J Biomol NMR (2013) 56:393–399
DOI 10.1007/s10858-013-9758-2

NMR STRUCTURE NOTE

PHD domain from human SHPRH

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Received: 8 May 2013 / Accepted: 22 June 2013 / Published online: 2 August 2013

Biological context

SHPRH (SNF2, histone linker, PHD, RING, helicase) is a 193 kDa member of SWI2/SNF2 family of ATP-dependent chromatin remodeling enzymes (Flaus et al. 2006), containing the C_3HC_4 (Cys3-His-Cys4) RING-finger characteristic of E3 ubiquitin ligases. The human SHPRH gene maps to the chromosomal region 6q24 whose allelic loss or deletions are observed in many types of cancer, leading to the hypothesis that this region may encode putative tumor suppressor gene(s) (Sood et al. 2003). Consistent with this notion, SHPRH has been subsequently shown to play a key role in the error-free branch of DNA damage tolerance—a mechanism by which cells can bypass replication blocks caused by sites of DNA damage (lesions) and/or fill post-replicational lesion-containing single-stranded DNA gaps (Chang and Cimprich 2009; Unk et al. 2010).

SHPRH, and the closely-related protein, helicase like transcription factor (HLTF), are functional homologues of *S. cerevisiae* Rad5, acting as ubiquitin ligases in Ubc13-Mms2-dependent poly-ubiquitination of proliferating cell nuclear antigen (PCNA) (Motegi et al. 2006, 2008; Unk et al. 2006). Human PCNA is a ring-shaped DNA-sliding clamp that provides a binding platform for various proteins involved in DNA replication and damage response (Moldovan et al. 2007). PCNA ubiquitination at replication forks that are stalled by DNA damage regulates switching between normal DNA replication and the two distinct modes of DNA damage tolerance (Chang and Cimprich 2009; Unk et al. 2010; Waters et al. 2009). Thus, PCNA mono-ubiquitination at K164 by the Rad6 (E2)/Rad18 (E3) pair triggers the exchange between replicative (polδ and polε) and low-fidelity translesion synthesis (TLS) DNA polymerases (Rev1, polη, polι, polκ and polζ) that constitute the *error-prone* branch of DNA damage tolerance responsible for the majority of mutagenesis in eukaryotic cells (Waters et al. 2009). Subsequent extension of K63-linked poly-ubiquitin chain by Ubc13-Mms2 (E2)/Rad5, SHPRH or HLTF (E3) promotes *error-free* DNA lesion bypass occurring via the template switching (TS) mechanism, where the newly synthesized strand of the sister duplex is used as a template for DNA synthesis across the lesion (Chang and Cimprich 2009; Unk et al. 2010).

Beyond their function as E3 ubiquitin ligases, Rad5 and HLTF act as ATP-dependent double-stranded DNA translocases capable of replication fork reversal (Chang and Cimprich 2009; Unk et al. 2010). In the process of fork reversal the Y-shaped DNA structure with homologous arms is converted into a four-way Holliday junction ('chicken-foot' intermediate) where one of the arms consists of the two newly synthesized daughter DNA strands. The formation of such an intermediate allows switching from the lesion-containing to the undamaged DNA template, facilitating error-free bypass replication across sites

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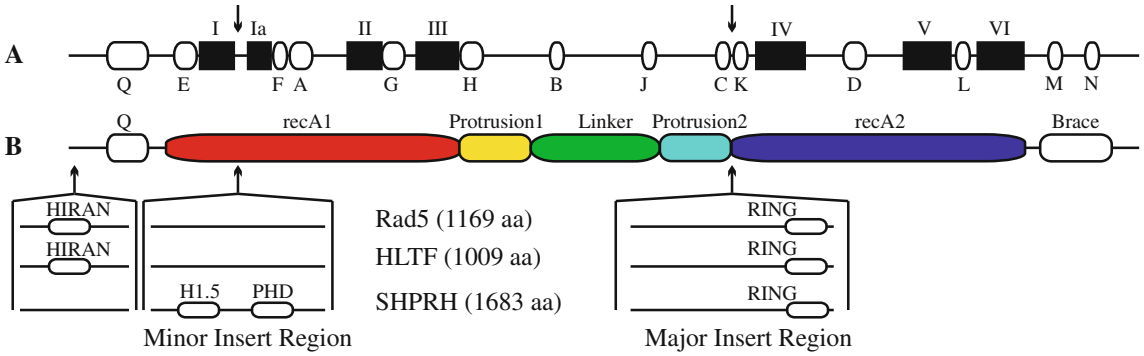

Fig. 1 **a** Conserved motifs in SWI2/SNF2 family of chromatin remodeling enzymes: seven original helicase motifs *I*, *Ia*, *II*, *III*, *IV*, *V* and *VI* and additional short conserved blocks labeled *Q* to *N* (Flaus et al. 2006). **b** Structural sub-domains of a core helicase-like domain of SWI2/SNF2 family enzymes, including two recA-like domains separated by the two helical protrusions and a linker domain flanked by the N-terminal Q-motif and the C-terminal brace domain (Flaus et al. 2006). Additional modular domains found in the 'major' and 'minor' insert regions and the N-terminal parts of *S. cerevisiae* Rad5, human HLTF and SHPRH are indicated in the *bottom* of the plot

of DNA damage (Chang and Cimprich 2009; Unk et al. 2010). It is likely that, similar to Rad5 and HLTF, SHPRH also possess a fork reversal activity, however this function remains to be demonstrated.

Rad5, SHPRH and HLTF share similar architectures that are characteristic of SWI2/SNF2 family members, including seven short conserved helicase motifs I, Ia, II, III, IV, V and VI (Flaus et al. 2006) spread over the length of each protein separated by insert regions of up to several hundred amino acids long (Fig. 1a). Such a discontinuous arrangement for a long time obscured identification of the domain structure for this protein family. The recently-determined spatial structures of SWI2/SNF2 family members, such as zebrafish Rad54 (Thoma et al. 2005), revealed that the helicase motifs define a core helicase-like domain, despite their long sequence separation, and consists of two recA-like domains and several other structural elements (Flaus et al. 2006) (Fig. 1b). Embedded in the helicase-like domain are so-called 'minor' and 'major' insert regions that may harbor additional accessory domains (Flaus et al. 2006). Thus, the 'major' insert region of Rad5, SHPRH and HLTF contains the embedded C_3HC_4 RING-fingers, while the 'minor' insert region of SHPRH includes the H1.5 (linker histone H1 and H5) domain and the C_4HC_3 (Cys4-His-Cys3) PHD (plant homeodomain) finger (Fig. 1b) (Chang and Cimprich 2009; Flaus et al. 2006; Unk et al. 2010). Additionally, the N-terminal parts of Rad5 and HLTF preceding the helicase-like domain contain $\sim$120 amino-acid region known as the HIRAN domain, which is not found in SHPRH (Chang and Cimprich 2009; Unk et al. 2010) (Fig. 1b).

Consistent with their roles as E3 ubiquitin ligases responsible for PCNA poly-ubiquitination, human SHPRH and HLTF interact with PCNA, Ubc13-Mms2 (their E2), and Rad18 (E3 transferring the first ubiquitin moiety) (Motegi et al. 2006, 2008; Unk et al. 2006). SHPRH and HLTF were also shown to bind one another (Motegi et al. 2008). Recent report suggests that SHPRH, but not HLTF, can interact with TLS DNA polymerases polη and polκ, and thereby participate in error-prone DNA lesion bypass (Lin et al. 2011). At this time, however, protein domains that mediate these interactions remain unknown. It is likely that beyond ubiquitin ligase and DNA translocase activities (Chang and Cimprich 2009; Unk et al. 2010) SHPRH and HLTF might have other functions, and that the list of their interaction partners will be extended as these functions are identified.

Here we report the solution NMR structure of the PHD-finger embedded in the 'minor' insert region of human SHPRH (Fig. 1b), which, along with the adjacent H1.5 domain, are not found in its homologue HLTF. PHD zinc fingers are small modules of approximately 50–80 residues stabilized by the two zinc atoms anchored by 8 zinc-coordinating residues, C_4HC_3 (Cys4-His-Cys3), found in proteins involved in chromatin remodeling and transcription regulation (Bienz 2006; Sanchez and Zhou 2011). PHD domains are generally considered as epigenome readers that recognize lysine and arginine methylation of the histone H3 tail, primarily H3K4 (three- and di-methylated, K4me3 and K4me2, vs. non-modified, K4me0) (Sanchez and Zhou 2011). Therefore, we have also investigated SHPRH PHD interaction with the peptides derived from the histone H3 tail with different methylation states of K4 residue. Surprisingly, no specific binding has been observed, suggesting that SHPRH PHD may belong to a small group of PHD-fingers that recognize other histone modifications, or bind other yet unknown molecular targets.

Methods and results

Protein expression and purification

The gene encoding PHD domain from human SHPRH (residues 652–716) was sub-cloned into PGEX-4T3 vector

using SalI and NotI restriction sites. The recombinant protein was overexpressed in *E. Coli* BL21(DE3) cells transformed with SHPRH PHD plasmid. Cell culture was inoculated overnight at 37 °C in 50 ml LB medium and then transferred to 1 L M9 medium with $^{15}NH_4Cl$ and ^{13}C-glucose used as sole nitrogen and carbon sources, respectively. Cells were grown at 37 °C to OD_{600} of 1.0–1.2, at which point 0.05 mM $ZnCl_2$ was added to the medium. After an additional 1 h growth to OD_{600} of ~1.4 protein expression was induced by 1 mM IPTG overnight at 20 °C. Cells were harvested by centrifugation, resuspended in PBS buffer (Na_2HPO_4 10 mM pH 7.5, KH_2PO_4 1.8 mM, NaCl 140 mM) and lysed by sonication. Soluble fraction was collected, loaded onto GST-column and incubated for 30 min, followed by protein elution with 50 mM Tris–HCl buffer, pH 8.0, containing 10 mM reduced glutathione. GST-tag was cleaved with thrombin at room temperature overnight, followed by protein purification on a HiLoad Superdex 75 column (GE Healthcare). The final protein sample used for NMR structure determination contained 0.7 mM $^{15}N/^{13}C$ labeled protein, 50 mM HEPES, 150 mM NaCl, 0.05 mM $ZnCl_2$, 1 mM DTT, pH 7.0. The same protocol was used to express and purify ^{15}N labeled SHPRH PHD domain for the H3 histone binding experiments (see below) with the exception that cells were grown in M9 medium containing ^{12}C-glucose.

NMR resonance assignment and protein structure calculation

NMR spectra for the backbone and side-chain resonance assignment and spatial structure determination of the SHPRH PHD domain were acquired at 20 °C on 11.7 and 18.8 T Agilent VNMRS spectrometers (500 and 800 MHz ^1H frequencies) equipped with cold probes. The resonance assignments were obtained from 2D ^1H–^{15}N HSQC, ^1H–^{13}C HSQC and 3D HNCO, HNCACB, CBCA(CO)NH, HBHA(CO)NH, HC(C)H-TOCSY, (H)CCH-TOCSY, ^{15}N- and ^{13}C-edited NOESY-HSQC (150 ms mixing time) spectra (Sattler et al. 1999). The spectra were processed with NMRPipe (Delaglio et al. 1995) and analyzed in CARA software. Nearly complete (98 %) set of the assigned backbone and side-chain chemical shifts was deposited to BioMagResBank (BMRB accession number 19229). The annotated assigned ^1H–^{15}N HSQC spectrum of the SHPRH PHD is shown in Fig. 2.

It was previously shown that PHD finger is a zinc-dependent fold and that zinc removal from the purified domain by addition of the excess EDTA results in the loss of the domain structure (Pascual et al. 2000). To ascertain that the SHPRH PHD domain in our samples is bound to zinc we have additionally recorded ^1H–^{15}N HSQC spectrum of 0.2 mM PHD domain in the presence of 10 mM

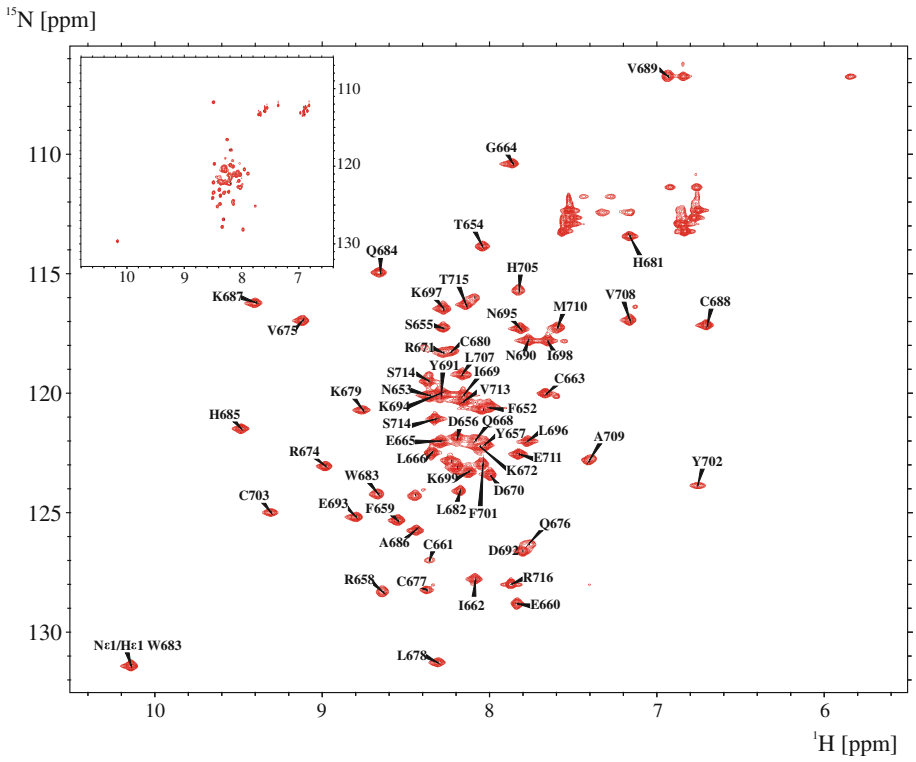

Fig. 2 ^1H–^{15}N HSQC spectrum of the SHPRH PHD domain (residues 652–716) acquired at 11.7 T magnetic field, 20 °C with *peaks* annotated with the backbone amide resonance assignments. The spectrum of 0.2 mM ^{15}N labeled domain in the presence of 10 mM EDTA is shown as *inset*

EDTA (Fig. 2, inset). Addition of EDTA resulted in significant precipitation of the SHPRH PHD sample. The remaining protein displays NMR spectrum characteristic of disordered protein (Fig. 2, inset), suggesting that apo-form of the SHPRH PHD domain is unstable in solution.

Structure calculation for the SHPRH PHD domain was performed in CYANA software (Guntert 2003) using (1) the backbone ϕ and ψ angle restraints obtained from chemical shifts with TALOS + program (Shen et al. 2009), (2) ^{1}H–^{1}H distance restraints based on correlations observed in NOESY spectra, and (3) hydrogen bond restraints added on the basis of NOE analysis. NOE cross-peaks were peak-peaked manually and assigned automatically in CYANA (Guntert 2003). Additional restrains for distances between zinc-ions and atoms of zinc-coordinating residues were introduced to enforce tetrahedral geometries of the two zinc centers based on zinc center geometries observed in high-resolution crystal structures (Alberts et al. 1998). A total of 100 structures of the domain were generated, followed by refinement of lowest-energy 20 models in CNS software (Brunger et al. 1998) using constrained molecular dynamic simulations in explicit solvent. Atomic coordinates of the final SHPRH PHD structural ensemble were deposited in the Protein Data Bank (PDB entry 2M85).

Spatial structure of SHPRH PHD domain

Table 1 summarizes NMR-based restraints used for the SHPRH PHD structure determination and structure refinement statistics. The ensemble of 20 least-energy structures of the domain shown in Fig. 3a exhibits mean pairwise RMSD of 1.2 Å calculated for heavy atoms of residues 657–712, displays minimal violations from idealized molecular geometry, and agrees well with the input experimental data (Table 1). The overall backbone fold for the least-energy model of SHPRH PHD domain is illustrated in Fig. 3b. The domain adopts a canonical PHD-finger fold with a central two-stranded anti-parallel β-sheet (residues 674–677 and 682–685) flanked on both sides by the two interleaved zinc binding sites formed by residues C661, C663, H685, C688 (ZnI site) and C677, C680, C703, C706 (ZnII site) (Fig. 3). The β-sheet region harbors two pairs of zinc-coordinating residues: one in the end of strand β1 and in a short β1–β2 connector loop (C677, C680 from ZnII site) and the other in the end of and immediately after strand β2 (H685, C688 from ZnI site). Loop 1 between the first pair of zinc-coordinating residues from ZnI site (C661, C663) and strand β1 lacks regular secondary structure, while loop 2 connecting strand β2 and the last pair of zinc-coordinating residues from ZnII site (C703, C706) includes two short helices, α1 (residues 686–688) and α2 (residues 693–697). Additionally, the C-terminal part of the domain forms α-helix α3 spanning the residues 704–710.

Table 1 NMR-based restraints used for the SHPRH PHD domain structure calculation and structure refinement statistics

NMR distance and dihedral restraints	PHD (652–716)
Distance restraints	
Total NOE	1,343
Intra-residue	824
Inter-residue	519
Sequential ($\|i - j\| = 1$)	824
Medium range ($\|i - j\| < 4$)	200
Long range ($\|i - j\| > 5$)	319
Protein-zinc restraints	46
Hydrogen bonds	14
Total dihedral angle restrains (ϕ, ψ)	87
Deviations from idealized geometry	
Bond lengths (Å)	0.02
Bond angles (°)	1.3
Ramachandran plot	
Residues in most favored regions (%)	75.1
Residues in additional allowed regions (%)	23.7
Residues in generously allowed regions (%)	0.3
Residues in disallowed region (%)	0.9
Mean pairwise rmsd (Å)	Residues 657–712
Heavy	1.18 ± 0.14
Backbone	0.5 ± 0.11

Figure 3c shows sequence alignment of the SHPRH PHD domain and the two groups of PHD fingers: one that recognizes K4-methylated tail of histone H3, H3K4me3 or H3K4me2, over non-modified H3K4me0 (red), and another that preferentially binds H3K4me0 (blue) (Sanchez and Zhou 2011). Structure analysis of various PHD-finger complexes with H3-derived peptides revealed amino-acid residues responsible for H3K4 recognition (Sanchez and Zhou 2011), marked by red and blue in Fig. 3c. Thus, recognition of the tri- or di-methyl ammonium group of H3K4 occurs via an aromatic cage formed by 2–4 residues with aromatic and hydrophobic side-chains (Fig. 3c, red). Among the cage-forming residues, the highly conserved tryptophan in strand β2 is present in all PHD fingers that read H3K4me3 and H3K4me2 modifications. Additional cage-forming aromatic residues are located in the loop 1 and the beginning of strand β1, or in the N-terminal part preceding the first two zinc-coordinating residues of ZnI site (Fig. 3c, red). In contrast, recognition of non-modified H3K4me0 does not require an aromatic cage, and occurs via hydrophobic and acidic amino-acids occupying the same positions as cage-forming residues in PHD domains that preferentially bind methylated H3K4 (Fig. 3c, blue).

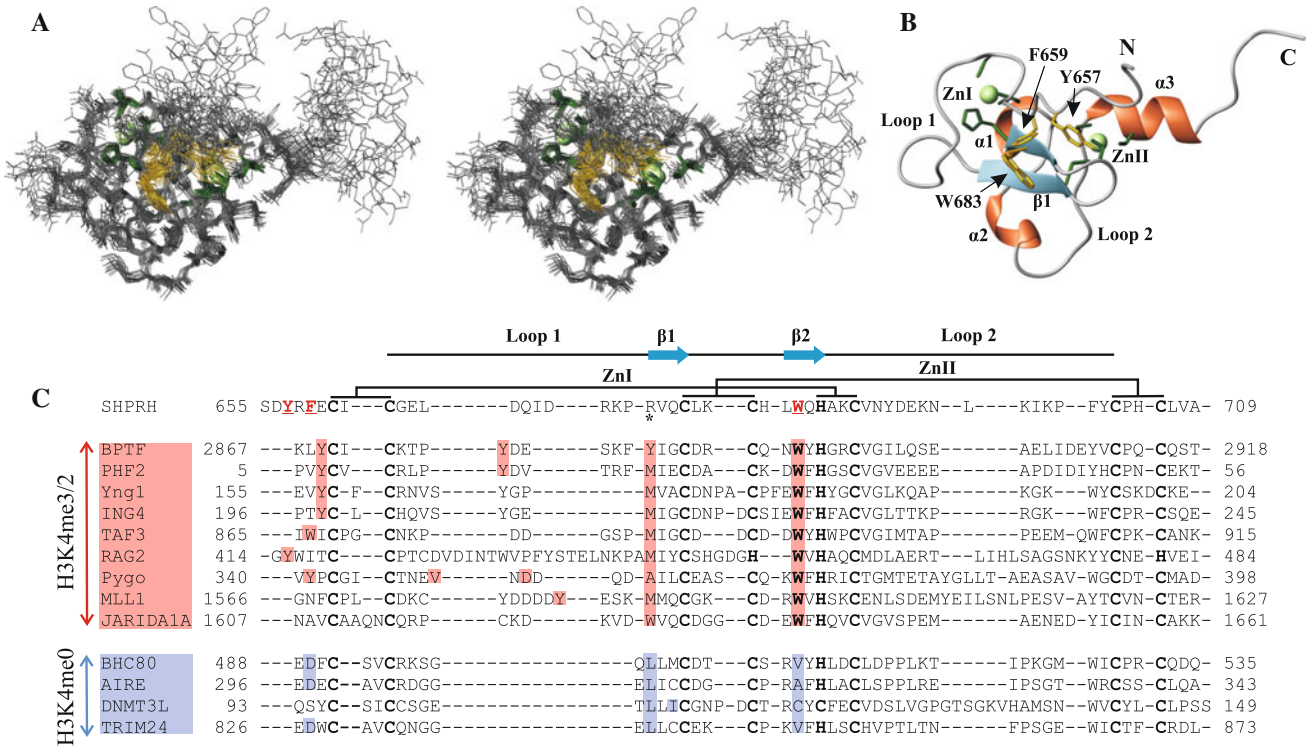

Fig. 3 **a** Stereo-view of the NMR-derived ensemble of 20 least-energy structures, and **b** the backbone conformation for the least-energy model of the SHPRH PHD domain (residues 652–716). Zinc-ions and side-chains of zinc-coordinating residues are shown in *green*, while the cluster of spatially closed aromatic residues Y657, F659 and W683 is shown in *yellow*. **c** Sequence alignment of the SHPRH PHD domain with PHD fingers that preferentially bind methylated H3K4me3 and H3K4me2 (*red*), or non-modified H3K4me0 (*blue*) histone H3 tail (Sanchez and Zhou 2011). Residues involved in recognition of H3K4 modifications are highlighted by *red* and *blue colors*, respectively

The SHPRH PHD domain contains tryptophan residue, W683, at the position highly conserved in PHD fingers that recognize tri- or di-methylated H3K4, as well as two aromatic residues, Y657 and F659, N-terminal to the first pair of zinc-coordinating cysteins of ZnI site, C661 and C663 (Fig. 3c, first line). The presence of these spatially close aromatic amino-acids that could potentially form an aromatic cage (Fig. 3a, b, yellow) might prove consistent with the ability of the domain to recognize K4-methylated tail of H3 histone.

SHPRH PHD domain is not a reader of H3K4 methylation state

To test the hypothesis that SHPRH PHD finger may bind and preferentially recognize histone H3 methylated at K4 we have performed a series of NMR titration experiments. In these experiments 25 mM solutions of custom synthesized (GenScript) 11 residue peptides derived from the H3 tail, ART**K**QTARKST, non-modified, di- or tri- methylated at K4 were gradually added to ~0.2 mM samples of ^{15}N labeled SHPRH PHD domain. The titrations were continued until protein to peptide ratio of 1:28 (H3K4me0 and H3K4me2 peptides) or 1:36 (H3K4me3 peptide) and were monitored by recording 11.7 T ^{1}H–^{15}N HSQC spectra at each titration step.

Surprisingly, no chemical shift changes consistent with specific binding were observed upon SHPRH PHD titrations with either of the H3-derived peptides irrespective of the K4 methylation state. Furthermore, no changes in the backbone $^{1}H^{N}$–^{15}N and side-chain $^{1}H^{\varepsilon 1}$–$^{15}N^{\varepsilon 1}$ chemical shifts were observed for W683, whereas the indole ring of a conserved tryptophan at this position in PHD fingers that bind tri- and di-methylated H3K4 is involved in cation-π and van der Waals interactions with the K4 methyl ammonium group crucial for stabilization of the complex (Sanchez and Zhou 2011). It should be noted that in all titration experiments we have noticed small chemical shift changes for the backbone amide of H705 at large excess of the added peptide (data not shown). Similar changes were observed in ^{1}H–^{15}N HSQC spectra of SHPRH PHD upon small variations in the sample pH. NMR titration experiments clearly suggest that, despite the presence of a conserved tryptophan and two more aromatic residues that could potentially form an aromatic cage, the SHPRH PHD finger *is not* capable of specific binding H3 histone tail

irrespective of the methylation state of K4 residue. One possible explanation for the lack of H3K4 binding by the SHPRH PHD domain is the presence of positively charged R674 in the beginning of β1-strand (marked by star in Fig. 3c) in a conserved position occupied by a hydrophobic residue in PHD domains that recognize H3K4 modifications.

Discussion and conclusions

In this work we have determined NMR structure of the PHD domain from the 'minor' insert region of human SHPRH (Figs. 1, 3), an E3 ubiquitin ligase responsible for poly-ubiquitination of DNA-sliding clamp PCNA and a tentative ATP-dependent DNA translocase that plays an important role in error-free branch of DNA damage tolerance (see "Biological context"). PHD fingers have been reported as universal readers of methylation state of H3 histone tail (Sanchez and Zhou 2011). Most of the domains described so far preferentially bind tri- or di-methylated H3K4, H3K4me3 and H3Kme2, while a smaller group recognizes non-modified H3K4me0 (Fig. 3c). Several PHD fingers were also identified that discriminate between methylation states of H3R2, H3R2me0 versus H3R2me2 (Sanchez and Zhou 2011). PHD domains are also known to interact with proteins other than histones (Bienz 2006), and some PHD domains such as the second PHD finger from AIRE do not recognize any histone H3 modifications (Gaetani et al. 2012). Here we have shown with the use of NMR titration experiments that SHPRH PHD domain does not bind either K4-metylated or unmodified histone H3 tail, contrary to what might be anticipated from its amino-acid composition (Fig. 3c). Thus, SHPRH PHD domain may have evolved another function distinct from reading histone H3 methylation state, or might be involved in recognition of a different histone modification.

Plant homeodomain fingers are structurally similar to RING domains characteristic of E3 ubiquitin ligases, which poses an intriguing possibility that PHD fingers might also function as components of E3 ligases. The PHD domain dependent ubiquitin ligase activity has indeed been reported for several nuclear proteins (Dul and Walworth 2007). Furthermore, PHD domain from the KAP1 co-repressor binds Ubc9 (SUMO E2 conjugating enzyme) and acts as E3 ligase for sumoylation of the adjacent bromodomain (Ivanov et al. 2007). Although E3 ubiquitin ligase activity of SHPRH is dependent on its RING domain that is embedded in the 'major' insert region (Motegi et al. 2006) (Fig. 1), it is conceivable that PHD finger might play an accessory role in Ubc13-Mms2-SHPRH ubiquitin-conjugation cascade. Other potential functions of SHPRH PHD might involve mediating interactions with various proteins of DNA damage tolerance pathways such as TLS DNA polymerases and/or Rad18 (E3 ligase responsible for PCNA mono-ubiquitination) (Lin et al. 2011; Motegi et al. 2006, 2008).

These and other possibilities need to be systematically addressed in future studies aimed at establishing functional role and identifying interaction partners of SHPRH PHD domain.

Acknowledgments This work was supported by UCHC startup funds and Agilent Technologies Foundation Grant #2730 to D.M.K., grants from FAPERJ, INCT-INBEB and Science without Boarders (Ciências sem Fronteiras) CNPq program to F.C.L.A., NIH Grant ES016486 to K.A.C., and CAPES sandwich Ph.D. fellowship to L.E.F.M. A.C.K. was funded by NIH training Grant T32CA09151 and American Cancer Society fellowship PF-12-156-01-DMC.

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