



Histidine phosphorylation in metalloprotein binding sites

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ABSTRACT

Post-translational modifications (PTMs) are invaluable regulatory tools for the control of catalytic functionality, protein-protein interactions, and signaling pathways. Historically, the study of phosphorylation as a PTM has been focused on serine, threonine, and tyrosine residues. In contrast, the significance of mammalian histidine phosphorylation remains largely unexplored. This gap in knowledge regarding the molecular basis for histidine phosphorylation as a regulatory agent exists in part because of the relative instability of phosphorylated histidine as compared with phosphorylated serine, threonine and tyrosine. However, the unique metal binding abilities of histidine make it one of the most common metal coordinating ligands in nature, and it is interesting to consider how phosphorylation would change the metal coordinating ability of histidine, and consequently, the properties of the phosphorylated metalloprotein. In this review, we examine eleven metalloproteins that have been shown to undergo reversible histidine phosphorylation at or near their metal binding sites. These proteins are described with respect to their biological activity and structure, with a particular emphasis on how phosphohistidine may tune the primary coordination sphere and protein conformation. Furthermore, several common methods, challenges, and limitations of studying sensitive, high affinity metalloproteins are discussed.

1. Introduction

Almost 60 years ago, Boyer et al. first reported the incidence of mammalian protein histidine phosphorylation in succinyl-CoA synthetase using ^{32}P labelling studies, years before specific evidence of phosphoserine (pSer) and phosphotyrosine (pTyr) emerged [1–5]. While phosphohistidine (pHis) is estimated to account for at least 6% of protein phosphosites, thorough investigations of the regulatory roles of pHis have only been initiated more recently [6]. The phosphorylation of mammalian protein histidine residues is increasingly being recognized as a component of regulatory pathways, protein-protein interactions, and cellular signaling (Scheme 1) [7,8]. Similarly, pHis has since been implicated as a crucial intermediate in numerous enzymatic reactions and diseases of cellular misregulation (e.g., carcinomas) [1,9–12]. However, in spite of histidine's rich activity both as a regulatory agent and ligand, only a few examples of histidine phosphorylation at or near metal binding sites are presently known.

It has been estimated that one-third of proteins are associated with a bound metal ion, and meta-analyses of transition metal binding proteins registered in the RCSB protein data bank revealed that histidine is the most common coordinating residue for divalent Mn, Zn, Fe, Ni, Cu, and Co [13–16]. Interestingly, studies of the eukaryotic phosphoproteome have indicated that phosphorylation is the most common post-translational modification (PTM) made to proteins [17–19]. Considering that studies of post-translational modifications on tyrosine (e.g., phosphorylation, sulfation) have been shown to have direct effects on

the metal ion affinity of metalloproteins, it is apparent that the intersection of protein metal binding sites and phosphorylation is a rich area for research [20–23]. The overlap between phosphoproteomics and metalloproteomics is highly diverse based on the number of residues that undergo reversible phosphorylation, however, studies specific to histidine phosphorylation have historically faced additional challenges due to the hydrolytic instability of pHis [24]. Recent advancements and optimization of peptide purification and LC-MS/MS conditions for sensitive pHis-containing samples have greatly improved accurate identification and localization [25]. For example, use of metal affinity (immobilized Cu^{2+}) facilitates selective enrichment of pHis peptides, which are subsequently identified using MALDI-TOF MS [26]. Further, the development of stable pHis mimics and specific antibodies have facilitated the identification of new histidine kinases, phosphatases, and provided initial insights into their regulatory roles [8,27,28]. These anti-pHis and anti-3pHis monoclonal antibodies have successfully detected pHis containing proteins by immunofluorescence staining and act to enrich peptide samples via immunoaffinity purification [27].

Studies exploring the dephosphorylation activity of protein histidine phosphatase PHPT1 have revealed a handful of metalloprotein substrates [8]. Complementary work exploring the phosphoproteome of zebra fish and its conservation in humans suggested additional pHis sites that have yet to be fully explored [29]. Evidence supporting the presence of metal binding sites at or near the site of histidine phosphorylation has been reported for all of the entries listed in Table 1. The proteins listed in Table 1 fall into several general categories including having activity in

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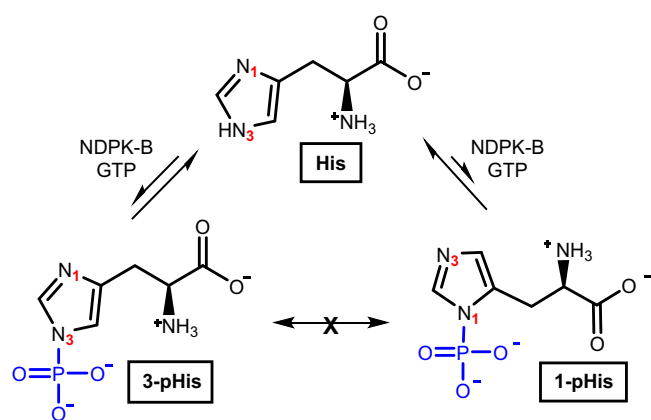
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Scheme 1. Formation of the two pHis isomers, 3-pHis and 1-pHis. 3-pHis is considered to be the more stable, thermodynamic product. Unimolecular interconversion of isomers (phosphotransfer) is not observed in the absence of enzyme by ^{31}P NMR spectroscopy.

Table 1
Metalloproteins featuring histidine phosphorylation in the binding pocket.

Identity	PDB ID	pHis site(s)	Physiological role
KCa3.1 [30]	3CNM	358	K ⁺ Channel; Immune Response
Zinc Finger Protein 526 [29]	–	378	Zinc Finger; Unknown
Double plant homeodomain finger 2 [8]	3IUF	227, 232, 303, 353	Zinc Finger; Transcription
Four and a Half LIM Domains Protein 2 [8]	2D8Z	60, 123, 182, 244	Zinc Finger; Transcription
Protein phosphatase Protein 1C A [8]	4MOV	66, 125, 173, 248	Phosphatase; Metabolism
Protein phosphatase Protein 1C B [8]	IS70	65, 124, 172, 247	Phosphatase; Metabolism
Carbonic Anhydrase 2 [8]	4WL4	64, 67, 94, 96, 119	Bicarbonate Synthesis
Carbonic Anhydrase 8 [8]	2W2J	87, 118, 141	Inactive Enzyme; Unknown
Pyruvate Kinase M2 [8]	4B2D	78	Kinase; Metabolism
Kinase Suppressor of Ras 1 [8]	–	348, 381	Kinase; Signaling
Prune [8]	–	107, 108	Phosphodiesterase; Proliferation

immune response, cellular proliferation, transcription, metabolism, and enzymatic transformation of small molecules. The convergence of histidine phosphorylation and metal binding sites containing histidine ligands has only recently been appreciated for its potential roles in protein regulation. Considering the propensity of histidine residues to both bind metal ions and undergo reversible phosphorylation, histidine phosphorylation is likely at the heart of several metal controlled regulatory mechanisms. The aim of this review is to highlight the intersection of metal binding and histidine phosphorylation in eleven proteins, with a focus on protein structure in and around the metal binding site. Therefore, the metalloproteins listed in Table 1 will be discussed in relation to their activity, structure, and the impact histidine phosphorylation has on each protein, if known.

2. Examples of co-localization of metal binding sites and histidine phosphorylation.

Considering the diverse nature of proteins presented in Table 1, in the following subsections each entry is categorized into one of three classes for simplicity: Ion Channels (2.1), Zinc Fingers (2.2), and Enzymes (2.3). A brief overview of the biological functions of each protein, associations with disease states, metal binding properties, and

hypotheses on how pHis may act in a regulatory capacity are included, if known.

2.1. Ion channels

The intermediate conductance calcium-gated potassium ion channel KCa3.1 (IK-1, SK4) assembles as a homotetramer with six trans-membrane domains in each subunit. KCa3.1 detects and responds to intracellular Ca²⁺ levels and correspondingly effluxes K⁺ [31,32]. KCa3.1 has been shown to participate in the immune response, activating T cells, B cells, and mast cells [33,34]. It has also been implicated in cancers, cardiovascular disease, and glucose intolerance [31,32,35].

In 2018, Lee and MacKinnon employed cryogenic electron microscopy (cryo-EM) to successfully resolve the KCa3.1 structure (Fig. 1) [36]. They proposed a calmodulin (CaM) dependent mechanism in which N-lobe of CaM interacts with the linker between S4 and S5 eliciting a large conformational change that controls efflux through the pore [36]. Complementary work from Srivastava et al. observed that unlike the other three KCa channels (KCa2.1–2.3) which only require Ca²⁺ binding to CaM for activation, KCa3.1 has a more nuanced mechanism and is antagonized by Cu²⁺ binding at H358 in the intracellular, four helix bundle region (Fig. 1) [33]. Phosphorylation of H358 relieves copper-mediated inhibition and allows activation of the channel upon structural rearrangement. As a model for the copper binding site in the coiled-coil four helix bundle, Ji et al. crystallized the C-terminus of the protein (M376 through L414) in complex with Cu²⁺ [37]. The homotetrameric structure chelates Cu²⁺ between four identical His residues (H389) with an axial water molecule in square pyramidal geometry and C4 symmetry [37].

The regulatory role of this copper binding site was demonstrated by treating KCa3.1 with 100 μM CuCl₂ in the absence of phosphorylation, which lead to a decrease in channel current in HEK 293 cells seen by whole-cell patch clamp electrochemical monitoring [30]. Addition of the metal chelator TPEN (*N,N,N',N'*-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine) correspondingly increased current, while treatment with other metal ions such as Zn²⁺ had no effect [30]. In the H358N mutant, copper dependent inhibition was abrogated, indicating that H358 is likely the site of copper binding in vivo [30]. Based on these findings, the authors propose that Cu²⁺ is bound to the C-terminal bundle at H358 until Ca²⁺ changes in concentration and reversibly binds CaM, thus eliciting a conformational change. Srivastava et al. propose that this conformational event transpires near H358, causing the alpha helix to move from a parallel position to the perpendicular one observed in the apo-cryo EM structure. This conformational change both destabilizes the primary coordination sphere of Cu²⁺ and exposes H358 for phosphorylation.

From these data, it is clear that KCa3.1 activity is mediated by histidine phosphorylation, copper chelation at H358 in the cytoplasmic 4-helix bundle, and Ca²⁺ concentration for CaM activation [30,33]. The relevance of copper binding to H398 instead of reversibly phosphorylated H358 in the model coiled-coil is not clear. It has been proposed that H389 acts as a Cu²⁺ shuttle, and the active destination is H358 [30]. However, many questions about the role of copper binding to the channel and the effects of histidine phosphorylation remain.

2.2. Zinc fingers – DPF2, FHL2, ZFP526

Zinc finger proteins have been extensively studied and usually contain well-characterized, zinc binding histidine residues. For example, the C2H2 family of zinc fingers has been thoroughly characterized and explored for its DNA binding ability and relies on the two His and two Cys residues in its metal binding region to chelate Zn²⁺ in a tetrahedral environment [38]. Studies on C2H2 family members show that mutation of coordinating residues leads to a disruption of the primary Zn²⁺ coordination sphere and misregulation of biological functions including apoptosis and cell differentiation/proliferation [39]. One of the three

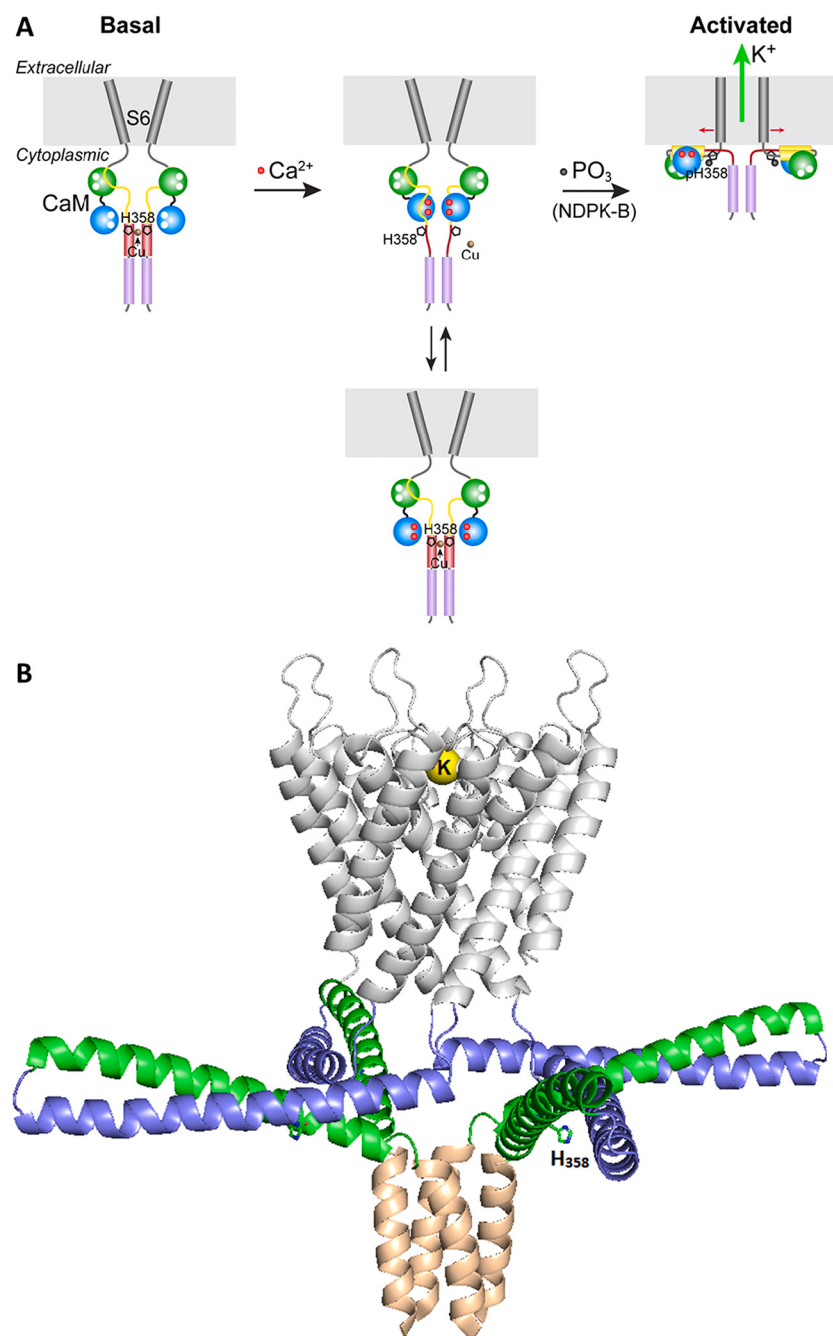


Fig. 1. (A) Mechanism of KCa3.1 proposed by Srivastava et al. [30]. In the basal (inactivated) state, H358 binds Cu²⁺ (purple sphere) and K⁺ efflux through the membrane channel is not observed. Ca²⁺ binding and H358 phosphorylation result in channel opening conformational changes. (B) Simplified cryo-EM structure of KCa3.1 in the absence of Cu²⁺; the four symmetrical H358 residues are oriented away from the C-terminal four helix bundle beige. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

zinc finger proteins listed in Table 1 belongs to the C2H2 family (Zinc Finger Protein 526, ZFP526), whereas DPF2 (Double Plant Homeodomain Finger 2) contains multiple zinc finger domains, and FHL2 (Four and a Half LIM Domains Protein 2) binds zinc using three cysteines and a single histidine (C3H).

A 2019 study exploring the phosphoproteome of zebra fish identified H378 in ZFP526 as a phosphosite, however, the metal binding properties and biological activity of this residue have yet to be explored [29]. While ZFP526 has not been crystallographically characterized in complexation with Zn²⁺, the familial conservation of residues in this region strongly suggests the sequence has metal binding ability (NH₂-YLCVDCGRGFG-TELTVAHRRAH-CO₂H) [40]. In this sequence the proposed coordinating residues of ZFP526 are underlined, and the site of histidine phosphorylation (H378) is indicated with bold typeface. The sequence of ZFP526 adheres to the prescribed C2H2 zinc finger pattern, Y-X-C-

X₂₋₅-C-X₃-F-X₅-ψ-X₂-H-X₃₋₅-H (X: any amino acid, ψ: hydrophobic residue), suggesting that metal binding would be favorable in this protein including a histidine that both coordinates Zn²⁺ and is reversibly phosphorylated [41]. Based on the sensitivity of zinc finger proteins to changes in the coordination environment, the addition of a large phosphate group (as shown in Fig. 2) would be expected to have a significant effect on the structure and function of this protein [39].

Unlike ZFP526, the two other zinc fingers DPF2 and FHL2 are well known in the literature and have published crystal structures from which structural hypotheses about the impact of histidine phosphorylation can be drawn. Double plant homeodomain finger 2 (DPF2) possesses multiple Zn²⁺ binding sites and belongs to the conserved PHD (plant homeodomain) family of proteins [42]. This protein acts as a transcription factor and is implicated in apoptosis, myeloid differentiation, and cancer [43–45].

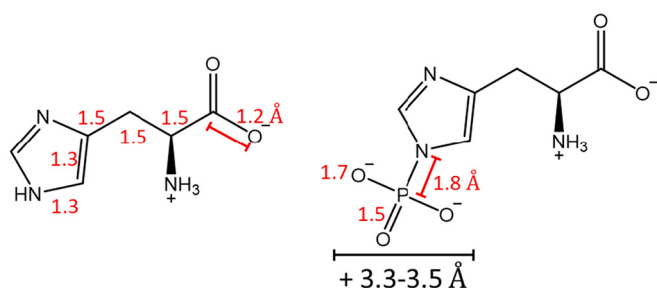


Fig. 2. Addition of a phosphate group on histidine elongates the residue by over 3 Å, potentially perturbing metal coordination.

Structurally, DPF2 has three domains: an N-terminal requiem domain which interacts with BAF, a C2H2 zinc finger which interacts with DNA, and finally a C-terminal dual PHD zinc finger which recognizes PTMs on histones and binds to nucleosomes [44,46–48]. The C2H2 zinc finger domain extends from D206-H232 and includes two possible sites of histidine phosphorylation at H227 and H232, both of which coordinate Zn^{2+} directly (Fig. 3) [8,46,47]. The other two proposed sites of histidine phosphorylation occur in the tandem PHD domain, at H303 and H353 (Fig. 3). It is worth noting that the potential modes of pHis regulation are different between the C2H2 domain and the PHD domain. At DPF2's C2H2 site, histidine coordinates Zn^{2+} differently than the PHD sites as shown in Fig. 3. Both C2H2 histidine residues are associated with the hydrophobic core of DPF2, which in addition to their Zn^{2+} binding may provide evidence that pHis serves in a regulatory capacity to

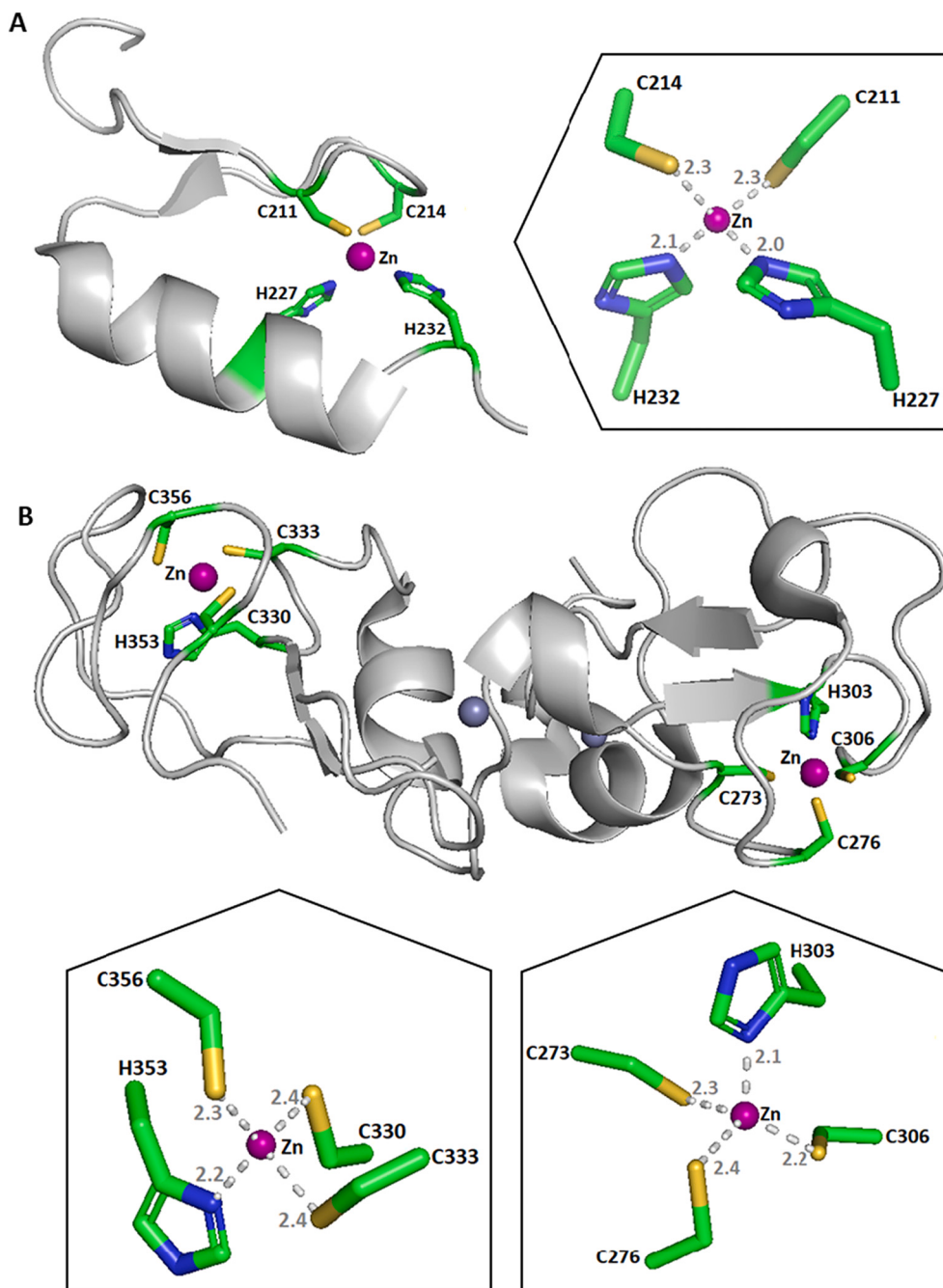


Fig. 3. The multiple Zn^{2+} binding sites of DPF2 shown with secondary structures and isolated primary coordination spheres – PDB: 3IUF (A), 5B79 (B) [46,47] Sites of proposed pHis present as coordinating ligands: H227, H232, H303, H353.

control DNA binding [47]. In contrast to the C2H2 domain, the dual PHD domain has been shown to specifically recognize the acetylated tails of histone H3, H4, and crotonylation [42,46]. Mutations of the PHD domain in 8 unique variants were also observed to impair DPF2's ability to recognize these modifications which in turn modifies the BAF chromatin remodeling complex [44]. In this report, the authors propose that one explanation for the decreased recognition is a loss of structural integrity in the PHD domain [44]. This mode of misregulation might also be a good descriptor of possible conformational disruption caused by pHis at H303 and H353 in the PHD domain. In either instance, histidine phosphorylation may act by disrupting the Zn^{2+} binding landscape in the primary coordination sphere causing metal binding to become strongly disfavored or by contributing to strain and conformational changes near the Zn^{2+} -binding pocket.

The final zinc finger listed in Table 1, FHL2 (Four and a half LIM domains protein 2), is structurally defined by its unique LIM domain which is a double zinc finger [49,50]. Similar to other zinc fingers, LIM domains are active regulators of gene transcription and cell

differentiation [50]. In addition, FHL2 has been implicated in cardiovascular disease, inflammation, and cancer [49,51,52].

LIM domains are cysteine rich and follow a conserved pattern of $\text{C-X}_2\text{-C-X}_{17-19}\text{-H-X}_2\text{-C-X}_2\text{-C-X}_2\text{-C-X}_{15-19}\text{-C}$ (X: any amino acid) and bind two equivalents of Zn^{2+} [50]. Three sites of histidine phosphorylation in FHL2 have been proposed: H123, H182, and H244 [8]. In all three instances, these residues are Zn^{2+} coordinating in a C3H pattern. Notably, all three His sites directly coordinate Zn^{2+} , as shown in Fig. 4. In each instance of Zn^{2+} coordination for ZFP526, DPF2 and FHL2, the orientation of imidazole and its ligand nitrogen are likely pivotal in determining the mechanistic ramifications of pHis on downstream biological activity considering the extraordinary sensitivity of Zn^{2+} to its desired tetrahedral environment.

2.3. Enzymes – PP1CA, PP1CB, CA2, CA8, PKM2, KSR1, Prune

Protein phosphatase 1 (PP1) is a serine/threonine phosphatase which contains multiple catalytic subunits - PP1C A, PP1C B, and PP1C C

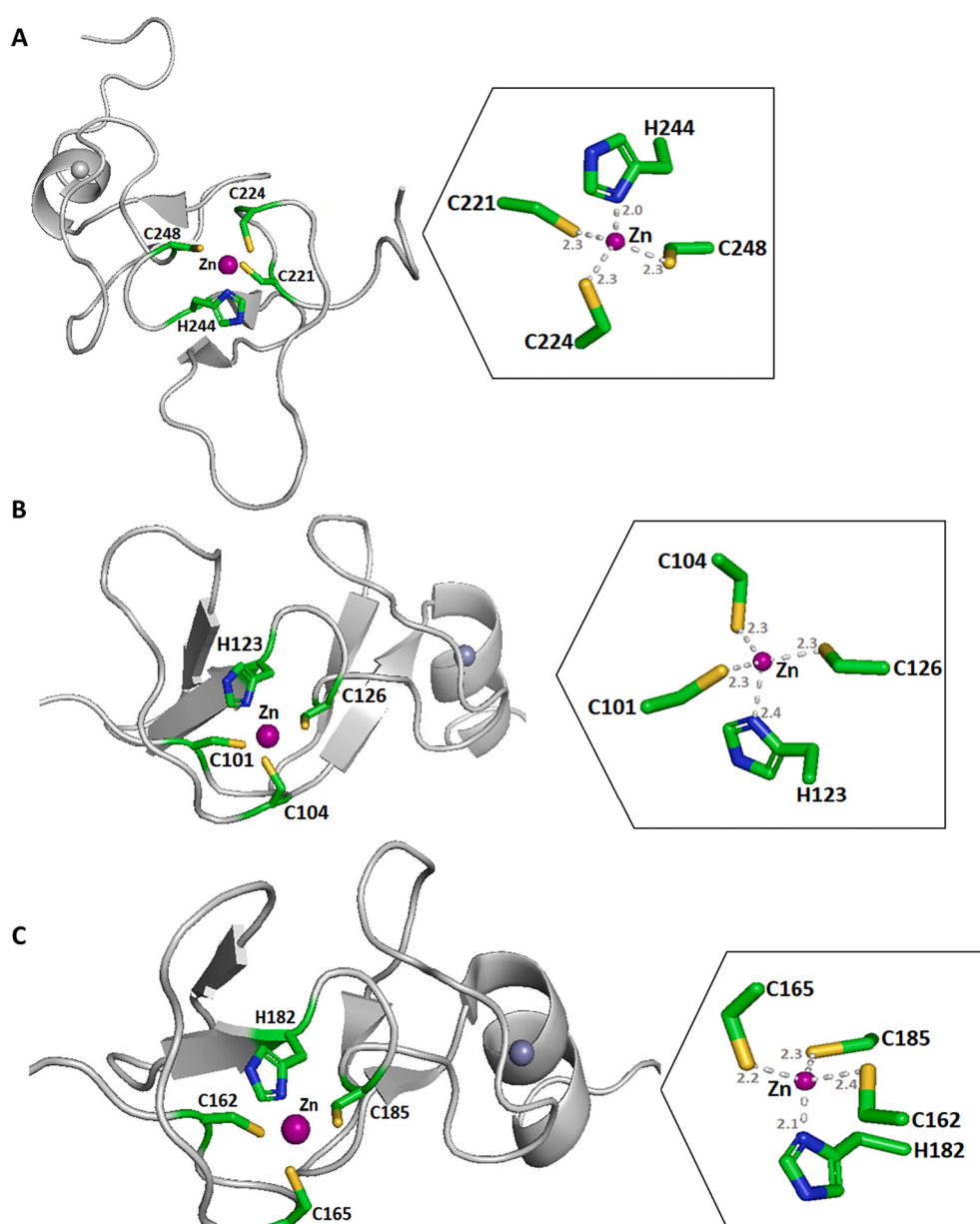


Fig. 4. Binding sites of FHL2 shown with secondary structures and isolated primary coordination spheres – PDB: 1X4L (A), 1X4K (B), 2D8Z (C). Sites of suggested pHis present as coordinating ligands: H123, H182, H244.

[53]. This protein is highly conserved in eukaryotes and the catalytic domains act by similar mechanisms [54]. Previous work has demonstrated that inhibitory proteins act on PP1 by blocking the catalytic active site which contains two metal ions [55,56]. PP1 has been implicated in numerous biological processes including metabolism by acting as a regulator of glycogen synthetase [57].

Structurally, the catalytic site of PP1 lies at the interface of the hydrophobic, acidic, and C-terminal regions [56]. The bimetallic active site may contain Mn^{2+} , Fe^{2+} , and/or Zn^{2+} depending on how the protein is expressed [58,59]. Of the three catalytic subunits, PP1C A and PP1C B possess sites of suggested histidine phosphorylation that may contribute to metal binding and subsequent catalytic activity [8]. PP1C A, the alpha subunit, has been structurally characterized by X-ray Diffraction Crystallography (XRD) in the presence of two coordinating Mn^{2+} ions in its active site (Fig. 5) [60]. The four possible sites of histidine phosphorylation at H66, H125, H173, H248 are proximal to the metal binding site, however only H66, H173, and H248 are close enough to coordinate either Mn^{2+} ion [8]. Importantly, the presence of H125 near the binding site is relevant in two capacities. First, the phosphorylation of this residue may spatially impact the binding pocket with the inclusion of a charged PTM. Whether or not a phosphohistidine positively or negatively impacts metal binding is yet to be determined and will likely differ on a case-by-case basis. Second, H125 is conserved in PP1C B as H124. While the exact role of H125/H124 is unknown, its proximity to the binding site and proposed ability to undergo reversible phosphorylation may suggest its involvement in regulating metal binding and/or enzyme activity.

Similarly to PP1C A, the beta subunit of the PP1 catalytic domain, PP1C B, coordinates two metal ions across multiple histidine residues and is suggested to undergo reversible histidine phosphorylation (Fig. 6) [61]. Four proposed sites of histidine phosphorylation at H65, H124, H172, H247 are proximal to the metal binding site, however only H65, H172, and H247 are close enough to coordinate either Mn^{2+} [8]. While H124 is close to the metal binding site with a distance of 3.9 Å (Fig. 6, dark gray dash), it is unlikely to serve as a coordinating ligand at this distance [62]. Phosphorylation of H124 may have meaningful impacts on the landscape of the metal binding pocket, as the presence of a large phosphate group could significantly alter the coordination sphere as

described in Fig. 2. Of the three coordinating histidine residues, H65 and H172 coordinate $Mn^{2+}(1)$ and H172 coordinates $Mn^{2+}(2)$ (Fig. 6). As described for PP1C A, the mixed identities of the coordinating imidazole nitrogen atoms may have presently unknown regulatory activity.

Another class of enzyme, carbonic anhydrases (CA), catalyze the reversible hydration of carbon dioxide to bicarbonate. For the protein CA2, this function is critical to maintain pH and electrolyte balance, malfunctions of which can result in osteopetrosis, acidosis, and calcification [63]. In contrast, its family member CA8 is not catalytically active for the conversion of $CO_2 \rightleftharpoons HCO_3^-$ [64]. Of the known carbonic anhydrases, there are three non-catalytic subtypes (CA8, CA10, CA11) all of which are characteristically defined by the absence of a coordinating histidine residue [64].

In CA2, a single Zn^{2+} ion is chelated by three histidine residues: H94, H96, and H119 (Fig. 7); completing the tetrahedron, Zn^{2+} also coordinates one molecule of H_2O [65]. It has been previously suggested that H64, H67, H94, H96, and H119 near the Zn^{2+} binding site undergo histidine phosphorylation in CA2, although only H94, H96, and H119 are directly implicated as coordinating ligands [8]. As shown in Fig. 7, the sites of proposed histidine phosphorylation - H94, H96, and H119 - bind Zn^{2+} directly. Interestingly, this protein is highly sensitive to mutations of histidine at positions 64, 94, 107, and 119 and suggests that control of this protein may be strongly tied to histidine phosphorylation [66–70]. In these studies, loss of histidine is associated with a decrease in substrate turnover and numerous disease states.

Structurally, the non-catalytic CA8 closely resembles CA2 with a few key differences (Fig. 8). First, the channel by which Zn^{2+} , CO_2 , and HCO_3^- travel to and from the active site is comparatively narrower owing to the presence of two bulky residues, R116 and I224, at the entrance. Additionally, unlike the inward facing orientation of H94 in CA2, its replacement R116 is repelled by the highly polar nature of the binding site. This replacement leaves only the possible pHis sites H118 and H141 to coordinate a hypothetical Zn^{2+} ion [8]. In order to better understand the significance of this replacement, Picaud et al. refined the crystal structure of CA8 (PDB: 2W2J) to fit a chloride ion (Fig. 8) [64]. It is clear from the structural data that the bond distances required for H118 and H141 to coordinate Zn^{2+} would be approximately 3.0 Å, which exceeds a likely interatomic range for this bonding interaction

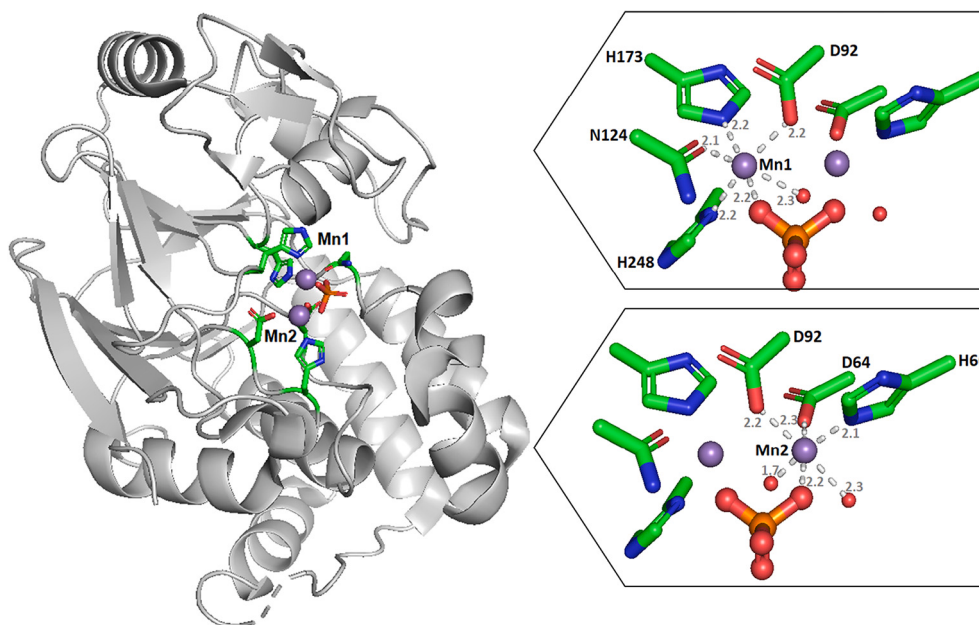


Fig. 5. Active site of PP1C A bound to two Mn^{2+} ions, shown with secondary structure and (inserts) isolated primary coordination spheres – PDB: 4MOV [60]. Red Spheres: H_2O . Sites of proposed pHis present as coordinating ligands: H66, H173, H248. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

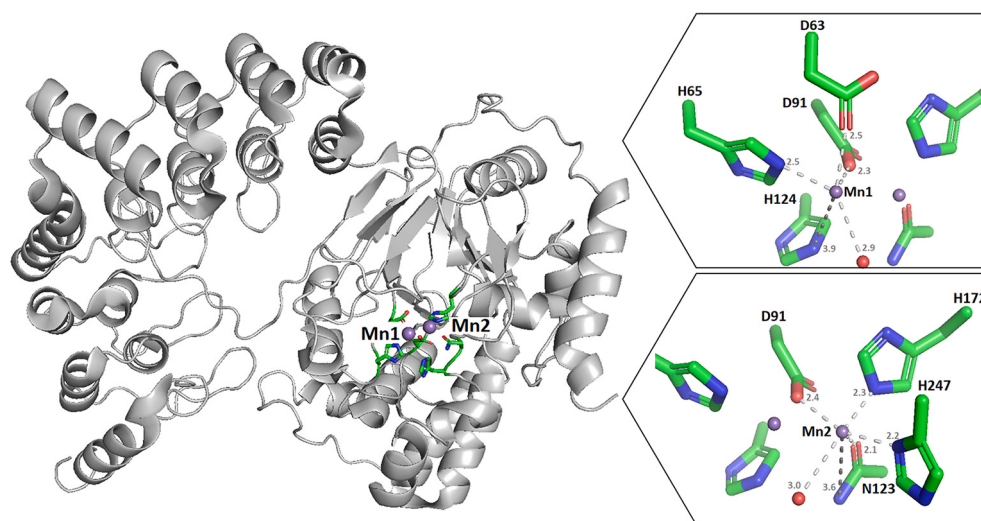


Fig. 6. Binding site of PP1C B bound to two Mn^{2+} ions shown with secondary structure and the isolated primary coordination spheres – PDB: 1S70 [61]. Red Spheres: H_2O . Sites of suggested pHis present as coordinating ligands: H65, H124 (dark gray dash), H172, H247. While H124 is proximal to the metal binding site at 3.9 Å, it is unlikely a primary coordination sphere ligand. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

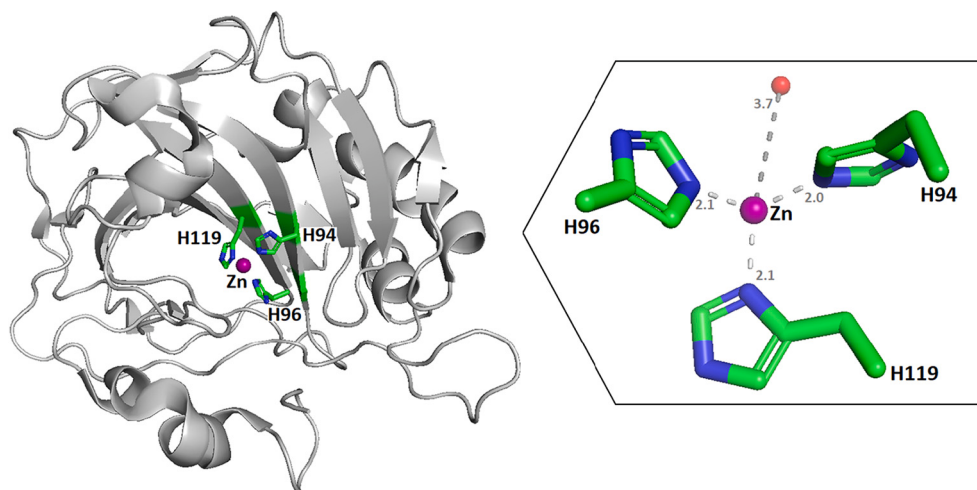


Fig. 7. Binding site of CA2 highlighting its secondary structure and primary coordination sphere - PDB: 4WL4 [64]. Red Sphere: H_2O . Sites of proposed pHis present as coordinating ligands: H94, H96, H119. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[64]. One interpretation of the data would be that loss of a single coordinating histidine residue (H94) is sufficient to negatively regulate catalysis in CA8. This would be consistent with the observation that mutations of CA2 that include coordinating histidine residues lead to decreased catalytic activity.

Another enzymatic protein currently known to experience metal binding and reversible histidine phosphorylation is Pyruvate Kinase M2 (PKM2). PKM2 is implicated in glucose consumption, cell proliferation, and transcriptional regulation [71,72]. In one report from Dombrackas et al. the structure of tetrameric PKM2 was elucidated by XRD in complex with multiple metal ions, an allosteric activator, and an inhibitor which suggests regulation of this protein is likely results from a complex mixture of chemical stimuli [71].

The tetrameric structure of PKM2 is a dimer of dimers, in which each monomer consists of four domains labelled A → D [71]. The active site of PKM2 resides at a cleft between the A and B domains [71]. In order to successfully promote the transformation of phosphoenolpyruvate into pyruvate + ATP, two metal ions are required – a monovalent metal cation (e.g., K^+) and two divalent cations (e.g., Mg^{2+} , Mn^{2+}) [71]. In addition to the requirement for metal cofactors, there is a rich body of evidence suggesting multiple PTMs including phosphorylation allosterically control enzyme activity [73–76]. In Fig. 9, binding of one K^+ and one Mg^{2+} is observed by XRD, however the histidine residue suggested

to undergo phosphorylation (H78) is not located in the primary coordination sphere of either metal [8,77]. While this suggests that metal binding isn't directly inhibited by reversible histidine phosphorylation, pHis may play a role in mediating cofactor binding due to its proximity to the coordination sphere as shown in Fig. 9. The precise role of reversible histidine phosphorylation in PKM2 regulation therefore remains unknown.

The final two enzymes presented in Table 1 also act as signaling proteins - KSR1 (Kinase Suppressor of Ras 1) and Prune. Human Prune belongs to the DHH (Asp-His-His) family of phosphoesterases, active in the regulation of cAMP and cGMP [78]. Prune has been implicated in cell motility and cancers, including breast, melanoma, and liver metastases [78,79]. The DHH sequence of Prune is conserved across several species, including *H. sapiens*, *M. mulatta*, and *M. musculus* [78]. The two sites of proposed histidine phosphorylation, H107 and H108, are not only conserved between species but are closely associated with the metal binding site which contains two ions, Mg^{2+} and/or Mn^{2+} [8,79]. Mutation studies aimed at discovering which residues are crucial for Prune activity revealed that D28, D126, H127, R128, P129, and D179 are essential for phosphodiesterase activity, consistent with each residue contributing to catalysis [79]. This finding may suggest that pHis at H107 and H108 could indirectly regulate activity, however as the crystal structure of Prune has yet to be elucidated the extent to which pHis

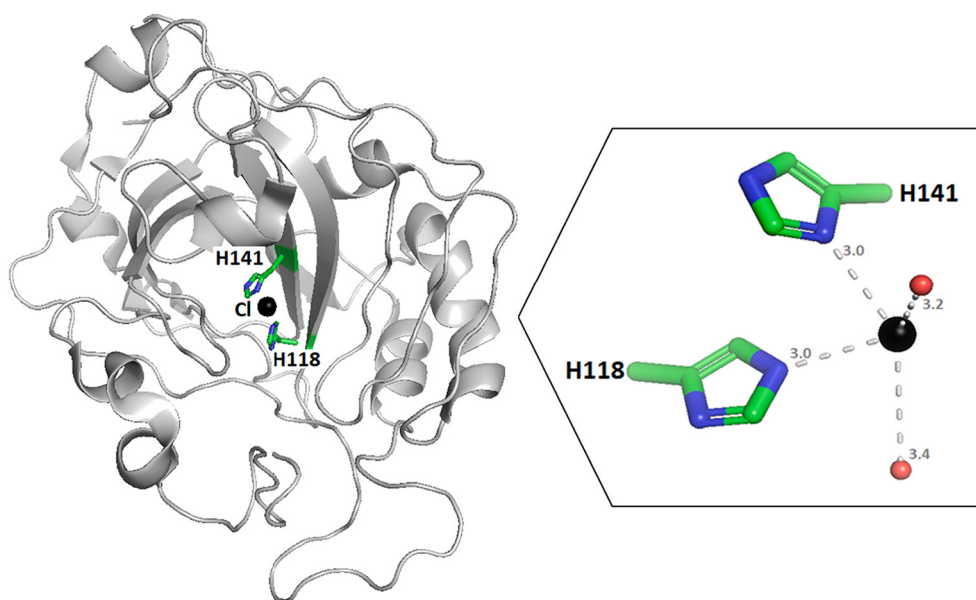


Fig. 8. Binding site of CA8 highlighting its secondary structure and primary coordination sphere - PDB: 2W2J [64]. Red spheres: H_2O ; black sphere (Cl): hypothetical zinc binding site. Distances shown correspond to a single Chloride ion found in the crystal structure. Sites of suggested pHis present as coordinating ligands: H118, H141. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

might impact the protein structure remains unclear. In other DHH proteins such as RecJ (PDB: 1IR6), a known metal binding site containing histidine residues H160 and H161 directly coordinate Mn^{2+} at distances of 2.2 and 3.2 Å, respectively [80]. Regrettably, based on the poor alignment between RecJ and Prune, significant conclusions cannot be extrapolated; however, the potential of pHis to regulate the phosphoesterase activity of Prune remains.

In contrast to Prune, the RAF (Rapidly Accelerated Fibrosarcoma) family member KSR1 is a protein pseudokinase active as a positive regulator in the Ras signaling pathway which mediates cell growth, differentiation, and motility [81,82]. Misregulation of the intricate RAF family pathways is strongly implicated in cancers [83]. The CA3 (Conserved Area) domain of KSR1 encompasses residues 303–397 and is a cysteine rich domain that is zinc finger-like and contains two sites of proposed histidine phosphorylation – H348 and H381 [8]. While the structure of KSR1 in a metal-bound or apo state has not yet been elucidated, KSR1 possesses a high degree of conservation with its family members BRAF and KRas, as shown in Scheme 2 and Fig. 10 [84]. In each instance, three cysteines and a single histidine coordinate Zn^{2+} in two separate binding sites. Both histidines suggested to undergo phosphorylation serve as ligands to Zn^{2+} in KRas, and using KRas as a model for KSR1, it is likely that KSR1 binds Zn^{2+} similarly (Fig. 10) [85]. This conservation suggests that H348 and H381 in KSR1 which may be reversibly phosphorylated may also bind Zn^{2+} directly, thereby participating in protein regulation.

3. Challenges and implications for studying histidine phosphorylation

There are several innate challenges to studying systems employing pHis in a regulatory capacity, one of which is the relative hydrolytic instability of the phosphoramidate (P–N) bond of phosphohistidine [24]. Phosphohistidine has two biologically relevant isomers, 1-pHis and 3-pHis (Scheme 1) which are highly sensitive to pH and temperature [25]. While 3-pHis is more stable than 1-pHis, it is still readily hydrolyzed to lose phosphate under mildly acidic conditions with a large, favorable ΔG° of -12 to $-14 \text{ kcal}\cdot\text{mol}^{-1}$ [9,25,86–88]. This fact has historically made detection, verification, and exact positional assignment of phosphohistidine in proteins challenging, leading to its

underestimation and underrepresentation in the literature [89]. To our knowledge, no incidents of mammalian metalloproteins containing pHis within coordination proximity of a metal ion have been documented crystallographically, thus complicating elucidation of the molecular basis for pHis regulatory mechanisms in these proteins. One route to overcome instability has been the development of pHis mimics capable of withstanding time, heat, and low pH, which have enabled investigators to identify new histidine kinases, phosphatases, and regulatory roles [8,27,28,90]. Application of mimics can provide strong circumstantial evidence if a comparison of a control protein (native histidine) and pHis mimic protein result in remarkably different activity. For example, a chemical approach could involve K_{eq} determination for a metal binding reaction. If a pHis mimic significantly reduces metal ion affinity versus a native His control, phosphorylation may impact the steric and electronic ligand properties guiding metal binding at the affected residue. Alternatively, if phosphohistidine causes a significant alteration in protein secondary structure, examining a native histidine control in comparison to a pHis mimic by Circular Dichroism (CD) spectroscopy could highlight how the PTM modifies structure on a larger scale. Even though approaches utilizing mimics do not exactly replicate pHis systems, they can be used to drive mechanistic hypotheses.

One unique challenge arising from the multiple isomers of pHis is precise determination of histidine's multiple modes of coordination (N_1 vs N_3). Just as histidine phosphorylation can occur on either nitrogen of imidazole (1-pHis vs 3-pHis, Scheme 1), metal coordination can occur at either imidazole site as shown in Fig. 11 [91]. The pyridine-like N_1 nitrogen is a strong σ -donor, modest σ -acceptor, and a weak π -acceptor [92]. As the lone pair of N_1 is oriented outward, instead of contributing to aromaticity, it is a strong Lewis base (and σ -donor) which favorably interacts with strong Lewis acids such as transition metals. The pyrrole-like N_3 nitrogen contributes its lone pair in an unhybridized p orbital to establish the aromaticity of the ring, however the electron rich nature of the ring's π -system also interacts strongly with Lewis acidic metal ions (Fig. 11). As a diamagnetic metal ion with a ground state d^{10} electron configuration, Zn^{2+} is an intermediate acid as per hard-soft acid-base theory (HSAB) and imidazole is an intermediate base. Therefore, preference for coordination site may instead depend on steric factors that restrict free rotation or alter the geometric environment [93]. In the context of histidine phosphorylation, both steric and electronic factors

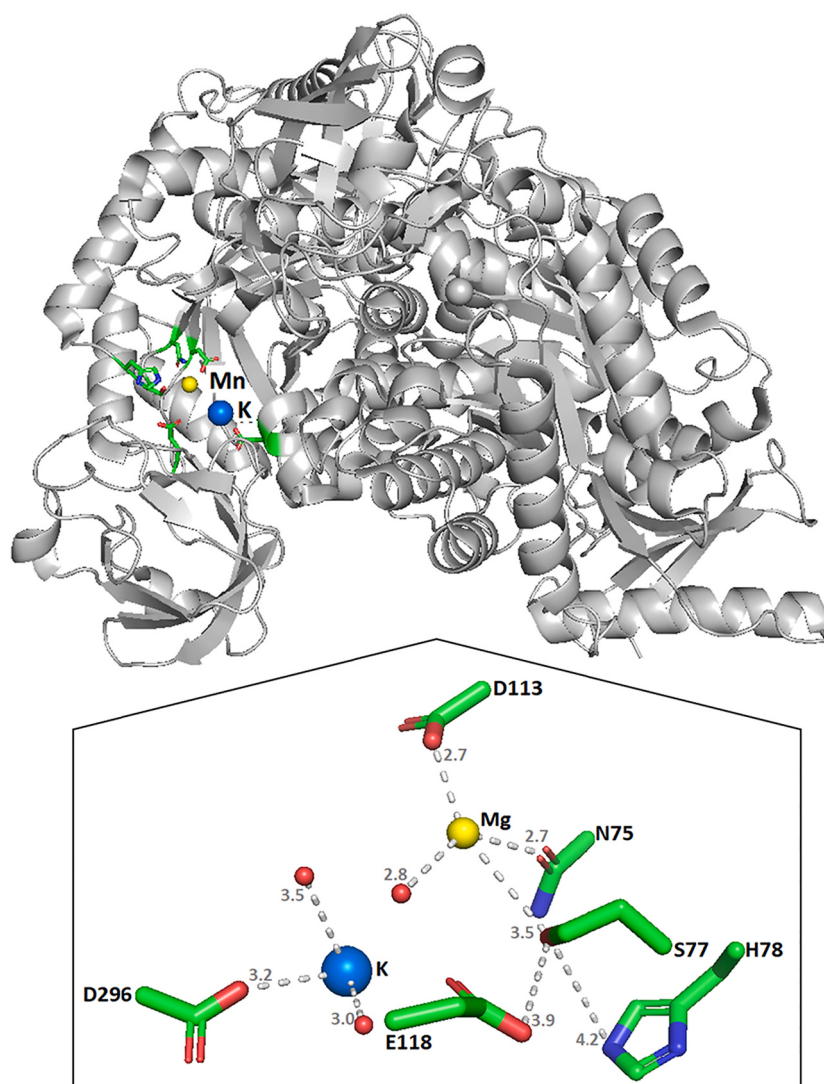


Fig. 9. Binding site of PKM2 highlighting its secondary structure and primary coordination sphere - PDB: 3BJF [77]. Red spheres: H₂O. Sites of suggested pHis present in the metal binding landscape: H78. The Mg²⁺-coordinating inhibitor, oxalate, was omitted for clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

THRFSTKS WLSQVCHVCQKSMIFGVKCKHCRLKCHNKCTKEAPAC
 THNFVRKTFFTLAFCDFCRKLLFQGFRCQTCGYKFHQRCSTEVPLMC
 THNFARKTFLKLAFCDICQKFLNNGFRQCQTCGYKFHEHCSTKVPTMC

Scheme 2. Sequences for KSR1 (top; UniProt: Q8IVT5), BRAF (middle; UniProt: P15056), and KRas (bottom; PDB: 6XI7) demonstrating the conserved cysteine rich domain with the phosphorylated histidine residues, H348 and H381 [84].

would undoubtedly play a key role in determining the effects upon metal coordination. Whether pHis can serve as a good ligand is an open question. Phosphorylation at the N₁ vs N₃ site would impact the primary coordination sphere of the metal ion significantly, as would the conversion of a neutral imidazole ligand to a negatively charged phosphate. It is worth noting that other instances of residue phosphorylation have been shown to increase a metal's affinity for its protein target, and that the instability of pHis in short peptides may not be predictive of its stability in a folded peptide [20].

Finally, as a result of being a relatively new area of study, not every instance of histidine phosphorylation is found in a well-studied system

such as KCa3.1 which has been thoroughly structurally characterized using cryo-EM and XRD. One alternative method to develop and explore mechanistic hypotheses is to examine the degree of conservation in a family of proteins. For example, in Table 1 three zinc fingers are listed including C3H and C2H2 types (C3H: 3 cysteine, 1 histidine; C2H2: 2 cysteine, 2 histidine). The C2H2 zinc finger is a common DNA-binding motif with a highly conserved β-β-α structure following the pattern (F/Y)-X-C-X₂₋₅-C-X₃-(F/Y)-X₅-ψ-X₂-H-X₃₋₅-H where X is any amino acid and ψ is a hydrophobic residue [41]. In a survey of human genes containing C2H2 zinc fingers, it was found that site specific mutation of one coordinating histidine to tyrosine was detected in skin cutaneous

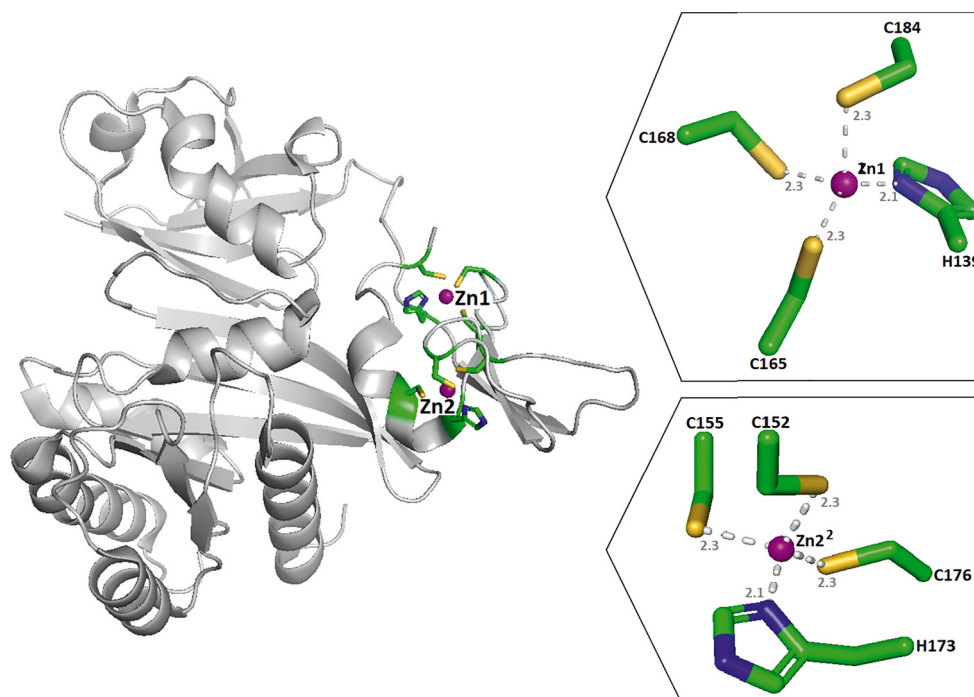


Fig. 10. Zn^{2+} binding sites of KRas (PDB: 6XI7) highlighting the secondary structure and the primary coordination spheres [84]. Based on the conservation of KRas and KSR1 shown in Scheme 2, it is plausible that the imidazole coordination seen in KRas is similar to that of KSR1, and H348 and H381 in KSR1 likely serve as Zn^{2+} binding residues.

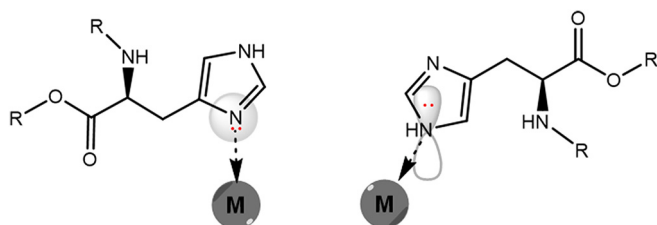


Fig. 11. Histidine is known to coordinate metals through different sites. In free histidine, the carboxyl and amine groups can bind, however when incorporated into a protein structure, histidine typically binds metal ions via one of its two imidazole nitrogen atoms.

melanomas [39]. Substitution of a bulky and poorly coordinating tyrosine residue for histidine sufficiently disrupts zinc binding and leads to loss of function, consistent with the observation that the namesake Zn^{2+} ion strongly prefers a tetrahedral environment [39,94]. Though the precise effects of histidine phosphorylation on a zinc-coordinating residue are not yet known, based on the detrimental substitution of tyrosine for histidine, it is likely that pHis would directly and negatively impact the primary Zn^{2+} coordination sphere. Thus even if a new protein is not thoroughly studied, hypotheses can be made if the protein family is highly conserved.

4. Discussion

In this focused review, eleven metalloproteins known to undergo reversible histidine phosphorylation have been highlighted with respect to their metal binding sites. In several instances the histidine residues known or proposed to undergo phosphorylation are also in the primary coordination sphere of the metal ions serving as ligands. However, the proximity of any pHis residue raises questions about how reversible histidine phosphorylation acts on metal binding sites, either through direct occupation of a primary coordination sphere ligand or indirect

manipulation of the metal binding pocket through steric accommodation of a phosphate group. In order to more fully understand how pHis regulates metalloproteins, experimental evidence for (a) the exact site of phosphorylation and (b) the pHis isomer present (1-pHis vs 3-pHis) are necessary. The location and isomer of phosphohistidine are key pieces of evidence to understand the mechanism of regulation in its own right as the relative stability of each isomer may dictate how persistent it is in the metal binding site. For the remaining examples that do not suggest direct coordination of histidine to a metal, the means of regulation are even less obvious. Their proximity to a binding site may result in pHis mediated conformational changes that reversibly activate the protein, or create a less favorable environment for cofactors to bind. Unequivocally determining how pHis acts in a regulatory capacity in each system presents unique challenges, one of which is the hydrolytic instability of pHis itself. With the emergence of stable pHis mimics, a significant hurdle to chemically interrogating these proteins has already been mitigated. However, judicious application of mimics and careful experimental design are paramount to accurately determine and extrapolate the role of pHis in proteins. As new sites of pHis are discovered in metalloproteins, these tools and techniques will help elucidate the means by which this long overlooked PTM exerts its regulatory influence.

Declaration of Competing Interest

None to declare.

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