Domain-based Bioinformatics Analysis and Molecular Insights for the Autoregulatory Mechanism of Phafin2

Mahmudul Hasan

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Daniel G. S. Capelluto, Chair
Michael Klemba
Iuliana Lazar
Mike Zhang

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Academic Abstract

Phafin2, an adaptor protein, is involved in various cellular processes, such as apoptosis, autophagy, endosomal cargo transportation, and macropinocytosis. Two domains, namely, PH and FYVE, contribute to Phafin2's cell membrane binding. Phafin2 also contains a poly aspartic acid (polyD) motif in its C-terminal region that can specifically autoinhibit the PH domain binding to membrane phosphatidylinositol 3-phosphate (PtdIns3P). Firstly, the study investigated the domain-based evolutionary pattern of PH, FYVE, and polyD motif of Phafin2 among its orthologs and Phafin2-like proteins. Using different bioinformatics tools and resources, it was concluded that the polyD motif only evolved in Phafin2 and PH- or both PH-FYVE-containing proteins of animals, highlighting the association in cellular functions that might have evolved uniquely in animals. Moreover, PH domain-free FYVE-containing proteins lack polyD motifs. Secondly, intramolecular autoregulatory and membrane binding properties of Phafin2 were studied by employing liposome co-sedimentation assay, isothermal titration calorimetry, and nuclear magnetic resonance spectroscopy. The residues Gly38, Lys45, Leu45, Lys51, Ala52, and Arg53 of the PH domain form a positively charged binding pocket that can bind the negatively charged polyD motif. The mutated Phafin2 PH domain (K51A/R53C and R53C) was unable to bind to synthetic polyD peptides, establishing the significance of those residues for the interaction between the PH domain and polyD motif. Moreover, the study also concluded that Phafin2-mediated membrane binding is not curvature-dependent.

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General Audience Abstract

Phafin2 is a protein that plays a crucial role in several important cellular functions, including cell death, recycling of cellular components, and transporting materials within cells. The protein's ability to attach to cell membranes is mainly due to two of its specific regions, the PH and FYVE domains. Additionally, Phafin2 has a section called the polyD motif that can block the PH domain from binding to specific cell membrane molecules. This study explored how these regions of Phafin2 have evolved across different species, focusing on the PH, FYVE, and polyD motifs. The findings suggest that the polyD motif is unique to Phafin2 and similar animal proteins, potentially indicating a unique role in animal cell functions. Further experiments examined how Phafin2 regulates itself and binds to cell membranes. The study identified specific amino acids in the PH domain crucial for interacting with the polyD motif. When these amino acids were altered, Phafin2 could no longer bind to synthetic polyD peptides, highlighting their importance. Finally, the research determined that Phafin2's ability to bind to membranes does not depend on the shape or curvature of the membrane.

Dedication

This dissertation is dedicated to my wife, Momotaj Begum Jui, and

My son Nisarga Ninad Swarabarna.

Acknowledgments

I am grateful for my family's unconditional support, particularly during my final years at Virginia Tech. These words can never fully express my gratitude for their relentless encouragement, devotion, and dedication, which helped me navigate the most challenging times.

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Thank you.

Mahmudul Hasan

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List of Abbreviation

EAPF	Endoplasmic reticulum-associated apoptosis-involved protein containing PH and FYVE domains
EEA1	Early endosomal antigen 1
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FYVE	Fab 1, YOTB, Vac1, and EEA1
ITC	Isothermal titration calorimetry
LAPF	Lysosome-associated apoptosis-inducing protein containing PH and FYVE domains
LAMP-2A	Lysosome-associated membrane protein type 2A
mTOR	Mechanistic target of rapamycin
mTORC2	Mechanistic target of rapamycin complex 2
NMR	Nuclear magnetic resonance
PDK1	Phosphoinositide-dependent protein kinase 1
PH	Pleckstrin homology
Phafin	PH domain and FYVE domain-containing proteins
PI	Phosphoinositide
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PLEKHF2	Pleckstrin homology and FYVE domain containing 2
PtdIns(3)P	Phosphatidylinositol 3-phosphate
PtdIns(4)P	Phosphatidylinositol 4-phosphate
PtdIns(5)P	Phosphatidylinositol 5-phosphate
PtdIns(3,4)P2	Phosphatidylinositol (3,4)-bisphosphate
PtdIns(3,5)P2	Phosphatidylinositol (3,5)-bisphosphate
PtdIns(4,5)P2	Phosphatidylinositol (4,5)-bisphosphate
TEM	Transmission electron microscopy

Chapter 1: Introduction

1.1 The Phafins Protein Family

Phafin proteins, including Phafin1 and Phafin2, contribute to vital cellular membrane dynamics related to nutrient uptake and intracellular cargo trafficking. They are associated with a process involving membrane phosphoinositides (PIPs), which interact with cytosolic proteins through their PIP-binding domains (Jarsch et al., 2016; Paolo & De, 2006; Schink et al., 2016). This membrane rearrangement occurs with the recruitment of plasma membrane, the endoplasmic reticulum (ER), membrane-enclosed organelles like endosomes the Golgi apparatus and lysosomes. The process is essential for the uptake, transport, and processing of extracellular nutrients and membrane-bound macromolecules, facilitating intracellular cargo trafficking. This process allows cells to transport and distribute essential molecules and organelles to maintain cellular structure and function (Conner & Schmid, 2003; Platta & Stenmark, 2011).

1.1.2 Phafin1

Phafin1, also referred to as LAPF (a lysosome-associated apoptosis-inducing protein containing the PH and FYVE domains) and PLEKHF1 (Pleckstrin homology, and FYVE domain containing 1), is ubiquitously expressed in fat, spleen, and, to a lesser extent, other tissues (Linn Fagerberg et al., 2014). It carries 279 amino acids; in humans, its correspondent gene is located on chromosome 19q12. There are two phosphatidylinositol 3-phosphate-binding domains in Phafin1, namely, an N-terminal PH domain and a central FYVE domain. Phafin1 has a shorter polyD motif compared to Phafin2, and it is followed by a C-terminal tail (Figure 1). Phafin1 is a pro-apoptotic protein that triggers caspase-independent apoptosis through the lysosomal-mitochondrial pathway after translocating to lysosomes (Chen et al., 2005).

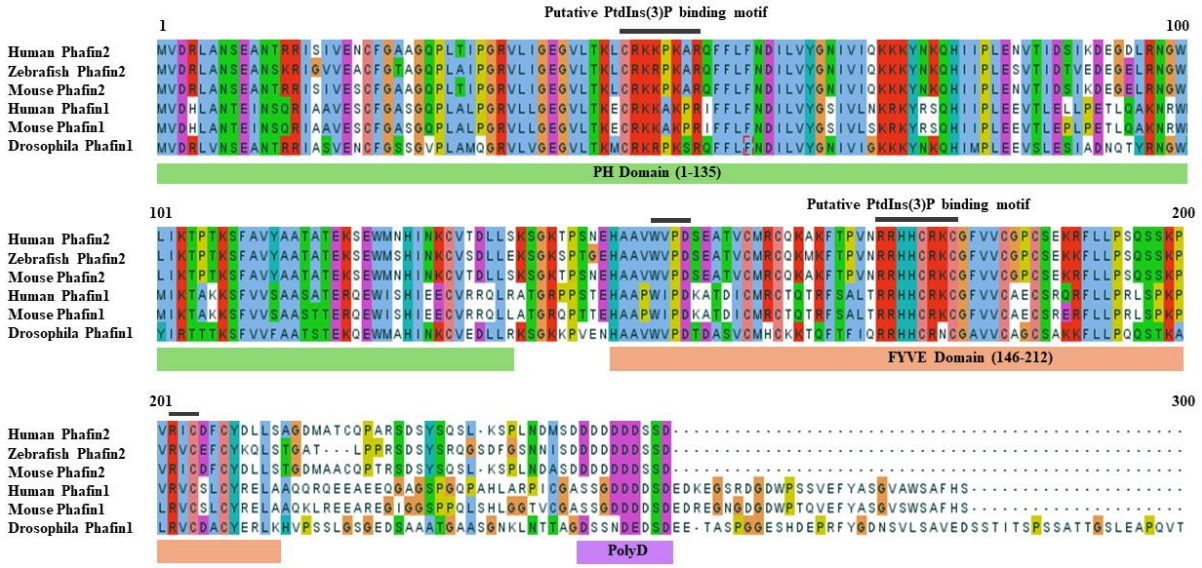

Figure 1.1: The modular organization of Phafin1 and Phafin2 proteins. PH domain, FYVE domain and polyD motif are shown alongside putative PtdIns(3)P binding site. Sequences were retrieved from UniProt Databases. Sequence Alignment was constructed by ClustalOmega and is colored using JalView.. UniProt Accession Numbers; Homo sapiens Phafin2: Q9H8W4, Mus musculus Phafin2 Q91WB4, Danio rerio Phafin2: Q7ZUV1, Homo sapiens Phafin1 Q96S99, Mus musculus Phafin: Q3TB82, Drosophila melanogaster Phafin1: O76902.

In addition, phosphorylated p53 could be recruited to the liposome by the adaptor role of Phafin1 (Nan et al., 2007). In autophagy, Phafin1 is heading to lysosomes through the Rab7-dependent signaling pathway. In HEK293T cells, introducing both hLC3A (microtubule-associated protein light chain 3, an autophagosome marker) and Phafin1 together is enough to trigger autophagy. In HEK293T cells, it was found that Phafin1 and hLC3A can induce autophagy (Kim & Cunningham, 2015; Lin et al., 2012).

1.1.2 Phafin2

Phafin2 is a 249 amino acid protein, and, in humans, its gene is located on chromosome 8q22. Phafin2 is also known as EAPF (an endoplasmic reticulum-associated apoptosis-involved protein containing PH and FYVE domains) and PLEKHF2 (pleckstrin homology and FYVE domain containing 2). Phafin1 and Phafin2 share similar structural architecture, having an N-terminal PH domain, a central FYVE domain, and a polyD motif. Both PH and FYVE domains are involved in lipid binding (Jarsch et al., 2016; Knævelsrud et al., 2013; Wang et al., 2019). The *phafin2* gene has a higher expression level than *phafin1*, and the higher expression level has been identified in bone marrow, lymph nodes, and other tissues (Doherty & McMahon, 2009). Phafin2 is directly involved in the lysosomal induction of autophagy and regulation of endosomal cargo trafficking (Knævelsrud et al., 2013). Phafin2 contributes to the biogenesis of endosomes. Additionally, the formation of enlarged endosomes is influenced by the overexpression of the Phafin2 FYVE domain. Even when the PH domain is absent in a Phafin2 construct, it can still associate with and promote the enlargement of endosomes; however, this activity is impaired in the absence of the FYVE domain (Lin et al., 2010). Phafin2 also plays significant roles in macropinocytosis and autophagy. A recent study also described the anti-bacterial properties of Phafin2 (Schink et al., 2021). It can help cellular machinery to recognize the gram-positive and gram-negative bacteria in the initial development of zebrafish embryos (Ren et al., 2022).

1.2 Structural Features and Phosphoinositide-binding Domains of Phafin2

Phosphorylated derivatives of phosphatidylinositol (PtdIns) are the key signaling molecules in cellular function, including membrane trafficking, cell survival, signaling transduction, and cytoskeletal dynamics (Kutateladze, 2010; Picas et al., 2016; Poccia & Larijani, 2009). When the myo-inositol headgroup of phosphatidylinositol is phosphorylated at a single position or in

different positions, this conversion can produce seven different PIPs: PtdIns(3)P; PtdIns(4)P; PtdIns(5)P; PtdIns(3,4)P_2; PtdIns(3,5)P_2; PtdIns(4,5)P_2; and PtdIns(3,4,5)P_3 (Olivença et al., 2018; Simonsen et al., 2001; Wallroth & Haucke, 2018). Phafin2 has two PIP-binding domains, the PH and FYVE domains (**Figure 1)**. The FYVE domain is unregulated for PtdIns3P binding, whereas the PH domain binding to PtdIns3P is autoregulated by its C-terminal acidic region (Tang et al., 2020). The PH domains have observed a lower range of amino acid sequence homology (7-30%), but the three-dimensional structures are similar. PH domains exhibit a distinct structure comprising seven β-strands, which combine to create two antiparallel β-sheets. These sheets are concluded at one end by a C-terminal α-helix (Auguin et al., 2004; Lenoir et al., 2010; Thomas et al., 2002; Yoon et al., 1994).

The PH domain can facilitate the recruitment of proteins to specific cellular membranes. The negative head group of the phospholipids available in the membrane causes the major binding space for the positive binding pocket of PH domains (Lemmon & Ferguson, 2000). Diverse specificity has been observed in the interaction of PH domains to phospholipids. PH domains can bind PtdIns(4,5)P_2 (Lemmon et al., 1995), PtdIns(3,4,5)P_3 (Frech et al., 1997; Manna et al., 2007), PtdIns(3)P (Agorio et al., 2017); PtdIns(4)P (Sugiki et al., 2012), PtdIns(5)P (Zwolak et al., 2013); and PtdIns(3,5)P_2 (Ghai et al., 2017). The Phafin2 PH domain usually binds PtdIns(3)P, but it can bind PtdIns(4)P and PtdIns(5)P (Matsuda-Lennikov et al., 2014; Schink et al., 2021). A highly basic sequence (KXn(K/R)XR) is identified in the β1/β2 loop of the human Phafin2 PH domain, and it is proposed that this basic sequence motif could be a binding point for PH domain and membrane (Singh et al., 2021; Yamamoto et al., 2020). Phafin2 was also found as a coincidence detector of both PtdIns(3)P and PtdIns(4)P in the progression of macropinosome in the

macropinocytic pathway (Schink et al., 2021). In addition, Phafin2 PH also interacts with F-actin and JIP4 (Schink et al., 2021; Tan et al., 2021).

FYVE domains are highly conserved, and they recruit protein in the PtdIns(3)P-enriched endocytic membranes. The interaction of the FYVE domain to PtdIns(3)P is more specific.(Kutateladze, 2006; Stenmark & Aasland, 1999; Stenmark et al., 1996). The double FYVE finger (2×FYVE) is usually used to detect the distribution of PtdIns(3)P in eukaryotic cells (Gillooly et al., 2001; Xu et al., 2016; Nascimbeni et al., 2017). The three-dimensional structures of FYVE domains form two double-stranded β-sheets where two antiparallel β-strands form each β-sheet. Three conserved PtdIns(3)P-binding motifs are found in FYVE domains; an N-terminal WxxD (in single letter amino acid code; x, any amino acid) motif, a central (R/K)(R/K)HHCR motif, and a C-terminal RVC motif (Kutateladze & Overduin, 2001; Mao et al., 2000; Misra & Hurley, 1999). The crystallographic structure of FYVE domain was solved in *Drosophila* Hrs and *Saccharomyces cerevisiae* Vps 27p protein. These structures showed similar coordination of two double-stranded β-sheets and a C-terminal α-helix (Gillooly et al., 2001). The FYVE domain usually contains eight cysteine residues (four CxxC motifs) coordinating two Zn^{2+} ions (Kutateladze, 2006). FYVE domains exist in both monomeric and homodimer states. For example, homodimer structures were revealed in Hrs and EEA1 FYVE domains. On the other hand, the Vps27p FYVE domain exists in a monomeric structure (Dumas et al., 2001; Mao et al., 2000; Misra & Hurley, 1999).

1.3 Major Functional Roles of Phafin2

Phafin2 is contributes directly or indirectly in many cellular functions, such as, cell death, recycling damaged cytoplasmic organelles, guiding traffic inside cells, and even anti-bacterial functions. Understanding its functions is vital for knowing more about the normal cell activities and disease development.

a) Apoptosis

Apoptosis is a collective approach to maintaining cellular homeostasis by discarding damaged or dying cells (Hengartner, 2000) . This event is also termed programmed cell death (PCD), which is usually led by different morphological changes, including chromatin condensation, plasma membrane blebbing, cell shrinkage, and apoptotic bodies (Carneiro & El-Deiry, 2020; Green, 2019). Two main apoptotic pathways exist: the death receptor-initiated extrinsic pathway and the mitochondria-initiated intrinsic pathway. However, the tumor necrosis factor-α (TNF-α) can induce both the death receptor and the mitochondrial pathways (Carneiro & El-Deiry, 2020; Green, 2019). Phafin2 is reported to induce TNF-α-initiated cellular apoptosis. The overexpression of Phafin2 triggers an ER-mitochondrial apoptotic pathway. It can also enhance the TNF-α-induced activity of caspase 3. Silencing of Phafin2 expression protects the cells from TNF-α-initiated apoptosis induction (Changfei et al., 2008). Two mutants of Phafin2, Phafin2 delta-PH (deletion of the N-terminal PH domain) and Phafin2 delta-FYVE (deletion of the central FYVE domain) were unable to translocate themselves to the ER after TNF-α treatment (Changfei et al., 2008), suggesting that both PH and FYVE domains contribute to the localization of Phafin2 to the ER. This localization event causes elevated Ca^{2+} levels in cytosol and reduced protein unfolding responses in the ER (Kim et al., 2002).

b) Autophagy

Autophagy, also known as the 'self-eating process of cells', is a degradative and recycling process. It involves delivering damaged cellular components like misfolded proteins or dysfunctional organelles to vacuoles or lysosomes for degradation and recycling (Keller et al., 2020; Kundu & Thompson, 2008; Levine & Deretic, 2007; Levine et al., 2011). It is a highly conserved building process of new cellular components from the metabolites produced by removing and recycling

damaged cellular organelles. It maintains cellular homeostasis in normal growth conditions. Autophagy can be selective or non-selective. Cytoplasmic bulky proteins are usually degraded non-selectively, where predetermined cellular organelles can be degraded selectively in the presence of different stress conditions, such as, growth factor deprivation, nutrient starvation, and infection (Jang & Lee, 2016; Kawabata & Yoshimori, 2016).

There are three types of autophagy: microautophagy, macroautophagy, and chaperone-mediated autophagy (Boya et al., 2013; Noguchi et al., 2020). Microautophagy directly engulfs cytoplasmic cargo components in a non-selective lysosomal degradative way. In macroautophagy, an isolated membrane structure or phagophore forms double membrane autophagosomes that lead to the engulfment of cytoplasmic components. Autophagosomes are recruited to lysosomes, forming autolysosomes. The engulfed cytoplasmic components are employed by the lysosomal hydrolytic enzymes for degradation, and the degradation products are delivered to the cytosol (Kundu & Thompson, 2008). The Hsc70 chaperone, found in both the cytosol and lysosomes, typically controls the process of chaperone-mediated autophagy. This process involves identifying cytosolic proteins that carry a specific sequence, like the pentapeptide KFERQ. The Hsc70 chaperone can also localize proteins across the lysosomal membrane with the help of an integral membrane receptor called LAMP-2A (lysosome-associated membrane protein type 2A) (Shintani & Klionsky, 2004). *Autophagy-related genes (ATG)* encode autophagy-related proteins, associated with autophagic machinery. The alteration of autophagy-related proteins can impair the normal process of cellular adaptation and cellular remodeling process (Choi et al., 2013), leading to various diseases, such as cancer (Mowers et al., 2018), microbial infection (Keller et al., 2020), neurodegeneration (Hara et al., 2006), myopathies (Nishino et al., 2000) , and aging (Lipinski et al., 2010).

PtdIns(3)P plays a significant role in autophagy induction. Many PtdIns(3)P-binding proteins are associated with autophagy progression, for example, the DFCP1 (double FYVE-containing protein 1) and WIPI (WD repeat domain phosphoinositide-interacting) protein families are indispensable to the autophagosome formation (Jang & Lee, 2016; Levine & Deretic, 2007; Levine & Kroemer, 2008; Levine et al., 2011) Phosphoinositide 3-kinase (PI3K) phosphorylates the 3'-OH group of the inositol ring of PIP. Phafin2 is reported as the critical initiation factor of autophagy (Hirata et al., 2018; Matsuda-Lennikov et al., 2014; Noguchi et al., 2014). Lysosomal localization of Phafin2 alongside the serine/threonine kinase Akt (also known as protein kinase B, PKB) forms the vital induction step of autophagy machinery. Akt proteins contribute to cell growth, vesicular trafficking, cell survival, transcriptional regulation, and cytoskeletal organization (Ebner et al., 2017; Manning & Toker, 2017).

There are three types of Akt proteins, Akt1, Akt2, and Akt3. In HEK293 cells, Akt1 and Akt2 can interact with Phafin2. PI3K-Akt-mTOR (mechanistic target of rapamycin) pathway is a prototype pathway in autophagy. After using rapamycin to induce autophagy, Phafin2 is translocated to lysosomes by binding to PtdIns(3)P, translocating Akt to these compartments (Matsuda-Lennikov et al., 2014). Akt has three domains: an N-terminal PH domain, a kinase domain in the middle, and a C-terminal regulatory domain. The PH domain controls autophagy in a dual regulatory function. The PH domain of Akt inhibits its kinase domain from the functional state, putting Akt in an inactive state called "PH-in." When the PH domain associates with PtdIns(3,4,5)P_3, it changes to an active state called "PH-out," and then PDK1 (phosphoinositide-dependent protein kinase 1) gets access to phosphorylate the Akt's kinase domain at T308, which leads to a partial activation of Akt. Therefore, Akt requires another phosphorylation at S473 by the action of mTORC2 (mechanistic target of rapamycin complex 2), leading to a fully functional state (Mowers et al., 2018).

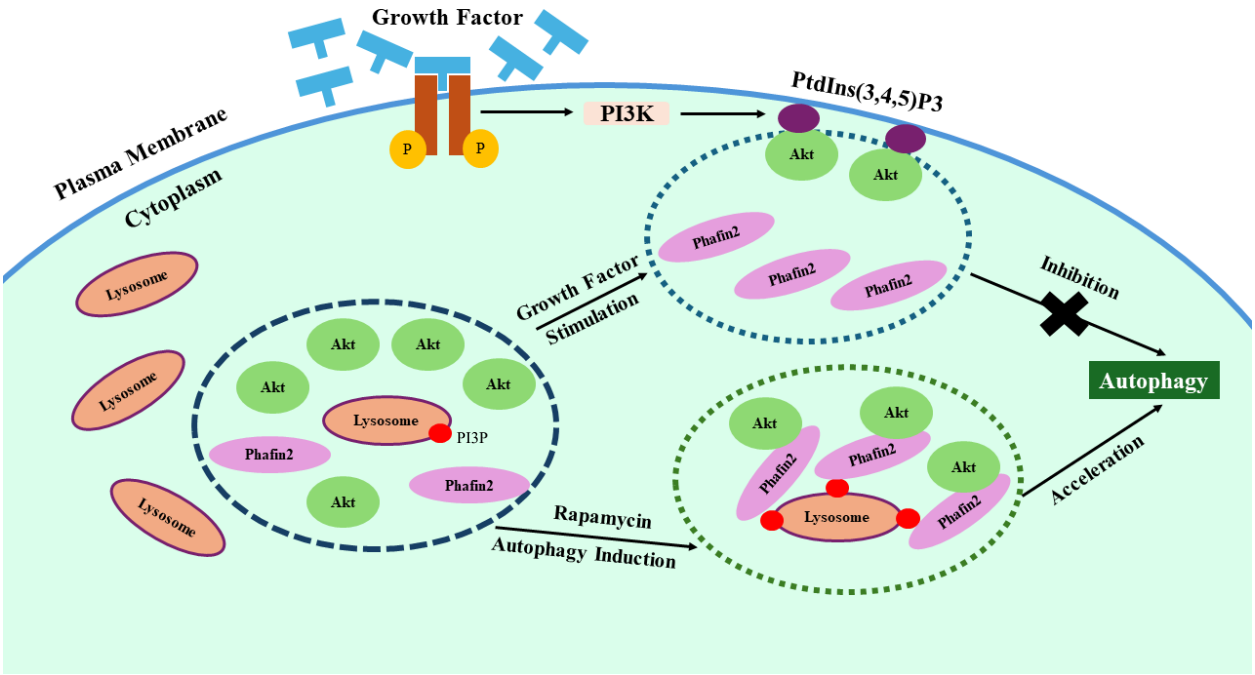

Figure 1.2: Role of Phafin2 and Akt proteins in the induction and regulation of autophagy. During autophagy induction, Phafin2 and Akt localize to lysosomes. On the other hand, the activity of Akt through phosphorylation plays a critical role in the downstream regulation of autophagy. The figure is adapted from Tang et al., 2023.

Activated class I PI3K causes increased PtdIns(3,4,5)P_3 levels at the plasma membrane, leading to the interaction of Akt PH domain with PtdIns(3,4,5)P_3. This event translocates Akt to the plasma protein, switching on its kinase activity through necessary conformational changes (Ebner et al., 2017). Functional Akt can inhibit autophagy by phosphorylating the downstream substrates associated with the autophagy pathway (**Figure 2)** (Mowers et al., 2018) .

c) Endocytic Trafficking

Cellular endocytic trafficking is a crucial process within cells, responsible for internalizing, sorting, and delivering various molecules to multiple destinations. It begins with the formation of vesicles at the cell membrane through endocytosis, where extracellular substances are engulfed and enclosed within vesicles. These vesicles then undergo sorting within the cell, where specific cargo molecules are directed to different destinations, such as lysosomes, for degradation or recycling back to the cell surface. The trafficking of vesicles is tightly regulated by a complex network of proteins and signaling pathways, ensuring efficient cargo transport to its targeted location. Dysregulation of endocytic trafficking can lead to various cellular dysfunctions and contribute to the pathogenesis of diseases such as cancer, neurodegenerative disorders, and infectious diseases(Ameen et al., 2007; Platta & Stenmark, 2011; Schreij et al., 2016).

Cell surface receptors contribute significantly to the complex network of proteins and signaling pathways. The major cell-surface receptors are (a) ion-channel coupled receptors, (b) G-protein-coupled receptors, and (c) enzyme-coupled receptors. The epidermal growth factor receptor (EGFR), an enzyme-coupled receptor, is internalized by endocytosis when ligand binds, leading to the degradation of activated receptor complexes. Hence, activated EGFR protects the cell environment from excessive stimuli (Brankatschk et al., 2012; Miaczynska et al., 2004; Raiborg & Stenmark, 2009). Phafin2 is associated with EGFR degradation by mediating endosomes fuses. It also contributes to endosome function by interacting with other binding partners. EGFR degradation slows down in Phafin2-depleted cells, indicating that Phafin2 associates in receptor trafficking (Nina et al., 2012).

Besides its role in EGFR degradation, Phafin2 modulates the insulin receptor (InsR) density on the cell surface [14]. Increased InsR levels appear on plasma membranes when Phafin2 is

overexpressed in the cell. Moreover, the decreased amount of InsRs on the cell surface were found in the downregulation of Phafin2 expression using Phafin2 siRNA (small interference RNA). These findings show that when Phafin2 levels go up, it inhibits InsR from internalization, causing more InsR to remain at the plasma membrane (Lin et al., 2010; Nina et al. et al., 2012).

d) Macropinocytosis

Macropinocytosis is a cellular process crucial for ingesting extracellular fluids and large molecules by cells, facilitated by an actin-dependent mechanism. This mechanism leads to the formation of large vesicles known as macropinosomes, which serve as compartments for internalizing these materials. Interestingly, under certain circumstances, macropinocytosis can also enable the uptake of bacteria and viruses, allowing them to enter cells. This process has gained increasing interest due to its significant roles in immune defense and the clearance of apoptotic bodies (Commisso et al., 2013; Mercer & Helenius, 2009; Swanson, 2008).

A new study found that Phafin2 is translocated to the newly formed macropinosomes, and it is driven through the interaction between Phafin2 and PtdIns(3)P (**Figure 3)**. Phafin2 interacts with a protein called Filamin A, which contributes to the cross-linking structure of actin protein and subsequent maturation of the macropinocytosis process on the outer surface of macropinosomes.

In Phafin2-depleted cells, the number of macropinosomes decreases significantly. JIP4, a motor-binding protein, is brought to the macropinosomes by Phafin2 to promote the tubulation of

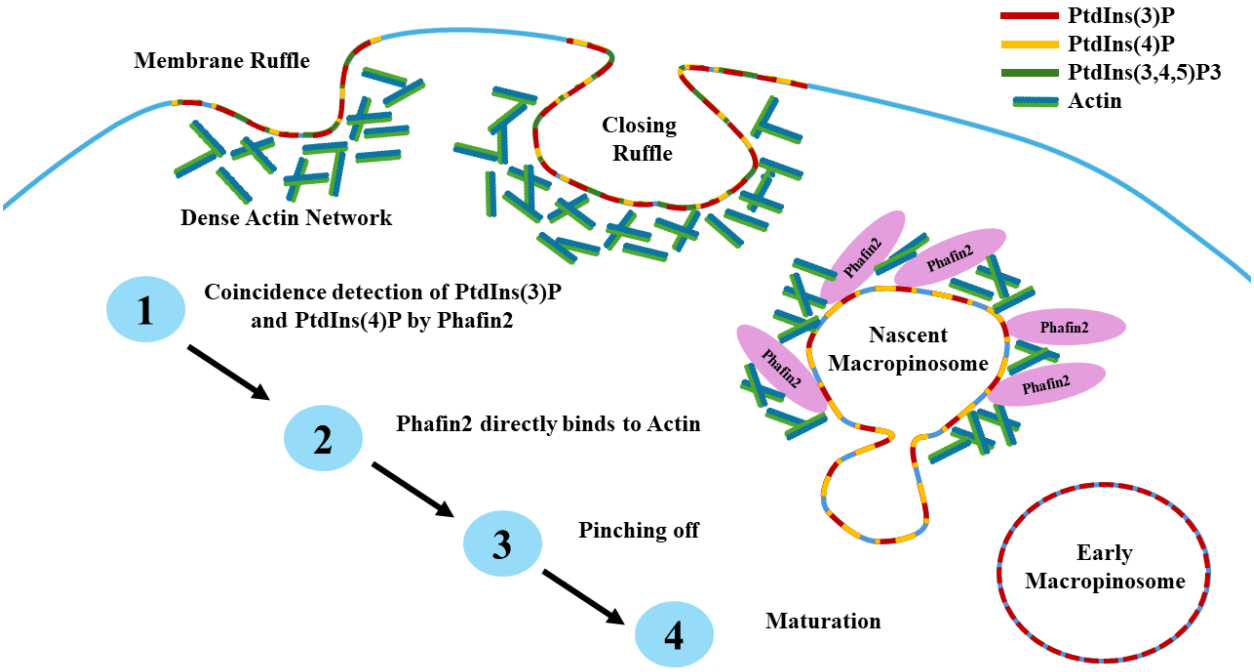

Figure 1.3: Critical role of Phafin2 in the process of macropinocytosis. Phafin2 competes with FilaminA to bind actin, leading to the pinching off of a newly formed macropinosome. The figure is adapted from Tang et al., 2023.

macropinosomes. The PH domain, but not the FYVE domain, is critical for the interaction of Phafin2 with JIP4. When Phafin2 or JIP4 levels decrease or their interaction with PtdIns(3)P is impaired, the formation of these macropinosomal tubes is reduced (Tan et al., 2021).

e) Anti-bacterial Functions and Cancer

Phafin2 acts as a type of determining pattern in the zebrafish's innate immune system. A recent study showed that recombinant Phafin2 can bind to bacterial marker molecules *in vitro*, such as

lipopolysaccharides, peptidoglycans, and lipoteichoic acid (Kutateladze, 2010). Phafin2 can act as the anti-bacterial agent against Gram-positive and Gram-negative bacteria, which might be very critical to the initial development of zebrafish embryos (Ren et al., 2022). Moreover, higher level expression of Phafin2 in different immune cells (as CD19+ B-cells and BDCA+ dendritic cells) also indicates to the association of Phafin2 in the immune response mechanism (Schink et al., 2021; Wu et al., 2016).

The cellular expression of Phafin2 in breast cancer, bladder/urinary tract cancer, and prostate cancer is higher than 15% (Lin et al., 2012). The different expression profile of the *phafin2* gene is detected in estrogen-responsive breast cancer cells. The *phafin2* gene is screened out from a set of 61 genes identified through transcriptomics data analysis of estrogen receptor-positive and estrogen receptor-negative human breast cancer cell lines, indicating distinct Phafin2's expression in both estrogen receptor-positive and estrogen receptor-negative cells (Weisz et al., 2004). According to the publicly available *Bioportal Cancer Genome* database, data screening suggests that both Phafin1 and Phafin2 expression levels are higher in many types of cancers (Schink et al., 2021). Also, the expression of Phafin2 mRNA is much higher in hepatocellular carcinoma tumors than normal livers, suggesting that the Phafin2's expression is upregulated in liver cancer (Lin et al., 2010).

Chapter 2

The PH domain and C-terminal polyD motif of Phafin2 exhibit a unique concurrence in animals

Mahmudul Hasan [1,2] and Daniel G. S. Capelluto[1]**

[1]Protein Signaling Domains Laboratory, Department of Biological Sciences, Fralin Life Sciences Institute and Center for Soft Matter and Biological Physics, Virginia Tech, Blacksburg VA, 24061, United States.; capellut@vt.edu; mahmudulh20@vt.edu

[2]Department of Pharmaceuticals and Industrial Biotechnology, Sylhet Agricultural University, Sylhet-3100, Bangladesh; mahmudulh20@vt.edu *

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Correspondence: mahmudulh20@vt.edu (M.H.); capellut@vt.edu (D.G.S.C.)

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2.1 Abstract

Phafin2, a member of Phafin family proteins, contributes to a plethora of cellular activities including autophagy, endosomal cargo transportation, and macropinocytosis. The PH and FYVE domains play key roles in Phafin2's membrane binding, whereas the C-terminal poly aspartic acid (polyD) motif specifically autoinhibits PH domain binding to membrane phosphatidylinositol 3-phosphate (PtdIns3P). Since the Phafin2 FYVE domain also binds PtdIns3P, the role of the polyD motif remains unclear. In this study, bioinformatics tools and resources were employed to determine the concurrence of the PH-FYVE module with the polyD motif among Phafin2 and PH-, FYVE-, or polyD-containing proteins from bacteria to humans. FYVE was found to be an ancient domain of Phafin2 and related proteins that are present in both prokaryotes and eukaryotes. Interestingly, the polyD motif only evolved in Phafin2 and PH- or both PH-FYVE-containing proteins of animals. PolyD motifs are absent in PH domain-free FYVE-containing proteins, which usually display cellular trafficking or autophagic functions. Moreover, prediction of the Phafin2-interacting network indicates that Phafin2 primarily cross-talks with proteins involved in autophagy, protein trafficking, and in neuronal function. Taken together, the concurrence of the polyD motif with the PH domain may be associated with complex cellular functions that evolved specifically in animals.

2.2 Introduction

Phafin2, also known as PLEKHF2 or EAPF, is a member of the Phafin proteins family that contains an N-terminal PH domain, a central FYVE domain, and a poly aspartic acid (polyD) motif at the C-terminus (Tang et al., 2020). Structurally, Phafin2 is a moderately elongated monomer primarily composed of both α-helical and β-strand elements, but it also has an estimated large contribution of random coil regions (Tang et al., 2017). Quantitative transcriptomics analysis shows that the human *Phafin2* gene, localized on chromosome 8q22, is broadly expressed in bone marrow and lymph nodes, among other tissues (Fagerberg et al., 2014). Amplification of the *phafin2* gene is associated with reduced survival in patients with prostate cancer (Shamsara & Shamsara, 2020).

Phafin2 is involved in multiple cellular functions, including endosomal cargo trafficking, apoptosis, macropinocytosis, and autophagy (Li et al., 2008; Lin et al., 2010; Matsuda-Lennikov et al., 2014; Schink, 2017; Schink et al., 2021). Similarly, the Phafin2 homolog Phafin1 targets lysosomes to promote autophagosome formation (Lin et al., 2012). The multiple functions of Phafin2 (and Phafin1) are associated with its ability to bind the phosphoinositide phosphatidylinositol 3-phosphate (PtdIns3P) through both the PH and FYVE domains (Matsuda-Lennikov et al., 2014; Tang et al., 2020). These domains are thermodynamically coupled (Tang et al., 2017), suggesting interdomain contacts. PtdIns3P is primarily found on both early endosomes and lysosomal surfaces and serves as a point of recruitment for PtdIns3P-binding effectors. For example, Phafin2 has been reported to control endosomal structure and function, primarily *via* its FYVE domain, in a Rab5-dependent manner (Lin et al., 2010). Further studies established that Phafin2 associates with early endosomes (Pedersen et al., 2012), where it co-localizes with PtdIns3P-and Rab5 (Lin et al., 2010) and interacts with the endosomal protein early endosome autoantigen 1 (EEA1), regulating endosomal fusion and protein trafficking (Pedersen et al., 2012).

Likewise, the *Drosophila* Phafin2 homologue Rush controls endosomal and lysosomal cargo trafficking by interacting with a Rab GDP dissociation inhibitor and PtdIns3P (Gailite et al., 2012).

Both the Phafin2 PH and FYVE domains are required for the induction of apoptosis (Li et al., 2008) and autophagy (Matsuda-Lennikov et al., 2014). To trigger apoptosis, Phafin2 is recruited to the endoplasmic reticulum, where it suppresses the unfolding protein response in this compartment and, simultaneously, it stimulates an increase of Ca^{2+} levels in the cytosol (Li et al., 2008). In the case of autophagy, both the PH and FYVE domains of Phafin2 associate with lysosomal PtdIns3P when coupled with the serine/threonine kinase AKT (Matsuda-Lennikov et al., 2014), which phosphorylates Phafin2 (Saei et al., 2021), and possibly depends on the serine/threonine kinase activity of the vaccinia-related kinase-2 (Hirata et al., 2018).

Recently, it has been reported that, during macropinocytosis, Phafin2 binds newly formed macropinosomes in a process that requires the presence of two distinct pools of PtdIns3P and by PtdIns4P (Schink et al., 2021). The FYVE domain is required for localization of Phafin2 to both the early and late steps of macropinosomes maturation, whereas the PH domain is only essential during the early steps (Schink, 2017). To participate in this process, the Phafin2 FYVE domain specifically associates with PtdIns3P, whereas the PH domain binds PtdIns3P, PtdIns4P, and actin. These types of functions facilitated by FYVE and PH domains can regulate the entire actin filament dynamics (Schink et al., 2021).

Recruitment of Phafin2, in turn, allows Filamin A, an actin filament cross-linking protein, to facilitate the reorganization of the actin cytoskeleton (Schink, 2017). Also, the Phafin2 PH domain is necessary to recruit the coiled-coil protein JIP-4 at macropinosomes, promoting membrane tubulation (Tan , 2020).

More recently, attention has been focused on the C-terminal polyD motif of Phafin2. Removal of the polyD motif does not alter Phafin2 function during macropinosome formation (Schink, 2017) but it is required to prevent nonspecific association of the protein to the plasma membrane (Schink et al., 2021). The polyD motif is required to downregulate Phafin2 PH domain binding to PtdIns3P (Tang et al., 2020), suggesting that the polyD motif prevents Phafin2 binding to other phosphoinositides at the plasma membrane; however, it becomes dispensable when the Phafin2 PH domain engages with nascent vesicles generated from macropinocytosis. Here, we employed a bioinformatics approach, focusing on the simultaneous presence of a PH domain and a polyD motif in Phafin2 orthologs from bacteria to mammals. Bioinformatics tools were used to observe the evolutionary trend of PH, FYVE, and polyD regions in the retrieved Phafin2 homologs from different databases. Moreover, the construction of a protein-protein interaction network based on human Phafin2 gave an insight into its experimentally reported binding partners or the proteins that crosstalk in the common pathways of Phafin2. Although PH domains are found in all organisms studied, only animal Phafin2 and other related PH-, FYVE-, and polyD-containing proteins simultaneously bear both a PH domain and a polyD motif, suggesting that the PH domain-polyD motif pair appeared more recently in evolution.

2.3 Material and Methods

a) Prediction of the Three-Dimensional Structure of Phafin2

The three-dimensional coordinates of all human Phafin2 heavy atoms were predicted by Alphafold (https://alphafold.ebi.ac.uk; accessed on 21 June 2022). To predict the structure of Phafin2, Alphafold employed the amino acid sequence of protein homologs as input [16].

b) Sequence retrieval and acquisition

PH-, FYVE- and polyD- containing proteins were retrieved from NCBI (https://www.ncbi.nlm.nih.gov), Ensembl genome browser (https://www.ensembl.org), and UniProt (https://www.uniprot.org) using the human Phafin2 amino acid sequence (Accession No: NP_078889) as the query sequence. In our study, we employed blastp (protein-protein BLAST) and PSI-BLAST (Position-Specific Iterated BLAST) with default parameters whenever we retrieved sequences from NCBI database. We might consider Quick BLAST, but it works better if the target percent identity is 50% or more. That is why the study design skipped Quick BLAST. Moreover, as the study had no intention to do any specific pattern-based search, that was why PHI-BLAST (Pattern Hit Initiated BLAST) or DELTA-BLAST (Domain Enhanced Lookup Time Accelerated BLAST) was also not considered._Orthologous sequences of human Phafin2 were retrieved from UniProt database.

c) **Functional protein module search**

Orthologous sequences of human Phafin2 were screened using the MEME (Bailey et al., 2015) database for identifying different motifs. The web-based domains search toolkits Pfam (Sonnhammer et al., 1997), Hmmscan (Finn et al., 2011), and InterPro (Hunter et al., 2009) were employed to search for the presence of modules in PH-, FYVE- or polyD-containing proteins from bacteria, archaea, fungi, plants, nonhuman mammals, human, and model organisms.

d) Multiple sequence alignment and phylogenetic analysis

Retrieved PH-, FYVE- or polyD-containing protein sequences were investigated using the multiple sequence alignment tool Clustal Omega v2.0.12 (EMBL-EBI, Wellcome Genome Campus, Cambridgeshire, UK, https://www.ebi.ac.uk/Tools/msa/clustalo/ (Sievers & Higgins, 2014) (last access date: December 27, 2021). After the assessment by MSA, phylogeny analysis

was allowed for understanding the diversity and evolutionary trends of orthologous sequences of human Phafin2 proteins. The analyses were achieved using the Molecular Evolutionary Genetics Analysis X (MEGA X) [University of Pennsylvania, Pennsylvania, USA) (Kumar et al., 2018) (last access date: December 15, 2021)]. An unrooted phylogenetic tree was generated with 203 retrieved Phafin2 orthologous sequences using the maximum likelihood method of MEGA X platforms. Bootstrapping with 1,000 replicates were performed to ensure the maximum reliability of individual branches of the tree. Newmikh file generated from MEGA X was employed to iTOL website to display, annotation, and management of our phylogenetic tree. The PH, FYVE, and polyD sequence patterns were displayed in the constructed tree by using advanced data set settings of iTOL (Letunic & Bork, 2019).

e) Protein-protein interactions analyses

The protein-protein interactions network of human Phafin2 was predicted using the STRING database. Human Phafin2 was used as the query search in the STRING database, and molecular interactions with a confidence score ≥90% were considered to avoid false-positive results (Szklarczyk et al., 2019).

2.4 Results and Discussion

a) Genomic features of the polyD motif in Phafin2's human orthologues

Orthologous and homologous proteins are usually the preliminary platforms used for evolutionary studies and functional annotation of proteins (Mier et al., 2015). MEME identified ten highly conserved regions in Phafin2, including the C-terminal polyD motif (**Figure 2.1)**. Most of the conserved sequences are found within the PH and FYVE domains. The genomic features of the

polyD motif of human Phafin2 has been described in comparative view of its orthologous proteins. A total of 203 orthologues of human Phafin2 (**File S1**) were employed in the phylogenetic study, and results indicated that Phafin2 and other related PH-, FYVE-, or polyD-containing proteins of *Gorilla gorilla* (gorilla), *Pan troglodytes* (chimpanzee), and *Pan paniscus* (bonobo) were evolutionarily convergent orthologues of human Phafin2 (**Figure 2.2**). Most orthologues were found to have PH-, FYVE- or polyD modules, but vase tunicate, channel catfish, atlantic salmon, green spotted puffer, burtons mouthbrooder, and common mallard have no polyD motif encoded with their PH-FYVE modules (**Figure 2.2**). Moreover, Hoffmanns two-toed sloth and Egyptian zebra contain a PH domain, but they lack both FYVE and polyD modules (**Figure 2.2**). The retrieved orthologues sequences from the *human genome browser 105* includes 26 species of primates, 32 species of rodents, 43 species of carnivores, ungulates, and insectivores, 106 species of mammals, 69 species of reptiles and birds, and 86 species of fishes. Among 203 sequences, 78 orthologues were from mammals, which indicates a relevant pool of Phafin2 proteins available in mammalian species **(Table S1).** Most importantly, Multiple Sequence Alignment (MSA) analysis shows that all human Phafin2 orthologues in other organisms usually carry the C-terminal acidic polyD region alongside the PH and FYVE domains **(File S2)**. The polyD motif of human Phafin2 is ten-amino acids long (240-DDDDDDDSSD-249), but variable amino acid positioning was identified in protein orthologues as follows: position # 240:D/S/W/Q; 241:D/S/E; 242:D/S/E; 243:D/S/E/G/N; 244:D/S/E; 245:D/E; 246:D/S/N; 247:S/D/E; 248:S/D/A/E/K; and 249:D/S/N/E. Although highly conserved, serine residues can also be present in other positions within the motif (**Figure 2.1**).

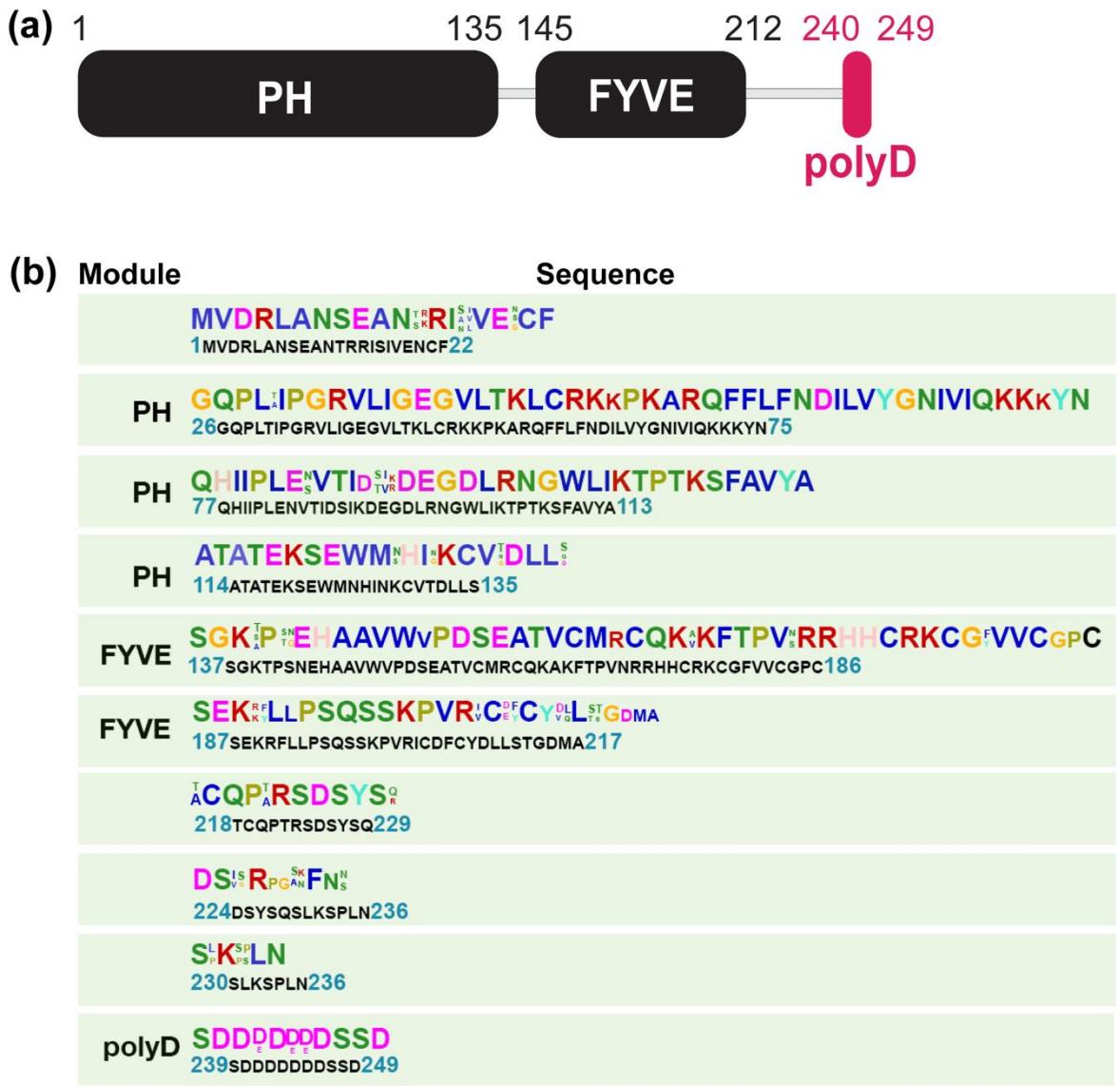

Figure 2.1 Consensus sequences found in mammalian Phafin2 and PH-, FYVE-, and polyD-containing proteins. Sequences associated to the PH, FYVE, and polyD modules are labeled. Logo sequences were obtained from protein sequences of human origin.

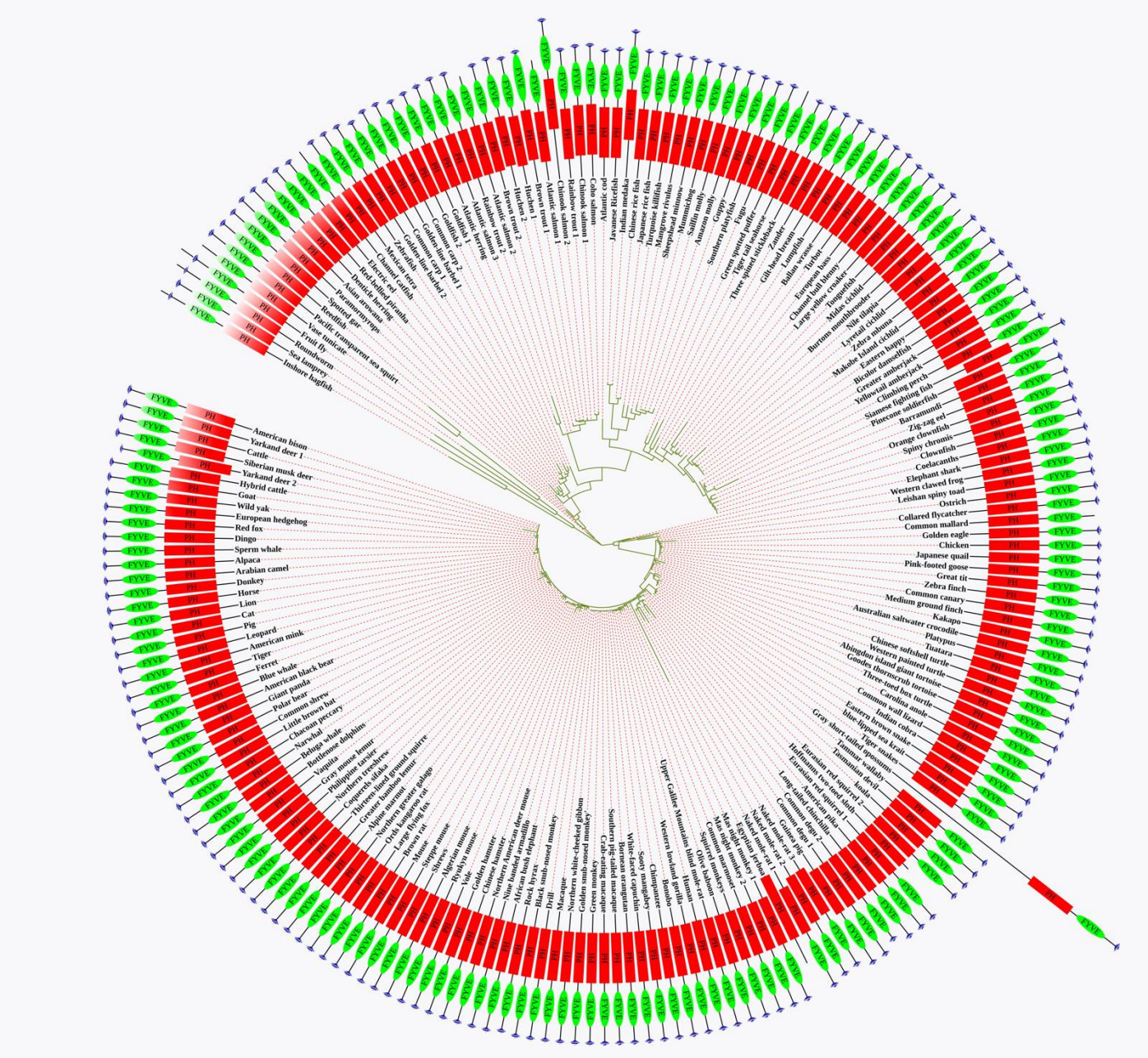

Figure 2.2 Phylogenetic analysis of Phafin2 orthologues retrieved using the Ensembl genome browser. The analysis of Phafin2 ancestral sequences was generated using the MEGA X software and domain structure was constructed using the I-TOL software.

Quantitative phosphoproteomic studies indicate that human Phafin2 serine residues at positions 239, 247, and 248 are phosphorylated (Olsen et al., 2010), emphasizing the role of these residues

within the polyD region. In addition, aspartic acid is often found to be replaced by glutamic acid within the polyD motif (**Figure 2.1**), thus, indicating that the conserved acidic nature of the motif is relevant for the function of Phafin2.

b) Concurrence of the polyD motif with the PH and FYVE domains in Phafin2 and other related PH-, FYVE-, or polyD-containing proteins

The presence of both the PH and FYVE domains is a signature of the modular architecture of Phafin2. However, other proteins having only PH or FYVE domains are also expressed in both prokaryotes and eukaryotes (Dai et al., 2007; Nakagawa et al., 2004). The presence of the polyD motif in human Phafin2 raises the question as to whether it is exclusively found only in human and other mammals, or whether it evolved or is evolutionarily expressed in Phafin2 and other related PH-, FYVE-, or polyD-containing proteins of other kingdoms. Database searches sometimes provide WGS sequences that might be contaminated from environmental sources. However, filtering WGS sequences did not affect our data analysis. Analysis of the evolution and concurrence of the polyD motif with the PH and FYVE domains in Phafin2 and other related PH-, FYVE-, or polyD-containing proteins in different organisms is provided in the following sections.

Bacteria

Unlike canonical PH domains and polyD motifs, FYVE domains of unknown function were found in about 40 bacterial proteins. Other protein domains, such as Vps27, Hrs, STAM (VHS), phosphatidylinositol 3- and 4-kinases, and ubiquitin-associated (UBA)-like domains, were found to be encoded together with the FYVE domains (**Table 2.1**, **Figure 2.3**, **and Files S3 and S4**).

Table 2.1 Different modules found in bacterial and archaea PH- and FYVE domain containing proteins.

Life forms	*UniProt ID*	*gene*	*Organism*	*Protein*	*Modules*
Bacteria	A0A1Y6CWR2_9GAMM	*samn02949497_2038*	*Methylomagnum ishizawai*	FYVE zinc finger	FYVE
	A0A2E9XWQ4_9RICK	*cmp47_14530*	*Rickettsiales bacterium*	Uncharacterized protein	VHS; FYVE
	A0A2E4CAT1_9ACTN	*cl450_07530*	*Acidimicrobiaceae bacterium*	Uncharacterized protein	FYVE; Phosphatidylinositol 3- and 4-kinase
	A0A0Q9PCH9_9GAMM	*asg87_06725*	*Frateuria sp. Soil773*	FYVE-type domain-containing protein	FYVE
	A0A2E8CGN4_9DELT	*cl926_13625*	*Deltaproteobacteria bacterium*	Uncharacterized protein	UBA-like; FYVE
Archaea	A0A482RXE8_9ARCH	*eon64_13770*	*Archaeon*	Uncharacterized protein	RhoGEF; FYVE; PH
	A0A482S4E7_9ARCH	*eon65_55335*	*Archaeon*	FYVE-type domain-containing protein	FYVE; FERM
	A0A482SL25_9ARCH	*eon65_20320*	*Archaeon*	Uncharacterized protein	FYVE; WW
	A0A482SVF7_9ARCH	*eon65_02420*	*Archaeon*	FYVE-type domain-containing protein	FYVE
	A0A482SZF2_9ARCH	*eon65_10680*	*Archaeon*	FYVE-type domain-containing protein	Lipase (class 3); FYVE
	A0A482RGE2_9ARCH	*eon67_05280*	*Archaeon*	FYVE-type domain-containing protein	IPT/TIG; FYVE

A0A482RTU5_9ARCH	*eon64_18385*	*Archaeon*	FYVE-type domain-containing protein	FYVE; Ankyrin repeat
A0A482SVG8_9ARCH	*eon65_11985*	*Archaeon*	FYVE-type domain-containing protein	FYVE; Tropomyosin

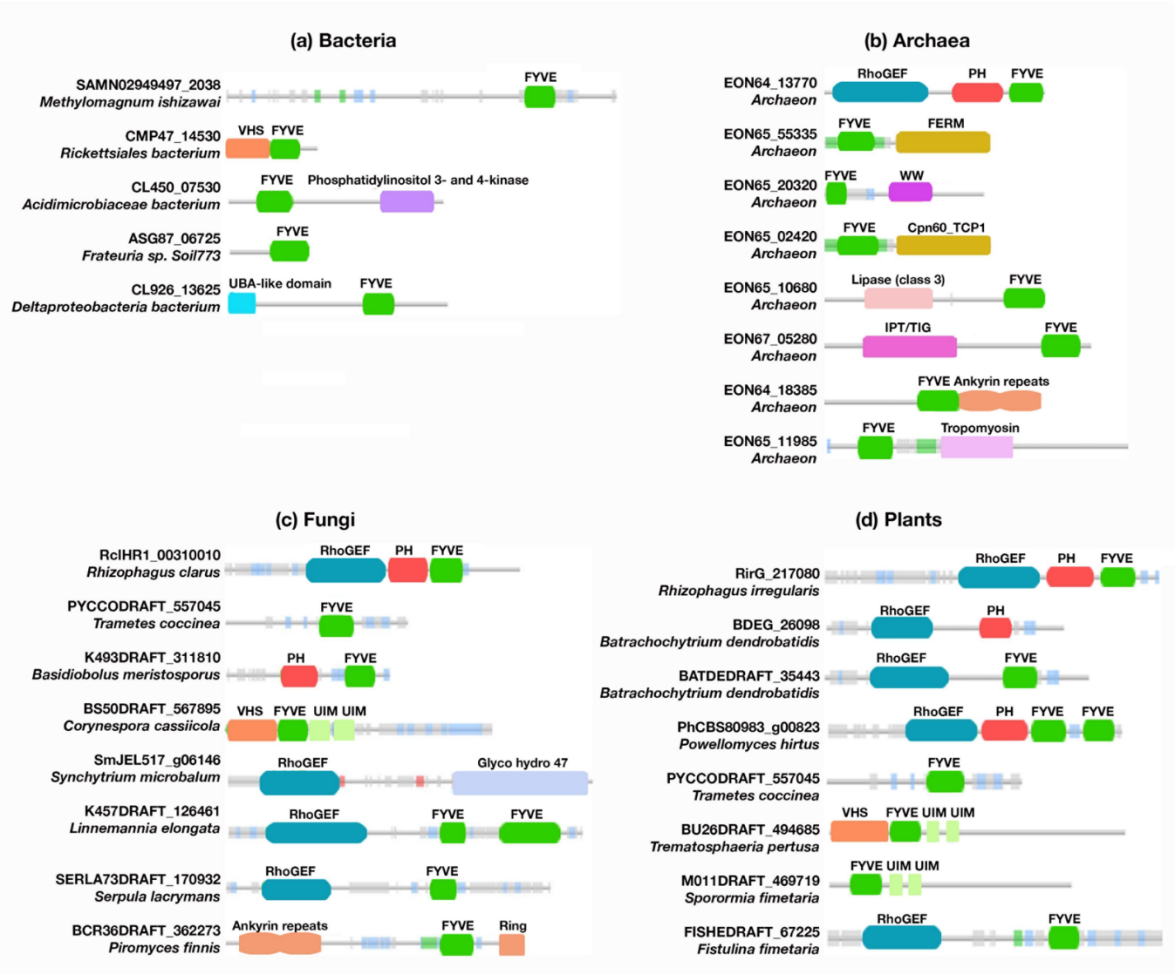

Figure 2.3 Schematic representation of PH- and FYVE-containing modules found in proteins from bacteria, archaea, fungi, and plants. Other unique domains are also shown.

Whereas eukaryotic VHS domains are involved in vesicular trafficking by either ubiquitinated cargo recognition or through phosphorylated receptor binding (Roach et al., 2021), bacterial VHS domains facilitate the subcellular localization of protein effectors (Pruneda et al., 2016). Likewise, UBA-like domains are associated with the ubiquitin/proteasome pathway in eukaryotes, but are uncommon modules of unknown function in bacteria (Su & Lau, 2009; Zientara-Rytter & Subramani, 2019). The same applies for phosphatidylinositol 3- and 4-kinases, which phosphorylate phosphoinositides in eukaryotes and whose activities are associated with cell cycle regulation, DNA recombination, and DNA damage checkpoint, among other functions (Boura & Nencka, 2015; Engelman et al., 2006; Roymans & Slegers, 2001). A PH-like domain (PH*b*) was reported in bacterial species; however, our database search did not recognize it as there were genomic dissimilarities with canonical PH domains (Xu et al., 2010). Genome wide studies of *Bacillus subtilis* reported three paralogs of PH*b*, which indicates common functional features of PH*b* proteins (Bunai et al., 2003; Kobel et al., 2002; Wang et al., 2002; Tang et al., 2006). PH*b* is also found in the Min1 phage from the nematode pathogen *Microbacterium nematophilum* (ORF77) (Akimkina et al., 2007) and *Lactococcus bacteriophage* ul36 (ORF124) (Labrie & Moineau, 2002). PH*b* homologs were also identified in other bacteria, such as *Oceanobacillus iheyensis48* (Swiss-Prot: Q8ELK9) and *Streptomyces coelicolor47* (SCO3793, Swiss-Prot: Q9F325). The availability of PH-like domains in bacterial species highlights that eukaryotic PH domains do not represent a new feature in these organisms; rather, they may be evolved or diverged from prokaryotes (Xu et al., 2010). Despite there are two categories of *PHb*, known as *PHb1* and *PHb2,* based on their size and ring structure symmetry, they are assumed to have the same function in bacterial cells. Genome wide study found that three paralogs of PH*b*, YjqA, YozO, and YvbH, are expressed in *B. subtilis.* YozO is associated to cell stress responses, whereas YvbH is a

membranal peripheral protein (Kobel et al., 2002; Wang et al., 2002; Wiegert et al., 2001). A YozO homolog, involved in cell envelope stress response activities, was detected in *Bacillus licheniformis* (Wecke et al., 2006). However, *PHb1* was confirmed in *Lactococcus bacteriophage ul36* (ORF124)45 and Min1 phage, which indicates that *PHb* might be involved in phage life cycle (Akimkina et al., 2007). Phosphoinositides are considered absent in *E.coli* and only are found in small bacterial and archaeal groups (Botero et al., 2019). The unavailability of a canonical PH domain in bacteria, such as the one found in Phafin2, indicates that eukaryotic PH domains might evolve in response to a natural selective pressure, and some gained the ability to bind phosphoinositides as a mechanism for organelle localization.

Archaea

Interestingly, while PH domains were found in tandem with FYVE domains, the polyD motif was not encoded in 68 archaeal proteins retrieved from the database (**Table 1**, **Figure 2.3, and Files S3 and S4**). The classic phylogeny study by Woese and co-workers, and a subsequent evolutionary study of universal genes, suggested that archaea are evolutionarily more convergent with eukaryotic organisms than with bacteria, and it was assumed that eukaryotic cells were emerged from an ancient archaeon cellular system (Pace, 2009; Woese et al., 1990). The presence of PH domains in archaea clearly indicates that these modules emerged among archaea species as an ancestor of all PH domains that were further evolved or emerged in other eukaryotic organisms. A Rho guanine nucleotide-exchange factor (GEF)-FYVE-PH tandem module was identified as predominant among archaea proteins (**Figure 2.3**). In eukaryotes, the RhoGEF domain regulates G protein signaling, where it stimulates GDP release, and consequently, GTP binding for the activation of specific Rho family proteins [21, 22].

Interestingly, FERM N-terminal, WW, lipase (class 3), Ig-like, plexins, transcription factors (IPT), and tropomyosin domains as well as ankyrin repeats were also found in tandem with archaea FYVE domains. In eukaryotes, the FERM domain is associated with protein localization to the plasma membrane, accelerating signaling pathways (Jung et al., 2012; Sulzmaier et al., 2014). Curiously, the FERM domain of human Kindlin-2 contains an inserted PH domain that specifically binds PtdIns3P and phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3]. This insertion may allow Kindlin-2 to target the plasma membrane, facilitating the regulation of integrin receptors (Liu et al., 2012). On the other hand, lipase (class 3), IPT, and tropomyosin domains as well as ankyrin repeats, are universally distributed in prokaryotes, plants, and animals and are involved in a wide range of cellular functions. For example, lipase class 3 is associated with lipid degradation, esterification, and transesterification, whereas the tropomyosin domain is required for the actin-myosin interaction (Nurniwalis et al., 2015; Oguchi et al., 2011). The ankyrin repeats are repetitive short sequences involved in protein-protein interactions, mediating a plethora of functions (Al-Khodor et al., 2010). Thus, it is possible that membrane binding of archaea PH- and FYVE-containing proteins are required for lipid and protein interactions.

Protozoans

To investigate protozoan PH-, FYVE-, or polyD-containing proteins, we employed both literature-based approach and NCBI BLAST. Several reports highlighted the presence of PH and FYVE domain-containing proteins in protozoans. For example, eleven FYVE- and two PX domain-containing proteins were found as potential PtdIns3P-binding proteins and one single PH domain-containing protein was detected as a PtdIns4P-binding protein in the *Entamoeba histolytica* genome (Watanabe et al., 2021). Among the PtdIns3P-binding proteins, a predominant conserved modular organization is represented by RhoGEF-PH/FYVE or just by the FYVE or PX domains

only, but all lack a polyD motif. The protozoan parasite *Leishmania major* contains five putative FYVE domain proteins displaying functional PtdIns3P-binding sites (Mertens et al., 2007). The FYVE domain has also been identified and characterized in *Giardia lamblia* (Sinha et al., 2011). For the identification of a polyD motif in protozoans, an NCBI pBLAST was employed using human Phafin2 as a query sequence with the FYVE domain-containing sequences of *E. histolytica, L. major,* and *G. lamblia*. No sequences from *G. lamblia* were found to be aligned with human Phafin2 in the BLAST search. Interestingly, protozoan sequences aligned with human Phafin2 have no polyD motif, suggesting that their membrane binding properties through the PH domain may be modulated by an alternative route. The mostly aligned protozoans' protein sequence was from *E. histolytica* with 71% query cover and 43% identity among all retrieved sequences of *E. histolytica and L. major* together. The BLAST and MSA results of protozoan proteins are shown in **File S5**.

Fungi

Approximately forty fungi Phafin2-related proteins were retrieved from the database, and our study revealed that they lack the polyD motif. RhoGEF-PH-FYVE and VHS-FYVE-ubiquitin-interaction motif (UIM) tandem modules were identified in fungal proteins (**Table 2**, **Figure 2.3**, and **Files S3** and **S4**). In addition, glycosyl hydrolase family 47, ankyrin repeats, and ring-finger domains were detected in these organisms. Both UIM and ring-finger domains play significant roles in ubiquitin moiety interactions present in cargo (Mattioni et al., 2020). Thus, the presence of a FYVE domain may contribute to membrane recruitment and prelocalization of fungal proteins. The presence of a PH domain has been reported in the fungal Target of Rapamycin (TOR) complex. Fungal species present two isoforms, TORC1 and TORC2, which are also found in mammals. The physiological fate of mTOR signaling lies in the regulation of cell growth in response to nutrient

uptake or growth factors (Heitman et al., 1991; Shertz & Cardenas, 2011). Fungal TORC2 is composed of six subunits (Tor2, Avo1, Avo2, Avo3, Lst8, and Bit61) (Eltschinger & Loewith, 2016), with a PH domain only found in the Avo1 subunit. The PH domain of Avo1 binds phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], allowing the recruitment of TORC2 to the plasma membrane (Berchtold & Walther, 2009). Although no direct downregulation of Avo1 PtdIns(4,5)P_2 has yet been reported, it is speculated that calcineurin modulates mTORC2 function by an uncharacterized molecular mechanism (Mulet et al., 2006). As fungal TORC2 plays major roles in sensing, homeostasis, and modulation of the lipid/protein composition of the plasma membrane as well as for actin cytoskeleton and actin-driven endocytosis (Berchtold & Walther, 2009; deHart et al., 2002; Roelants et al., 2017), the presence of a PH domain would serve as a membrane anchor for carrying out these functions.

The phosphoinositide-specific PH domain-containing phospholipase C (PLC) has been studied in different fungal species (Barman et al., 2018). PLC catalyzes the hydrolysis of PtdIns(4,5)P_2, which leads to the production of the secondary messengers inositol 1, 4, 5-trisphosphate and diacylglycerol (Berridge & Irvine, 1984). The PH domain is localized at the N-terminus of PLCs and is responsible for membrane PtdIns(4,5)P_2 binding (Yamamoto et al., 1999). PLCs, such as those from PLC1 from *Alternaria alternata* (Huang et al., 2020) and *Magnaporthe oryzae* (RHO et al., 2009), encode a PH domain, which is in tandem with the EF-hand Ca^{2+}-binding, the catalytic X and Y, and the phospholipid-binding C2 domains. Regulation of phospholipid-binding of PLC is mediated by association of PtdIns4P 5-kinase and Ca^{2+} to the PH and C2 domains, respectively (Gresset et al., 2012). One of the molecular mechanisms suggested for the regulation of mammalian PLC PH domain binding to phosphoinositides is that Ca^{2+} controls phosphoinositide headgroup conformation and recognition (Bilkova et al., 2017). Not all fungal PLCs have a PH

domain. For example, *Botrytis cinerea* have two PLC-encoding genes, *bcplc1* and *bcplc2*, where bcplc1 only contains a PH domain (Schumacher et al., 2008), suggesting that membrane binding by bcplc2 is driven by its C2 domain. Similarly, *Coprinopsis cinerea*, a multicellular basidiomycete mushroom employed as a model organism in eukaryotes (Pukkila, 2011), has three putative PLC genes, *CcPLC1*, *CcPLC2*, and *CcPLC3*, with the first two encoding for a PH domain (Oh et al., 2012), whereas *Cryphonectria parasitica* PLC does not have a PH domain (Essen et al., 1996). In summary, few fungal PH domains have been reported to bind PtdIns(4,5)P_2 and the absence of a polyD motif in these proteins suggests the presence of an alternative regulatory mechanism for phosphoinositide binding.

Plants

Plants possess a vast number of functional activities related to PH- and FYVE-containing proteins. Our study found ~250 sequences from database searches, but it is possible that some of these sequences correspond to contaminants from fungal species. Moreover, no polyD motif was identified in plant proteins. Instead, the PH and FYVE domains were present in the form of RhoGEF-PH-FYVE and VHS-FYVE-UIM-UIM tandem modules. In addition, RING finger and ankyrin repeats were encoded with FYVE domains (**Table 2.2**, **Figure 2.3**, and **Files S3** and **S4**). The PH domain is the predominant lipid-binding domain of plants. About sixty PH domain-containing proteins have been detected in the *Arabidopsis* genome (de Jong & Munnik, 2021). Plant PH domains preferentially exhibit association to phosphoinositides and phosphatidic acid, leading to widely diverse functions. For example, the *Medicago truncatula* ZR1 protein regulates root and nodule development, which might be mediated by interactions of its PH and FYVE domains with phosphoinositides and phosphatidic acid (Bruijn, 2020). However, regulation of ZR1 interactions with phospholipids remains to be investigated. Other studies involve the role of the

PH domains of proteins that lack FYVE domains. *Arabidopsis thaliana* AtPH1 is a late endosomal, vacuolar, and multivesicular body protein that regulates vacuolar metal transporter localization through the interaction of its PH domain with PtdIns3P (Agorio et al., 2017). *A. thaliana* SEC3 is a PH domain protein that binds membrane PtdIns(4,5)P_2, which is required for pollen tube growth and provides the site of pollen germination (Bloch et al., 2016). Indeed, SEC3 function might be

Table 2.2 Different modules found in fungi and plant PH- and FYVE domain-containing proteins.

Life forms	***UniProt ID***	***gene***	***Organism***	***Proteins***	***Modules***
Fungi	A0A2Z6RA14_9GLOM	*rclhr1_00310010*	*Rhizophagus clarus*	Uncharacterized protein	RhoGEF; PH; FYVE
	A0A1Y2IJD5_PYCCO	*pyccodraft_557045*	*Trametes coccinea*	FYVE-domain-containing protein	FYVE
	A0A1Y1YYR8_9FUNG	*k493draft_311810*	*Basidiobolusmeristosporus*	Uncharacterized protein	PH; FYVE
	A0A2T2PC27_CORCC	*bs50draft_567895*	*Corynespora cassiicola*	*Vacuolar protein sorting-associated protein 27*	VHS; FYVE; UIM
	A0A507BWX7_9FUNG	*smjel517_g06146*	*Synchytrium microbalum*	*alpha-1,2-Mannosidase*	RhoGEF; Glycosyl hydrolase family 47
	A0A197JU28_9FUNG	*k457draft_126461*	*Linnemannia elongate*	*Uncharacterized protein*	RhoGEF; FYVE
	F8Q904_SERL3	*serla73draft_170932*	*Serpula lacrymans*	*Uncharacterized protein*	RhoGEF; FYVE
	A0A1Y1UY75_9FUNG	*bcr36draft_362273*	*Piromyces finnis*	*Ankyrin*	Ankyrin repeats; FYVE; Ring finger
Plants	A0A015IGQ3_RHIIW	*rirg_217080*	*Rhizophagus irregularis*	*Rom2p*	RhoGEF; PH; FYVE
	A0A177WRZ8_BATDL	*bdeg_26098*	*Batrachochytrium dendrobatidis*	*Uncharacterized protein*	RhoGEF; PH

F4P650_BATDJ	*batdedraft_35443*	*Batrachochytrium dendrobatidis*	*Uncharacterized protein*	RhoGEF; FYVE
A0A507EFA4_9FUNG	*phcbs80983_g00823*	*Powellomyces hirtus*	*Uncharacterized protein*	RhoGEF; PH; FYVE
A0A1Y2IJD5_PYCCO	*pyccodraft_557045*	*Trametes coccinea*	*FYVE-domain-containing protein*	FYVE
A0A6A6HW15_9PLEO	*bu26draft_494685*	*Trematosphaeria pertusa*	*Vacuolar protein sorting-associated protein 27*	VHS; FYVE; UIM
A0A6A6V864_9PLEO	*m011draft_469719*	*Sporormia fimetaria*	*Vacuolar protein sorting-associated protein 27*	FYVE; UIM
tr|A0A0D7A3M6|A0A0D7A3M6_9AGAR	*fishedraft_67225*	*Fistulina fimetaria*	*Uncharacterized protein*	RhoGEF; FYVE
tr|A0A1Y1UY75|A0A1Y1UY75_9FUNG	*bcr36draft_362273*	*Piromyces finnis*	*Ankyrin*	Ankyrin repeats; FYVE; Ring finger

restricted by subcellular localization since the protein can be regulated by both PtdIns(4,5)P_2-dependent and -independent mechanisms (Bloch et al., 2016). Moreover, plant enzymes bear PH domains, as they occur with the phosphatidylinositol-4-kinase, which produces PtdIns4P, the most abundant phosphoinositide required for the control of cell membrane identity (Simon et al., 2016).

Animals

In animals, the polyD motif is encoded in Phafin2 and other related PH-, FYVE-, or polyD-containing proteins (**Files S1 and S2**). Our analysis focused on the presence of Phafin2 and other related PH-, FYVE-, or polyD-containing proteins in nonhuman mammals, since they are the closest relatives to humans. In nonhuman mammals, the polyD motif is consistently found at the C-terminus, downstream of both the PH and FYVE domains. However, PH domain-containing

proteins that lack both FYVE and polyD modules were also identified (**Figure 2.4**). In addition, the squalene synthase catalytic activity and the presence of a RhoGEF domain are found in some proteins with PH, FYVE, and polyD modules (**Table 2.3**, **Figure 2.4**, and **Files S3** and **S4**). In mammals, membrane bound squalene synthases are associated with the cholesterol biosynthetic pathway (Lello et al., 2005). RhoGEF domains, which regulate the function of GTPases, are predominantly found in tandem with the Dbl-homology (DH), PH, and FYVE domains leading to a wide range of cellular activities (Cerione & Zheng, 1996; Fort & Blangy, 2017).

About 170 homologous Phafin2 sequences are associated with humans. Unlike PH-, FYVE-, or polyD-containing proteins, the polyD motif is encoded together with the PH and FYVE domains in Phafin1 and 2 (**File S4**). Phafin1 (PLEKHF1; **Figure 4**) shares a high sequence similarity with Phafin2, but it presents an additional tail of 27 amino acids downstream of the polyD motif (Lin et al., 2012).

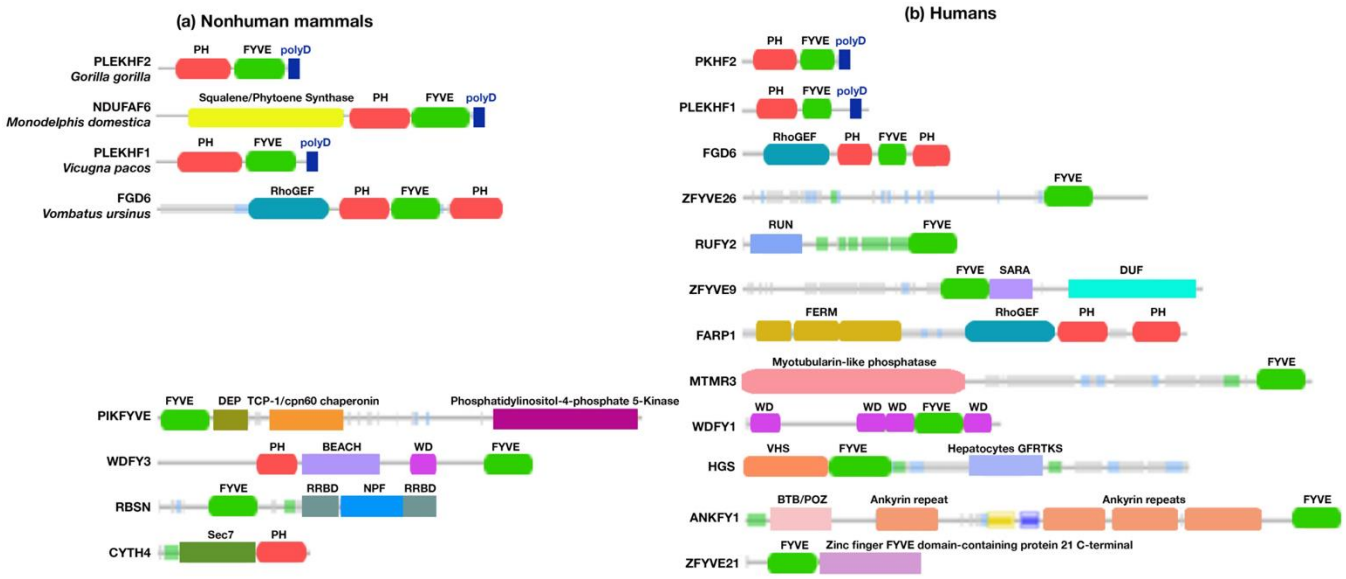

Figure 2.4 Schematic representation of PH-, FYVE-, and polyD-containing modules found in proteins from nonhuman mammals and humans. Other unique domains are also shown.

Phafin1 is not only involved in autophagosome formation (Lin et al., 2012), but is also linked to apoptosis (Li et al., 2007), caveolae-associated endocytosis, bacteria removal in macrophages, and the induction of innate immunity (Li et al., 2019). Human FYVE domains are often associated with other domains rather than PH domains (**Table 2.3**, **Figure 2.4**, and **File A3**). Other polyD-free PH- and FYVE-containing proteins contain alternative tandem modules, such as RhoGEF-PH-FYVE-PH, FERM N-terminal FERM-central-FERM C-terminal-PH-like-FERM adjacent, RhoGEF-PH-PH, RUN-FYVE, myotubularin-like phosphatase-FYVE, WD-G-β repeat-WD-G-β repeat-WD-G-β repeat-FYVE-WD-G β repeat, VHS-FYVE-hepatocyte growth factor-regulated tyrosine kinase substrate (GFRTKS), ankyrin repeats-FYVE, FYVE-Zinc finger FYVE domain-containing protein 21 C-terminus, FYVE-DEP-TCP-1/cpn60 chaperonin family-PtdIns4P 5-kinase, PH-BEACH-WD-G-β repeat-FYVE, FYVE-Rabenosyn Rab binding domain (RRBD)-Rabosyn-5 repeating NPF sequence-motif-RRBD, and Sec7-PH domain modules. PH domains are predominantly found in human proteins that lack both FYVE domains and polyD motifs and alternative mechanisms of regulation of PH domains are proposed (Nagel et al., 1998). Phosphoinositide synthesis and turnover as well as post-translational modifications are the most common mechanisms for modulation of PH domain function, as occurs

Table 2.3 Different modules found in mammalian Phafin2 and PH-, FYVE-, and polyD-containing proteins.

UniProt ID	***gene***	***Organism***	**Protein**	***Modules***
G3RXJ3_GORGO	*plekhf2*	*Gorilla gorilla*	Pleckstrin homology and FYVE domain containing 2	PH; FYVE; polyD
F6Q9Z2_MONDO	*ndufaf6*	*Monodelphis domestica*	Uncharacterized protein	Squalene/phytoene synthase; PH; FYVE, polyD
A0A6J0AU76_VICPA	*plekhf1*	*Vicugna pacos*	pleckstrin homology domain- containing family F member 1	PH; FYVE; polyD

A0A4X2MDN9_VOMUR	*fgd6*	*Vombatus ursinus*	FYVE, RhoGEF and PH domain containing 6	RhoGEF; PH; FYVE
PKHF2_HUMAN	*pkhf2*	*Homo sapiens*	Pleckstrin homology domain-containing family F member 2	PH; FYVE; polyD
K7ELB8_HUMAN	*plekhf1*	*Homo sapiens*	Pleckstrin homology domain-containing family F member 1	PH; FYVE; polyD
A4FVC4_HUMAN	*fgd6*	*Homo sapiens*	FGD6 protein	RhoGEF; PH; FYVE
G3V2D8_HUMAN	*zfyve26*	*Homo sapiens*	Zinc finger FYVE domain-containing protein 26	FYVE
RUFY2_HUMAN	*rufy2*	*Homo sapiens*	RUN and FYVE domain-containing protein 2	RUN; FYVE
ZFYV9_HUMAN	*zfyve9*	*Homo sapiens*	Zinc finger FYVE domain-containing protein 9	FYVE; SARA; DUF
FARP1_HUMAN	*farp1*	*Homo sapiens*	FERM, ARHGEF and pleckstrin domain-containing protein 1	FERM N-terminal; FERM central; FERM C-terminal PH-like; FERM adjacent (FA); RhoGEF; PH
G5E953_HUMAN	*mtmr3*	*Homo sapiens*	Phosphatidylinositol-3-phosphate phosphatase	Myotubularin-like phosphatase; FYVE
WDFY1_HUMAN	*wdfy1*	*Homo sapiens*	WD repeat and FYVE domain-containing protein 1	WD, G-b repeat; FYVE
A0A7I2YQD1_HUMAN	*hgs*	*Homo sapiens*	Hepatocyte growth factor-regulated tyrosine kinase substrate	VHS; FYVE; Hepatocyte growth factor-regulated tyrosine kinase substrate
ANFY1_HUMAN	*ankfy1*	*Homo sapiens*	Rabankyrin-5	Ankyrin repeats; FYVE
ZFY21_HUMAN	*zfyve21*	*Homo sapiens*	Zinc finger FYVE domain-containing protein 21	Zinc finger FYVE-containing protein 21 C-terminus

FYV1_HUMAN	*pikfyve*	*Homo sapiens*	1-phosphatidylinositol 3-phosphate 5-kinase	FYVE; DEP; TCP-1/cpn60 chaperonin family; PtdIns4P 5-kinase
A7E293_HUMAN	*wdfy3*	*Homo sapiens*	WDFY3 protein	PH domain associated with BEACH; BEACH; WD; G-b repeat; FYVE
RBNS5_HUMAN	*rbsn*	*Homo sapiens*	Rabenosyn-5	FYVE; Rabenosyn Rab binding; Rabenosyn-5 repeating NPF sequence-motif
CYH4_HUMAN	*cyth4*	*Homo sapiens*	Cytohesin-4	Sec7; PH

with mTOR proteins (Liu et al., 2015). Interestingly, an alternative mechanism has been reported for the human PH-containing protein Cytohesin-4, which is involved in vesicular trafficking pathways (Venkateswarlu, 2003). Cytohesin-4 contains a tandem of Sec7-PH modules, and instead of a polyD motif, presents an N-terminal coiled-coil domain that autoinhibits PH domain binding to membrane phosphoinositides (Hiester & Santy, 2013). Likewise, the PH domain-containing oxysterol binding proteins (OSBPs) are cholesterol transfer proteins that modulate the Golgi apparatus organization and function (Ngo & Ridgway, 2009). It has been proposed that, in the absence of sterols, the OSBP sterol-binding domain intramolecularly interacts with the PH domain, inhibiting PtdIns4P binding and restricting the protein at the endoplasmic reticulum (Mohammadi et al., 2001). Sterol binding promotes an open conformational state in OSBP, which relieves the autoinhibition of its PH domain consequently facilitating binding to Golgi apparatus membranes *via* PtdIns4P interactions (Ngo & Ridgway, 2009). A recent study estimates that about 50% of human PH domain-containing proteins do not bind phosphoinositides (Singh et al., 2020). For example, the human FERM, ARH/RhoGEF, and pleckstrin domain protein 1 (FARP1) protein

displays a modular FERM N terminal-FERM central-FERM C terminal PH-like-FERM adjacent-RhoGEF-PH-PH modular unit. Membrane lipid binding of FARP1 appears to be FERM domain-dependent but PH domain-independent (Kuo et al., 2018). The second PH domain of FARP1 has been suggested to intramolecularly modulate the binding of the first PH domain and the DH domain to Rho GTPases (He et al., 2013).

The presence of a polyD motif in Phafin2 and its orthologues facilities a unique regulatory mechanism of PH domains. Thus, the Phafin2 C-terminal polyD motif downregulates the binding of the PH domain to PtdIns3P (Tang et al., 2020), impairing spurious plasma membrane targeting (Schink et al., 2021). Phafin2 forms a complex with lysosomal PtdIns3P and AKT, inducing autophagy (Matsuda-Lennikov et al., 2014). AKT presents an N-terminal PH domain that binds PtdIns(3,4,5)P_3 with high affinity (Landgraf et al., 2008). Similar to that found in Phafin2, the C-terminal tail of AKT is enriched in acidic residues, which can be phosphorylated at Ser473, Ser477, and Thr479. Ser473 phosphorylation is required to displace the PH domain from the kinase domain, leading to an increase of its catalytic activity (Chu et al., 2018). On the other hand, phosphorylation of the AKT C-terminal acidic tail at Ser477 and Thr479 is required to reduce the affinity of the PH domain for PtdIns(3,4,5)P_3 (Chu et al., 2018), by targeting the PH domain C-terminal α-helix (Chu et al., 2020). Interestingly, the soy bean-derived peptide Lunasin, which contains a C-terminal polyD motif, inhibits the anti-autophagic phosphorylation of AKT at Thr308 and Ser473 (Shidal et al., 2017), a cellular process required for the binding of AKT to PtdIns(3,4,5)P_3. Other PH domains can be modulated by poly acidic regions. For example, the cell cycle transcription factor DP1 contains an acidic region that binds to the PH domain of the p62 subunit of the transcription factor IIH, serving as a mechanism of transcriptional activation (Okuda et al., 2016). However, it is not known whether this association controls p62 PH domain membrane

binding through PtdIns3P and PtdIns5P (Lello et al., 2005). Thus, it is possible that poly acidic regions regulate the activity of PH domains, independent of the presence of a FYVE domain.

c) The presence of Phafin2 or related proteins in model organisms and their structural features

The study also extended to different nonmammalian model organisms, such as *Arabidopsis thaliana*, *Saccharomyces cerevisiae* (yeast), *Danio rerio* (zebrafish), *Drosophila melanogaster* (fruit fly), *Xenopus tropicalis* (western clawed frog), and *Caenorhabditis elegans* (nematode worm). Unlike *A. thaliana*, *S. cerevisiae*, *D. melanogaster*, *X. tropicalis*, and *C. elegans*, our search indicates that only *D. rerio* contains a gene encoding a polyD motif together with the PH-FYVE modules.

Data analysis indicates that in *Arabidopsis* species, we found a similar pattern of PH- or FYVE modules to those described before for other plants and fungal species. Both PH and FYVE domains were found in *Arabidopsis*, but never encoded together. Consistent with that found in plants, the polyD motif is absent in *Arabidopsis*. The mostly abundant domain modules were FYVE-, PH-, Las17-binding protein actin regulator-FYVE, phosphatidylinositol-4-phosphate-5-kinase-TCP-1/cpn60 chaperonin family-FYVE, BAR domain of APPL family, putative GTPase-activating protein for Arf, and ankyrin repeats. The autophagy-linked FYVE (Alfy) protein contains a tandem of FYVE, BEACH, and WD40 modules, allowing association to the autophagy receptor p62 and PtdIns3P (Clausen et al., 2010; Filimonenko et al., 2010). Interestingly, *Arabidopsis* Alfy-like proteins contain both PH and BEACH domains, but lack a FYVE domain (Chung, 2019; Teh et al., 2015). In *A. thaliana*, the BEACH domain-containing proteins (BDCPs) are expressed by the gene *spirrig* (SPI) (De Lozanne, 2003; Saedler et al., 2009). Plant BDCPs are known to initiate membrane-dependent cellular processes (Saedler et al., 2009). SPI contains a tandem of PH-

BEACH domains followed by five WD40 repeats. Unexpectedly, the SPI PH domains do not bind phospholipids (Steffens et al., 2017), suggesting that binding of SPI to endosomal membranes is phospholipid-independent. About fifteen genes encoding FYVE domain-containing proteins are reported in the *Arabidopsis* genome (Wywial & Singh, 2010), where thirteen of them do not have homologous sequences in yeast and mammals (Shen et al., 2020). Two *Arabidopsis* FYVE domain-containing PtdIns(3,5)-kinases, FAB1A and FAB1B, play a significant role in the plant cellular environment, and depletion of their expression leads to pollen abortion, delayed endocytosis, acidification, or abnormal vacuole formation (Hirano et al., 2011; Shen et al., 2020; Whitley et al., 2009). The plant FYVE-domain protein FREE1 has been reported for cargo membrane protein sorting, vacuole formation, multivesicular body biogenesis, and autophagic-mediated degradation (Gao et al., 2014; Gao et al., 2015; Kolb et al., 2015). Similarly, the cell death-related endosomal FYVE/SYLF protein 1 (CFS1) was found to be associated with endosomal trafficking and autophagy (Sutipatanasomboon et al., 2017).

Both PH and FYVE domains were found to be encoded together without a polyD motif in *D. melanogaster*. FYVE domain encoded with RUN, WD, phosphatidylinositol-4-phosphate 5-kinase, Rabenosyn Rab binding domain, and VHS were frequently found in *Drosophila,* and these multimodular proteins are mainly associated with membrane trafficking, endocytic cargo transport, and autophagy (Kamalesh et al., 2017; Kitagishi & Matsuda, 2013; Mao et al., 2000; Morrison et al., 2008; Nocker & Ludwig, 2003). No PH domains nor polyD motifs were identified in *S. cerevisiae*. The FYVE domain was found to be encoded in proteins with phosphatidylinositol-4-phosphate 5-kinase, VHS, UIM, and Rabenosyn Rab binding domains, in agreement with our findings in other fungi species. *D. rerio* is the only model organism that we identified as bearing a protein (NP_956538) with a polyD motif at the C-terminus, downstream of the PH and FYVE

modules. The sequence of NP_956538 was stored in the UniPort database as the part of NIH - Zebrafish Gene Collection (ZGC) project. Despite we did not find any experimental study associated with a Phafin2 expressed in *D. rerio*, the *Gene Ontology annotation of EMBL-EBI* suggested its function as human Phafin2, that is, as a phosphatidylinositol binding, and associated with early endosomes (Gaudet et al., 2011). In zebrafish, other functional domains, such as RUN, RhoGEF, WD, phosphatidylinositol-4-phosphate 5-kinase, Rabenosyn Rab binding, myotubularin-like phosphatase, and Beige/BEACH domains were identified to be encoded with PH, FYVE, or PH-FYVE modules (**Figure 2.5**).

In *C. elegans*, we found a protein (NP_499183) that encodes PH and FYVE domains together, but it lacks the polyD motif. Despite this protein was stored at the NCBI database as an uncharacterized protein (Consortium*, 1998), the presence of both PH and FYVE domains suggests similar Phafin2 function. Other proteins are PH-free but with a FYVE domain encoded together with VHS, RhoGEF, phosphatidylinositol-4-phosphate 5-kinase, myotubularin-like phosphatase, rabenosyn Rab binding, WD and Beige/BEACH domains (Figure 2.5 and File S6). The *blastp (protein-protein BLAST) and* PSI-BLAST (Position-Specific Iterated BLAST) search did not show any PH-, FYVE-, or polyD-containing proteins in *X. tropicalis*. However, homologues of ALFY were reported in *X. tropicalis*, with a role in assisting autophagic progression by selectively degrading protein aggregates (Reinhart et al., 2021). The FYVE domain containing protein EEA1 was also reported in *X. tropicalis* (Posiri et al., 2019). A homologue of human ANKFY1 protein was found in *X. tropicalis*, a FYVE domain-

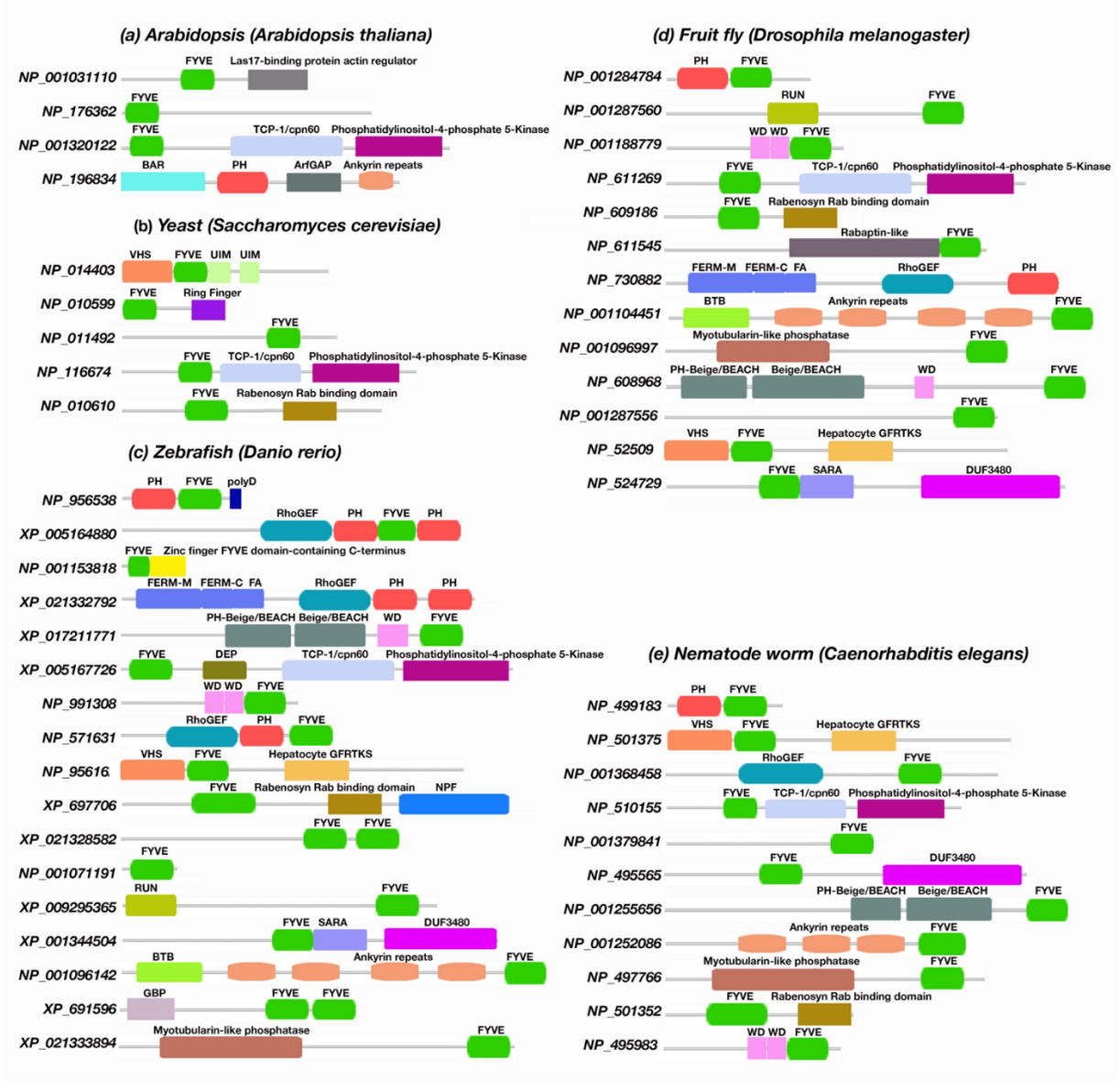

Figure 5. Availability of PH-, FYVE-, and polyD-containing modules in model organisms. (a) *Arabidopsis thaliana*, (b) *Saccharomyces cerevisiae*, (c) *Danio rerio*, (d) *Drosophila melanogaster*, and (e) *Caenorhabditis elegans*.

containing protein involved in a variety of cellular functions, including Rab5 regulation, motor function, and early endosomal maturation (Hermle et al., 2018; Maekawa et al., 2017).

d) Protein-protein network and functional lineage of human Phafin2

The protein-protein interaction network of human Phafin2 was retrieved using the STRING database, and proteins involved in this network were analyzed for their reported functions. The proteins AKT1, AKT2, AKT3, Beclin-1 (BECN1), PtdIns3P 5-kinase (PIKFYVE), PHD finger protein 20-like protein 1 (PHF20L1), neuronal acetylcholine receptor subunit □-2 (CHRNA2), lysosomal-associated transmembrane protein 4B (LAPTM4B), coiled-coil domain-containing protein 24 (CCDC24), and Kelch-like family member 33 (KLHL33) were found in the protein-protein network of human Phafin2 (**Figure 2.6**). Interestingly, several of these proteins participate in autophagy. Both AKT1 and AKT2 are found to interact with Phafin2, and interaction of AKT2 with Phafin2 plays a critical role in the induction of lysosomal autophagy (Matsuda-Lennikov et al., 2014). BECN1 is also considered a key player in autophagy, facilitating the PI3K complex formation, leading to multiple membrane trafficking pathways and the initiation of autophagosome formation (Liu et al., 2016). BECN1 stimulates the aggregation of cofactors to generate the BECN1–PIK3C3–PIK3R4 complex, which induces the autophagy protein cascade (Han et al., 2018). It is not currently known whether Phafin2 participates in this process. In addition, BECN1 was found to be in complex with VPS34 and AMBRA1 for autophagy induction and that downregulation of AMBRA1 limits the function of BECN1 in the induction complex (Fimia et al., 2007). LAPTM4B, another member of Phafin2 protein network, plays a significant role in lysosomal function in autophagy. Lower levels of LAPTM4B cause impairment of the autophagosome–lysosome fusion and decreasing of autophagy (Usman et al., 2021; Vergarajauregui et al., 2011).

Other predicted Phafin2-associated proteins are involved in functions outside of autophagy. For example, PIKFYVE participates in endosomal cargo transport, endomembrane homeostasis, and

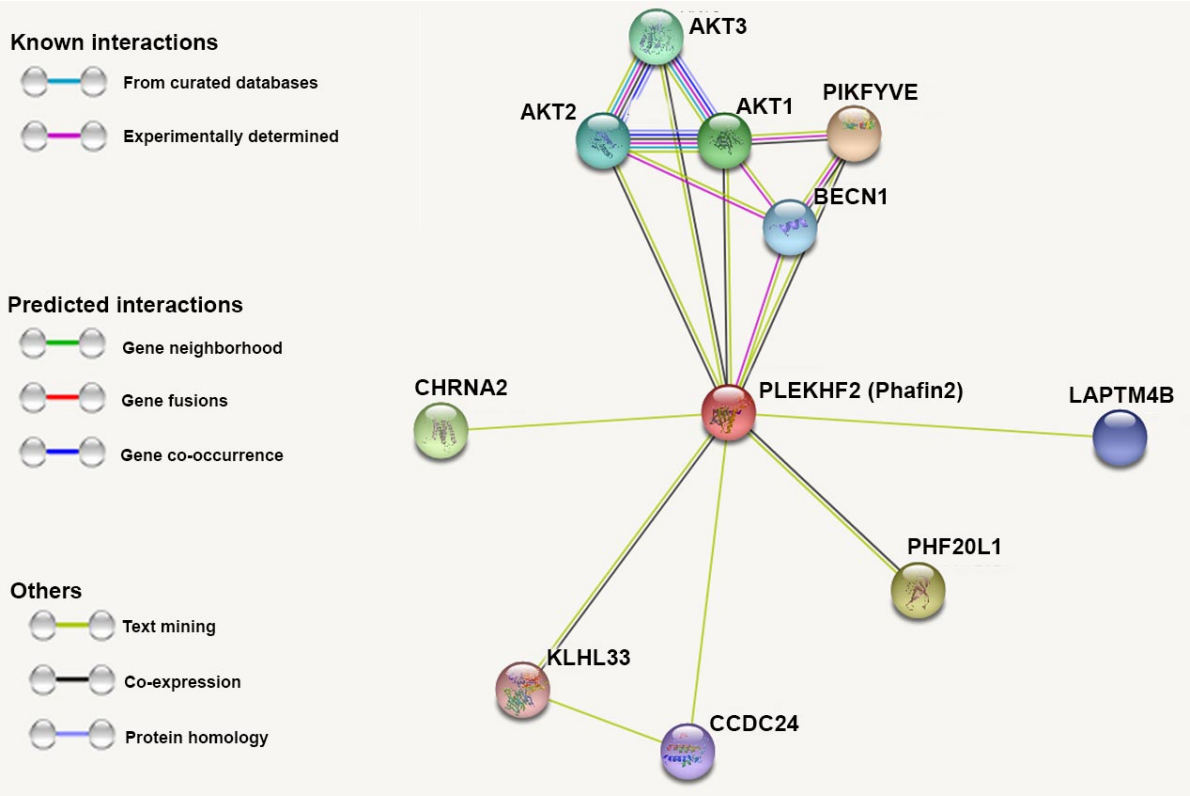

Figure 2.6. The protein-protein interaction network of human Phafin2 was generated using the STRING database. The color of the lines connecting the proteins indicates the typology of the protein–protein interaction. BECN1, Beclin-1; PIKFYVE, PtdIns3P 5-kinase; PHF20L1, PHD finger protein 20-like protein 1; CHRNA2, neuronal acetylcholine receptor subunit α-2; LAPTM4B, lysosomal-associated transmembrane protein 4B; CCDC24, coiled-coil domain-containing protein 24; KLHL33, Kelch-like family member 33.

lysosomal trafficking (Desale & Chinnathambi, 2021; Karabiyik et al., 2021). This prediction aligns with early work on Phafin2 function, in which the protein was associated with cargo trafficking and endosomal and lysosomal function (Lin et al., 2010; Pedersen et al., 2012). Unrelated protein networks include the prediction of Phafin2 association to PHF20L1, which interacts with proteins that are mono-methylated in their lysine residues, and is crucial for

transcriptional repression (Carr et al., 2017). However, methylations have not been yet reported on Phafin2. Another predicted Phafin2-interacting protein is CHRNA2, which is linked with the hippocampus-dependent learning and memory processing (Okada, 2021; Sharp & Brannigan, 2021). Interestingly, CHRNA2 and Phafin2 are common targets of the androgen receptor upon binding with androgen; however, the receptor represses CHRNA2 expression but upregulates Phafin2 transcription (Chen et al., 2010). CCDC24, which is linked to hyperactivity disorder (Qi et al., 2019) and Kelch-like protein 33, which is associated with neuronal proteins and linked with Mendelian diseases (Dhanoa et al., 2013), are proteins of unknown function. Nonetheless, except for AKTs, none of the predicted Phafin2-interacting proteins have yet been reported to physically contact Phafin2. Thus, this protein network remains to be confirmed experimentally.

2.5 Conclusions

Whereas many functions have been characterized for the PH and FYVE domains of Phafin2, there is limited information about the role of the C-terminal polyD motif in the protein. In this bioinformatics analysis, we primarily focused on the evolutionary history and functional lineage of the polyD motif in Phafin2 and other related PH-, FYVE-, or polyD-containing proteins. Interestingly, the polyD motif was absent in PH-, FYVE-, or both PH-FYVE-containing proteins of bacteria, archaea, fungi, and plants. Moreover, PH domains were not found in tandem with FYVE domain-containing proteins in bacteria. Thus, the FYVE domain might be the ancient functional domain of PH-, FYVE-, and polyD-containing proteins. The PH domain emerged for the first time in archaea species, and later, it was distributed among the different life forms with other protein domains. The polyD motif is exclusively found in animals. However, functional diversity of Phafin2 and other related PH-, FYVE-, or polyD-containing proteins is more consistent

in eukaryotic organisms including in cellular trafficking, autophagy, membrane remodeling, apoptosis, signal transduction, and transcription regulation. Interestingly, human homologues of Phafin2 possess few additional functional modules (WD, BEACH, Rabosyn-5 repeating NPF sequence-motif, and Sec7), which are only found in eukaryotes. Phafin2 was predicted to interact with AKT1, AKT2, and AKT3, which are known as Phafin2 partners, BECN1, and LAPTM4B, and that all these proteins are linked to autophagy. Other putative Phafin2 interactors, such as PIKFYVE, contributes to endosomal cargo transport, endomembrane homeostasis, and lysosomal trafficking, whereas others such as CHRNA2, CCDC24, and KLHL33, are associated with neuronal functioning. In closing, the polyD motif likely displays a functional association with both the PH and FYVE domains in animal Phafin2 proteins. Although the C-terminal polyD motif of Phafin2 regulates binding of the PH domain to PtdIns3P and provides membrane specificity, it remains unknown, and intriguing, whether this acidic region controls the function of other PH domain-containing proteins.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/membranes12070696/s1, Table S1. Different modules found in bacterial and archaea PH- and FYVE domain-containing proteins. Table S2: Summary of homologs of human Phafin2. Figures S1–S6: Phafin2-related protein sequence alignments using Jalview; Files S1–S6.

Chapter 3: Autoinhibitory Mechanism of Phafin2

3.1 Abstract

Autoinhibition is a regulatory mechanism in many proteins and is vital for maintaining their functionality. Phafin2 is an adaptor protein involved in diverse cellular processes, and here, we investigated Phafin2's autoinhibitory mechanism mediated by the conserved C-terminal polyD motif. Using Nuclear Magnetic Resonance (NMR) spectroscopy and bioinformatics analysis, we identified critical amino acid residues within the PH domain responsible for interaction with the polyD motif. Residues Gly38, Lys45, Lys51, Ala52, and Arg53, within the PH domain, form a positively charged binding pocket that can accommodate the negatively charged polyD motif. Further, using site-directed mutagenesis. We demonstrated the requirement of Lys51 and Arg53 for the PH domain-polyD interaction through isothermal titration calorimetry. Additionally, our study explores the membrane binding properties of Phafin2 and indicates that its interaction with membranes is independent of its curvature, highlighting the Phafin2 relevance across various membranous compartments. Overall, our findings shed light on the molecular mechanisms underlying Phafin2 autoinhibition and provide insights into its functional regulation of the protein in membrane-associated cellular processes.

3.2 Introduction

Autoinhibition is a self-regulatory mechanism conserved in many proteins. It is commonly observed that two domains or functional sites of a protein interact to maintain a balance between functional and non-functional states, thereby regulating protein functions. Post-translational modifications, interactions with molecules, irreversible proteolysis, or changes in environmental factors, can also facilitate this event (Khan & Goult, 2019; Pufall & Graves, 2002). The accuracy of autoinhibitory coordination is vital for maintaining a protein molecule's cellular functions. Any change or mutation interfering with this regulation can disrupt functional harmony, leading to profound health complexities. For example, tyrosine-kinase Src (c-Src) is an autoinhibitory protein whose disruption can be oncogenic (Boggon & Eck, 2004).

PH domain-containing Protein kinase B or Akt is an excellent example of an autoinhibitory protein. Akt has an N-terminal pleckstrin homology (PH) domain, a kinase domain (KD) in the middle, and a C-terminal hydrophobic motif. The PH domain of Akt interacts with its KD domain, leading to the negative regulation of Akt kinase activity. However, Akt activation occurs when the PH domain binds to increased PtdIns(3,4,5)P_3 levels at the plasma membrane. This binding causes a conformational change in Akt, and it becomes available for the PDK1 (phosphoinositide-dependent protein kinase 1) and mTORC2 (mechanistic target of rapamycin complex 2), which phosphorylate Akt at Thr308 and Ser473, respectively (Nam et al., 2018; Ebner et al., 2017; Yudushkin, 2020). Being an adaptor protein, Phafin2 contributes to endosomal cargo trafficking (Nina et al., 2012), macropinocytosis (Schink et al., 2017; Tan et al., 2021), and autophagy (Matsuda-Lennikov et al., 2014). Interaction between Phafin2 and PtdIns(3)P plays a significant role in recruiting Phafin2 in the membrane of specific cellular organelles. Though the Phafin2

FYVE domain constitutively interacts with PtdIns(3)P, the PH domain cannot make the interaction with PtdIns(3)P because the C-terminal polyD motif blocks it (Tang et al., 2020). In autoinhibitory proteins, intrinsically disordered regions (IDRs) contribute to autoinhibition alongside catalysis, binding affinity, and the regulation of oligomerization (Basu et al., 2020; Mol et al., 2004; Pedersen et al., 2012). The function of IDRs usually relies on binding to a structured domain, which might compete with another binding partner or trigger allosteric changes elsewhere in the structure (Boggon & Eck, 2004; Chakrabarti & Chakravarty, 2022; Trudeau et al., 2013). Phafin2 has about 40% of its structure made up of disordered or random coil regions including a flexible C-terminal polyD (eight repeated aspartic acidic residues and two serine residues) tail (Tang et al., 2017). Having this disordered region is indicative of the autoinhibitory mechanism through intramolecular interactions. Enriched intrinsic disorder regions are also consistent with other proteins having an autoinhibitory mechanism (Trudeau et al., 2013). It has already been reported that the conserved C-terminal acidic motif autoinhibits Phafin2 binding to PtdIns3P (Tang et al., 2020). Now, the question is, what regions or amino acid residues of the PH domain are responsible for this interaction?

This chapter discusses the critical amino acids required for the binding between Phafin2, the PH domain, and its conserved C- C-terminal motif. We employed nuclear magnetic resonance spectroscopy (1H-15N Heteronuclear Single Quantum Coherence) and a Multiple Sequence Alignment study to reveal the critical amino acids needed for the intramolecular inhibitory mechanism in Phafin2. We also discussed whether Phafin2's ability to bind to membranes depends on the curvature of the membrane. In recent years, membrane remodeling research has focused on its association with membrane trafficking and regulating flux through subcellular compartments

(Knævelsrud et al., 2013; Legendre-Guillemin et al., 2004). We utilized the liposome co-sedimentation assay with Phafin2 constructs to evaluate Phafin2's capacity for inducing membrane curvature across liposomes of varying sizes.

3.3 Materials and Methods

a) Materials

The synthetic polypeptide of the human Phafin2 C terminal polyD (residues 240-DDDDDDDSSD-249) region was purchased from Biomatik Corporation (Canada), which allowed for ITC and NMR experiments for the study.

b) Protein expression and purification

The human full-length Phafin2 (residues 1-249) cDNA was inserted into a pGEX4T3 vector (Cytiva). The cDNA encoding the Phafin2 PH domain (residues 1-135) was placed into a pGEX6P1 vector, while the cDNA encoding the Phafin2 FYVE domain (residues 145-212) was inserted into a pGEX4T3 vector. Recombinant proteins were produced in *Escherichia coli* (Rosetta; Stratagene) cells. Bacterial cells were cultured in Luria-Bertani media at 37°C until they reached an optical density of approximately 0.8. Induction of the glutathione S-transferase (GST) fused Phafin2, Phafin2 PH, or Phafin2 FYVE was initiated by adding 1 mM isopropyl β-D-1-thiogalactopyranoside followed by a 3-hour incubation at 25°C. After centrifugation at a *g*-force of 5163 RCF (5500 RPM), the cell pellets were stored at -80°C. The pellets were resuspended in the extraction buffer (50 mM Tris, 500 mM NaCl; pH 7.3). Various chemicals including 5 mM benzamidine, 1 mL lysozyme, 1 mM dithiothreitol (DTT), and 1-2 mL of 10% Triton X-100 were added to the resuspended pellets. Following sonication and centrifugation at a *g*-force of 9,960 RCF (12000 RPM), the supernatant was incubated with glutathione beads on a rocker at 4°C

for 1-2 hours. After subsequent washing and further processing, overnight digestion with protease (Thrombin for pGEX4T3 or HRV-3C for pGEX6P1) was done at 4°C. Protein sample was further loaded onto a Superdex 75 column (S75), equilibrated with a buffer solution of 50 mM Tris-HCl and 1M NaCl at pH 7. Fractions containing highly purified proteins (Phafin2/PH/FYVE) were pooled, concentrated and measured for further steps. . Protein concentrations were measured using a Nanodrop instrument (Nanodrop One, Thermo Fisher Scientific, Waltham, MA), measuring absorbance at 280 nm. Additionally, SDS-PAGE was performed by comparing 10 µg of each protein sample with purified GST, serving as a reference.

c) Liposome Co-sedimentation Assay

Dioleoyl-phosphatidylcholine (DOPC) and phosphatidylinositol 3-phosphate (PtdIns3P) were used in liposome preparation. DOPC and PtdIns3P were resuspended in two solvent mixtures: one consisting of chloroform and methanol in a 1:1 ratio (DOPC) and another consisting of chloroform, methanol and water in a 65:35:8 ratio (PtdIns3P). DOPC was mixed with or without 10% PtdIns3P and dried overnight in a desiccator. A solution containing 20 mM HEPES (pH 7.3) and 100 mM NaCl was then added to the PtdIns3P-DOPC mixture and incubated for 1 h at 42°C. The PtdIns3P-DOPC mixture allowed five freeze-thawing cycles using liquid nitrogen, each lasting 10-15 s. Then, homogenous liposomes were obtained by extrusion process through 50, 200, and 400 nm membranes (Avanti Polar Lipids). For the experiment, two hundred microliters of liposomes, with a lipid concentration of ~2 mg/ml were incubated with 200 µl of untagged protein (20 µM) and incubated on a rocker for 1 h at room temperature. Protein-liposome complexes were subsequently fractionated via ultracentrifugation at a *g*-force of 68040 RCF (45,000 RPM and radius of the rotor; 3 cm) for 1 h at 20°C. Analysis of protein distribution between pellet and supernatant fractions

was performed using SDS-PAGE. The SDS gel outputs were employed by Image Lab software to analyze the intensity of the protein bands.

d) Isothermal titration calorimetry

To conduct the isothermal titration calorimetry (ITC), a MicroCal PEAQ instrument (Malvern Panalytical Inc.) was employed. The experiments were set at 25°C in a buffer comprising 20 mM HEPES (pH 7.0) and 100 mM NaCl. Dissolving the polyD peptide in the same buffer and adjusting it to pH 7.0 with protein sample preceded the experiments. Loading the ITC sample cell with 75 μM of Phafin2 PH or mutated Phafin2 PH facilitated titration with 750 μM polyD peptide from the syringe. The titration protocol included a single injection of 0.4 μL of polyD peptide followed by 13 injections of 3.0 μL each, with a 120-second interval between injections and a stirring speed of 750 rpm. Adjusting the raw titration data involved subtracting the heat changes from polyD peptide titrations to buffer. Fitting the binding curve to a one-site binding model was performed using the MicroCal PEAQ-ITC analysis software. The reported dissociation constant (K_D) value represents the average from three independent experiments.

e) Nuclear magnetic resonance spectroscopy

NMR experiments were conducted at 25°C using a Bruker Avance III 600 MHz NMR spectrometer (Virginia Tech) with a 5 mm z-gradient triple resonance probe. The ^{15}N-labeled Phafin2 PH domain (150 μM) was prepared in a solution consisting of 90% H_2O, 10% D_2O, 20 mM d_{11}-Tris-HCl (pH 7.0), 100 mM NaCl, 1 mM d_{18}-DTT, and 1 mM NaN_3. The polyD peptide, dissolved in the same buffer, was adjusted to pH 7.0. ^{1}H-^{15}N HSQC (heteronuclear single quantum coherence) spectra were acquired for the Phafin2 PH protein samples in the absence and presence of 1200 μM

Phafin2 polyD motif. Subsequently, the spectra were processed and analyzed using Mnova software (Mestrelab Research).

f) Multiple sequence alignment

The orthologue sequences of Phafin2 were retrieved from the UniProt database (Consortium, 2015). These sequences were employed for multiple sequence alignment by ClustalOmega (Sievers & Higgins, 2014). Alignment files were further checked manually, and conserved regions were identified and visualized using JalView software.

3.4 Results and Discussion

a) Phafin2-mediated membrane binding is not curvature-dependent.

In the study, different-sized liposomes were employed to mimic lipid bilayers. We used 50, 200, and 400 nm PtdIns3P-bound and PtdIns3P-free liposomes to incubate with Phafin2 and the Phafin2 FYVE domain. The bigger liposomes corresponded to the smaller lipid bilayer curvature and vice versa. The Phafin2 FYVE domain was used as a positive control since the FYVE domain can constitutively bind to PtdIns3P-bound liposomes (Tang et al., 2020) . The investigation showed no interaction between PtdIns3P-free liposomes and Phafin2 or FYVE, suggesting the critical role of PtdIns3P in the membrane binding of Phafin2. In the case of PtdIns3P-bound liposomes, binding of more than 90% was attained with either full-length Phafin2 or the FYVE domain. No significant variation was found in liposome size, as the binding was in a similar range in most cases (**Figure 3.1**). These findings suggested that Phafin2-mediated membrane binding is not curvature-dependent, indicating the availability of Phafin2 in both the early and later stages of the maturation process of any membrane-derived vehicles.

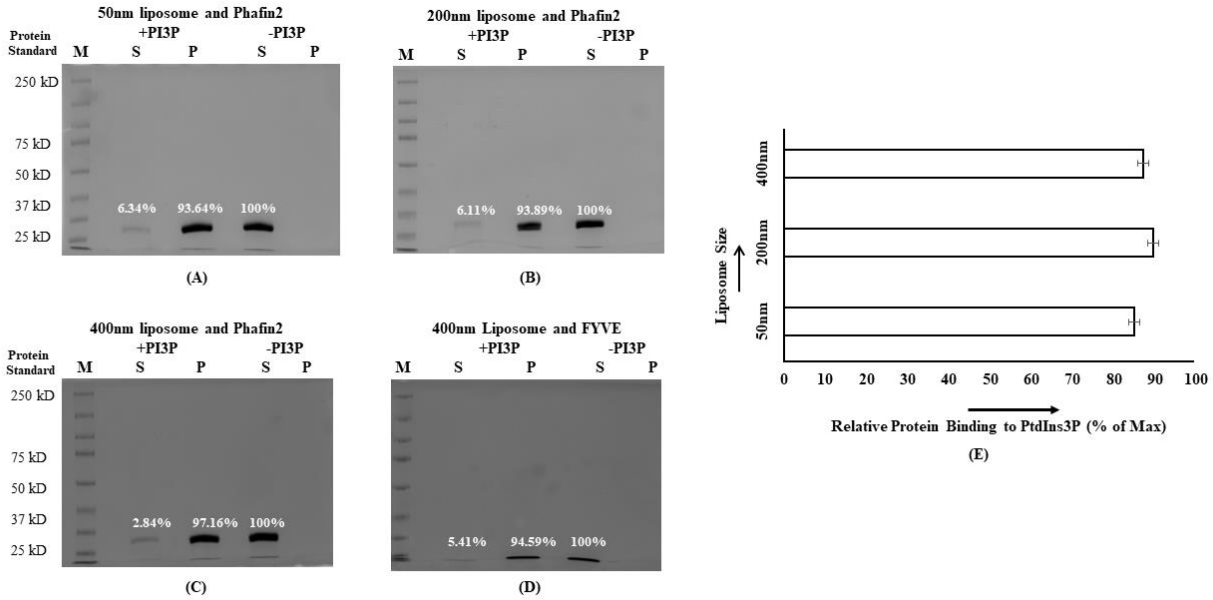

Figure 3.1 Phafin2-mediated membrane binding is not curvature-dependent. Different-sized liposomes, such as 50nm (A), 200nm (B), and 400nm (C), were incubated with Phafin2, and samples from pellets and supernatants were run at SDS-PAGE after ultracentrifugation. In addition, the FYVE domain was incubated with a 400nm liposome as a positive control (D). Image-Lab software calculated the relative protein binding to PtdIns3P liposomes and plotted it against the liposome size (E).

b) **Critical amino acids of Phafin2 PH domain required for binding to the polyD motif.**

It's been established that the FYVE domain of Phafin2 binds to endosomal PtdIns3P. However, the PH domain cannot do so because it is inhibited by the negatively charged polyD region, resulting in intramolecular autoinhibition. We employed two-dimensional NMR spectroscopy (^{1}H-^{15}N Heteronuclear Single Quantum Coherence) to determine the critical amino acid residues needed

to interact with the PH domain and the C terminal polyD region. We titrated 150 µM of ^{15}N-labeled Phafin2 PH domain with 1200 µM of unlabeled polyD peptide and compared this spectrum to only ^{15}N-labeled Phafin2 PH domain. Previously published ^{1}H, ^{15}N, and ^{13}C backbone resonance assignments of Phafin2 PH domain (Ellena et al., 2022) was used to locate each resonance. Upon comparing the spectra of the PH domain with and without the polyD peptide, significant perturbations were observed in specific signals of the PH domain when titrated with the polyD peptide **(Figure 3.2).**

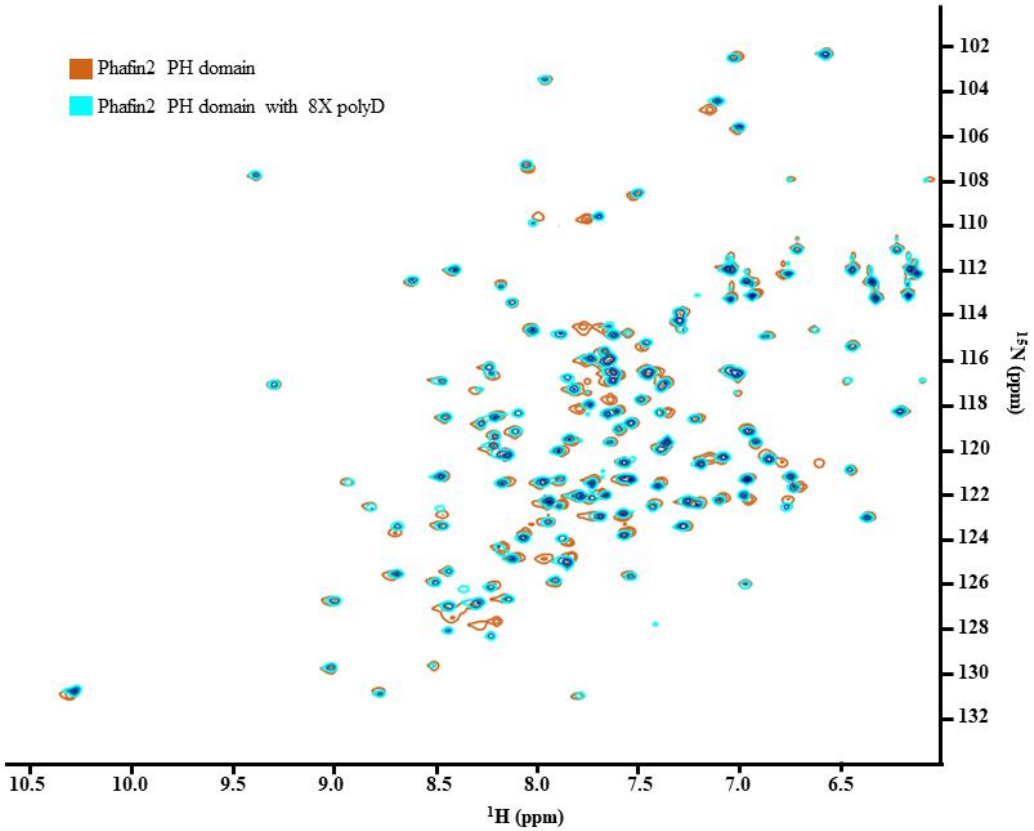

Figure 3.2 The superimposed ^{1}H-^{15}N HSQC spectra of the ^{15}N-labeled Phafin2 PH domain (cyan) and ^{15}N-labeled Phafin2 PH domain titrated with unlabeled polyD peptide (red).

We continued this experiment three times, calculated the average of differences, and considered the residues that consistently showed differences beyond the average of three experiments, including standard deviation. The results identified specific residues, including Gly38, Lys45, Lys51, Ala52, Arg53, Ile67, Ile90, Lys91, Thr104, Phe109, and Ala110. Gly38, Lys45, Lys51, Ala52, and Arg53 consistently exhibited perturbations across all experiments **(Figure 3.3 and Figure 3.4).**

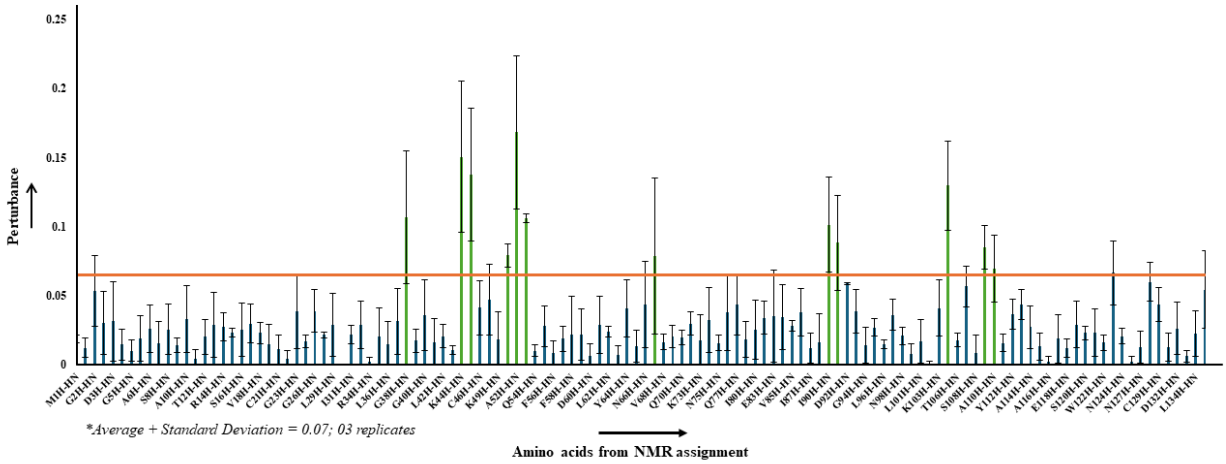

Figure 3.3 The perturbation of amino acid NMR resonances of the Phafin2 PH domain after the addition of polyD. The orange line shows the significance level (mean + SD). The NMR data has been processed using MNova and SPARKY software.

Moreover, the region spanning from Gly38 to Arg53 is notably rich in basic or neutral amino acid residues, forming a positively charged binding pocket suitable for accommodating the negatively charged C-terminal polyD region. In the three-dimensional structural view, all the identified amino acid residues are aligned in a parallel direction, potentially indicating a binding arrangement for the polyD peptide. Bioinformatics analysis of Phafin2 orthologues suggests that the region

spanning Gly38 to Arg53 is highly conserved among all Phafin2 orthologues containing the polyD region **(Figure 3.5).** This observation is consistent with our previous study, where the polyD motif evolved exclusively in Phafin2 and proteins containing both PH and FYVE domains in animals (Hasan & Capelluto, 2022).

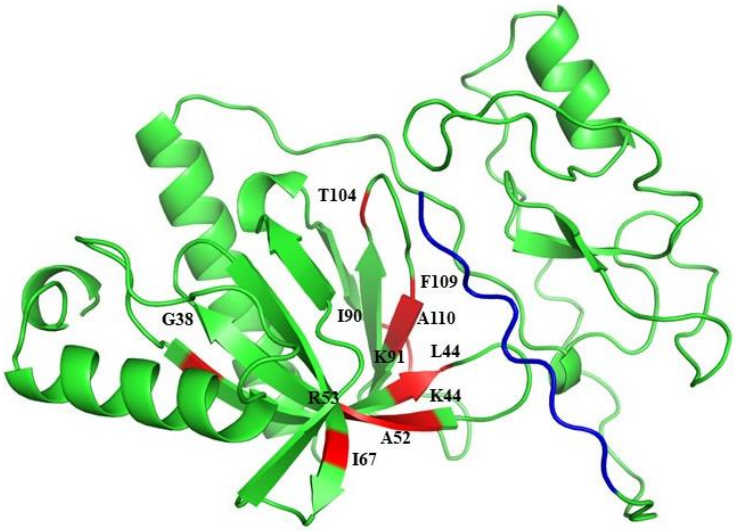

Figure 3.4 3D Structure of Phafin2 (AlphaFold). Labeled amino acids (red) whose NMR resonances are significantly shifted after the addition of the polyD motif (highlighted in blue in Phafin2).

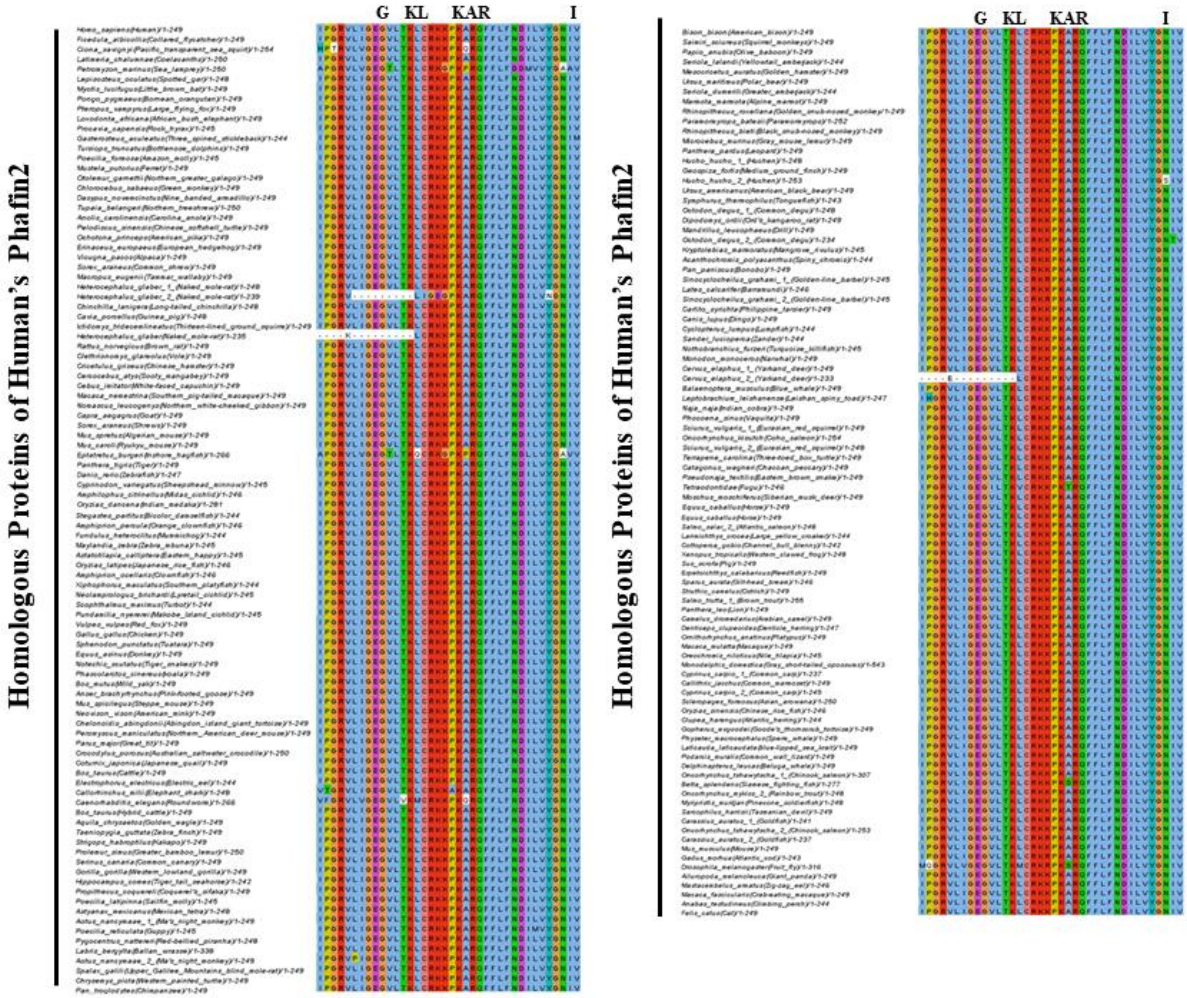

Figure 3.5 Multiple Sequence Alignment of Phafin2's orthologues showing critical amino acid residues of PH domain required for polyD binding.

Biophysical basis for the Phafin2 autoregulation by its polyD motif

After identifying the residues of the Phafin2 PH domain that were predominantly shifted in NMR experiments, the question arose regarding whether mutating these positions can alter the interaction between the mutated PH domain and the polyD peptide. We replaced Lys51 with the

neutral amino acid residue alanine and Arg53 with another neutral amino acid, cysteine. The intention was to decrease the overall positive charge of the region spanning from Gly38 to Arg53.

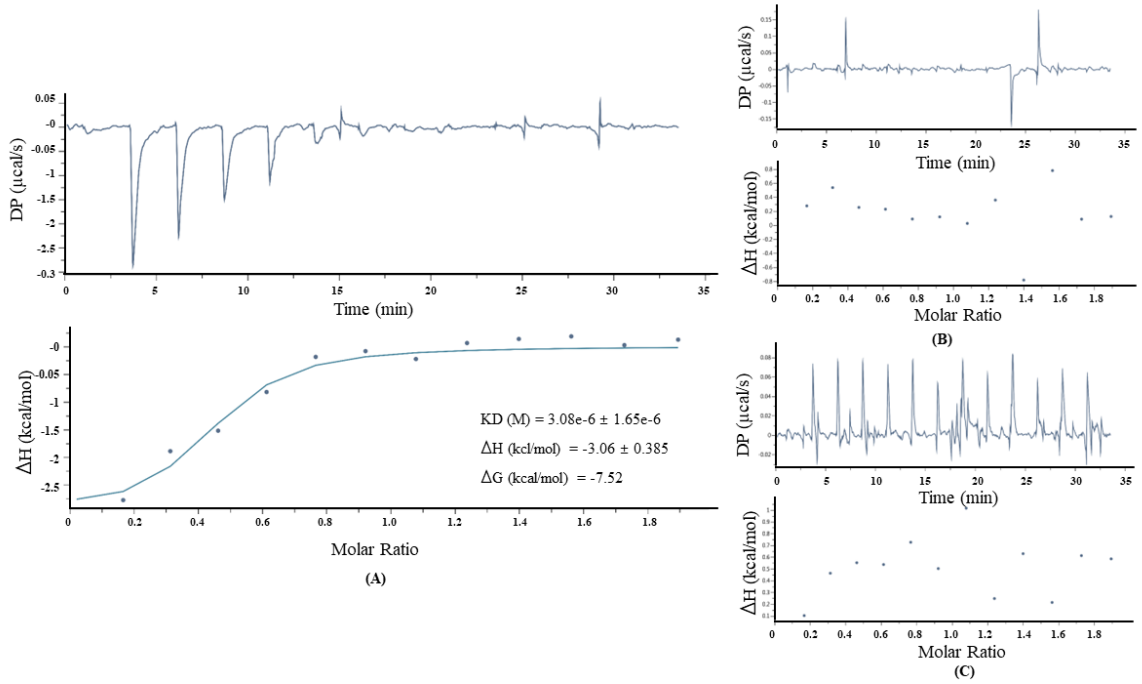

Figure 3.6 The Phafin2 polyD motif interacts with the Phafin2 PH domain (A). Mutated PH domains (K51A/R53C and R53C) do not bind to the polyD motif (B).

Two mutated PH domain constructs were generated: K51A/R53C and R53C. Interactions between the wild-type Phafin2 PH domain and the polyD peptide were also investigated as a positive control. The binding was exothermic with an enthalpy change (ΔH) of -3.25 kcal/mol and an estimated K_D of 3.08 ± 1.65 µM. This observation was consistent with the previously reported binding pattern between the PH domain and the polyD peptide **(Figure 3.6A)**. Interestingly, neither of the mutated constructs, K51A/R53C nor R53C, showed any binding with the polyD peptide in the ITC experiments **(Figure 3.6B & 3.6C)**. The results indicate that replacing the positively charged residues in the region spanning from Gly38 to Arg53 abolishes the binding ability of the Phafin2 PH domain to its polyD peptide.

3.5 Conclusions

In this chapter, we gained insight into the intramolecular autoinhibitory mechanism of Phafin2 by employing different experimental techniques, including NMR spectroscopy, ITC, and bioinformatics analysis. We have identified the critical amino acid residues within the PH domain essential for binding to the conserved C-terminal polyD motif. The findings reveal a positively charged binding region formed by specific residues, facilitating the interaction between the PH domain and the polyD motif, thereby regulating Phafin2 activity. Moreover, our investigation into the membrane binding properties of Phafin2 suggests that its PtdIns3P-mediated interaction with membranes is not dependent on membrane curvature. These findings will help to understand Phafin2's function in various cellular processes, including endosomal cargo trafficking, macropinocytosis, and autophagy. Future studies may focus on the functional consequences of Phafin2 autoinhibition and its role in cellular physiology, including exploring the potential way to develop therapeutic strategies targeting Phafin2-associated diseases.

Chapter 4

4. Conclusions

In this study, bioinformatics, biophysical, and structural biology approaches have been employed to understand the function and regulation of Phafin2, a multifaceted protein involved in various cellular processes. I explored valuable insights into Phafin2's domain-based molecular architecture, its interaction with cellular membranes, and the intramolecular autoregulatory mechanisms governing its activity.

While the polyD motif is absent in PH-, FYVE-, or both PH-FYVE-containing proteins of bacteria, archaea, fungi, and plants, it is exclusively found in animals. The results suggest a unique role for the polyD motif in animal Phafin2 proteins. In addition, PH domains were not found in tandem with FYVE domain-containing proteins in bacteria. Hence, the FYVE domain could serve as the ancestral functional domain among proteins containing PH-, FYVE-, and polyD motif. The study suggested the first emergence of the PH domain in archaea species. Interestingly, the functional diversity of Phafin2 and its other related PH-, FYVE-, or polyD-containing proteins is more consistent in eukaryotic organisms, particularly in processes like cellular trafficking, autophagy, membrane remodeling, apoptosis, signal transduction, and transcription regulation. Additionally, human homologs of Phafin2 possess additional functional modules not found in other organisms, indicating species-specific adaptations. The findings suggest that the polyD motif has evolved among animals and might play a crucial role in regulating Phafin2 function. However, further research is needed to fully understand the functional implications of the polyD motif in Phafin2 and other PH domain-containing proteins.

My study explores Phafin2's autoinhibitory mechanism, a self-regulatory process crucial for maintaining protein function. The elucidation of Phafin2's molecular architecture and regulatory mechanisms holds significant implications for understanding its role in cellular physiology and disease pathogenesis. I employed experimental techniques to identify critical amino acid residues within the PH domain essential for binding to the C-terminal polyD motif. The findings illuminated a positively charged binding pocket spanning Gly38 to Arg53, considering a critical region for accommodating the negatively charged C-terminal polyD motif. In addition, Gly38, Lys45, Lys51, Ala52, and Arg53 are highly conserved among the Phafin2's orthologues. Structurally aligned PH domains that lack polyD reveal distinct patterns of sequence conservation, suggesting that the emergence of polyD maintains conserved regions in PH domains. Identifying critical amino acid residues within the PH domain opens avenues for a better understanding of Phafin2-associated molecular functions. It provides a potential target for further therapeutic intervention, offering promising avenues for drug discovery.

Moreover, the investigation into Phafin2's membrane-binding properties provided valuable insights into its interaction with cellular membranes. I concluded that Phafin2-mediated membrane binding is not curvature-dependent, suggesting its availability across various stages of membrane maturation. This investigation will assist further research to better understand Phafin2-associated molecular mechanisms.

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