Pah1 function in vivo [83,84] (Figs. 3 and 4). For the nuclear/ER localization of Pah1, the rest of its sequence (i.e., intrinsically disordered regions (IDRs), RP (*r*egulation of *p*hosphorylation) domain, and C-terminal acidic tail) mediates the interaction with Nem1-Spo7 [58,83,85]. The IDRs contain almost all of the phosphorylation sites that serve for interaction with Nem1-Spo7, and the phosphorylation of numerous sites is regulated by the RP domain [85]. The acidic tail, which is rich in negatively charged amino acids, interacts with Nem1-Spo7 [58] through ionic interaction with the positively charged amino acids of the C-terminal basic tail of Spo7 [86].

Based on available information, the following events for the phosphorylation/dephosphorylation-mediated regulation of Pah1 localization is postulated [87] (Fig. 2). The phosphorylation-mediated masking of the amphipathic helix is required for Pah1, which lacks a membrane targeting sequence, to associate specifically with the nuclear/ER membrane [57]. Phosphorylated Pah1 in the cytosol cannot associate with the membrane due to the masking of the amphipathic helix, but it interacts with Nem1-Spo7 through its C-terminal acidic tail and phosphorylated residues [58]. Through this interaction, phosphorylated Pah1 in the cytosol is localized to the surface of the nuclear/ER membrane where Nem1-Spo7 resides. The acidic tail, which is rich in negatively charged amino acids, interacts with Nem1-Spo7 [58] through the interaction with the basic tail of Spo7 [86]. The phosphorylated residues of Pah1 are recognized by Nem1-Spo7 as the substrates for dephosphorylation [13,68,72,74]. Subsequent dephosphorylation by Nem1-Spo7 results in the exposure of the amphipathic helix. Dephosphorylated Pah1, which is released from Nem1-Spo7, associates with the nuclear/ER membrane via its amphipathic helix. Pah1 then scoots along the membrane surface to recognize and dephosphorylate its substrate PA [73]. After multiple rounds of catalysis, the dephosphorylated enzyme, or enzyme phosphorylated by protein kinase C [64], dissociates from the membrane surface and is subject to proteasomal degradation [70]. The phosphorylation-mediated inhibition of the amphipathic helix coupled with the acidic tail-mediated protein-protein interaction with Nem1-Spo7 ensures that Pah1 translocates from the cytosol specifically to the nuclear/ER membrane [57,68,74].

4. Catalytic core

In their seminal work that identified *Lpin1* as a mutated gene responsible for the defects in lipid metabolism of the fatty liver dystrophy (*fld*) mouse [88], Reue and co-workers [34,41] recognized N-LIP (N-terminal lipin) and C-LIP (C-terminal lipin) as conserved domains in mammalian lipin proteins and their homologs, including *S. cerevisiae* Pah1 (previously known as Smp2). In general, N-LIP and C-LIP are separated by sequences that are largely disordered (i.e., IDRs) (Fig. 3). Whereas N-LIP is located at the N-terminus of all homologous proteins, C-LIP is found at the C-terminus of most lipin proteins but is found in the middle of the yeast homolog [34] (Fig. 3). Subsequently, *S. cerevisiae* Pah1 and human lipin 1γ were revealed to be the Mg^{2+}-dependent PA phosphatases possessing the D*X*D*X*(T/V) catalytic motif in C-LIP and belong to the HAD-like phosphatases [1], and thus, C-LIP containing the catalytic motif is commonly referred to as the HAD-like domain in Pah1 and lipin proteins [89,90]. When mutations of Pah1 in the conserved aspartate residues (D398E and D400E) within the D*X*D*X*(T/V) catalytic motif of HAD-like domain and a conserved glycine residue (G80R) of N-LIP caused near total loss of PA phosphatase activity, it became evident that the N-LIP and the HAD-like domains likely comprise the catalytic core of the enzyme [80]. Insight into the structure of the catalytic core of Pah1, as well as that of lipin 1, has come from structural information derived from the crystal structure of the *T. thermophila* Pah2 homolog [82] and from AlphaFold2 predictions [91,92] (Fig. 4).

4.1. HAD-like domain

The characteristic domain of Mg^{2+}-dependent PA phosphatases is the HAD-like domain, which is found in a family of proteins encompassing a diverse group of enzymes that catalyze the same or similar reactions on a broad range of substrates [93–96]. Aravind and colleagues [93] examined the diversity of the enzymes and hypothesized that at least 5 distinct HAD proteins can be traced to the last universal common ancestor of all extant life. The HAD superfamily was originally named for the HAD enzymes, but includes many phosphatases, ATPases, phosphonatases, and phosphomutases [97]. The HAD-like enzymes catalyze phosphoryl or, more rarely, carbon group transfers, and these activities are dependent on the Rossmann-like fold specific to the protein superfamily [93]. The *S. cerevisiae* genome is predicted to contain at least 45 genes encoding enzymes with HAD-like domains [97]. Of these enzymes, Pah1 is the only one that has Mg^{2+}-dependent PA phosphatase activity [1]. Interestingly, App1 is another protein in *S. cerevisiae* that exhibits Mg^{2+}-dependent PA phosphatase activity, but its catalytic domain was noted to be fungal-specific and distinct from the HAD-like domain found in Pah1 [9].

The Rossmann fold is canonically defined as a 6 parallel β sheet structure (with a 321,456 topology for the β sheet) that is surrounded by α-helices [98,99]. Rossmann folds show some variation in the number of β strands (i.e., 5 or 7 β strands), and these variants are often referred to as Rossman-like or Rossmannoid folds [100]. The Rossmann fold is hypothesized to be an evolutionarily ancient structural motif that positions the core catalytic residues needed for substrate and cofactor orientation and reaction intermediate stabilization at the end of the loops separating the alternating α and β motifs to form the active site (Fig. 5) [100,101]. The Rossmann-like fold is readily observed in the *T. thermophila* Pah2 as 7 β strands forming the central β sheet with the last two β strands (β6 and β7) antiparallel to the rest of the strands (Fig. 5C) [82]. Interestingly, in the AlphaFold2-predicted structure, Pah1 contains 6 β strands in which only β6 is antiparallel to the rest (Fig. 5A), showing a difference from lipin 1α that contains 5 β strands in which the β5 sheet is antiparallel (Fig. 5B). It is yet unclear whether the differences in the Rossmann-like folds of Pah2, Pah1, and lipin 1α reflect any change in enzyme function within the cell or the Mg^{2+}-dependent PA phosphatase reaction.

HAD phosphatases are also characterized by the presence of a cap domain, which is a mobile element that covers the active site and contributes to substrate specificity [93,102]. The cap domain is inserted between topological components of the HAD domains and falls into multiple categories based on its size and insertion location [93,97]. Intriguingly, there is no evidence to indicate that Pah1 or any of lipins contains a cap domain. Their predicted structures as well as the solved structure of Pah2 indicate that their catalytic function is mediated by a capless HAD-like domain (Fig. 4C) [82]. This suggests that the membrane surface itself may play a major role in substrate specificity and that other structural elements of PA phosphatase contribute to the specificity for PA. In vitro studies with PA incorporated into unilamellar phospholipid vesicles composed of the major nuclear/ER membrane phospholipids indicate that the fatty-acyl moiety of PA has no effect on activity [73].

There are also two other structural signatures of HAD proteins, the squiggle and flap components, which are hypothesized to be related to the transition of the active site cavity between "open" and "closed" states [93,100]. The open and closed states of the active site are related to solvent exposure and solvent occlusion, respectively. The squiggle element of HAD enzymes are in a helical conformation located immediately downstream of the first β strand of the Rossmann-like fold [93]. This helical squiggle appears to alternate between tightly and loosely wound states, which alters the position of the flap that is next to the active site. The flap movement is what causes a transition between the open and closed conformations of the active site [93]. In some cases, the cap domains have been inserted in the flap (i.e., C1 caps), suggesting that the squiggle may be related to cap mobility in these instances. Pah1 and lipin 1α seem to contain a squiggle (Fig. 4A and B), but they do not show a structure that is similar to the flap domain of other HAD-like phosphatases such as deoxy-D-mannose-octulosonate 8-phosphate phosphatase from *Haemophilus influenzae* [93,103].

The mechanism of phosphoryl transfer in HAD-like phosphatases is a multistep process that involves the transition between the open and closed states of the active site [90,104–108]. The enzyme in the open state allows the substrate (generally a phosphoester) and a Mg^{2+} ion to access the active site. When the active site transitions to the closed conformation, the Mg^{2+} ion interacts with the negatively charged phosphate group, preparing it for a nucleophilic attack by the first conserved Asp (i.e., Asp-398 in Pah1 or Asp-678 in lipin 1α). Importantly, the closed state occludes solvent from the active site, which facilitates the nucleophilic attack of the Asp. This nucleophilic attack forms a phosphoryl-aspartate intermediate with the carboxyl group of Asp bound to the phosphate. The enzyme transitions back to the open configuration, allowing the product (i.e., DAG in the PA phosphatase reaction) to leave and solvent to access the active site again. A water molecule is deprotonated by the second conserved Asp in the active site (i.e., Asp-400 in Pah1 or Asp-680 in lipin 1α), which allows for the hydrolysis of the phosphoryl-aspartate intermediate and returns the active site to its original state.

For Pah1 and lipins, it is largely unknown how the catalytic reaction is affected by the membrane surface. Considering that PA phosphatase lacks the cap and flap domains, the membrane surface may act as a structure that closes the active site, excluding solvent from the first step of the reaction. This raises the question of what "opens" the active site back up to allow for solvent entry. One possibility is that the enzyme is held in association with the membrane by the amphipathic helix and other structural components, and its binding to PA "closes" the active site using the membrane to occlude water molecules. Upon the phosphoryl-aspartate intermediate formation and release of DAG back into the membrane, the HAD-like domain could "peek" up and "open" the active site back up, allowing the water molecule to enter and regenerate the active site for the next round of catalysis. The effect of the membrane surface on the reaction mechanism, the presence of the squiggle or flap structure, and the diversity of Rossmann folds in PA phosphatases represent open questions that have yet to be investigated about the HAD-like domain of Pah1 or lipins.

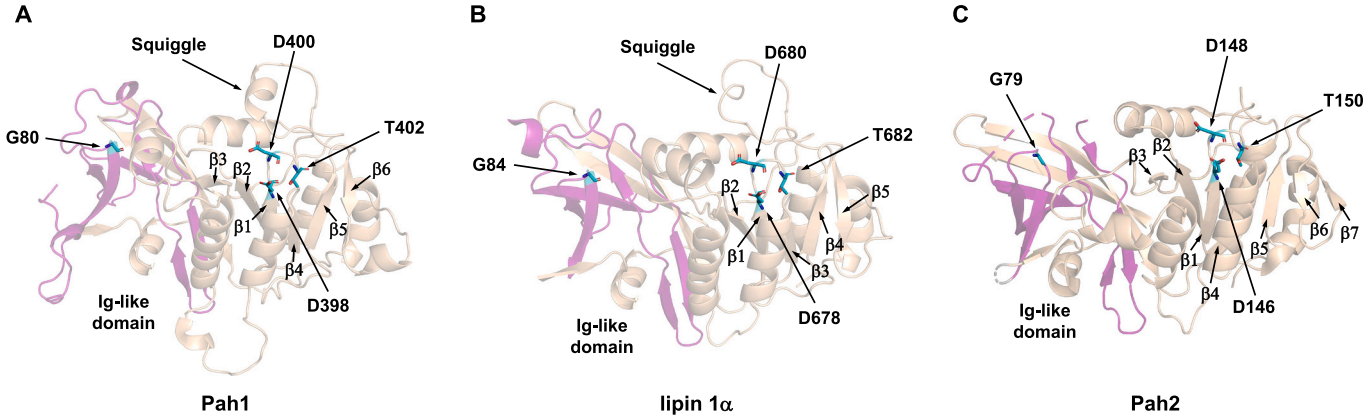


Fig. 5. Predicted structures of the catalytic cores of *S. cerevisiae* Pah1 and human lipin 1α. The diagram generated from the AlphaFold2 structures displays the co-folding of the N-LIP and HAD-like domains of Pah1 (*A*) and lipin 1α (*B*). Experimental evidence for the co-folding of the N-LIP and HAD-like domains is provided by the crystal structure of *T. thermophila* Pah2 (*C*). The residues from the D*X*D*X*T catalytic motif are indicated and shown as a stick diagram to see the orientation of the residues in the active site. The conserved glycine residue in the N-LIP domain is also indicated. The Ig-like domain, originally identified in *T. thermophila* Pah2, is shown along with the suggested location of the squiggle motif. The β strands that form the central β sheet of the Rossman-like fold are numbered, and their positions are indicated.

4.2. N-LIP domain

N-LIP is another characteristic domain of conserved Mg^{2+}-dependent PA phosphatase enzymes [34]. The *fld*^{2J} mouse [88] has a single substitution mutation (G84R) in the N-LIP domain of lipin 1 that is responsible for the *fld* mutant phenotypes [34]. This glycine residue is conserved in homologous sequences from diverse organisms including humans and *S. cerevisiae* (Fig. 5). As indicated above, the G80R mutation in *S. cerevisiae* Pah1 results in near total loss of PA phosphatase activity, indicating the importance of the conserved glycine to catalysis [80]. The crystal structure of *T. thermophila* Pah2, as well as the AlphaFold2 predictions of *S. cerevisiae* Pah1 and human lipin 1α shows that the N-LIP and HAD-like domains co-fold with each other to form a catalytic core (Figs. 4 and 5). The major difference between Pah2 and Pah1/lipins is the lack of IDRs that separate the N-LIP and HAD-like domains [82]. The Pah2 structure contains an atypical "split immunoglobulin (Ig)-like" domain [82]. The term domain in this context refers to a structural motif that is not a necessarily specific contiguous segment of the amino acid sequence [82]. This Ig-like domain, which is shown in the AlphaFold2 structures of Pah1 and lipin 1α, is split in that part of it is derived from N-LIP and the other is from the HAD-like domain (Fig. 5). Together this implies that this "split" Ig-like domain must co-fold to produce the catalytic core of PA phosphatase [82]. Hydrogen deuterium exchange-mass spectrometry analysis of Pah2 indicates that the split Ig-like domain interacts with the membrane surface [82]. Despite this recent work, the role of the N-LIP domain in catalysis remains unclear.

5. Amphipathic helix

The amphipathic helix of *S. cerevisiae* Pah1 is an essential region to its membrane association, and therefore its catalytic function at the nuclear/ER membrane. The presence of an amphipathic helix in PA phosphatases, which is known to mediate reversible binding of proteins to membrane surfaces [109], was first identified in *S. cerevisiae* Pah1 [57]. The N-terminal 18 amino acid residues of Pah1 constitute the amphipathic helix required to maintain the membrane surface interaction [57]. It is composed of a single alpha helix with a clearly identifiable hydrophobic region. Pah1 is activated through its dephosphorylation by Nem1-Spo7, which allows association with the nuclear/ER membrane predominantly through the amphipathic helix; this suggests that the amphipathic helix is exposed for interaction with the membrane only under the dephosphorylated state of the protein [57]. The exposure of the amphipathic helix through Pah1 dephosphorylation is supported by the Nem1-Spo7-independent function of the phosphorylation-deficient variants of Pah1 such as Pah1-7A (alanine substitutions for 7 sites phosphorylated by Pho85 [61], Pah1-ΔRP, Pah1-CR (conserved regions), and Pah1-CC (catalytic core) [57,68,85,87]. These phosphorylation-deficient variants all display altered localization within the cell. While not formally studied, the amphipathic helix is conserved in the mammalian lipin proteins (Fig. 3).

6. Acidic tail

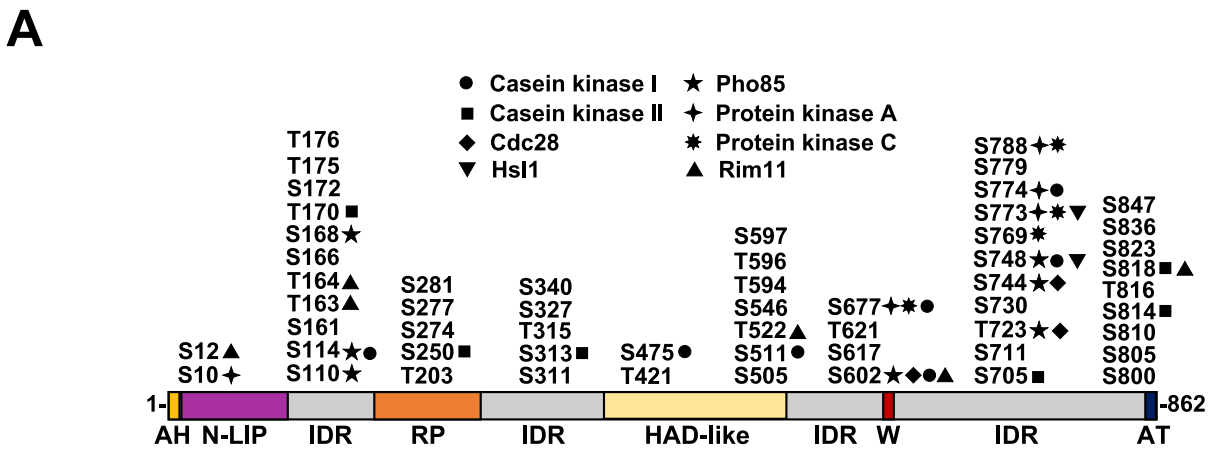
The C-terminal acidic tail of *S. cerevisiae* Pah1 is highly enriched with aspartate and glutamate residues [58]. The essential nature of the acidic tail to the localization and regulation of Pah1 function has been elucidated through the analysis of acidic tail and phosphorylation-deficient mutant forms of the enzyme [58,87]. This feature provides the mechanism by which phosphorylated Pah1 is specifically recruited to the nuclear/ER membrane through interaction with Nem1-Spo7 [58]. The acidic tail interacts with the basic tail of the regulatory subunit Spo7, which then facilitates the dephosphorylation of Pah1 by the catalytic subunit Nem1 [86]. The acidic tail is not required for the membrane association of the unphosphorylated form of Pah1, but without it, the specificity for the nuclear/ER membrane localization of the protein is lost [58,87]. The acidic tail is not found in the mammalian lipin proteins, which like Pah1 are subject to multiple phosphorylations [2,110,111] and are substrates for the CTDNEP1-NEP1R1 complex, the mammalian homolog of Nem1-Spo7 [19,112]. Additionally, the regulatory subunit NEP1R1 does not contain a basic tail analogous to that present in the *S. cerevisiae* Spo7 [86]. The implications are that lipin is recruited to and interacts with its phosphatase complex in a manner distinct from that of Pah1. The basis for the recruitment and interaction of lipin 1 to CTDNEP1-NEP1R1 remains uninvestigated.

The amphipathic helix and acidic tail are not shown in the crystal structure of *T. thermophila* Pah2, although for different reasons (Fig. 4). The presence of the amphipathic helix at the N-terminus of Pah2 and its importance to membrane surface association is supported by hydrogen deuterium exchange-mass spectrometry analyses [82]. *T. thermophila* Pah2, which lacks IDR sequences, does not contain the acidic tail, which explains its absence in the crystal structure. Interestingly, Pah2 lacks a region for phosphorylation similar to that found in Pah1, raising a question about the control of its cellular function. Unlike *S. cerevisiae pah1*Δ cells, *T. thermophila* cells deficient in Pah2 exhibit neither growth defects nor deleterious phenotypes [81]. With the exception of a very weak complementation of the respiratory-deficient phenotype displayed by the *S. cerevisiae pah1*Δ mutant, the *T. thermophila PAH2* gene does not complement the mutant for its defects in lipid synthesis and nuclear/ER membrane structure [81]. Interestingly, the *S. cerevisiae* Pah1-CC variant complements classic *pah1*Δ mutant phenotypes [81,87]. The substantial architectural difference between *T. thermophila* Pah2 and *S. cerevisiae* Pah1-CC is the presence of the WRDPLVDID domain (see below) in the latter protein. Whether this inclusion is sufficient to explain the differential complementation abilities has not been investigated.

7. Intrinsically disordered regions

A large portion of the *S. cerevisiae* Pah1 sequence represents multiple IDRs [1,34,58,83], which lack a stable and consistent tertiary structure (Figs. 3 and 4) [113]. Sequences that constitute the IDRs are not conserved and sometimes referred to as non-conserved regions or linker regions [1,34,58,83]. From a structural perspective, IDR-containing proteins are difficult to work with due to their inherently disordered nature [114,115]. Yet, the results of biochemical, molecular genetics, and cell biological imaging studies reveal their importance to the regulation of Pah1. In one sense, the IDRs may be the most well characterized component of Pah1 due to the extensive work detailing the effects of phosphorylation of the enzyme. Indeed, the IDRs are the regions where nearly all the phosphorylation sites are located (Fig. 6A), the importance of which is to control enzyme location as mediated by the amphipathic helix, RP domain, acidic tail, and Nem1-Spo7 [57,58,68,72,74,83,85]. Global phosphoproteomics studies have shown that Pah1 is a substrate for ~20 protein kinases and contains 56 phosphorylation sites [8,116] (Fig. 6A). The protein kinases that have been characterized for Pah1 phosphorylation include the cyclin dependent kinases Cdc28 [62] and Pho85 [61], casein kinases I [66] and II [65], protein kinases A [63] and C [64], glycogen synthase kinase 3β homolog Rim11 [67], and the septin-associated Hsl1 kinase [117] (Fig. 6A). With this information, analyses of cells bearing phosphorylation-deficient mutations at specific sites have provided insight into the contributions each protein kinase has to the regulation of Pah1 location, activity, and stability [8]. Some phosphorylation sites (e.g., Ser-10, Ser-511, and Ser-814) are unique to specific protein kinases while others (e.g., Ser-602, Ser-677, and Ser-748) are common to multiple protein kinases [8] (Fig. 6A). Some phosphorylations are hierarchical in nature where the phosphorylation at one site affects the phosphorylation at another site [8,67]. Additionally, phosphorylations by some protein kinases stimulate (e.g., casein kinase I) or inhibit (e.g., Pho85, Rim11, and Hsl1) PA phosphatase activity [61,66,67,117].

AlphaFold3 was used to predict the structure of the phosphorylated form of Pah1 (Fig. 6B). The phosphorylation of all the serine/threonine



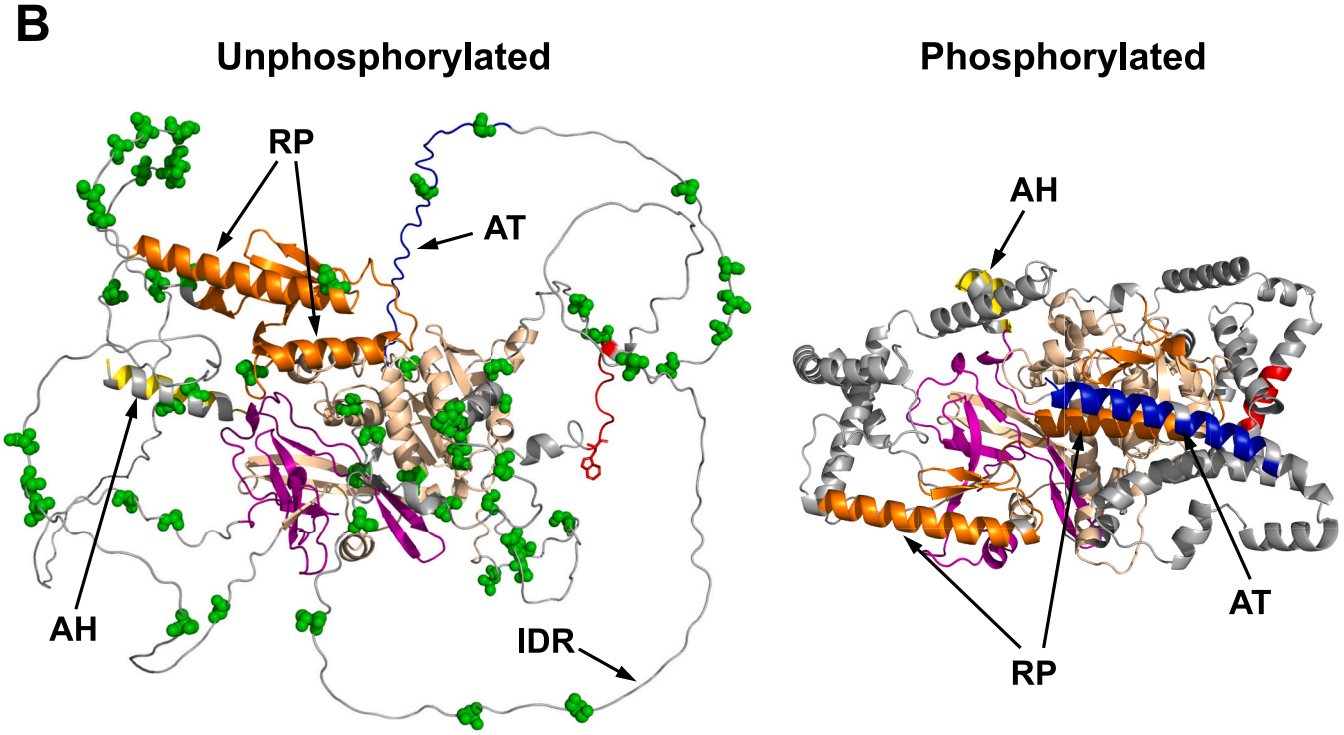


Fig. 6. Phosphorylation sites in Pah1. The serine and threonine residues known to be phosphorylated [61–68,84,117,140–150] are grouped at their approximate regions in the Pah1 protein (*A*). The sites phosphorylated by casein kinase I (*CKI*) [66], casein kinase II (*CKII*) [65], Cdc28 [62], Hsl1 [117], Pho85 [61], protein kinase A [63], protein kinase C [64], and Rim11 [67] are indicated. AlphaFold3 predictions of the unphosphorylated and phosphorylated forms of Pah1 (*B*). The green dots on unphosphorylated Pah1 represent all of the serine/threonine residues that are targets of phosphorylation. The phosphorylated structure represents Pah1 with all serine/threonine residues in their phosphorylated state. *S*, serine; *T*, threonine; *CKI*, casein kinase; *PKA*, protein kinase A; *PKC*, protein kinase C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sites alters the structure of the enzyme. The IDRs in the unphosphorylated form adopt a more ordered structure when the serine/threonine residues are phosphorylated. Notable changes in the phosphorylated structure include a kink introduced in the amphipathic helix, the acidic tail adopting an alpha helical conformation, and a repositioning of the RP domain to associate with the catalytic core. While the structures of the unphosphorylated and phosphorylated forms of Pah1 are predictions, their differences correlate with the roles the acidic tail and amphipathic helix play in the recruitment, dephosphorylation, and membrane association of the enzyme, as well as the stability of the phosphorylated form with respect to its protection against proteasomal degradation.

Lipin proteins also contain IDRs with multiple sites of phosphorylation [2,110,111,118,119]. Lipin 1, like Pah1, is regulated by controlling its subcellular localization through the posttranslational modifications phosphorylation and dephosphorylation [110]. Compared with Pah1 phosphorylation, protein kinases responsible for the phosphorylation of lipin enzymes and their effects are less characterized. Best characterized is the mTOR-mediated insulin-dependent phosphorylation of lipin 1 that induces its cytosolic localization with the reduction of enzyme activity,

which is similar to the effects shown by the Pho85-mediated phosphorylation of Pah1 [61,110,118]. In contrast, the phosphorylation of lipin 2 has not been implicated in the regulation of the enzyme in a manner similar to that of lipin 1 or Pah1. The phosphorylation of lipin 2 does not regulate its membrane interaction or PA phosphatase activity [120]. As discussed above, the phosphatase complex CTDNEP1-NEP1R1 mediates the dephosphorylation of lipin 1, allowing its association with the nuclear/ER membrane surface with increased catalytic competence [19,112,121,122]. The activity of CTDNEP1-NEP1R1 on lipin is highly specific for Ser-106 phosphorylated by the mTOR complex 1 [122], which is similar to the specificity of Nem1-Spo7 for the 7 sites of Pah1 that are phosphorylated by Pho85 [72]. It is interesting that Ser-106 is located in the structured N-LIP domains of lipin 1 and lipin 2 [82]. The emergent question then is whether CTDNEP1-NEP1R1 preferentially dephosphorylates the structured regions of lipin unlike Nem1-Spo7 whose preferred sites of Pah1 phosphorylation by Pho85 are located in the IDRs [61]. The phosphorylations of Pah1 and lipins are not directly comparable because of differences in protein kinases, their regulations, and their effects on the localization, stability, catalytic activity, and hierarchical phosphorylation of the enzymes.

8. RP domain

The RP domain, which was identified through bioinformatics, molecular genetics, and biochemical approaches, regulates the phosphorylation state of Pah1 [85]. The domain is located within the IDR that separates the N-LIP and HAD-like domains (Figs. 3 and 4). The RP domain is predicted to be comprised of 2 α-helices and 4 β-strands (arranged topologically α1 β1 β2 α2 β3 β4), with the 4 β-strands forming an antiparallel β-sheet. Mutational analysis indicates that the RP domain facilitates the phosphorylation of Ser-511, which is a site phosphorylated by casein kinase I [66]; Ser-602, a site phosphorylated by Pho85 [61], Cdc28-cyclin B [62], casein kinase I [66], and Rim11 [67]; Ser-773, which is phosphorylated by protein kinases A [63] and C [64], and Hsl1 [117]; and Ser-774, which is phosphorylated by protein kinase A [63] and casein kinase I [66] (Fig. 6). The site of Pah1 most affected by the ΔRP mutation is Ser-602, the major phosphorylation site [84] and a main target of the Pho85 protein kinase [61]. Reduction in the phosphorylation of Ser-602 is primarily responsible for gain-of-function phenotypes (e.g., bypass of Nem1-Spo7 for membrane recruitment and dephosphorylation) imparted by the ΔRP mutation [85]. The precise nature of how the RP domain regulates the phosphorylation of Pah1 is unclear at this time. One possibility is that the RP domain interacts with other regions of Pah1, perhaps not dissimilar to the N-LIP interacting with the HAD-like domain, to alter the tertiary structure of the enzyme by either masking or exposing phosphorylation sites.

9. WRDPLVDID domain

The C-terminal IDR of Pah1 contains the conserved WRDPLVDID domain (residues 637–645) that is important for its in vivo function [83] (Fig. 3). In this sequence, the residue that has the major effect on Pah1 function is Trp-637 [83]. Although the residue is not required for PA phosphatase activity per se, it is required for Pah1 function in the cell to dephosphorylate PA to produce DAG for TAG synthesis [83]. Based on mutational analysis, Trp-637 causes reduced phosphorylation of residues at the N-terminal region (e.g., Thr-203 and Ser-277) and the middle of the protein (e.g., Thr-596 and Ser-602), but elevated phosphorylation at the C-terminal region (Ser-744, Ser-773, Ser-774, Ser-779, and Ser-814). The Trp-637-mediated effects on phosphorylation influence Pah1 interaction with the membrane, however, it is unclear whether these phosphorylations enhance and/or decrease the interaction of the C-terminal acidic tail with Nem1-Spo7 [58] and/or of the N-terminal amphipathic helix with the membrane [57]. Furthermore, this region of Pah1 is predicted to be intrinsically disordered, adding to both the importance of IDRs to PA phosphatase function and to the complexity of elucidating the mechanism by which Pah1 operates (Fig. 4).

The prediction of the Pah1 structure by AlphaFold2 [91] shows the positions of Trp-637 in yeast Pah1 and conserved Trp-873 in human lipin 1α in relation to the N-LIP and HAD-like domains (Fig. 4A and B). According to this model, Trp-637 and Trp-873, and their respective catalytic residues (i.e., Asp-398 and Asp-400 in yeast Pah1 and Asp-678 and Asp-680 in human lipin 1α) lie in almost the same plane, suggesting that the tryptophan residues are important to properly position the catalytic residues for substrate recognition at the membrane surface. The conserved tryptophan residue is not present in the *T. thermophila* Pah2 structure, and thus, no insight into its role is available [82].

The WRDPLVDID domain contains a putative destruction box motif (i.e., R*X*PL*XX*I) that might be involved in the ubiquitin-mediated degradation of the enzyme [123]. Pah1 is partially stabilized in the stationary phase of mutants defective in the ubiquitin degradation pathway [69]. Since the destruction box is known to be important for the ubiquitin pathway of protein degradation [124], mutations in the R*X*PL*XX*I motif could be used to address the hypothesis that Pah1, in addition to being degraded by a ubiquitin-independent mechanism [70], is also degraded by a ubiquitin-dependent mechanism.

10. Differences between *S. cerevisiae* Pah1 and human lipins might be exploited to control lipid metabolism and growth of fungal pathogens

The conservation of the catalytic cores in *S. cerevisiae* Pah1 and mammalian lipin 1 indicates that Pah1 is a good model for studying lipins in higher eukaryotes. This may be true when it comes to the mode of action and kinetics of PA phosphatase activity, but important regulatory aspects of these enzymes differ. The acidic tail required for Pah1 to interact with Spo7 [86] of the Nem1-Spo7 complex [58] and the RP domain located between the N-LIP and HAD-like domains to control Pah1 phosphorylation [85] are not conserved in human lipins (Fig. 3). In contrast, the M-LIP domain found within the large IDR of lipin 1 for its dimerization and membrane association [125] and a nuclear localization signal found in the IDR region near the N-LIP domain for nuclear import of lipin 1 [34,126,127] are not found in Pah1 (Fig. 3). Differences between the *S. cerevisiae* Pah1 and mammalian lipins are reflected in the observation that full-length human lipin 1 does not fully complement the temperature sensitivity caused by the *S. cerevisiae pah1*Δ mutation [128]. As in *S. cerevisiae*, the localization of lipin 1 in mammalian cells is controlled through its phosphorylation within its IDR by multiple protein kinases [2,19,110,118,129,130] and its dephosphorylation by the CTDNEP1-NEP1R1 protein phosphatase complex [19]. However, the phosphorylation sites in lipin 1 differ from those in Pah1 [2,111] and the phosphorylated form of lipin 1 expressed in *S. cerevisiae* may not be recognized by the Nem1-Spo7 complex. Moreover, the lack of the acidic tail needed for the interaction with Nem1-Spo7 at the nuclear/ER membrane compromises lipin 1 for its translocation specificity. Thus, the attributes necessary for lipin 1 to completely complement *pah1*Δ in *S. cerevisiae* are absent.

Fungal pathogens represent an under-recognized threat to public health and agriculture, and the growing frequency of anti-fungal resistant infections requires increased attention and novel strategies to combat them [131–133]. While the ultimate function of the yeast Nem1-Spo7/Pah1 and human CTDNEP1-NEP1-R1/lipin 1 phosphatase cascades are the same, namely, to convert PA to DAG, the mechanisms and regulations involved are not identical. The structural aspects of Pah1 that are not found in the homologous human proteins are conserved in yeast, and in particular, some opportunistic pathogenic yeast (e.g., *Aspergillus fumigatus*, *Candida albicans*, *Candida auris, Cryptococcus neoformans, Fusarium oxysporum* and *Kluyveromyces lactis*) that infect humans [134–136]. Thus, targeting the fungal-specific attributes will likely inhibit the growth of yeast pathogens without affecting the function of the CTDNEP1-NEP1-R1/lipin 1 phosphatase cascade in humans. In fact, PA phosphatases have been suggested as possible

therapeutic targets due to their importance to growth and pathogenesis in multiple fungal pathogens [137–139].

11. Concluding remarks

S. cerevisiae Pah1, the PA phosphatase responsible for providing the DAG needed for the synthesis of TAG, is a complex multi-domain/region protein whose function is dependent on its subcellular localization as mediated by phosphorylation and dephosphorylation. PA phosphatase, as a key enzyme to produce DAG, has profound effects on lipid metabolism, cellular homeostasis, and growth. Structure-function analyses of Pah1 have benefited from mutagenic analyses of the domains/regions and phosphorylation sites in the enzyme, the Pah1 AlphaFold2 structure, and the bona fide *T. thermophila* Pah2 structure. Many questions related to the structure, function, and interplay of the domains/regions remain open to investigation. For example, what is the exact function of the N-Lip domain in the catalytic core, how is the exposure of the amphipathic helix controlled, how does the RP domain regulate phosphorylation, how does the WRDPLVDID domain regulate function, and how does phosphorylation regulate Pah1 structure and vice versa? Whereas the mode of action of yeast and mammalian PA phosphatases is the same, mechanisms controlling their regulation are different and might be utilized in a practical context.

CRediT authorship contribution statement

Geordan J. Stukey: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gil-Soo Han:** Writing – review & editing, Investigation, Formal analysis, Data curation, Conceptualization. **George M. Carman:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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