# A splice variant of the human phosphohistidine phosphatase 1 (PHPT1) is degraded by the proteasome 

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#### Abstract

Regulation of protein activity by phosphorylation is central in many cellular processes. Phosphorylation of serine, threonine and tyrosine residues is well documented and studied. In addition, other amino acids, like histidine can be phosphorylated, but neither the mechanism nor the function of this modification is well understood. Nevertheless, there is a 14 kDa enzyme with phosphohistidine phosphatase activity, named PHPT1, found in most animals, but not in bacteria, plant or fungi. There are a few splice variant transcripts formed from the human PHPT1 locus and some of them are predicted to form variant proteins, but studies of these proteins are lacking. In order to get insight into the possible function of the variant transcripts encoded at the PHPT1 locus, ectopic expression of PHPT1 transcript variant 6, predicted to be degraded by the non-sense mediated mRNA decay pathway, in HeLa cells was undertaken. In HeLa cells the splice variant protein was degraded by the proteasome, unlike the wild type protein. Using an in silico modeling approach the variant C-terminal end of the proteins were predicted to form different secondary structures that might explain the different properties of the two proteins. The specific degradation of the PHPT1 splice variant indicates that at least for the PHPT1 protein, the quality control and the self-guarding of the cellular system works at two levels, first at the RNA level, aberrant transcripts are degraded by the non-sense mediated mRNA decay pathway, and the small amount of proteins that are formed will be degraded by the proteasome.


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## 1. Introduction

Regulation of protein activity by phosphorylation is central in many cellular processes. Phosphorylation of serine, threonine and tyrosine is well documented and studied. Also other amino acids, like histidine (His), can be phosphorylated. However, the mechanism of His phosphorylation and function of the phosphorylated histidine ( pHis ) residues have remained enigmatic. In prokaryotes, fungi and plants histidine phosphorylation is an important signaling mechanism in the two-component system, while in higher eukaryotes very little is known about the function of His phosphorylation (Gonzalez-Sanchez et al., 2013). A 14 kDa enzyme capable of reversing pHis was purified in 2002 and thus the human phosphohistidine phosphatase (PHPT1) gene, encoding

[^0]the enzyme could be identified (Ek et al., 2002, Klumpp et al., 2002). The PHPT1 protein is ubiquitously expressed in mammalian cells (Zhang et al., 2009) (http://www.proteinatlas.org/search/PHPT1). In addition, proteins with homology to the PHPT1 protein can be found in most animals, but is lacking in bacteria, plant or fungi (Ma et al., 2005; Klumpp et al., 2010; Besant and Attwood, 2012).

Mutational analysis has identified conserved amino acid residues that are important for the enzymatic activity of the PHPT1 protein (Ma et al., 2005; Klumpp et al., 2010). Moreover, a deletion study revealed that the C-terminal part of the protein is necessary for the activity, since when five or more residues are removed from the C-terminal part of the protein phosphatase activity was drastically reduced (Klumpp et al., 2010). There are four predicted isoforms (isoforms 2-5) of the human PHPT1 protein resulting from various alternative splicing events. Isoform 3 is the wild type PHPT1 protein and there is just a two amino acid insertions that differs between isoform 3 and 4, while isoform 2 and 5 have
completely different C-terminal tails (http://www.ncbi.nlm.nih. gov/protein/?LinkName=gene_protein_refseq\&from_uid=29085). In addition, there are two transcripts originating from the PHPT1 gene locus that have been annotated as long non-coding RNAs (lncRNAs) in the NCBI database (http://www.ncbi.nlm.nih.gov/ nuccore/?LinkName=gene_nuccore_refseqrna\&from_uid=29085). These two transcripts (isoforms 6 and 7) are considered as non-coding due to their particular alternative splicing pattern, which renders these transcripts as likely candidates for nonsense-mediated mRNA decay (NMD). When a nonsense codon is introduced more than 50 nt away from the $3^{\prime}$ splice site in a transcript due to an aberrant splicing event it will cause the ribosome to pause during translation. This pause leads to degradation of the transcript by the nonsense-mediated decay (NMD) pathway (Kervestin and Jacobson, 2012). NMD is an evolutionary conserved quality control mechanism that selectively degrades mRNAs with premature termination codons to ensure that truncated protein variants will not be translated in the cell. Transcripts that escape the NMD quality control can give rise to non-functional proteins that may be degraded by protein quality control mechanism known as ubiquitin-proteasome system (UPS) in the cells (Sontag et al., 2014). Ubiquitin addition to proteins that targets them for degradation is catalyzed by sequential action of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). Among the described E3 ligases, the Cullin-RING E3 ubiquitin-Ligases (CRL) are recognized as the most eminent group of E3 ubiquitin-ligases. The essential proteins in the CRLs are the Cullins (Cul1-7 in mammals), which function as the molecular scaffolds to position the substrate proteins in close proximity to the E2 enzymes, which in turn attach the ubiquitin moiety to the proteins (Petroski and Deshaies, 2005).

In the present study we have performed a biochemical characterization of the PHPT1 protein isoforms 3 and 6 in prokaryotic and eukaryotic cells. Our results indicate that the PHPT1 protein isoform 6 [hereafter referred to as PHPT1(Spl)] is prone for proteasomal degradation in HeLa cells via Cul2- and Cul5-containing CRL complexes. This is in contrast to the PHPT1 protein isoform 3 [hereafter referred to as PHPT1(wt)], which remains resistant to proteasomal degradation. Moreover, we find that the PHPT1(Spl) protein forms high molecular weight species both in bacterial ( $E$. coli), fission yeast and in mammalian (HeLa) cells. The different properties of the proteins might be explained by the different secondary folding of the C-terminal part of the proteins detected using an in silico modeling approach. Our results suggests that in addition to the NMD-mediated degradation of the PHPT1 isoform 6 mRNA, also the UPS quality control mechanism is involved in the elimination of the aberrant PHPT1 isoform 6 protein in mammalian cells.

## 2. Material and methods

### 2.1. Plasmid construction

The coding regions of the PHPT1 wild type and splice variant 6 were synthesized by GeneArt, Life Technology and inserted into the bacterial expression vector pET11a (Merck Millipore) generating plasmids pET11a(T7)PHPT1(wt) and pET11a(T7)PHPT1(Spl). The genes was cut out with BamHI and ligated into the Schizosaccharomyes pombe expression vector pREP3X (Forsburg and Sherman, 1997) generating plasmid pREP3XPHPT1(wt) and pREP3XPHPT1(Spl). The two coding regions were also amplified by PCR with primers containing EcoRI and XhoI restriction sites and cloned into corresponding EcoRI and XhoI sites in pcDNA3.1 (Invitrogen) vector containing Flag epitope tag sequence. The generated plasmids pcDNA3.1(Flag)PHPT1(wt)
and pcDNA3.1(Flag)PHPT1(Spl) allow the expression of the PHPT1 proteins as N -terminal Flag-tagged fusion proteins in mammalian cells. Dominant negative Cullin1-5 (DN-Cul1-5) plasmids have been published before (Inturi et al., 2013).

### 2.2. Bacterial and fission yeast expression

Plasmids pET11a(T7)PHPT1(wt) and pET11a(T7)PHPT1(Spl) were transformed into E. coli bacterial strain BL21(DE3)pLysS along with an empty pET11a vector control. Cells were subcultured from overnight cultures and protein expression was induced with Isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM after $\mathrm{OD}^{600} 0.6$ was reached in 10 ml cultures. After 3 h of protein expression at $30^{\circ} \mathrm{C}$, with aeration, the culture was aliquoted into two 5 ml parts. One part of the culture was collected at 4000 g for 10 min to obtain a $70-100 \mathrm{mg}$ wet cell pellet and re-suspended in $400 \mu \mathrm{l}$ mild lysis buffer ( 250 mM sucrose, 50 mM HEPES, pH $7.5,150 \mathrm{mM} \mathrm{NaCl}, 5 \mathrm{mM}$ EDTA, $1 \%$ Tween-20, Complete Protease Inhibitor Cocktail (Roche) with $250 \mu \mathrm{~g} / \mathrm{ml}$ lysozyme. The cells were lysed on ice for 1 h with periodical vortexing every 20 min . The solution was then centrifuged at $21,000 \mathrm{~g}$ for 20 min and the supernatant was collected. The other part of the cell culture was collected at 4000 g for 10 min and re-suspended in $400 \mu \mathrm{l}$ of strong $1.5 \%$ SDS RIPA buffer ( 50 mM Tris, $\mathrm{pH} 8.0,150 \mathrm{mM} \mathrm{NaCl}, 1 \%$ Triton X-100, $1.5 \%$ SDS, 2 mM EDTA, 50 mM NaF, 0.1 mM sodium vanadate, $20 \mathrm{mM} \beta$ mercaptoethanol) and boiled for 10 min at $98{ }^{\circ} \mathrm{C}$. The supernatant was collected after centrifugation for 20 min at $21,000 \mathrm{~g}$.

The fission yeast strain PJ121 ( $h^{+}$ura4-D18 leu1-32 ade6M216) was transformed with the empty vector pREP3X alongside pREP3XPHPT1(wt) and pREP3XPHPT1(Spl). The transformed yeast strains were cultured in minimal PMG-leu media (http://www-bcf. usc.edu/ forsburg/media.html) to select for the plasmid and protein was extracted in 50 mM HEPES, $\mathrm{pH} 7.5,150 \mathrm{mM} \mathrm{NaCl}, 1 \%$ Tween-20, Complete Protease Inhibitor Cocktail (Roche) by beadbeating with glass beads at top speed in the Bio101 FastPrep machine.

### 2.3. PHPT1 enzymatic activity measurements

PHPT1 enzymatic activity measurements were performed as described previously (Beckman-Sundh et al., 2011).

### 2.4. Cell culture and transient transfection

HeLa cells obtained from ATCC (www.atcc.org) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10\% FCS (PAA) and penicillin-streptomycin solution (Gibco). Cells were transiently transfected with plasmids pcDNA3.1(Flag)PHPT1(wt) and pcDNA3.1(Flag)PHPT1(Spl) by using the Turbofect reagent (Thermo Scientific) at 60-80\% cell confluence on 6-well or 24 -well cluster plates according to the manufactures instructions. Proteasome inhibitor MG132 (Sigma, C2211, dissolved in DMSO) was used to treat the cells at final concentration of $25 \mu \mathrm{M}$ for 5 h . Control cells were treated only with DMSO.

### 2.5. Western blot analysis

For Western blot analysis of protein expressed in bacteria, $5 \mu \mathrm{~g}$ of protein from both extraction protocols were boiled in $4 x$ Laemmli sample buffer for 10 min at $98^{\circ} \mathrm{C}$, loaded onto a precast $4-20 \%$ SDS-PAGE gel (BioRad) and run at 120 V for $60-80 \mathrm{~min}$ at room temperature (RT). Wet transfer was performed on PVDF membrane (Immobilon-FL, Millipore) at 200 mA for 40 min at RT. The primary mouse anti-T7 (Novagen, 69522) and mouse anti-GAPDH (Abcam, ab125247) antibodies were used for immunoblotting with
a dilution of 1:5000 and 1:1000, respectively. As secondary antibody, ECL Mouse IgG HRP-linked whole Ab (GE Healthcare, Cat. No LNA931 V/AG) was used with a dilution of 1:5000 for primary antiT7 antibody and 1:2500 for anti-GAPDH. Membranes were exposed for $1-5 \mathrm{~min}$ using ChemiDoc ${ }^{\mathrm{TM}}$ Imaging System with Image Lab ${ }^{\mathrm{TM}}$ Software.

HeLa cells were collected 36 h post-transfection and were lysed thereafter in 2XSDS-PAGE Loading Dye ( $2.5 \%$ SDS, $25 \%$ glycerol, 125 mM Tris-HCl ( pH 6.8 ), $0.01 \%$ bromophenol blue, $5 \%$ $\beta$-mercaptoethanol) at RT for 10 min . The protein samples were heated at $95^{\circ} \mathrm{C}$ for 5 min and loaded on $15 \%$ SDS-PAGE. The proteins were transferred onto nitrocellulose membrane (Protan), blocked in Odyssey blocking buffer (LI-COR) and thereafter incubated with the primary antibodies diluted in the blocking buffer overnight at $4^{\circ} \mathrm{C}$. The following primary antibodies were used for Western blotting: anti-c-Myc (Santa Cruz, sc-42), anti-Flag (Sigma, M2) and anti-goat Actin (Santa Cruz, sc-1616). After intensive washing with PBS $+0.1 \%$ Tween 20 (PBS-T), the membranes were incubated with the fluorescent secondary antibodies (IRDye ${ }^{\circledR}$, LI-COR) for 30 min at RT. The membranes were scanned and the fluorescence signals were detected by using the Odyssey scanner (LI-COR). The protein expression analysis was done by using Image Studio Software (LI-COR).

### 2.6. Indirect immunofluorescence assay

Indirect immunofluorescence assays were carried out as described previously (Inturi et al., 2013). Briefly, HeLa cells were grown on fibronectin coated coverslips and transfected for 24 h with the indicated plasmids. Cells were fixed with $3 \%$ paraformaldehyde in PBS for 15 min at RT and permeabilized with $0.1 \%$ Triton X-100 in PBS-T for 15 min at RT. The coverslips were blocked with $2 \%$ Bovine Serum Albumin/PBS-T solution followed by immunostaining with anti-Flag (M2, Sigma, 1:1000) antibody. Flag-tagged proteins were visualized with anti-TRITCconjugated anti-mouse IgG (Sigma, T5393, 1:1000) secondary antibody. Nucleus was detected by DAPI $(1 \mu \mathrm{~g} / \mathrm{ml})$ staining supplemented into the Fluoromount-G mounting media (Southern Biotech). Coverslips were analyzed with fluorescence microscope (Nikon eclipse 90i) and the images were processed with NISelements (AR 3.10, Nikon) software.

### 2.7. Modeling

The PHPT1 homology model was constructed using the AMBER suite, version 12 (Salomon-Ferrer et al., 2013). The 3D model of PHPT1 was based on the X-ray determined crystal structure of PHPT1 (2NMM, Protein Data Bank). An initial 3D structure of PHPT1 was obtained by transferring the backbone coordinates from the template residues to aligned residues of 2 NMM . The C terminal part of PHPT1 was manually aligned. The three-dimensional model was subjected to molecular mechanics energy minimization calculations using the AMBER force field as implemented in the AMBER suite. The refinement of the homology model was carried out through energy minimization: 500 iterations of steepest descent calculation were performed and then the conjugated gradient calculation was carried out until achieving $0.1 \mathrm{kcal} / \mathrm{mol} \AA-1$ of convergence on the gradient.

Molecular dynamics simulations were performed using the AMBER suite in a canonical NVT ensemble, where the number of atoms, volume and temperature remain constant. The protein was solvated in a cubic periodic box containing explicit single point charge water molecules in order to perform simulations in a properly simulated aqueous environment. The solvated model was refined by subsequent energy minimizations in an effort to
equilibrate the water molecules of the solvent. The time step was 2 fs and all simulations were conducted at 300 K .

The model was first equilibrated for a time of 100 ps keeping the whole protein fixed to allow the water molecules to relax. A subsequent 100 ps of equilibration followed, where full degrees of freedom were allowed to the complete molecular system. The actual molecular dynamics simulation that followed was set to 300 nanoseconds. All molecular dynamics simulations were performed on a set of two nVidia GTX780 graphics cards connected using the scalable interface (Vlachakis et al., 2014).

## 3. Results

### 3.1. The PHPT1(Spl) protein forms insoluble aggregates in E. coli

The PHPT1 transcript variant 6 [PHPT1(Spl)] is annotated as an IncRNA (NCBI Accession number 109807.1). The use of an alternative splice site results in an intron retention introducing a stop codon 332 nt away from the terminal splice site (Fig. 1A). A stop codon more than 50 nt from the $3^{\prime}$ end of the exon makes the mRNA a target for NMD-mediated degradation (Kervestin and Jacobson, 2012). However, at least one protein will be synthesized from the variant transcript before the NMD-mediated decay, and if the NMD is not completely efficient additional PHPT1(Spl) proteins can accumulate in the cells. The protein produced from transcript variant 6 is predicted to have an alternative C-terminal amino acid sequence when compared to the PHPT1 transcript variant 3 [PHPT1(wt)] protein (Fig. 1B). In order to investigate whether the PHPT1 splice variant 6 also has activity against pHis, the PHPT1(wt)and PHPT1(Spl) proteins were expressed in E. coli. Thereafter the cell extracts induced for the PHPT1(wt) and PHPT1(Spl) protein


Fig. 1. Transcript 6 from the PHPT1 locus is a result of a variant splicing event and renders the transcript a target for the nonsense mediated mRNA decay.
(A) Top shows the PHPT1 wild type transcript and bottom the PHPT1 transcript 6, transcript is (blue) and coding region (red). Boxes are exons and lines are introns. A stop codon is introduced in the splice variant 332 nt away from the $3^{\prime}$ end of the exon.
(B) Transcript 6 produces a protein with an alternative C-terminal. Alignment using CLC Work Bench between the PHPT1 wild type protein [PHPT1(wt)] and the protein produced from Transcript 6 [PHPT1(Spl)].


Fig. 2. PHPT1(wt) but not PHPT1(Spl), is soluble and has dephosphorylation activity against pHis.
(A) Dephosphorylation of Ac-Val-Arg-Leu-Lys-pHis-Arg-Lys-Leu-Arg-pNA by PHPT1(wt) (grey) and PHPT1(Spl) (black). The specific activity of PHPT1(wt) was $6.6 \mathrm{nmol} / \mathrm{min} / \mathrm{mg}$ protein and the dephosphorylation activity of PHPT1(Spl) was the same as for E. coli extract with empty plasmid. (B) Left panel: a mild $1 \%$ Tween, EDTA and lysozyme extraction buffer was used to extract soluble cytosolic proteins from BL21(DE3)pLysS E. coli cells after T7-PHPT1(wt) and T7-PHPT1(Spl) protein expression. Right panel: BL21(DE3)pLysS E. coli cells were boiled in a harsh $1.5 \%$ SDS RIPA buffer to extract total protein content after T7-PHPT1(wt) and T7-PHPT1(Spl) protein expression. T7-PHPT1(wt) and T7-PHPT1(Spl) proteins were detected by Western blot using anti-T7 antibody and equal protein loading was confirmed using anti-GAPDH antibody. Asterisk ( ${ }^{*}$ ) indicates a potential dimer of the PHPT1(Spl) protein.
expression were analyzed for pHis dephosphorylation activity. In agreement with previous reports the PHPT1(wt) protein containing extract could release phosphate groups from pHis, with a specific activity of $6.6 \mathrm{nmol} / \mathrm{min} / \mathrm{mg}$ protein (Ma et al., 2005; BeckmanSundh et al., 2011) (Fig. 2A). This was in contrast to the PHPT1(Spl) containing extract did not show this enzymatic activity (Fig. 2A).

To confirm the presence and compare the expression levels of the PHPT1(wt) and PHPT1(Spl) proteins in the E. coli extracts, a Western blot analysis was undertaken. Using a mild cell lysis buffer containing $1 \%$ Tween, same as used for enzymatic activity measurements, the PHPT1(wt) protein was detected at the predicted size of 14 kDa , whereas the PHPT1(Spl) protein signal was not detected (Fig. 2B, left panel). When a more harsh cell lysis buffer (containing $1.5 \%$ SDS ) was used, the PHPT1(Spl) could also be detected at its predicted size of 14 kDa . However, as this cell lysis buffer causes protein denaturation, the cell lysates were not suitable for enzymatic activity measurements. Notably, the PHPT1(Spl), and to less extent the PHPT1(wt) protein, formed high molecular weight species, perhaps corresponding to dimer formation, when expressed in E. coli (Fig. 2, right panel). We also tested whether the PHPT1(Spl) could be expressed as a soluble protein using the fission yeast, Schizosaccharomyces pombe, as a host. However, also in S. pombe the PHPT1(Spl) protein was aggregating and could only be extracted using the harsh cell lysis buffer (data not shown). This was unlike the wild type PHPT1 protein that was easily extracted and showed enzymatic activity also when expressed from the yeast host.

The PHPT1(Spl) protein is expressed in E. coli and S. pombe, but in an insoluble form, probably due to protein aggregation, therefore no conclusions on a possible enzymatic activity of the splice variant protein could be drawn.


Fig. 3. Proteasome inhibition stabilizes the PHPT1(Spl) protein.
HeLa cells grown on 6 -well plate were transiently transfected with the FlagPHPT1(wt) and Flag-PHPT1(Spl) proteins expressing plasmids for 36 h . MG132 at final concentration of $25 \mu \mathrm{M}$ was added to the cells 5 h before harvesting the cells. The expression levels of the Flag-PHPT1(wt) and Flag-PHPT1(Spl) proteins were detected by Western blot by using anti-Flag antibody. Anti-actin and anti-c-Myc antibodies were used to monitor equal protein loading and the MG132 treatment, respectively. Asterisk (*) indicates a potential dimer of the PHPT1(Spl) protein.

### 3.2. The PHPT1(Spl) protein stability is affected by the proteasome in HeLa cells

The PHPT1 protein is considered to be a ubiquitously expressed protein in mammalian cells, therefore, we decided to test the expression pattern of the PHPT1(Spl) protein in HeLa cells. For this purpose, we generated plasmids encoding the PHPT1(wt) and PHPT1(Spl) proteins as N-terminal Flag-tag fusion proteins and transfected them into HeLa cells. Similarly to the bacterial cell extracts (Fig. 2B) the HeLa cell extracts transfected with the Flag-PHPT1(Spl) encoding plasmid did not show pHis dephosphorylation activity due to the used mild protein extraction conditions (data not shown). However, the Flag-PHPT1(Spl) protein expression was detectable in HeLa cell lysates when more stringent cell lysis approach was applied (Fig. 3). Interestingly, the FlagPHPT1(Spl) protein was forming higher molecular weight species, corresponding to protein aggregation, which were not detected in Flag-PHPT1(wt) expressing HeLa cells (Fig. 3). The PHPT1(Spl) protein was not detectable under mild extraction conditions in HeLa cells, which might be due to insolubility of the protein. In the same experiment we tested whether the PHPT1(Spl) protein could be targeted for proteasomal degradation due to its aberrant accumulation pattern. For this purpose, we treated HeLa cells expressing the Flag-tagged PHPT1 proteins with the proteasome inhibitor MG132 (Lee and Goldberg, 1998) (Fig. 3). A clear stabilization of the Flag-PHPT1(Spl) protein was observed after 5 hours MG132 treatment (Fig. 3), whereas the Flag-PHPT1(wt) protein stability was not changed under the same experimental conditions. The effectiveness of the MG132 treatment was monitored by using c-Myc as a control, since this protein is highly


Fig. 4. Cullin 2 and 5 regulate the stability of the PHPT1(Spl) protein. HeLa cells grown on 24 -well plate were transiently transfected with the plasmids encoding for the Flag-PHPT1(Spl) (A) or Flag-PHPT1(wt) proteins (B) and DN-Cullin (DN-Cul 1-5) proteins for 36 h . The PHPT1 and DN-Cul1-5 proteins contain Flag-tag, which was used to detect the respective proteins by Western blotting. Anti-actin antibody staining was used to monitor equal protein loading.
unstable due to degradation by the proteasome (Fig. 3, middle panel).

### 3.3. The PHPT1(Spl) protein stability is controlled by Cullin proteins in HeLa cells

To further analyze the mechanism controlling the PHPT1(Spl) protein stability, we were interested to identify the potential E3 ubiquitin ligases responsible in this process. We concentrated our efforts on Cullin-RING E3 ubiquitin-ligases (CRL), as they are the most prominent class of E3 ligases in mammalian cells. An effective way to inactivate the function of Cullin-containing E3 complexes is to overexpress the dominant-negative Cullin (DNCul) proteins in the cells (Inturi et al., 2013). We took advantage of five available DN-Cul plasmids (DN-Cul 1-5), which were coexpressed along with the PHPT1(Spl) protein encoding plasmid in HeLa cells. Using this approach a stabilization of the PHPT1(Spl) protein was observed mainly in the presence of the DN-Cul2 and DN-Cul5 proteins (Fig. 4A). When the experiment was performed in the PHPT1(wt) protein expressing cells only a minor stabilization of the PHPT1(wt) protein was detected in the presence of the DN-Cul2 and DN-Cul5 proteins (Fig. 4B). Taken together, our data indicates that the PHPT1 (Spl) protein is targeted for proteasomal degradation by the Cul 2-and Cul 5-containing E3-ubiquitin ligases in HeLa cells.


Fig. 5. Proteasome inhibition affects subcellular localization of the PHPT1(Spl) protein.
HeLa cells were transiently transfected with the respective plasmids for 24 h and treated with DMSO or MG132 for additional 5 h . The protein localization was thereafter detected by using indirect immunofluorescence analysis. Nuclei were stained with DAPI dye (blue) and the PHPT1 proteins were stained with anti-Flag antibody (red). Scale bars correspond to $10 \mu \mathrm{~m}$.

### 3.4. Altered subcellular localization of the PHPT1(Spl) protein in HeLa cells

Available data indicates that the PHPT1(wt) protein localizes in the cytoplasm as well as in the nucleus (Zhang et al., 2009) (http://www.proteinatlas.org/search/PHPT1). Therefore it became of interest to analyze the localization pattern of the PHPT1(Spl) protein in mammalian cells. For this purpose we performed indirect immunofluorescence assay in HeLa cells expressing the FlagPHPT1(wt) and Flag-PHPT1(Spl) proteins. As shown in Fig. 5, the Flag-PHPT1(Spl) protein showed a slightly different localization pattern when compared to the Flag-PHPT1(wt) protein. The FlagPHPT1(Spl) protein had less nuclear staining and clearly had a punctuated cytoplasmic localization pattern, potentially indicating formation of protein aggregates. This statement is strengthened by monitoring the PHPT1 protein localization pattern in MG132 treated cells. Indeed, the Flag-PHPT1(Spl) protein displayed a less speckled cytoplasmic pattern in MG132 treated HeLa cells, while the protein detection from the nucleus increased. However, the Flag-PHPT1(wt) protein localization pattern was not affected by proteasome inhibition (Fig. 5).

### 3.5. Modeling predicted an altered secondary structure for the C-terminal tail

To explain the difference in properties between the wild type PHPT1 protein and the splice variant we decided to investigate whether the alternative C-terminal tail caused differences in the secondary structure of the proteins. To this end we used a determined X-ray structure of the PHPT1 protein (Protein Data Bank number 2NMM) to build a model of the wild type and the splice variant proteins. The wild type protein (Fig. 6A, magenta) aligned nicely to the X-ray structure (Fig. 6A, orange) except for a loop between two $\beta$-strands that was smaller in the X-ray structure. The gap in the sequence alignment for this part had no effect on the arrangement of the secondary elements of the


Fig. 6. Homology modeling of the PHPT1 (Spl) protein.
Homology models of the PHPT1 proteins (A) or the variant C-terminal tail (B) shown in magenta for the PHPT1(wt), turquoise for the PHPT1(Spl), while the 2NMM X-ray structure template is in orange color.
tertiary structure of PHPT1. However, the re-modeled C-terminal region of the PHPT1 variant revealed a significant change in the 3D conformation. Both the wild-type PHPT1 3D model and the X-ray determined structure, contained a set of two extended $\beta$-strands that was coupled by a third shorter $\beta$-strand in parallel direction to the closest of the two $\beta$-strands. The PHPT1 splice variant had the same set of the two extended $\beta$-strands, but completely lacked the third anti-parallel $\beta$-strand that was replaced by a $\alpha$-helix conformation. This difference in the predicted secondary structure might explain the different properties of the two protein variants that we have revealed in this study.

## 4. Discussion

Protein phosphorylation is a major signaling pathway in all cell types. Our knowledge about serine, threonine and tyrosine phosphorylation is well documented, while publications covering phosphorylation on His residues are sparse. A His phosphatase, PHPT1, has been identified in most animals, although the reports on the functions of this protein remains scarce and practically nothing is known about the splice variants of the PHPT1 protein. In addition to the four validated PHPT1 isoforms (2-5), there are two PHPT1 transcripts (isoforms 6 and 7) that are annotated as lncRNA due to premature stop codons in their pre-mRNA sequence. To understand the possible functional importance of the various transcripts originating from the human PHPT1 gene locus we characterized the noncoding transcript number 6. This particular transcript is capable of encoding a PHPT1 protein variant [PHPT1(Spl)] with an alternative C-terminal tail (Figs. 1 and 6B). When the splice variant was expressed alongside the wild type PHPT1 protein in bacteria, fission yeast and mammalian cells the PHPT1(Spl) was forming insoluble aggregates, while the PHPT1(wt) could easily be extracted (Figs. 2, 3 and 5 and data not shown). The intention of the recombinant protein expression was to elucidate whether the splice variant protein had enzymatic activity against pHis, but since the PHPT1(Spl) protein was insoluble in all the systems tested we were not able to draw any conclusions on a possible enzymatic activity.

In our hands the PHPT1(Spl), and to less degree, PHPT1(wt) formed higher order protein structures, perhaps corresponding to dimer formation, when protein samples were separated using SDSPAGE. This was surprising since previous reports on expression and SDS-PAGE separation of the recombinant PHPT1(wt) protein show one single band corresponding to the 14 kDa monomer of PHPT1 (Ma et al., 2005; Baumer et al., 2007). Although, a propensity of the recombinant wild type PHPT1 protein to form aggregates in solution has been reported (Ludwig et al., 2011). In the case of PHPT1(Spl) protein, the higher migrating band was detected in extracts from E. coli and HeLa cells, whereas for the PHPT1(wt) a weak extra band only occurred when expressed in E. coli (Fig. 2B and 3). Moreover, in HeLa cells expressing the PHPT1(wt) or the PHPT1(Spl) proteins the splice variant was found in cytoplasmic speckles, indicative of protein aggregation, while the wild type protein displayed a more uniform staining throughout the cell. When the HeLa cells were treated with the proteasome inhibitor MG132, the PHPT1(Spl) protein changed localization, from the cytoplasm to a pronounced nuclear staining. This could be due to a relocalization of the splice variant to the nucleolus, which has been shown to occur for a number of proteins, including ubiquitinated forms, when cells are treated with proteasome inhibitors (Latonen, 2011). In summary, the tendency of the splice variant protein to form aggregates was stronger as compared to the PHPT1(wt) protein. Finally, in HeLa cells the PHPT1(Spl) protein, was specifically degraded by the proteasome via Cul2- and Cul5- associated E3 ubiquitin ligase complexes, while the PHPT1(wt) protein levels were only modestly altered (Fig. 4). Cullin-2 and Cullin-5 interacts with substrates using the same Elongin-BC adaptor so overlapping substrates for these two Cullins are not unexpected (Petroski and Deshaies, 2005).

Taken together the results indicate that the variant C-terminal tail makes the PHPT1(Spl) more sticky as compared to the wild type PHPT1 protein and indeed the variant C-terminal tail is much more hydrophobic as compared to the wild type. In our molecular modeling study, the PHPT1(Spl) protein aligned with the X-ray structure and with the 3D model of the PHPT1(wt) protein except for the C-terminal tail (Fig. 6). Interestingly, the wild type protein forms a $\beta$-strand in the C-terminal end while the splice variant protein forms a completely different secondary structure having a $\alpha$-helix instead (Fig. 6B). Upon extended molecular dynamics simulations, we noticed that the axis of the unique $\alpha$-helix seems to be somewhat tilted away from its original position. This could be caused by the establishment of new interactions between the short $\alpha$-helix element of the C-terminal part and the nearby, longer $\alpha$-helix, in an antiparallel arrangement. This 7 Angstrom apart anti-parallel conformation of $\alpha$-helices is very typical of coil-coil conformations that lead to anti-parallel four helix bundles and are responsible for protein-protein interaction (Palaiomylitou et al., 2008). Therefore, we suggest that the C-terminal splice variant of PHPT1 may be capable of further protein-protein interactions that could explain that PHPT1(Spl) has a stronger tendency to form protein aggregates as compared to the PHPT1(wt) protein (Fig. 2B, 3, 5 and 6B).

The development of new Next Generation Sequencing techniques during the last decades has revealed a higher transcriptional activity in eukaryotic cells than previously anticipated (Cech and Steitz, 2014). We also begin to understand functional importance of some of the non-coding transcripts that are formed in the cells. Several lncRNAs have highly specific functions, regulating the gene expression either at the transcription initiation or posttranscriptional mRNA stability levels (Cech and Steitz, 2014). Many of the IncRNAs have not been assigned a function and might be considered as spurious. However, a recent report indicates that IncRNAs associate with and are translated by polyribosomes akin to functional mRNAs. Many of these transcripts are targeted for degradation by the translation-dependent NMD quality control
mechanism (Smith et al., 2014). Intriguingly, transcripts targeted for NMD-mediated decay can be still detected at low levels as well as be translated into proteins in the cell. To avoid accumulation of the non-functional proteins the UPS pathway will induce degradation of these proteins. Indeed, this double quality control mechanism by the NMD and UPS pathways has been described for the mammalian cMyBP-C nonsense mRNA and protein (Carrier et al., 2010).

The cells has evolved systems like the NMD pathway to suppress the effect of the non-functional transcription, but there is a lot that remains to be elucidated in how the cell differentiates between functional and non-functional transcripts. Perhaps the hydrophobic C-terminal tail of the PHPT1(Spl) protein has evolved to enhance aggregation of the unwanted protein species, which in turn can facilitate degradation. We suggest that transcripts from the PHPT1 gene locus can be used as a good model system to understand the possible functions of aberrant transcript variants and how the cell cope with unwanted proteins formed from spurious transcription events as PHPT1 is ubiquitously expressed at high levels in mammalian cells and have many different isoforms and lncRNAs.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel. 2014.10.009.

## References

Baumer N, Maurer A, Krieglstein J, Klumpp S. Expression of protein histidine phosphatase in Escherichia coli, purification, and determination of enzyme activity. Methods Mol Biol 2007;365:247-60.
Beckman-Sundh U, Ek B, Zetterqvist O, Ek P. A screening method for phosphohistidine phosphatase 1 activity. Ups J Med Sci 2011;116:161-8.
Besant PG, Attwood PV. Histone H4 histidine phosphorylation: kinases, phosphatases, liver regeneration and cancer. Biochem Soc Trans 2012;40:290-3.

Carrier L, Schlossarek S, Willis MS, Eschenhagen T. The ubiquitin-proteasome system and nonsense-mediated mRNA decay in hypertrophic cardiomyopathy. Cardiovasc Res 2010;85:330-8.
Cech TR, Steitz JA. The noncoding RNA revolution-trashing old rules to forge new ones. Cell 2014;157:77-94.
Ek P, Pettersson G, Ek B, Gong F, Li JP, Zetterqvist O. Identification and characterization of a mammalian 14-kDa phosphohistidine phosphatase. Eur J Biochem 2002;269:5016-23.
Forsburg SL, Sherman DA. General purpose tagging vectors for fission yeast. Gene 1997;191:191-5.
Gonzalez-Sanchez MB, Lanucara F, Helm M, Eyers CE. Attempting to rewrite History: challenges with the analysis of histidine-phosphorylated peptides. Biochem Soc Trans 2013;41:1089-95.
Inturi R, Thaduri S, Punga T. Adenovirus precursor pVII protein stability is regulated by its propeptide sequence. PLoS One 2013;8:e80617.
Kervestin S, Jacobson A. NMD: a multifaceted response to premature translational termination. Nat Rev Mol Cell Biol 2012;13:700-12.
Klumpp S, Hermesmeier J, Selke D, Baumeister R, Kellner R, Krieglstein J. Protein histidine phosphatase: a novel enzyme with potency for neuronal signaling. J Cereb Blood Flow Metab 2002;22:1420-4.
Klumpp S, Ma NT, Baumer N, Bechmann G, Krieglstein J. Relevance of glycine and cysteine residues as well as N - and C-terminals for the activity of protein histidine phosphatase. Biochim Biophys Acta 2010;1804:206-11.
Latonen L. Nucleolar aggresomes as counterparts of cytoplasmic aggresomes in proteotoxic stress. Proteasome inhibitors induce nuclear ribonucleoprotein inclusions that accumulate several key factors of neurodegenerative diseases and cancer. Bioessays 2011;33:386-95.
Lee DH, Goldberg AL. Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol 1998;8:397-403.
Ludwig K, Habbach S, Krieglstein J, Klumpp S, Konig S. MALDI-TOF high mass calibration up to 200 kDa using human recombinant 16 kDa protein histidine phosphatase aggregates. PLoS One 2011;6:e23612.
Ma R, Kanders E, Sundh UB, Geng M, Ek P, Zetterqvist O, et al. Mutational study of human phosphohistidine phosphatase: effect on enzymatic activity. Biochem Biophys Res Commun 2005;337:887-91.
Palaiomylitou M, Tartas A, Vlachakis D, Tzamarias D, Vlassi M. Investigating the structural stability of the Tup1-interaction domain of Ssn6: evidence for a conformational change on the complex. Proteins 2008;70: 72-82.
Petroski MD, Deshaies RJ. Function and regulation of cullin-RING ubiquitin ligases. Nat Rev Mol Cell Biol 2005;6:9-20.
Salomon-Ferrer R, Case DA, Walker RC. An overview of the Amber biomolecular simulation package. WIREs Comput Mol Sci 2013;3:198-210.
Smith JE, Alvarez-Dominguez JR, Kline N, Huynh NJ, Geisler S, Hu W, et al. Translation of small open reading frames within unannotated RNA transcripts in Saccharomyces cerevisiae. Cell Rep 2014;7:1858-66.
Sontag EM, Vonk WI, Frydman J. Sorting out the trash: the spatial nature of eukaryotic protein quality control. Curr Opin Cell Biol 2014;26:139-46.
Vlachakis D, Bencurova E, Papangelopoulos N, Kossida S. Current state-of-the-art molecular dynamics methods and applications. Adv Protein Chem Struct Biol 2014;94:269-313.
Zhang XQ, Sundh UB, Jansson L, Zetterqvist O, Ek P. Immunohistochemical localization of phosphohistidine phosphatase PHPT1 in mouse and human tissues. Ups J Med Sci 2009;114:65-72.


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