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Chemoproteomic identification of phosphohistidine acceptors: posttranslational activity regulation of a key glycolytic enzyme†

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Histidine phosphorylation, an unconventional and understudied posttranslational modification, often involves phosphohistidine (**pHis**) "acceptor" proteins, which bind to **pHis** residues and undergo phosphotransfer from **pHis**. While the roles of **pHis** acceptors are well-documented in bacterial cell signalling and metabolism, the presence and functions of additional **pHis** acceptors remain largely unknown. In this study, we introduce a chemoproteomic strategy leveraging a stable analogue of 3-**pHis** to identify 13 putative **pHis** acceptors in *Escherichia coli*. Among these, we identified phosphofructokinase-1 (**PfkA**), a central enzyme in glycolysis, as a **pHis** acceptor phosphorylated at His249 by phosphocarrier protein HPr (PtsH). This phosphorylation, modulated by carbon source availability, inhibited **PfkA**'s kinase activity, while the **pHis**-specific phosphatase signal inhibitory factor X (SixA) reversed the effect, restoring the kinase function. Our findings reveal a novel regulatory mechanism in which histidine phosphorylation dynamically controls a key glycolytic enzyme, implicating a broader role for **pHis** in bacterial metabolism.

Introductions

Histidine phosphorylation, an underexplored form of protein phosphorylation,[1] plays a role in various biological processes, including signal transduction, metabolism, and epigenetics.[2–4] However, phosphohistidine (**pHis**) is unstable and prone to dephosphorylation and phosphoryl transfer, particularly under acidic conditions, presenting challenges for its study using conventional techniques.[1,5–7]

Currently, the function of histidine phosphorylation is best understood within the context of cell signalling and metabolism. Bacterial histidine kinases, essential components of the two-component signal transduction system (TCS),[8] are potential drug targets to combat antibiotic resistance.[9–12] The phosphoenolpyruvate: sugar transferase system (PTS) features a phosphorelay where the phosphoryl group from **pHis** is transferred to another histidine and eventually to transported sugars.[13]

Recent advancements in **pHis**-specific antibodies[14–19] and phosphoproteomics have enabled the annotation of numerous **pHis** sites across both prokaryotic and eukaryotic proteomes.[20–22] However, the physiological roles and downstream pathways of these **pHis** sites remain poorly understood.

In canonical phosphorylation signalling, specialised "reader" proteins play a crucial role in mediating downstream effects. The readers specifically recognise and bind to phosphorylated amino acid residues, facilitating protein–protein interactions and signal transduction. Well-characterised examples include the Src homology 2 (SH2) domain for phosphotyrosine (pTyr),[23] 14-3-3 proteins for phosphoserine (pSer),[24] and the forkhead-associated (FHA) domain for phosphothreonine (pThr).[25] These readers form noncovalent complexes with their target phosphoproteins to mediate downstream functions (Fig. 1A).

In contrast, **pHis**-binding proteins can undergo phosphorylation through phosphotransfer from a target **pHis** residue. The high reactivity of **pHis** enables its phosphoryl transfer to a nucleophilic residue, a distinct molecular mechanism in TCS and PTS. In such cases, the **pHis** binders can be regarded as **pHis** "acceptors," distinct from "readers" (Fig. 1B).[26] To the best of our knowledge, no **pHis** reader lacking an acceptor function has yet been identified. Well-characterised **pHis** acceptors include the response regulators (**pHis** to phosphoaspartate (pAsp)) in the TCS and phosphorelay proteins (**pHis** to **pHis** or **pHis** to phosphocysteine (pCys)) in the PTS; however, the presence and functions of additional **pHis** acceptors remain largely unknown.

This unique phosphotransfer characteristic of **pHis** poses a major challenge in identifying novel **pHis** acceptors. For

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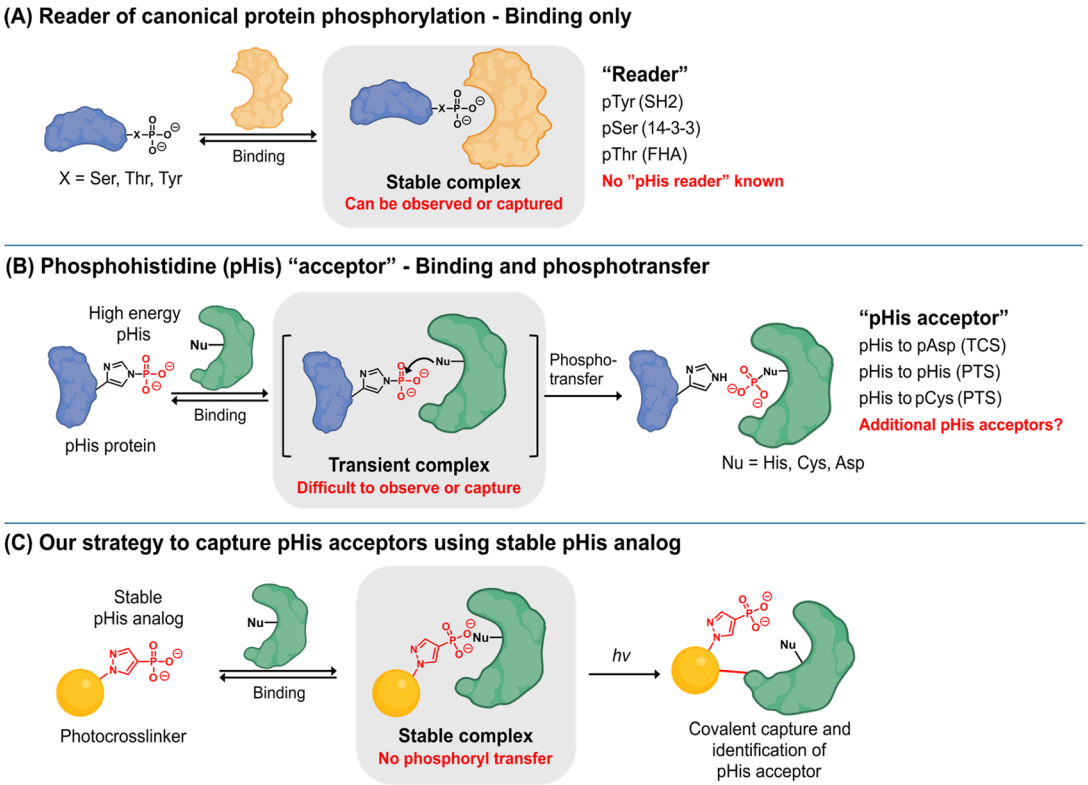

Fig. 1 (A) Schematic of canonical phosphorylation readers, which bind to phosphorylated residues to mediate downstream functions. (B) Unlike readers, phosphohistidine (**pHis**) "acceptors" undergo phosphorylation *via* phosphotransfer from **pHis** residues. The transient interaction between **pHis** and its **pHis** acceptor presents a challenge in identifying novel **pHis** acceptors. (C) Our chemoproteomic strategy to capture **pHis** acceptors using a photoaffinity probe based on a stable **pHis** analogue. Figures were created in https://www.BioRender.com.

canonical phosphorylation, the interaction between a reader and a phosphorylated residue forms a relatively stable complex (Fig. 1A), facilitating the identification of binding partners by affinity enrichment or photocrosslinking.[27–29]

In contrast, pulldown experiments using **pHis**-containing baits would be far more challenging, as the interaction between the bait and the **pHis** acceptor during the phosphotransfer is transient, making it exceedingly difficult to capture (Fig. 1B).[30]

A potential solution to this challenge is to use a stable **pHis** analogue as bait, which can bind to **pHis** acceptors more reliably by preventing phosphotransfer (Fig. 1C). A similar pulldown strategy using a non-hydrolysable pTyr mimic successfully enriched SH2-containing proteins from mammalian cell extracts.[31,32]

In this study, we developed a chemoproteomic workflow using chemical probes derived from a stable analogue of 3-**pHis** to identify novel **pHis** acceptor proteins (Fig. 1C). This approach identified putative **pHis** acceptors in *Escherichia coli* (*E. coli*), including phosphofructokinase-1 (**PfkA**), a crucial key node enzyme in glycolysis. We also elucidated the role of **pHis** in regulating **PfkA** activity and characterised the pathways mediating **PfkA** phosphorylation and dephosphorylation. These findings reveal a new posttranslational regulatory mechanism controlling a crucial glycolytic enzyme, indicating a broader role for histidine phosphorylation in metabolic regulation.

Results

Design and synthesis of pPyp-BP

To identify the **pHis** acceptors, we designed a chemoproteomic strategy using affinity-based probes (Fig. 1C). Due to its instability and susceptibility to phosphotransfer, **pHis** itself is unsuitable as bait. Instead, a probe (**pPyp-BP**) featuring a phosphonopyrazole-based stable analogue of 3-**pHis** and a benzophenone photocrosslinker was designed to covalently label the putative **pHis** binders (Fig. 2A). This **pHis** analogue has been successfully utilised as a **pHis**-mimicking hapten to generate **pHis**-specific antibodies, demonstrating its structural similarity to **pHis**.[18,19] An alkyne handle was also incorporated into the probe to enable in-gel visualisation and enrichment of labelled targets through the Cu-catalysed click reaction.[33] The probe was synthesised from pyrazole (ESI Fig. S1†).

pPyp-BP labels known pHis acceptor proteins *in vitro*

Next, we investigated whether **pPyp-BP** could effectively label known **pHis** acceptors. Since phosphorelay proteins in the PTS are well-established **pHis** acceptors, we selected *E. coli* phosphoenolpyruvate-protein phosphotransferase (PtsI) as a model. This PTS protein can accept the phosphoryl group from the histidine-phosphorylated phosphocarrier protein PtsH (PtsH–**pHis**).[34,35] We also examined signal inhibitory factor X

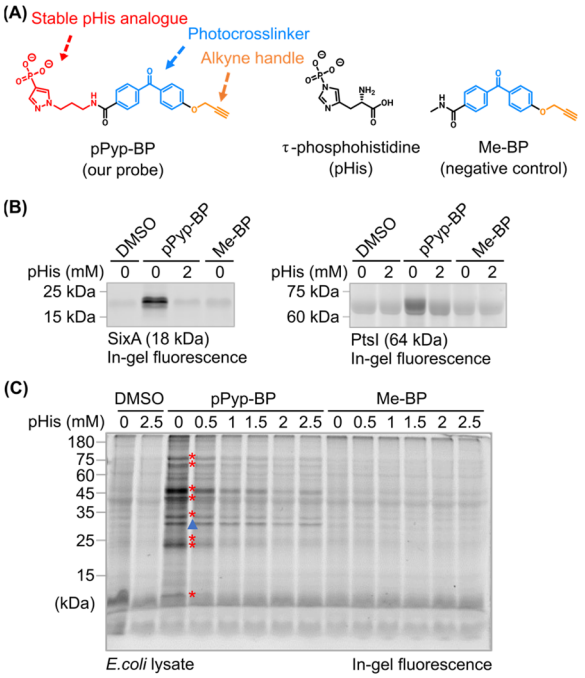

Fig. 2 (A) Structure of the **pPyp-BP** probe and the negative control **Me-BP**. (B) Fluorescent labelling of recombinant *E. coli* PtsI and SixA, two well-characterized **pHis** acceptors. Labelling was visualised *via* a click reaction with TAMRA-azide, followed by in-gel fluorescence imaging. Excess **pHis** reduced labelling intensity, confirming the probe's specificity for **pHis**-binding sites. (C) Labelling of LB-cultured *E. coli* lysates. Protein bands with reduced labelling by **pHis** competition are marked with red stars, indicating probe specificity. A nonspecific band (blue triangle) was unaffected by **pHis** competition. Minimal labelling was observed with the **Me-BP** control. (Coomassie-stained gels are shown in ESI Fig. S2 and S6.†).

(SixA), a **pHis**-specific phosphatase in *E. coli*, as its catalytic mechanism involves phosphotransfer from the substrate **pHis**, forming **pHis** at its active site, which is subsequently hydrolysed for catalytic turnover.

Both PtsI and SixA were successfully labelled *via* photocrosslinking with **pPyp-BP**, whereas no labelling was observed for **Me-BP**, a negative control probe lacking the **pHis** analogue moiety. Notably, labelling was inhibited by excess free **pHis** (Fig. 2B), suggesting that **pPyp-BP** and **pHis** share the binding sites. Furthermore, a **pHis**-accepting site mutant of PtsI (H189E) showed significantly reduced labelling, confirming that the **pPyp-BP** targets the **pHis**-binding site (ESI Fig. S3†).

The structural similarity between **pHis** and pTyr raised the possibility that **pPyp-BP** might also bind to pTyr readers.[36] To address this, we performed labelling experiments using a recombinant SH2 domain (ESI Fig. S4†).[37] As anticipated, only minimal background labelling was observed, which remained unaffected by the presence of **pHis** or pTyr.

These results indicate that **pPyp-BP** effectively and specifically labels **pHis** acceptors. However, the response regulators (*E. coli* OmpR and GlrR) in the TCS, other well-known **pHis** acceptors, were weakly labelled with **pPyp-BP**, but the labelling was not competed with **pHis**, indicating nonspecific interactions (ESI Fig. S5†). Although our probe may function for other response regulators, this finding suggests that its design may not be optimal for all **pHis** acceptors.

pPyp-BP labels putative pHis acceptors in *E. coli*

Having validated **pPyp-BP** with known **pHis** acceptors, we extended its application to identify new **pHis** acceptors. Numerous proteins in *E. coli* lysates were preferentially labelled with **pPyp-BP** than **Me-BP** (Fig. 2C). The labelling by **pPyp-BP** was gradually inhibited by increasing concentrations of free **pHis** (red stars, Fig. 2C and S7 ESI†) but not by excess pTyr, pSer, or pThr (ESI Fig. S8†), suggesting that these labelled proteins specifically interact with **pHis**. In contrast, some pPyp-binding proteins showed unchanged labelling (blue triangle, Fig. 2C) despite competition with free **pHis**, indicating they are artefacts that do not interact with **pHis**. The **pPyp-BP** labelling was also significantly reduced in urea-denatured lysates, indicating the labelling is specific for native proteins (ESI Fig. S9†).

To identify the labelled proteins, we subjected the **pPyp-BP**-labelled lysate to a click reaction with biotin-azide, followed by streptavidin-mediated enrichment. Streptavidin-based western blots of the biotin-labelled lysates showed similar labelling patterns to in-gel fluorescence imaging (ESI Fig. S10†). Proteomic analysis, compared with a lysate labelled with **Me-BP** (Fig. 3A, i *vs.* ii), identified 170 pPyp binders (Fig. 3B and ESI Table S1†).

Some of these binders bound to pPyp but not to native **pHis** (Fig. 2C, blue triangle). To eliminate such artefacts, we performed a competitive **pPyp-BP** labelling in the presence of excess **pHis** (Fig. 3A, i *vs.* iii). Among the 170 pPyp binders, 55 proteins showed reduced labelling under these conditions, indicating competition with **pHis** (Fig. 3C and Supplementary Table S2†). These 55 proteins were identified as putative **pHis** binders (Fig. 3D).

The identified **pHis** binders included both potential **pHis** readers and acceptors. Since **pHis** acceptors are targets for phosphoryl transfer, they are expected to be phosphoproteins (Fig. 1B).[26] Approximately 24% of the identified hits (13 out of 55) possessed previously annotated **pHis** sites[22] (Tables 1 and ESI S2†) and were classified as **pHis** acceptors (Fig. 3D). Among these, PtsI – a known **pHis** acceptor – was identified, validating the reliability of our chemoproteomic approach. However, response regulators of the TCS, another class of known **pHis** acceptors, were not detected, which is consistent with our *in vitro* results (ESI Fig. S5†). SixA, one of our model **pHis** acceptors, was also not detected, presumably due to its low abundance (20–60 copies per cell).[38]

Recombinant PfkA is selectively targeted by pPyp-BP

Next, phosphofructokinase-1 (**PfkA**), the top hit in our chemoproteomic analysis (Table 1), was selected for further validation. **PfkA** catalyses the phosphorylation of fructose 6-phosphate (F6P) to fructose 1,6-bisphosphate (F1,6BP), the first committed step in glycolysis. This reaction is a key regulatory point that determines overall glycolytic flux.[39–41] Therefore, investigating this enzyme could reveal a potential mechanistic link between

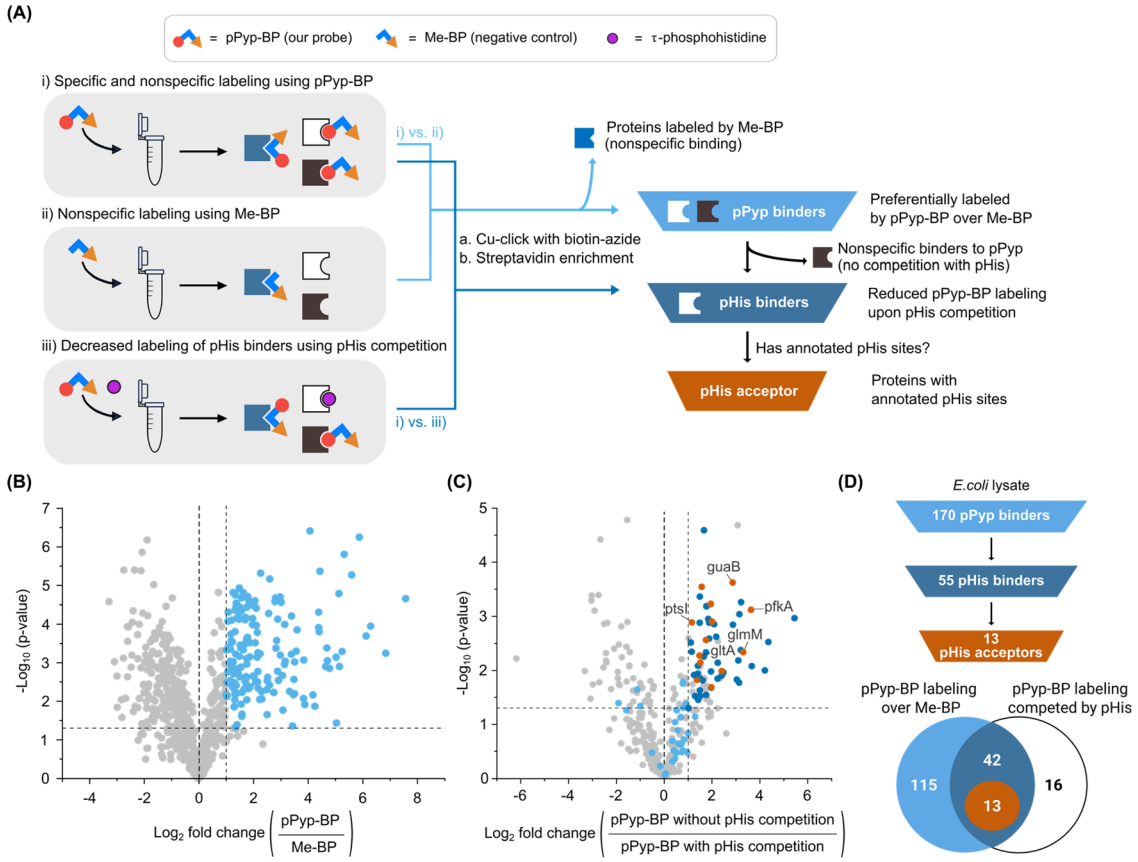

Fig. 3 (A) Overview of the chemoproteomic workflow to identify **pHis** acceptors. Probe-labelled *E. coli* lysates were subjected to a Cu-click reaction with biotin-azide and streptavidin agarose bead enrichment. The **pPyp-BP** probe photocrosslinks with pPyp binders, which are distinguished from nonspecific binders to the negative control **Me-BP** (experiments i *vs.* ii). Competitive proteome labelling with **pPyp-BP** in the presence of excess **pHis** identifies **pHis** binders (experiments i *vs.* iii) by eliminating proteins that interact with **pPyp-BP** but do not recognize native **pHis**. Proteins with annotated **pHis** sites are subsequently designated as **pHis** acceptors. (B) Identification of putative pPyp binders in *E. coli* grown in LB medium. Light blue dots represent 170 proteins preferentially labelled by **pPyp-BP** over **Me-BP**. (C) Identification of putative **pHis** acceptors in LB-grown *E. coli*. A total of 77 proteins that exhibited significantly reduced **pPyp-BP** labelling in the presence of **pHis** made the cutoff, indicating **pHis** specificity. Among them, 55 proteins (dark blue and orange dots) also showed preferential labelling by **pPyp-BP** over **Me-BP** (as in panel B) and were designated as **pHis** binders. Of these, 13 proteins (orange dots) with previously annotated **pHis** sites were further classified as putative **pHis** acceptors. (D) Selection process for **pHis** acceptors using data from panels B and C. The Venn diagram is color-coded to match the dot classifications in the volcano plots.

Table 1 Putative **pHis** acceptors identified in this study, ranked by log 2 fold changes between **pPyp-BP** labelling with and without **pHis** competition (see Fig. 3C and ESI Table S2)

Protein symbol	Protein name (UniProt)	Reported **pHis** site[22,44]	Log 2 fold change
PfkA	ATP-dependent 6-phosphofructokinase isozyme 1	His249	3.63
GlmM	Phosphoglucosamine mutase	His102, His387	3.30
GuaB	Inosine-5′-monophosphate dehydrogenase	His88	2.87
GltX	Glutamate-tRNA ligase	His128, His130, His131	2.40
AceE	Pyruvate dehydrogenase E1 component	His459	2.01
Rho	Transcription termination factor Rho	His294	1.98
GroEL	60 kDa chaperonin	His400	1.95
NrdA	Ribonucleoside-diphosphate reductase 1 subunit alpha	His331	1.75
PykA	Pyruvate kinase II	His46	1.57
ClpB	Chaperone protein ClpB	His566, His567	1.52
SucC	Succinyl-CoA ligase [ADP-forming] subunit beta	His3	1.48
GltA	Citrate synthase	His110, His114, His122, His229, His283	1.37
PtsI	Phosphoenolpyruvate-protein phosphotransferase	His188 (ref. 45)	1.16

histidine phosphorylation and central metabolic pathways. Although the allosteric regulation of **PfkA** by small molecule metabolites is well established,[42] its modulation through post-translational modification has yet to be elucidated.

We first repeated the photocrosslinking using recombinant **PfkA** *in vitro*. Notably, **PfkA** was efficiently photolabeled with **pPyp-BP** but not with **Me-BP**. Moreover, the labelling gradually decreased with increasing concentrations of free **pHis** (Fig. 4A). These results indicate that **pPyp-BP** targets **PfkA** at its **pHis**-recognition site. Subsequent LC-MS/MS analysis of the probe-labelled **PfkA** identified Met60 and Phe73 as the labelling sites (Fig. 4B and ESI Table S3†), both located near the substrate-binding pocket and His249, a known **pHis** site (ESI Fig. S11†).[22,43] This finding suggests that if **PfkA** is a **pHis** acceptor, a **pHis** donor might bind **PfkA** at this site, leading to His249 phosphorylation.

PfkA His249 is phosphorylated by PtsH and dephosphorylated by SixA *in vitro*

To confirm **PfkA** as a **pHis** acceptor, we incubated it with free **pHis** (ESI Fig. S13†). Western blot analysis demonstrated **PfkA** was indeed histidine-phosphorylated, and LC-MS/MS analysis confirmed phosphorylation at His249, a previously annotated **pHis** site (Fig. 4C and ESI Table S4†).[22,44] No additional phosphosites were detected, highlighting the unique reactivity of His249.

While promising, free **pHis** as an amino acid monomer is unlikely an endogenous phosphoryl donor for **PfkA**. We next examined proteins within the PTS, which are well-established **pHis** donors and acceptors (Fig. 4D).[13] Notably, PTS-mediated phosphoryl transfer can extend to non-PTS **pHis** acceptors, such as transcriptional regulators, to regulate carbohydrate metabolism.[46]

Gratifyingly, we observed histidine phosphorylation of **PfkA** following incubation with histidine-phosphorylated PtsH (PtsH–**pHis**), which was phosphorylated by PtsI–**pHis** (Fig. 4E). However, PtsI–**pHis** alone could not phosphorylate **PfkA**. Mutation of **PfkA** His249 to Ala reduced **pHis** signal by 78%, confirming His249 as the primary phosphorylation site *in vitro* (Fig. 4E).

We then investigated whether SixA, the only known **pHis**-specific phosphatase in *E. coli*,[47,48] could dephosphorylate **PfkA**–**pHis**. Notably, **PfkA**–**pHis** phosphorylated by PtsI/PtsH was efficiently dephosphorylated by SixA *in vitro* (Fig. 4E). Western

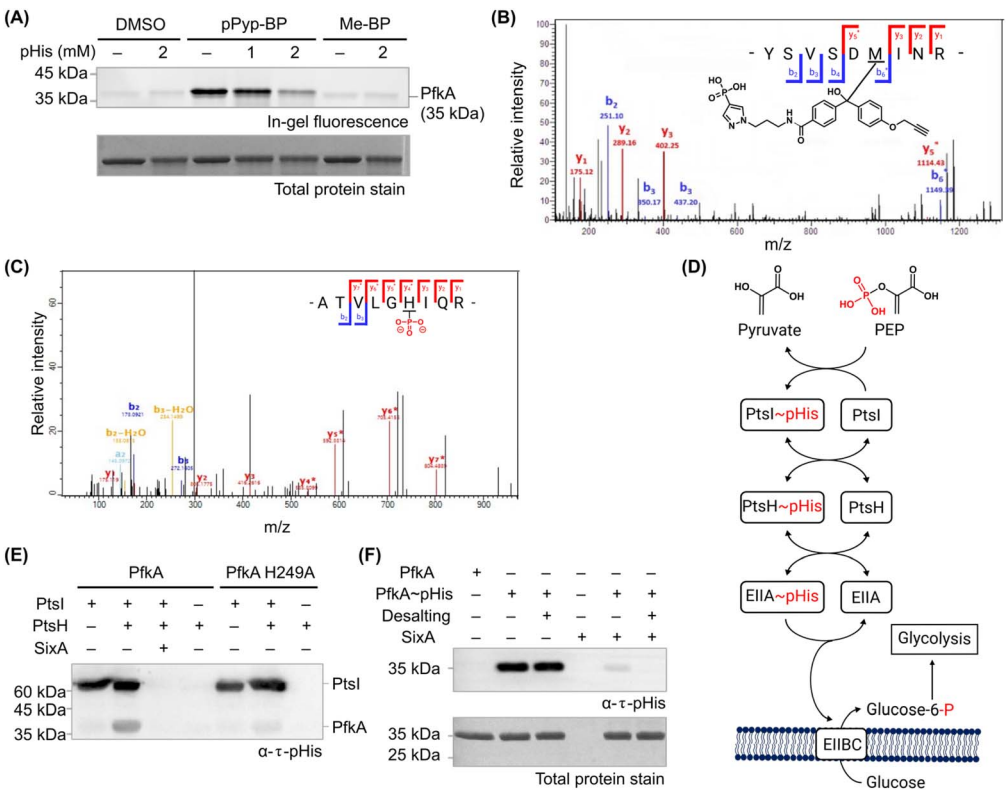

Fig. 4 (A) **pPyp-BP** labelling of recombinant **PfkA** decreased with increasing concentrations of **pHis**, indicating labelling specificity. (B) LC-MS/MS spectrum of **PfkA** labelled with **pPyp-BP**, identifying Met60 as the modification site. (C) LC-MS/MS spectrum confirming histidine phosphorylation at His249 in **PfkA**. (D) Schematic representation of the PTS phosphorelay: The phosphoryl group of phosphoenol pyruvate (PEP) is sequentially transferred to histidine residues of PtsI, PtsH, and EIIA, ultimately phosphorylating the incoming sugar (glucose) for glycolysis. (E) **PfkA** was histidine-phosphorylated by the PtsH/PtsI phosphorelay system. Minimal **PfkA** phosphorylation occurred without PtsH, confirming PtsH–**pHis** as the **pHis** donor. Reduced phosphorylation of the **PfkA** H249A mutant confirms His249 as the primary **pHis** site. Both **PfkA**–**pHis** and PtsI–**pHis** were dephosphorylated by SixA, suggesting the possibility of indirect **PfkA** dephosphorylation *via* unphosphorylated PtsI/PtsH. (F) SixA directly dephosphorylated **PfkA**–**pHis** without requiring PtsH or PtsI. (Coomassie-stained gels shown in ESI Fig. S12.†).

blot analysis revealed that PtsI–**pHis** was also dephosphorylated by SixA, suggesting that dephosphorylation of **PfkA–pHis** might be mediated indirectly *via* nonphosphorylated PtsI/PtsH. However, SixA directly dephosphorylated **PfkA–pHis** even in the absence of PtsI/PtsH (Fig. 4F).

Histidine phosphorylation of PfkA His249, mediated by PtsH and SixA, regulates the kinase activity in *E. coli*

With promising *in vitro* results, we next investigated the histidine phosphorylation of **PfkA** in *E. coli*. His_6-tagged **PfkA** was recombinantly expressed and purified from wild-type (WT) or *sixA* deletion (Δ*sixA*) strains of *E. coli* MG1655. Gratifyingly, **PfkA** from the Δ*sixA* strain showed robust histidine phosphorylation, whereas **PfkA** from the WT strain was unphosphorylated (Fig. 5A), indicating that SixA is the primary endogenous phosphatase for **PfkA–pHis**. Additionally, **PfkA** H249A mutant from the Δ*sixA* strain lacked **pHis** (Fig. 5B), verifying His249 as the *in vivo* phosphorylation site.

Next, we investigated the functional impact of His249 phosphorylation. His249, located within the substrate-binding pocket, forms a crucial hydrogen bond with its substrate F6P or product F1,6BP (Fig. 5C).[43] Mutation of His249 to Glu substantially reduced enzyme activity by decreasing its affinity for F6P, likely due to electrostatic repulsion between the phosphoryl group and the glutamate residue.[49] Therefore, we hypothesised that phosphorylation of His249 would similarly impair enzyme activity.

To test this, we performed *in vitro* kinase activity assays[50] using **PfkA** purified from different cellular conditions, reflecting the *in vivo* phosphorylation states (ESI Table S5†). **PfkA** isolated from the Δ*sixA* strain showed a 13-fold increase in **pHis** level and a 97% reduction in kinase activity compared with **PfkA** from the WT strain (Fig. 5D). Notably, treatment with recombinant SixA restored **PfkA** activity by dephosphorylating **PfkA–pHis** (Fig. 5D), thereby demonstrating the reversible inhibitory effect of histidine phosphorylation.

Finally, we evaluated PtsH as the endogenous phosphorylation donor of **PfkA**, as our *in vitro* results suggested. In *E. coli*, PtsH is well-known to exist as PtsH–**pHis** under non-PTS carbon sources, such as glycerol, but remains unphosphorylated in glucose media.[51] Notably, **PfkA** from glycerol-grown *E. coli* showed higher histidine phosphorylation and significantly lower kinase activity than **PfkA** from glucose-fed *E. coli* (Fig. 5E, left). Again, recombinant SixA restored **PfkA** activity by dephosphorylating **PfkA–pHis** (Fig. 5E, right). The catalytically

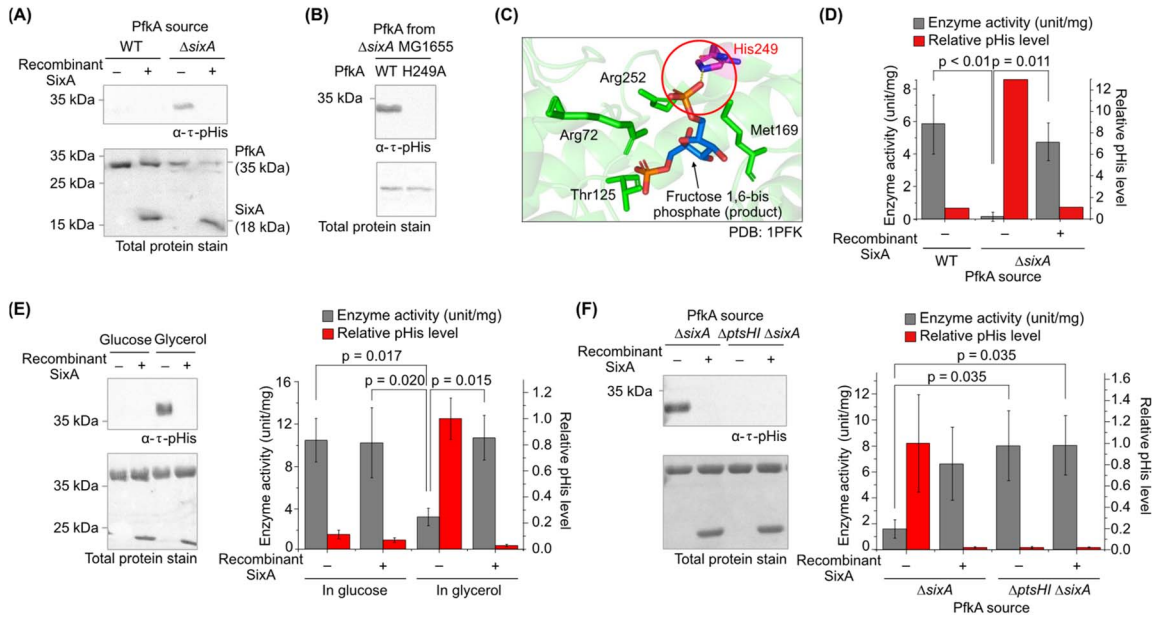

Fig. 5 (A) Western blot analysis comparing **PfkA** isolated from wild-type (WT) or *sixA* deletion (Δ*sixA*) *E. coli* MG1655 strains which were cultured in minimal media supplemented with glycerol. **PfkA** from the Δ*sixA* strain exhibited a higher **pHis** level than the WT strain, and treatment with recombinant SixA dephosphorylated **PfkA–pHis**. (B) The **PfkA** H249A mutant isolated from the Δ*sixA* strain lacked phosphorylation, confirming His249 as the *in vivo* phosphorylation site. Cells were cultured in minimal media supplemented with glycerol. (C) Crystal structure of **PfkA**'s active site (PDB: 1PFK), showing His249 interacting with the phosphoryl group of fructose 1,6-bisphosphate. (D) Quantification of **pHis** levels (from Figure 5A) and **PfkA** kinase activity from WT and Δ*sixA* strains. **PfkA–pHis** from the Δ*sixA* strain exhibited reduced enzyme activity and elevated histidine phosphorylation, while the addition of recombinant SixA restored the kinase activity and reduced **pHis** levels, demonstrating inverse correlation between these parameters. (E) Histidine phosphorylation and kinase activity of **PfkA** isolated from the Δ*sixA* strain cultured in glucose-*versus* glycerol-containing minimal medium. **PfkA** from glucose-grown bacteria showed negligible histidine phosphorylation and higher kinase activity, whereas **PfkA** from glycerol-cultured *E. coli* showed the opposite, implicating the PTS involvement in **PfkA** phosphorylation. (F) Significant reduction in **PfkA** phosphorylation and higher enzyme activity in the Δ*ptsHI* Δ*sixA* strain indicated that PtsH is responsible for **PfkA** phosphorylation *in vivo*. In all cases, the addition of recombinant SixA fully dephosphorylated **PfkA** and restored kinase activity. Cells were cultured in minimal media supplemented with glycerol. (Coomassie-stained gels and uncropped full membrane images are shown in ESI Fig. S14.† Raw kinetic data for the **PfkA** activity assays are provided in ESI Table S5.†).

inactive SixA H8A mutant failed to dephosphorylate **PfkA–pHis** or restore its enzyme activity, confirming that SixA's effect is strictly dependent on its phosphatase activity (ESI Fig. S15†). Furthermore, the deletion of the *ptsHI* genes abrogated **PfkA** phosphorylation, indicating that PtsH is responsible for **PfkA** phosphorylation *in vivo* (Fig. 5F).

Discussion

This study identified novel **pHis** acceptors in *E. coli* by developing a novel chemoproteomics workflow based on a stable **pHis** analogue. Despite the established significance of known **pHis** acceptors, the presence and function of additional **pHis** acceptors have remained unknown. Capturing the transient interaction during phosphotransfer has been a key challenge in discovering new **pHis** acceptors (Fig. 1B), which our approach successfully addresses. Given that **pHis** acceptors participate in phosphotransfer *via* nucleophilic attack, future probes may be improved by incorporating electrophilic warheads or transition-state analogues to target the transfer event.

Our findings demonstrate that the enzymatic activity of *E. coli* **PfkA** is posttranslationally regulated through histidine phosphorylation mediated by PtsH–**pHis** in the PTS (Fig. 4D). Beyond the canonical role in sugar uptake, PTS proteins also regulate other non-PTS proteins depending on their phosphorylation state. For example, unphosphorylated PtsH, also known as HPr, binds to and modulates the activity of several metabolic enzymes in *E. coli*, including pyruvate kinase, glucosamine-6-phosphate deaminase, glycogen phosphorylase, and phosphofructokinase-2 (PfkB), an isoenzyme of **PfkA**.[52–54]

In contrast, the regulatory functions of histidine-phosphorylated PTS remain less understood. Most identified **pHis** acceptors of PTS proteins are transcription regulators.[46] This study broadens the functional scope of PTS by demonstrating that **PfkA**, a key glycolytic enzyme, serves as a **pHis** acceptor for PtsH–**pHis**. Glycerol kinase (GlpK) in Firmicutes has also been reported to undergo histidine phosphorylation *in vitro*, mediated by PtsI and PtsH, but whether this phosphorylation occurs *in vivo* is unclear.[55]

The robust phosphorylation of overexpressed **PfkA**-His_6 in this study suggests that PtsI/PtsH possesses a phosphorylation capacity well beyond what is required for regulating endogenous **PfkA**. This raises the intriguing possibility that PtsI/PtsH may phosphorylate additional cellular targets beyond **PfkA**, potentially expanding its regulatory role. Further investigations will be necessary to explore this possibility.

PfkA is a unique **pHis** acceptor as it lacks previously known **pHis**-accepting domains such as HPr, EII, PTS regulation domains (PRD),[56] and the receiver domain (REC) of response regulators.[57] AlphaFold3 modelling[58] of the complex between PtsH–**pHis** and **PfkA** revealed that the **pHis**15 of PtsH–**pHis** and His249 of **PfkA** are well-positioned for phosphotransfer. This spatial arrangement was absent in the predicted interaction between **PfkA** and unphosphorylated PtsH (ESI Fig. S16†), suggesting a **pHis**-dependent interaction. Further structural studies of this interaction will be necessary to fully understand this mechanism.

We also demonstrated that SixA dephosphorylates and reactivates of **PfkA–pHis**. Previously, SixA was only known to target the phosphotransfer protein NPr[48] and the histidine kinase ArcB,[47] which are involved in nitrogen assimilation and anaerobic responses, respectively. Our findings suggest that SixA has a broader role in regulating central metabolism in *E. coli*.

Based on our findings, we propose a mechanistic model for how histidine phosphorylation of **PfkA** regulates glycolysis (Fig. 6). In the absence of glucose, elevated PtsH–**pHis** levels lead to **PfkA** phosphorylation at His249, leading to reduced enzymatic activity. As **PfkA** catalyses the first committed step in glycolysis, its inhibition would decelerate the glycolytic pathway.[41] In glucose-rich conditions, both PtsH and **PfkA** remain unphosphorylated, thereby facilitating glycolysis.

This regulatory mechanism may extend beyond *E. coli*. The PTS is widespread in bacteria, and sequence alignment reveals that His249 of **PfkA** is highly conserved across both Gram-positive and Gram-negative bacteria (ESI Fig. S17†). Notably, the homologous histidine (His250) in **PfkA** of *Staphylococcus aureus* is also a **pHis** site,[59] suggesting that **PfkA** may undergo similar posttranslational regulation in Gram-positive bacteria.

In *E. coli*, several enzymes are regulated by phosphorylation at serine, threonine, or tyrosine residues.[60] Our findings indicate that histidine phosphorylation similarly modulates enzyme activity. Notably, 11 of the 13 putative **pHis** acceptors identified are metabolic enzymes (Table 1), highlighting potential links between histidine phosphorylation and various metabolic pathways. Further studies are needed to determine whether the activities of these enzymes are regulated by histidine phosphorylation.

Our work also has practical applications in metabolic engineering. Phosphofructokinases are targeted to regulate metabolic flux through glycolysis, particularly through the Embden–Meyerhof–Parnas pathway (EMPP). Deletion of *pfkA* and/or *pfkB* in *E. coli* has been utilised to redirect metabolic flux toward the pentose phosphate pathway (PPP) and the Entner–Doudoroff

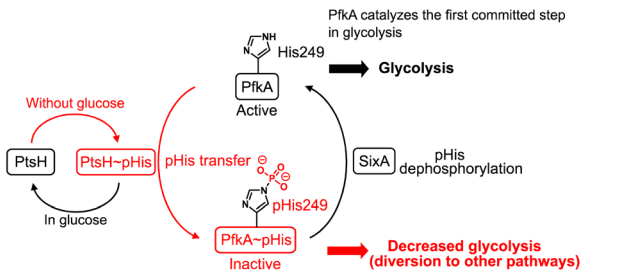

Fig. 6 A mechanistic model depicting the regulation of glycolysis by the interaction between PtsH and **PfkA** in *E. coli*. In glucose-rich conditions, PtsH remains dephosphorylated, allowing **PfkA** to retain its enzymatic activity. In this active state, **PfkA** phosphorylates F6P, facilitating its entry into the downstream Embden–Meyerhof–Parnas pathway (EMPP). Conversely, in the absence of glucose, PtsH is phosphorylated to PtsH–**pHis**, which phosphorylates **PfkA** at His249 to form **PfkA–pHis**. This phosphorylation inactivates **PfkA**, preventing F6P processing and redirecting the metabolic flux to alternative pathways.

pathway; however, these deletion mutants suffer from reduced growth rates.[61,62] While we have not directly measured metabolic changes in this study, insights from this study may offer an alternative approach for dynamically regulating **PfkA** activity and glycolytic flux in *E. coli*.

While this proof-of-concept study focused on *E. coli*, our method can be extended to eukaryotes, where numerous **pHis** sites have been identified.[21] In mammals, histidine kinases, including nucleoside diphosphate kinases (NDPKs),[63] and **pHis** phosphatases, such as phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP) and phosphohistidine phosphatase 1 (PHPT1), are implicated in cancer development and metastasis.[64,65] The identification of novel **pHis** acceptors in eukaryotes will help further elucidate the physiological functions of **pHis**.

Conclusions

We developed a chemoproteomic workflow utilising a stable **pHis** analogue as bait to identify **pHis** acceptors in *E. coli*. This study validated **PfkA**, a key glycolytic enzyme, as a *bona fide* **pHis** acceptor, of which catalytic activity is dynamically modulated by histidine phosphorylation. The identification of additional putative **pHis** acceptors, many of which are metabolic enzymes, hints at the potential of histidine phosphorylation as a regulatory mechanism in bacterial metabolism, warranting further investigations.

As the number of annotated **pHis** sites in the proteome grows, future studies should aim to elucidate the regulatory mechanisms and physiological roles of these phosphosites. This proof-of-concept study provides a foundation for addressing these challenges.

Data availability

Additional experimental details and data are provided in the ESI.† The proteomics raw data have been deposited to the ProteomeXchange Consortium (PRIDE, PXD053006 and PXD056526).

Author contributions

S. C., S. A., and J.-M. K. designed research and analysed data. S. C., S. A. performed the experiments. K. H. C. prepared the mutant *E. coli* strains. S. K. L. and J.-M. K. supervised the research. S. C., S. A., and J.-M. K. wrote the manuscript with inputs from all authors.

Conflicts of interest

There are no conflicts to declare.

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