MYPT1, the targeting subunit of smooth-muscle myosin phosphatase, is a substrate for the asparaginyl hydroxylase factor inhibiting hypoxia-inducible factor (FIH)

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INTRODUCTION

FIH [factor inhibiting HIF (hypoxia-inducible factor)] is an asparaginyl hydroxylase that contributes to oxygen-dependent regulation of hypoxically induced genes by hydroxylation of HIF [1,2]. Hydroxylation of the CAD (C-terminal transactivation domain) of HIF suppresses HIF activity by preventing the recruitment of the p300 (a transcriptional activator protein required to drive p53 expression)/CBP (CREB (cAMP-response-element-binding protein)-binding protein) transcriptional co-activators to HIF target genes [3,4].

Recently, several novel substrates of FIH have been identified that are targeted for asparaginyl hydroxylation within a protein–protein interaction motif known as the ARD [ankyrin repeat domain]. These experiments have demonstrated that asparaginyl hydroxylation occurs at multiple sites within the ARDs of several proteins, including MyPT1 (inhibitor of NF-κB (nuclear factor κB)), Notch-1–3, ASB4 (AR and SOCS box protein 4), Rabankyrin and RNASel [5–8]. Quantitative MS/MS (tandem MS) analyses have demonstrated that the extent of hydroxylation may vary between sites in the same ARD, and is generally incomplete at any given site, at least in cells grown under the standard tissue culture conditions used in these experiments. However, despite the identification of multiple ARD substrates of FIH, functional insights into the role of ARD hydroxylation have so far been limited [5,6]. Although effects of FIH have been reported on biological functions of some of these target proteins, it is not yet clear how they relate to hydroxylation [7,9].

ARDs contain variable numbers of ARs, which consist of paired antiparallel α-helices that stack upon one another to form a protein interaction surface [10]. Although hydroxylation of the Notch1 ARD promotes the formation of an intra-repeat hydrogen bond postulated to enhance structural stability, it does not alter the classical ARD conformation [6]. The clearest biological action of an FIH-mediated ARD hydroxylation defined to date is the cross-competition between Notch1 and HIF–CAD for asparagine hydroxylation. Thus expression of the ARD of Notch1 competitively inhibits FIH-mediated HIF–CAD asparagine hydroxylation, resulting in enhanced HIF–CAD activity [6].

Although yeast two-hybrid and proteomic screens have identified only a relatively small number of ARD substrates of FIH [5,6], the human genome encodes over 300 ARD proteins, most of which contain consensus motifs for FIH-mediated hydroxylation [SMART (Simple Modular Architecture Research Tool) database 11]. This raises important questions as to whether hydroxylation occurs on other ARD proteins and, if so, to what extent, what roles hydroxylation might play in hypoxia signalling, and what the implications are for cross-competition with HIF for asparagine hydroxylation.

To address these questions and to determine whether asparagine hydroxylation might occur on ARD proteins not captured in previous screens, we chose to study a bioinformatically identified
candidate substrate, MYPT1 (myosin phosphatase targeting subunit 1). MYPT1 is an ARD-containing protein with established functions in contractile regulation that are of potential interest to hypoxia biology [12], and for which there is structural information that might guide the analysis of functional effects [13].

We show that the ARD of MYPT1 contains three sites of asparaginyl hydroxylation and that these hydroxylations are FIH-dependent. For the first time we show that asparagine hydroxylation occurs in an ARD protein purified from an animal: this hydroxylation is incomplete, suggesting that previous results from tissue culture cells give a true reflection of ARD hydroxylation in vivo. Further, we show that MYPT1, and other ARD substrates of FIH, can modulate the transcriptional activity of HIF–CAD through competitive FIH inhibition.

**MATERIALS AND METHODS**

**Cell culture**

HEK-293T [human embryonic kidney cells expressing the large T-antigen of SV40 (simian virus 40)], HeLa and MCF7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) foetal calf serum, 2 mM L-glutamine, 50 IU · ml⁻¹ penicillin and 50 μg· ml⁻¹ streptomycin.

**Plasmids**

Human MYPT1 ARD (residues 1–296) was amplified by PCR and cloned into pEF1/V5-His A (Invitrogen). Wild-type FIH and D201A FIH plasmids were as described previously [6]. For reporter gene assays, full-length human MYPT1, human MYPT1 ARD (residues 1–296), mouse Notch1 ICD (intracellular domain; residues 1751–2531), full-length human Tankyrase1, full-length human p105, full-length human IκBα, full-length human Rabankyrin5 and full-length human RNaseL were amplified by PCR and cloned into p3xFLAG-CMV-10 (Sigma). The integrity of all constructs was verified by DNA sequence determination.

**Transfection and reporter gene assays**

Cells were transfected in dishes of 10 or 15 cm in diameter, using 4.5 or 10 μg of total plasmid DNA respectively and FuGENETM transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. Where applicable, MYPT1 ARD and FIH expression plasmids were co-transfected in the ratio 4:1. The transcriptional activity of HIF1α CAD was analysed in HeLa cells using a GAL4 DBD (DNA-binding domain)–HIF–CAD fusion protein and the UAS (upstream activating sequence)–luciferase reporter system as described previously [6].

**Knock-down of FIH expression using siRNA (small interfering RNA)**

Human FIH (target F1) and control dHIF (Drosophila HIF) duplexes have been described previously [14], and were synthesized by Ambion. Cells were transfected twice at 24 h intervals using a 20 nM dose of duplex and Oligofectamine reagent (Invitrogen), according to the manufacturer’s instructions.

**Antibodies**

Anti-FIH antibody was as described previously [14]. Anti-MLC (myosin light chain) (M4401), anti-β-tubulin (T5293) and anti-

**Immunoprecipitation and immunoblotting**

Cells were lysed in Jie’s buffer [100 mM NaCl, 20 mM Tris/HCl, pH 7.5, 5 mM MgCl₂ and 0.5% (v/v) Nonidet P40] supplemented with Complete™ EDTA-free protease inhibitor cocktail (Roche Applied Science). V5-tagged MYPT1 ARD was immunoprecipitated with anti-V5–agarose affinity gel (Sigma). Endogenous MYPT1 was immunoprecipitated by pre-incubation with anti-MYPT1 antibody followed by incubation with Protein A–agarose (Upstate Biotechnology). For immunoblotting of phospho-MLC, cells were lysed directly into 3 × SDS sample buffer and sonicated briefly. Lysates or immunoprecipitates were resolved by SDS/PAGE, electrophoretically transferred to PVDF membranes (Millipore) and probed using the indicated primary antibodies. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Dako) were used with either SuperSignal West Pico or SuperSignal West Dura (Pierce Biotechnology) to visualize immunoreactive species. Where necessary when immunoblotting immunoprecipitates, anti-rabbit TrueBlot horseradish peroxidase-conjugated secondary antibody (eBioscience) was used to avoid signal from immunoprecipitating immunoglobulin.

**Protein digestion and LC (liquid chromatography)-MS analysis**

After resolution by SDS/PAGE and staining, protein-containing bands were excised and digested in bicarbonate buffer using either sequencing-grade trypsin (Promega) or sequencing-grade endoprotease Glu-C [also known as staphylococcal (Staphylococcus aureus) V8 protease; Roche Applied Science] as described in [16]. LC was performed using an Ultimate system equipped with a Famos autosampler and C18 PepMap column (LC Packings). The chromatography system was connected directly to a UV flow cell (Ultimate) and a three-dimensional high-capacity ion-trap mass spectrometer (HCTplus; Bruker Daltonics) via a pneumatically assisted nano-electrospray source. Silica-coated Picotips (New Objective) were used for electrospray ionization. Instruments were controlled using HyStar 3.0 and EsquireControl 5.2 software (Bruker Daltonics). Raw chromatography data were processed and Mascot-compatible files were generated using DataAnalysis 3.2 software (Bruker Daltonics). Searches were performed using Mascot software (Matrix Science [17]) using the most recent releases of the Swiss-Prot or NCBI nr databases, following which MS/MS spectra and extracted ion chromatograms were analysed in detail using DataAnalysis 3.2.

**Protein purification**

Endogenous myosin phosphatase holoenzyme was purified from turkey gizzard as described in [18].

**Statistical analysis**

Unless otherwise stated, quantitative data are presented as the means ± S.E.M. for three experiments. Statistical significance was evaluated using Student’s t test.
MYPT1 is a substrate for FIH

Figure 1 MYPT1 is a potential FIH substrate

Alignment of known FIH substrates with four putative hydroxylation sites in human MYPT1: Asn-67, Asn-100, Asn-226 and Asn-288. Arrow indicates target asparagine residues. Sequences were aligned using ClustalW and shaded using BoxShade.

RESULTS

MYPT1 is a potential FIH substrate

FIH-dependent asparagine hydroxylation sites within ARDs conform to a L(−8)D/E(−2)XN motif specifically located at the β-hairpin loops that connect ARs [5–7]. Bioinformatic alignment with known FIH substrates (Figure 1) indicated that the targeting subunit of smooth-muscle myosin phosphatase, MYPT1, contains two potential hydroxylation sites within the β-hairpin loops of its ARD that match the L(−8)D/E(−2)XN consensus (Asn-67 and Asn-226) and a further two conforming to L(−8)N (Asn-100 and Asn-288). Three of these sites (Asn-67, Asn-100 and Asn-226) have additional similarities to previously identified substrates in the primary sequence (Figure 1).

The ARD of MYPT1 is hydroxylated in vivo

To determine whether any of the asparagine residues identified were hydroxylated in vivo we immunopurified V5-tagged human MYPT1 ARD from transfected HEK-293T cells. The purified ARD protein was resolved by SDS/PAGE and digested using either trypsin or endoprotease Glu-C before analysis by LC-MS/MS. Peptides containing all four candidate asparagine residues were observed, and MS/MS sequencing assigned hydroxylation at Asn-67, Asn-100 and Asn-226 (Figure 2 and Supplementary Figure S1 at http://www.BiochemJ.org/bj/420/bj4200327add.htm). No hydroxylation was detected at Asn-288 (results not shown), the site with the least similarity to known FIH substrates (Figure 1).

FIH is necessary and sufficient for hydroxylation of the MYPT1 ARD

In order to determine whether MYPT1 was a substrate for FIH we quantified hydroxylation at Asn-67, Asn-100 and Asn-226 by LC-MS under conditions of various FIH interventions. HEK-293T cells were transfected with siRNA duplexes targeting FIH or a control sequence (dHIF), then subsequently transfected with MYPT1 ARD plus pcDNA3 vector (EV), FIH or a hydroxylation-defective FIH mutant (D201A). After immunoprecipitation and digestion, the extent of hydroxylation at each site was determined by LC-MS. In the presence of endogenous levels of FIH each of the three sites was approx. 5–10 % hydroxylated at the target asparagine (Figure 3A). Overexpression of FIH increased the level of hydroxylation to approx. 90 %, whereas overexpression of the FIH D201A mutant had no effect. Suppression of FIH by siRNA reduced the extent of hydroxylation at all three sites. Although consistent across the three sites this effect of FIH suppression was not statistically significant. This is likely to reflect the low basal levels of MYPT1 hydroxylation present under the experimental conditions, which were approaching the limits of detection by MS, combined with the incomplete siRNA-mediated suppression of FIH (Figure 3B).

These results thus confirm bioinformatic predictions that MYPT1 contains multiple sites of FIH-dependent hydroxylation within its ARD, consistent with the hypothesis that FIH-mediated hydroxylation is a general phenomenon among ARD-containing proteins. They also indicate that, at least in transfected tissue culture cells, hydroxylation of individual ARs is incomplete.

Endogenous MYPT1 is hydroxylated

To test whether asparagine hydroxylation occurs in the full-length protein in vivo and to assess the extent of hydroxylation in normal tissue, we next sought to determine whether a sample of endogenous animal MYPT1 was hydroxylated. A sample of myosin phosphatase holoenzyme, purified from turkey gizzard,
was resolved by SDS/PAGE and stained with Coomassie Blue. The MYPT1 species was excised and digested with trypsin or Glu-C and then analysed by LC-MS/MS and LC-MS.

Although the turkey MYPT1 gene has not been sequenced, the amino acid sequence of the chicken protein is available (NCBI database, gi number 633038). As conservation of the MYPT1 ARD between human and chicken is greater than 96%, it seems reasonable to assume a high degree of conservation between the chicken and turkey sequences. Searching the MS/MS fragmentation data against the chicken protein sequence correctly identified many predicted peptides, validating this assumption. MS/MS sequencing confirmed hydroxylation at Asn-67 and Asn-100 (Supplementary Figure S2 at http://www.BiochemJ.org/bj/420/bj4200327add.htm), and LC-MS quantified the degree of hydroxylation at the Asn-67 and Asn-100 sites as 48 and 43% respectively (Figure 4). A peptide containing Asn-288 was observed by Glu-C digestion, but in agreement with the data from cell culture experiments, no hydroxylation was detected at this site (results not shown). A peptide containing Asn-226 was not detected in these experiments, probably due to a glycine to arginine substitution four residues N-terminal to Asn-226 in the chicken protein relative to human MYPT1, which results in a short tryptic fragment that is not amenable to detection by MS.

**FIH affects neither the interaction between MYPT1 and PP1cδ nor MLC phosphorylation**

We next sought to understand what the consequences of asparagine hydroxylation are on the function of myosin phosphatase. MYPT1 regulates MLC phosphorylation by recruiting the PP1cδ (protein phosphatase type 1cδ isoform), by imparting selectivity towards MLC and by fully activating the myosin phosphatase complex [19].

The structure of the N-terminus of MYPT1 in complex with PP1cδ shows that the main binding site for PP1cδ is the well-characterized PP1 binding motif (KVKK) located immediately N-terminal to AR1 [13]. Additional interactions are mediated by the ARD: the β-hairpin loops of the two groups of ARs form a "clamp" that stabilizes the C-terminal tail of PP1cδ (Supplementary Figure S3 at http://www.BiochemJ.org/bj/420/bj4200327add.htm). Several residues in the MYPT1 ARD interact with PP1cδ, including Val-71, which is adjacent to Asn-67, and Asp-229 and Trp-231, which are C-terminal to Asn-226; this raises the possibility that hydroxylation of the asparagine residues could modulate the interaction with PP1cδ. To test this we immunoprecipitated endogenous MYPT1 from HEK-293T cells transfected with pcDNA3 vector (EV), FIH, D201A FIH, control siRNA (dHIF) or FIH siRNA. Small amounts of overexpressed FIH were observed co-immunoprecipitating with endogenous MYPT1. However, neither FIH overexpression nor knock-down affected the interaction between MYPT1 and PP1cδ (Figure 5A).
MYPT1 is a substrate for FIH

Structural data indicate that binding of phosphorylated MLC to the myosin phosphatase holoenzyme is mediated by an acidic groove created by the catalytic cleft of PP1cδ and the α-helices of the MYPT1 ARs [13]. It is thought that this interaction promotes the activity and specificity of PP1cδ for phosphorylated MLC. To determine whether FIH-mediated MYPT1 ARD hydroxylation regulates PP1cδ activity towards phosphorylated MLC, we asked whether FIH overexpression or knock-down had any effect on MLC phosphorylation using antibodies specific for phosphorylated MLC (Ser-19 or Thr-18/Ser-19). FIH knock-down or overexpression did not alter the levels of phosphorylated MLC in HEK-293T, HeLa or MCF7 cells, neither did they significantly affect the level of MYPT1 (Figure 5B).

Therefore FIH-mediated asparagine hydroxylation does not appear to affect the function of myosin phosphatase dramatically, at least under the conditions tested.

MYPT1 and other ARD-containing proteins can influence HIF transcriptional activity through competition for FIH

We have previously shown that Notch1 overexpression competitively inhibits HIF–CAD hydroxylation [6]. This competition results in enhanced HIF–CAD activity that can be detected in a luciferase reporter assay based on UAS–luciferase and an HIF–CAD–GAL4 DBD fusion. To test whether MYPT1 also enhances HIF–CAD activity, and whether this property is observed more generally among ARD substrates of FIH, HeLa cells were transfected with HIF–CAD–GAL4 DBD, UAS luciferase and 3×FLAG-tagged ARD-containing proteins. As expected, extracts from cells transfected with Notch1 ICD displayed luciferase activity 5–6-fold higher than control samples expressing only HIF–CAD–GAL4 DBD and UAS luciferase, reflecting reduction in FIH-mediated suppression of HIF–CAD activity by Notch1.
contain at least one candidate FIH hydroxylation site that fulfils the dual requirements of conforming to the consensus and being located on the β-hairpin loop of the AR. We therefore infer that the hydroxylation of ARDs by FIH is likely to be common, although perhaps not universal.

Of the four predicted hydroxylation sites in the eight ARs of the MYPT1 ARD, only Asn-288 was shown not to be an FIH substrate. It is unclear at present whether this is due to the divergent sequence surrounding Asn-288 relative to other FIH substrates (Figure 1), or to the fact that AR8 does not adopt a canonical AR fold [13]. Interestingly, Asn-100 was hydroxylated at a similar level to Asn-67 and Asn-226, despite the absence of D/E in the previously described L−α2D/E−α2XN motif, showing that an acidic residue is not essential for hydroxylation at this position in all ARD substrates. Taken together the results presented here provide some insights into the primary sequence determinants that regulate FIH-mediated ARD hydroxylation, and suggest that structural factors may also contribute.

Despite the proximity of known interaction sites between MYPT1 and PPlcδ to two of the three asparagine hydroxylation sites characterized here, we did not observe any significant effect of FIH manipulation on the MYPT1–PPlcδ interaction, or on the activity of myosin phosphatase towards MLC. With respect to the MYPT1–PPlcδ interaction, this may be unsurprising since the high-affinity binding site is located N-terminal to the ARD [20–22]. Furthermore, Asn-67 and Asn-226 project away from PPlcδ (Figure 5A) and may therefore not be expected to modify the described interactions, despite their close proximity [13]. Likewise, the β-hairpin loops, which contain the hydroxylated asparagine residues, are located on the opposite face of the ARD from which they contributes to the acidic groove of the MLC-binding site, consistent with the absence of detectable regulation of MLC phosphorylation by FIH. Although the lack of effect of asparagine hydroxylation on MYPT1 signalling is consistent with similar findings for other AR substrates, including IκBα and Notch [5,6], it is possible that FIH regulates myosin phosphatase under other specific conditions.

One physical consequence of Notch1 ARD asparagine hydroxylation is the formation of a hydrogen bond between the newly created hydroxyl group and an acceptor residue two positions N-terminal to the target asparagine [6]. In all previously characterized FIH substrates the predicted acceptor group is the carboxylate of an aspartic acid residue [5–7]. However, most candidate FIH sites without the D/E of the L−α2D/E−α2XN motif still contain a residue capable of hydrogen-bonding at this position, including for example the L−α2N−α2L−XN motif of Asn-100 in MYPT1, suggesting that this may be a general property of FIH-mediated ARD hydroxylation. Analyses of the published MYPT1 ARD structure [13] suggest that all three FIH hydroxylation sites are predicted to create a hydrogen bond to the −2 residue (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/420/bj4200327add.htm). Studies on a synthetic AR protein have shown that asparaginyl hydroxylation can stabilize the ARD fold [23]. However, whether the three hydroxylated residues in MYPT1 act in synergy to increase the stability of the canonical ARD fold remains to be determined.

In contrast with IκBα and Notch1, which contain two target asparagine residues that are differentially hydroxylated [5,6], the three sites of MYPT1 are hydroxylated to approximately the same extent. Although in HEK-293T cells hydroxylation of the overexpressed MYPT1 ARD was at a relatively modest level (5–10%), this is likely to be due to saturation of the endogenous FIH: co-expression of exogenous FIH increased hydroxylation at all three sites essentially to completion. In support of this argument, hydroxylation of the two sites observed in endogenous

DISCUSSION

We have shown in the present study that the targeting subunit of the smooth-muscle myosin phosphatase holoenzyme, MYPT1, is a novel FIH substrate and that its ARD is targeted for asparagine hydroxylation at multiple independent sites. The initial candidacy of these sites was based on bioinformatic alignment with known FIH substrates. Similar analysis suggests that most ARD proteins

Figure 6 MYPT1 and other ARD-containing proteins can influence HIF transcriptional activity through competition for FIH

(A) HeLa cells were transfected with UAS–luciferase, HIF–CAD–GAL DBD, the indicated 3×FLAG-tagged ARD protein (mH1 ICD: mouse Notch1 ICD; TNKS1: Tankyrase1) and β-galactosidase as a transfection control. At 48 h post-transfection, cells were harvested and analysed in triplicate for luciferase and β-galactosidase activity. ** P < 0.01 relative to EV control sample. (B) Cell extracts were analysed by anti-FLAG immunoblotting to confirm similar expression of the ARD proteins. Molecular masses are indicated to the left in kDa.
MYPT1 purified from animal tissue was significantly higher than in the overexpressed ARD from tissue culture cells. Importantly, however, hydroxylation at these residues was still incomplete. This is consistent with observations made on ARD proteins purified from tissue culture cells [5,6], and indicates that incomplete hydroxylation of ARDs is likely to be the physiological norm. This could reflect the fact that endogenous FIH is also limiting in vivo, perhaps due to the ubiquity and abundance of its substrates.

Finally, we demonstrate that, similarly to Notch, MYPT1 effectively inhibits FIH-dependent suppression of HIF–CAD activity. Interestingly, overexpression of several other known ARD substrates of FIH also enhanced the activity of HIF–CAD, although this effect is not universal and different ARD substrates compete to different extents (Figure 6). It is possible that the ability of an ARD to promote HIF–CAD activity depends on its affinity for FIH, and therefore on the extent to which the ARD physically sequesters FIH away from HIF–CAD.

Given that FIH-mediated hydroxylation is likely to be very common among ARD proteins and that several ARD substrates apparently inhibit HIF–CAD hydroxylation, it seems plausible that, collectively, ARDs act as a ‘sink’ for FIH. This would explain not only the observations of limiting FIH activity towards HIF–CAD in tissue culture models [14] but also the incomplete hydroxylation of ARDs themselves, due to cross-competition.

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SUPPLEMENTARY ONLINE DATA

MYPT1, the targeting subunit of smooth-muscle myosin phosphatase, is a substrate for the asparaginyl hydroxylase factor inhibiting hypoxia-inducible factor (FIH)

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Figure S1 The ARD of MYPT1 is hydroxylated in vivo

(A) Representative MS/MS spectra showing the tryptic fragment of human MYPT1 containing Asn-67 (GADIN\text{YANVDGLTA}YANVDGLTA\text{V}) in unoxidized and hydroxylated states. Spectrum (i) shows the unoxidized peptide, whereas spectrum (ii) shows the hydroxylated peptide. In (ii) an additional mass of 8 Da is observed in the \text{y}^{2+} (doubly charged) ion series at \text{y}^{24+}, indicating hydroxylation on Asn-67. For clarity, ions detected in the \text{y}^{1+} series prior to \text{y}^{15+} are not shown. (B) Representative MS/MS spectra showing the Glu-C fragment of human MYPT1 containing Asn-100 (NGA\text{NINQPDNE}) in unoxidized and hydroxylated states. Spectrum (i) shows the unoxidized peptide, whereas spectrum (ii) shows the hydroxylated peptide. In (ii) an additional mass of 16 Da is observed in the \text{b} ion series from \text{b}7, and in the \text{y} ion series from \text{y}6, indicating hydroxylation on Asn-100. Intens., intensity.

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Figure S2  Endogenous MYPT1 is hydroxylated

(A) MS/MS spectra showing the tryptic fragment of turkey MYPT1 containing Asn-67 (GADIN(YANVDGLTA) in unoxidized and hydroxylated states. Spectrum (i) shows the unoxidized peptide, whereas spectrum (ii) shows the hydroxylated peptide. In (ii) an additional mass of 8 Da is observed in the y++ (doubly charged) ion series at y24++, indicating hydroxylation on Asn-67. For clarity, ions detected in the y+ series prior to y15++ are not shown. (B) MS/MS spectra showing the GluC fragment of turkey MYPT1 containing Asn-100 (NGANINQPDNE) in unoxidized and hydroxylated states. Spectrum (i) shows the unoxidized peptide, whereas spectrum (ii) shows the hydroxylated peptide. In (ii) an additional mass of 16 Da is observed in the b ion series from b7, and in the y ion series from y6, indicating hydroxylation on Asn-100. Intens., intensity.
MYPT1 is a substrate for FIH

Figure S3 Crystal structure of MYPT1 ARD (in red) complexed to PP1cδ (in blue)

The three hydroxylation sites identified in the present study are indicated. (PDB accession number 1S70, [1]).

REFERENCE


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Figure S4 Asparagine hydroxylation of MYPT1 ARD may result in the formation of intra-AR hydrogen bonding

Close-up views of (A) Asn-67, Asn-100 and (B) Asn-226 showing the potential for hydrogen-bond formation to the −2 residue after asparagine hydroxylation (PDB accession number 1S70, [1]).