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pHisphorylation; The Emergence of Histidine Phosphorylation as a Reversible Regulatory Modification

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Abstract

Histidine phosphorylation is crucial for prokaryotic signal transduction and as an intermediate for several metabolic enzymes, yet its role in mammalian cells remains largely uncharted. This is primarily due to difficulties in studying histidine phosphorylation because of the relative instability of phosphohistidine (pHis) and lack of specific antibodies and methods to preserve and detect it. The recent synthesis of stable pHis analogs has enabled development of pHis-specific antibodies and their use has started to shed light onto this important, yet enigmatic posttranslational modification. We are beginning to understand that pHis has broader roles in protein and cellular function including; cell cycle regulation, phagocytosis, regulation of ion channel activity and metal ion coordination. Two mammalian histidine kinases (NME1 and NME2), two pHis phosphatases (PHPT1 and LHPP), and a handful of substrates were previously identified. These new tools have already led to the discovery of an additional phosphatase (PGAM5) and hundreds of putative substrates. New methodologies are also being developed to probe the pHis phosphoproteome and determine functional consequences, including negative ion mode mass spectroscopy and unnatural amino acid incorporation. These new tools and strategies have the potential overcome the unique challenges that have been holding back our understanding of pHis in cell biology.

Introduction

Histidine phosphorylation was first reported in 1962 when Boyer [1] detected pHis in an enzyme intermediate of oxidative phosphorylation. The enzyme was subsequently revealed to be succinyl-CoA synthetase (SUCLG1), a key TCA cycle enzyme that couples formation of ATP from ADP with conversion of succinyl-CoA to succinate. Phosphorylation of histidine's imidazole nitrogen atoms (N1 or N3) forms high-energy phosphoramidate (P-N) bonds in contrast to the more stable phosphoester bonds (P-O) formed when serine (Ser), threonine (Thr) and tyrosine (Tyr) are phosphorylated. Thus, a high free energy of hydrolysis [2] makes pHis relatively unstable and the phosphoramidate bond rapidly hydrolyzes at low pH or when exposed to significant heat. A combination of moderate heat and acid (e.g. pH 6 and 60°C for 30 min [3]) or exposure to certain primary amines [2] also efficiently

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hydrolyzes pHis. Standard biochemical and proteomic procedures for phosphoester amino acids (pSer, pThr and pTyr [4]) fail to preserve and detect pHis. Consequently, the study of histidine phosphorylation over the last half century has lagged behind the study of pSer, pThr and pTyr (Fig. 1A).

The lack of well-established protocols and reagents, including pHis-specific antibodies (Abs) and phosphohistidine phosphatase-specific inhibitors, has also contributed to the sluggish pace of research. Until recently, pHis has been left in the dark; however, the last several years has seen an acceleration in interest and discovery of histidine phosphorylation's place in cell biology. Light has begun to be shed on its role as a regulator of protein function, in addition to its better known roles as a reactive enzyme intermediate and in prokaryotic signal transduction. This review will focus on recent advances in our understanding of the roles of pHis in mammalian systems that have been stimulated by the availability of new reagents, and the renewed interest and increased appreciation of pHis as an important form of reversible, posttranslational regulation of protein function.

Phosphohistidine Analogs and Antibody Generation

The recent acceleration in pHis research derives from the development of new chemical reagents that facilitated production of pHis-specific Abs. Early attempts at generating pHis Abs were fruitless, since pHis itself is hydrolyzed too rapidly to function as an immunogen. Indeed, our group made an unsuccessful attempt to raise pHis Abs in rabbits by using chemically phosphorylated poly-His [5]. Nevertheless, the fact that an early anti-pTyr Ab (2G8) cross-reacted with pHis in ATP citrate lyase (ACLY [Fig. 2]) gave credence to the idea that generation of pHis Abs should be possible [6]. A strategy of using chemically stable mimetics in place of genuine pHis was therefore needed to circumvent the stability issue. Histidine's two nitrogens can both be phosphorylated resulting in two distinct isomers; 1-phosphohistidine (1-pHis) and 3-phosphohistidine (3-pHis [Fig. 1B]), which makes pHis unique as a phosphoamino acid. In 1999, Schenkels et al. synthesized a stable phosphofurylalanine analog of 1-pHis (Fig. 1C)[7], but did not try to synthesize peptides or immunize animals with it. In 2010, Muir's group made a key breakthrough by synthesizing two phosphoryltriazolylalanine analogs (1-pTza and 3-pTza [Fig. 1C]) that mimic 1- and 3-pHis respectively, replacing the unstable phosphoramidate bonds with non-hydrolyzable phosphonate bonds (P-C) and mimicking the electrostatics and geometry of their respective pHis isomer [8,9]. Using a peptide derived from histone H4 (one of the first reported mammalian pHis substrates [10–12]) with His18 replaced by 3-pTza, they immunized rabbits and raised a sequence-specific anti-3-pHis Ab. Shortly thereafter, Webb's group also reported similar stable analogs compatible with synthetic peptide synthesis [13–15]. Muir subsequently developed "pan-specific" polyclonal 3-pHis Abs, using a 3-pTze analog coupled directly to KLH as antigen, but their usefulness in eukaryotic studies was limited by significant cross-reactivity with pTyr [16], which is structurally most similar to 3-pHis (Fig. 1B).

Inspired by this progress, we conceived of a unique approach that used Muir's pTza analogs embedded in degenerate peptide libraries coupled to KLH as immunogens (Fig. 1C) to promote generation of *sequence-independent* Abs. Rabbits immunized with either the 1-

pTza or 3-pTza library developed high titer polyclonal antibodies; subsequently, we used spleen cells from these rabbits to obtain multiple monoclonal antibody (mAb) clones specific for either 1-pHis or 3-pHis, and validated their usefulness in various immunological assays [3]. These pHis mAbs have been valuable for investigating pHis in mammalian cells [17,18] as well as in bacteria, where they have been used to study auto-phosphorylation of bacterial histidine kinases (*unpublished results*). Our proteomic and immunofluorescence analyses using these mAbs indicate that pHis has important functions in mammalian biology; 1-pHis appears to play a role in phagocytosis, while 3-pHis signals are elevated during mitosis and detected on specific mitotic structures (i.e. spindle poles, centrosomes and midbodies) [3]. Current efforts are focused on using these mAbs to enrich pHis phosphopeptides from digests of cell lysates followed by mass spectrometry (MS) analysis to define global histidine phosphorylation patterns and specific sites of histidine phosphorylation in proteins. The significant challenges that remain for pHis site detection by MS are discussed below (***Phosphohistidine Proteomics***).

Attempts to further refine the pHis analog-antibody strategy were recently made by Muimo [19] and Muir [20] who both developed pyrazole-based analogs (pPza and pPye respectively [Fig. 1D]) and used them to raise polyclonal rabbit Abs. It remains to be seen if these “second-generation” analogs will lead to production of pHis Abs with improved attributes and usefulness in detection and enrichment for pHis. We have cloned and expressed recombinant versions of our pHis mAbs and are using them to solve the co-crystal structures of Ab/antigen complexes. The structural and sequence information will inform targeted mutagenesis of key IgG variable domain residues to improve the affinity and sequence-independence attributes of these mAbs. As new pHis substrates and sites are identified and validated, *sequence-specific* pHis Abs can be raised against peptides containing stable pHis analogs corresponding to these defined sequences, and then used along with genetic analysis (e.g. nonphosphorylatable Asn mutants of pHis sites) to interrogate the functional consequences. Alternatively, non-cleavable 1-pHis or 3-pHis analogs could be incorporated site-specifically into proteins in cells using unnatural amino acid technology [21,22].

Metabolic Enzymes

Nucleoside diphosphate kinases (NDPKs or NMEs) are a highly conserved family of essential enzymes present in bacteria and eukaryotes (NDK is the bacterial homolog) that auto-phosphorylate to form exclusively 1-pHis intermediates that catalyze the transfer of phosphate from nucleoside triphosphates (e.g. ATP) onto nucleoside diphosphates (e.g. GDP)[23] or in some cases other proteins [24–29] (see ***Mammalian Histidine Kinases***). Biossan et al. recently demonstrated that NME1/2 and the mitochondrial specific NME4 provide localized GTP production for dynamin family proteins (e.g. DYN1, DYN2 and OPA1) for membrane remodeling (Fig 2) [23] and this may be true for other GTPases. NME7 localizes to centrosomes (Fig 2) and is part of the gamma-tubulin ring complex (γTuRC), and its kinase activity is required for nucleation of microtubules (Table I) [30]. The observation that NDPK contains a reactive phospho-intermediate was originally made in 1965 [31] and it was subsequently demonstrated to be 1-pHis [32]. Soon after discovery of the 3-pHis isomer in SUCLG1 (Fig. 2), several other 3-pHis intermediates were identified including; phosphoglycerate mutase (PGAM1 [Fig. 2]) in 1970 [33] and ATP citrate lyase

(ACLY) in 1971 [34,35]. Subsequently, Dixon's lab discovered that phospholipase D (PLD [Fig. 2]) and related family members in yeast and E. coli function via a pHis intermediate [36]. Burgos et al. used a crystallographic approach to show that nicotinamide phosphoribosyltransferase (NAMPT), an enzyme that recycles nicotinamide to NAD+, couples ATP hydrolysis with formation of 1-pHis on H247 that dramatically increases catalytic activity [37]. Other enzymes that rely on pHis intermediates may exist that have gone unnoticed due to their instability and difficulty of detection. While these enzymes are *selectively* phosphorylated at N1 or N3, it is possible that some His residues (not stabilized in a catalytic cleft) can accommodate either isomer. Furthermore, isomerization or phosphotransfer to another acceptor residue, which is known to occur ex vivo/in vitro [38,39], may also occur in vivo after His phosphorylation for certain protein substrates.

What enzymes phosphorylate histidine in proteins?

While there are abundant examples of histidine kinases in prokaryotes, only two, highly related proteins, NME1 and NME2, have been reported to have protein histidine kinase activity in mammalian cells. A histone H4 (Fig. 2) histidine kinase *activity* was identified in yeast cellular extracts, but the specific protein responsible was never purified [10,11,40]. NME1/2 histidine kinase activity may depend on its usage of the 1-pHis isomer as an intermediate vs. the more thermodynamically stable 3-pHis isomer used by other enzymes. A handful of substrates have been recently identified that are directly and reversibly phosphorylated by NME1/2 and PHPT1 (see ***Phosphohistidine Phosphphatases***) respectively. The best studied of which are the ion channels KCa3.1 [17,28,41,42] and TRPV5 [24]. A G-protein beta subunit, GNB1 [43,44] is also a direct substrate of NME1 kinase activity and PHPT1 phosphatase activity (Fig. 2). Other potential substrates that have not been as well characterized have been reported including; KSR1 [29,45] and annexin-I [46]. We identified over 780 potential pHis substrates using 1- and 3-pHis mAbs to enrich for pHis-containing proteins from HEK293 cell lysates. Although we controlled for non-specific interactions using denaturing lysis conditions and SILAC MS, further validation is required to confirm these substrates and identify specific pHis sites [3]. Given the potentially large size of the pHis proteome, we should be open to the possibility that additional His kinases exist.

What does histidine phosphorylation do?

Phosphorylation of His, other than in enzyme intermediates, could have several functional consequences and some of these are likely to be unique to His, while others are analogous to Ser, Thr and Tyr phosphorylation, and lead to altered protein conformation or the binding of another protein. While no pHis-specific binding domain has yet been identified, such a domain could be used to promote docking of proteins, similar to 14-3-3, WW or SH2 domains for pSer/pThr and pTyr, respectively [47]. Indirect evidence indicates that SH2 from Grb2 can bind pTza [48]; however, there is no evidence that SH2 binds pHis in vivo. Uniquely, His phosphorylation would cause a charge switch from +1 to −1.5 and this large change could in theory be a mechanism to regulate certain electrostatic protein-protein or protein-nucleic acid interactions.

Our SILAC MS data identified 239 out of 786 proteins involved in RNA and DNA/nucleic acid binding/processing [3]. The Skolnik and Hubbard groups [17] recently demonstrated that the ion channel activity of the tetrameric calcium-activated potassium channel KCa3.1 is stimulated by phosphorylation of His358, showing that this prevents chelation of a Cu^{2+} ion, which stabilizes the KCa3.1 tetramer in the inactive state by coordinating the four His358 residues in the cytoplasmic C-terminal tails, which form a 4-helix bundle. This intimates that cells may use His phosphorylation as a general mechanism to regulate coordination of metal ions by His that are often key to enzyme catalysis by stabilizing the transition state in the active site. Potential proteins of this sort that were enriched by pHis mAbs and are known to use His to coordinate metal ions include; PP1, FHL2, KSR1 [49], DPF2, PKM2, PRUNE and CA2/8 (Table II).

Phosphohistidine Phosphatases

Like phosphoester amino acids, pHis is reversible by specific phosphatase enzymes; three such mammalian enzymes are known; PHPT1 [24,25,42,50–53], LHPP [54,55] and the recently identified PGAM5 [18]. Known substrates for PHPT1 include; KCa3.1[42], TRPV5 [24], ACLY [53] and GNB1 (Fig. 2) [25,56]. No specific substrates have yet been identified for LHPP; however, it seems to play an important role in CNS function and disease. A SNP in LHPP has been linked with major depressive disorder (MDD) and alcohol dependence and risky behavior [57–59]. Other PGAM family members could potentially dephosphorylate pHis as well, since they share sequence homology and belong to the “histidine” phosphatase family that function via a pHis intermediate: STS-1 and STS-2 (TULA1/2; UBASH3B/A) [60]. The sole substrate for PGAM5 identified so far is NME2 (also known as NDPK-B), and PGAM5 exhibits selectivity for this NME family member (Fig. 2) [18]. Since we currently lack phosphohistidine phosphatase inhibitors, the best method for preservation of pHis in cell lysates is to use denaturing conditions to prevent all phosphatase activity, including those phosphoester phosphatases with “promiscuous” activity towards phosphoramidate bonds, which include PP1, PP2A and PP2C [61,62]. An open question is whether or not all pHis modifications *require* a specific phosphatase, or whether pHis can decay spontaneously acting as either an automatic timer or perhaps even a pH sensor? Conversely, some pHis sites may be unusually stable due to local environment.

Phosphohistidine Proteomics

Some time ago it was suggested that pHis represents as much as 6% of phosphorylation sites [63], but in reality we do not have a good estimate of how large the pHis proteome is. Phosphopeptide enrichment and phosphorylation site identification by MS has been essential to the study of pSer, pThr and pTyr. In addition to the common challenges of phosphoproteomics, like low stoichiometry, reduced ionization efficiency and neutral loss of phosphate, identification of pHis phosphopeptides from complex mixtures presents unique challenges. These include; acid-catalyzed hydrolysis under standard liquid chromatography (LC) conditions (pH 3), isomerization of 1-pHis and 3-pHis isomers [39], inter- and intra-peptide phosphotransfer from His to Asp [38] and inability to distinguish between the two pHis isomers. Previous efforts have been made to selectively enrich for pHis phosphopeptides prior to LC-MS using immobilized Cu II ions [64]). An alternative strategy of elevating LC pH to 5 (without enrichment) was employed as a compromise between

stabilization of pHis and ionization of peptides. 20 pHis phosphopeptides were identified; however, these have not yet been validated at the protein level [65,66]. Negative electron-transfer dissociation (NETD) developed by the Coon lab, which is run under alkaline conditions, is a promising new technique that has the potential to solve these issues [67–69]. We are currently using pHis mAbs to selectively enrich pHis phosphopeptides from digests of cell lysates and identify phosphorylation of specific His residues to gain a more accurate catalogue of the pHis proteome in mammalian cells.

Conclusions

While pHis currently has well established roles in prokaryotic two-component signal transduction and as a high-energy intermediate in various metabolic pathways, it remains to be seen if pHis plays as significant of a role in regulation of protein function and signal transduction as pSer, pThr and pTyr. There does appear to have been an evolutionary shift in transmembrane signaling and extracellular sensing mechanisms from prokaryotic histidine kinases to eukaryotic receptor tyrosine kinases. Since no pHis-binding domain has been identified, it is unclear if pHis can also serve as a protein-interaction docking site, but we now see that it has roles beyond simply serving as a means for intermolecular phosphate transfer. These roles may be unique to His since it is a common residue in metalloproteins, catalytic triads, and is used as a proton shuttle (e.g. carbonic anhydrase) and pHis could be a common mechanism to regulate these functions by simple interference with protonation of imidazole nitrogens.

Future efforts should focus on; refining tools and methods that have emerged in the last several years including pHis analog-antibody development, developing new reagents including phosphohistidine kinase and phosphatase inhibitors and improving proteomic and genetic methods, including NETD MS and unnatural amino acid technology. Selective pHis phosphopeptide enrichment using pHis-specific mAbs and site identification by MS will be instrumental in providing a more complete catalog of substrates and possibly His kinases and phosphatases. Knowledge of all the players should help give us a better understanding of the game they are playing in the cell.

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Highlights

- Histidine phosphorylation (pHis) is reversible by specific kinases and phosphatases
- pHis is heat and acid labile, but stable under alkaline conditions
- Stable pHis analogs have enabled development of pHis-specific antibodies
- pHis antibodies and refined methods have identified new functions and substrates
- Enrichment of pHis phosphopeptides for proteomics is the next critical step

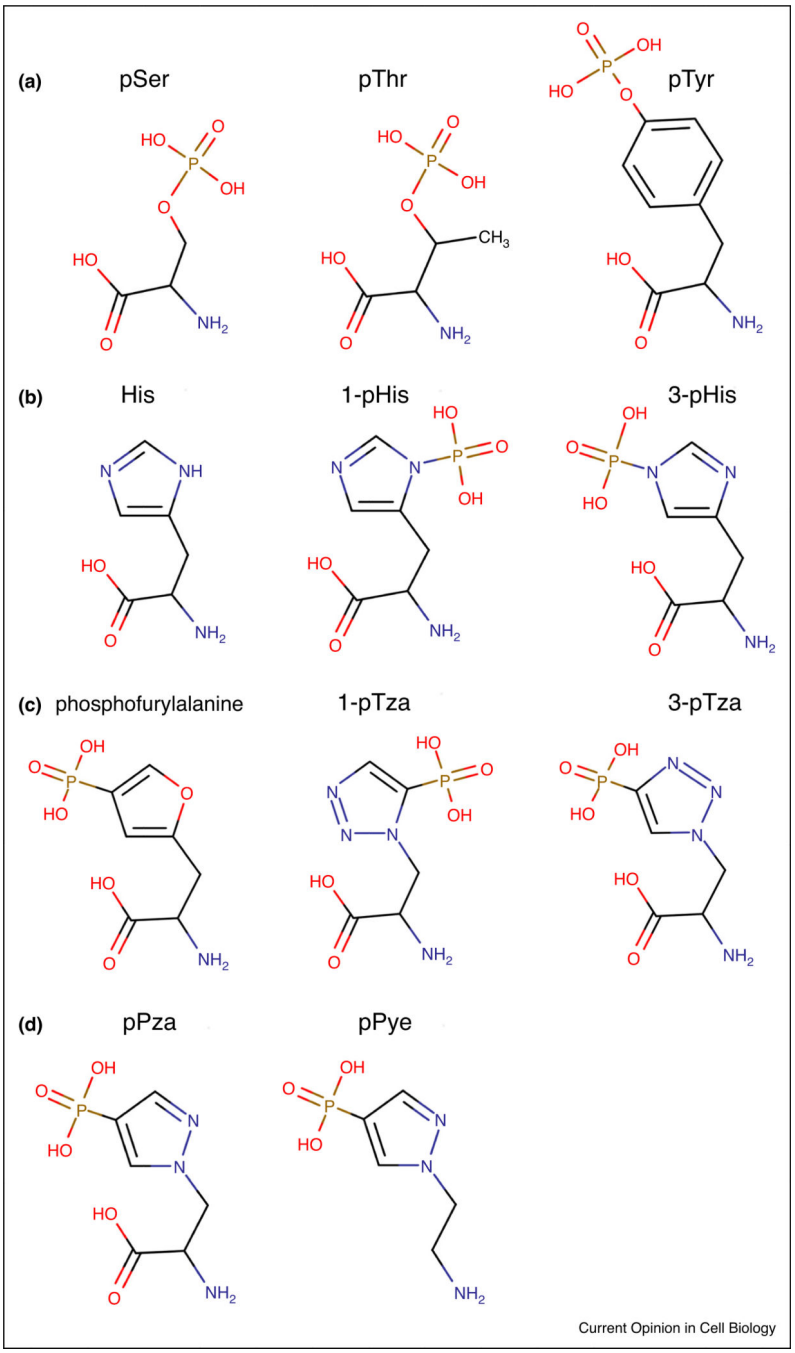

Figure 1. pHis Isomers and Structural Analogs

Structural drawings of (**A**) the phosphoester amino acids; pSer, pThr and pTyr are contrasted with (**B**) histidine, 3-phosphohistidine (3-pHis) and 1-phosphohistidine (1-pHis). Examples of phosphohistidine structural analogs designed for antibody generation include; (**C**) phosphofurylalanine and the two phosphoryltriazolylalanine analogs (3-pTza) and (1-pTza). (**D**) Second-generation, pyrazole-based pHis analogs; 4-Phosphopyrazol-2-yl alanine (pPza) and phosphono-pyrazolyl ethylamine (pPye).

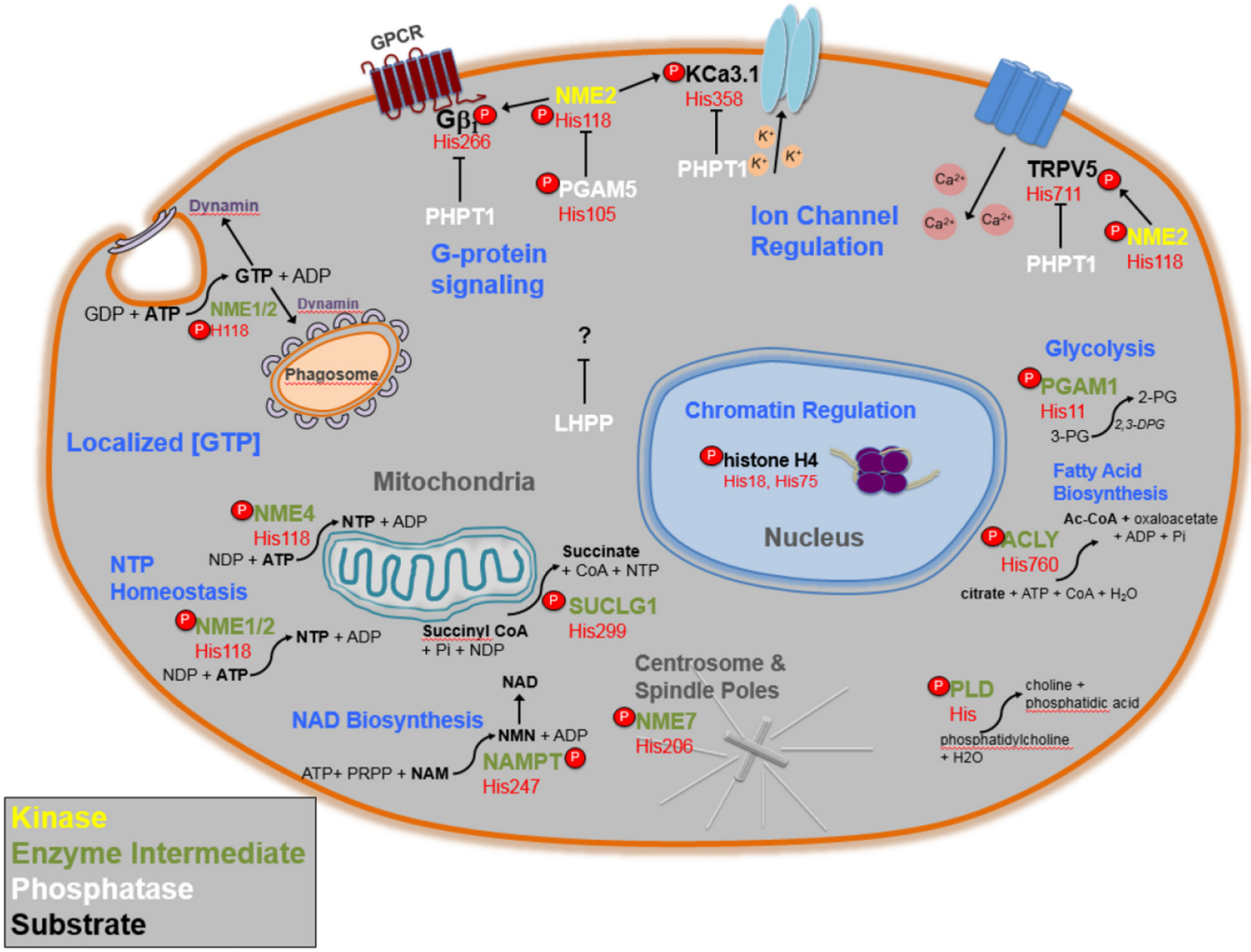

Figure 2. Summary of pHis Cellular Functions

An illustration of the pHis related proteins discussed in this review and their various functions, enzymatic reactions and subcellular localizations. NME1/2 protein histidine kinase functions are in *yellow*, pHis enzyme intermediates are in *green*, phosphohistidine phosphatases are in *white* and pHis substrates are in **bold**. Beneath each protein's gene name is the specific amino acid position number of the pHis residue in *red*. Cellular functions of specific pHis proteins are in *blue*. The subcellular localization of pHis related proteins and functions are in *grey*. Curved arrows represent reactions catalyzed by enzymes that utilize pHis intermediates. For LHPP, phospholysine and 3-phsphohistidine are substrates in vitro, however no known substrates have yet been identified in vivo.

Table 1

Mammalian pHis Related Proteins

Gene	Uniprot ID	Function	Site	N1 or N3
NME1	**P15531**	NDPK, His kinase	H118	1-pHis
NME2	**P22392**	NDPK, His kinase	H118	1-pHis
NME4	**O00746**	NDPK, mitochondrial	H151	1-pHis
NME7	**Q95YB8**	NDPK, centrosomal	H206	1-pHis
PGAM1	**P18669**	Glycolysis	H11	3-pHis
PGAM5	**Q96HS1**	Ser/Thr & His phosphatase	H105	3-pHis
SUCLG1	**P53597**	Succinyl-CoA ligase	H299	3-pHis
ACLY	**P53396**	ATP-citrate synthase	H760	3-pHis
PHPT1	**Q9NRX4**	pHis phosphatase	-	-
LHPP	**Q9H008**	pLys, pHis & pyrophosphate phosphatase	-	-
GNB1	**P62873**	GPCR signal transduction	H266	3-pHis
KCa3.1	**O15554**	Calcium-activated potassium channel	H358	3-pHis
TRPV5	**Q9NQA5**	Calcium channel, Ca^{2+} reabsorption	H711	3-pHis
HIST1H4A	**P62805**	Nucleosome, chromatin regulation	H18,	1/3-pHis
PLD	**Q13393**	Phospholipid metabolism, signaling	H94	?
NAMPT	**P43490**	NAD+ biosynthesis	H247	1-pHis

For each known pHis related protein discussed in the text of the review; the gene name, Uniprot ID number, a brief functional description, the specific pHis site and the specific pHis isomer (1-pHis [N1] or 3-pHis [N3]) is provided.

Table II

Potential Regulation of Metal Ion Coordination by pHis as A General Mechanism

Gene	Uniprot ID	Function	Site(s)	Ion(s)
KCa3.1	**O15554**	Calcium-activated potassium channel	H358	Cu^{2+}
PPP1CA	**P62136**	Ser/Thr Phosphatase, alpha subunit	H66, 125, 173, 248	Mn^{2+}
PPP1CB	**P62140**	Ser/Thr Phosphatase, beta subunit	H65, 124, 172, 247	Mn^{2+}
FHL2	**Q14192**	Transcriptional regulation	H60, 123, 182, 244	Zn^{2+}
KSR1	**Q8IVT5**	Ras/Raf/MAPK signaling scaffold	H348, 381	Zn^{2+}
DPF2	**Q92785**	Transcription factor, hematopoiesis	H227, H232, 303, 353	Zn^{2+}
PKM/PKM2	**P14618**	Glycolysis, Warburg effect, metabolism	H78	$Cu2^{+}$,K^{+}
PRUNE	**Q86TP1**	Phosphodiesterase, NME1 inhibitor	H107, 108	Mn^{2+},Mg^{2+}
CA2	**P00918**	Carbonic anhydrase	H64, 67, 94, 96, 119	Zn^{2+}
CA8	**P35219**	Carbonic anhydrase-like	H87, 118, 141	Zn^{2+}

Based on the intriguing finding that phosphorylation of histidine in KCa3.1 regulates its function via coordination of a metal ion (Cu^{2+})[17], we speculate that other proteins that were enriched by pHis mAbs [3] and have metal ion coordination sites involving histidine(s) may also be functionally regulated by histidine phosphorylation. The gene name, Uniprot ID number, a brief functional description, the potential pHis regulated site(s) and the metal ions coordinated by these sites are provided. The list of similarly regulated proteins could be much longer; however, at this point this list is hypothetical and further, direct evidence is necessary for validation.