Plant Homeodomain (PHD) Fingers of CHD4 Are Histone H3-binding Modules with Preference for Unmodified H3K4 and Methylated H3K9

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A major challenge in chromatin biology is to understand the mechanisms by which chromatin is remodelled into active or repressed states as required during development and cell differentiation. One complex implicated in these processes is the nucleosome remodeling and histone deacetylase (NuRD) complex, which contains both histone deacetylase and nucleosome remodeling activities and has been implicated in the silencing of subsets of genes involved in various stages of cellular development. Chromodomain-helicase-DNA-binding protein 4 (CHD4) is a core component of the NuRD complex and contains a nucleosome remodeling ATPase domain along with two chromodomains and two plant homeodomain (PHD) fingers. We have previously demonstrated that the second PHD finger of CHD4 binds peptides corresponding to the N terminus of histone H3, and we establish the histone-binding surface of this domain. This is the first instance where histone binding ability has been demonstrated for two separate PHD modules within the one protein. These findings suggest that CHD4 could bind to two H3 N-terminal tails on the same nucleosome or on two separate nucleosomes simultaneously, presenting exciting implications for the mechanism by which CHD4 and the NuRD complex could direct chromatin remodeling.

The N-terminal tails of histones are subject to many reversible covalent modifications in vivo, and different modifications have often been associated with either active or repressed chromatin states. According to prevailing ideas, the status of the cell is translated to chromatin in the form of specific post-translational modification (PTM) patterns on histone tails. This tagged chromatin is then recognized by effector proteins and complexes that regulate how the underlying genetic information is used. The complicated and intertwined processes of tagging the histone tails, recognizing the tags, remodeling chromatin into active (open) or repressed (compacted) states, and removing the tags requires the coordination of multiple protein functions.

The nucleosome remodeling and histone deacetylase (NuRD) complex is unique among nucleosome remodeling complexes in that it couples histone deacetylase activity with nucleosome remodeling ATPase activity (although the purpose of this enzymatic combination is currently unclear). The NuRD complex has traditionally been considered a transcriptional corepressor complex, consistent with the repressive function of histone deacetylation (reviewed in Refs. 2, 3). Several key NuRD complex components have been shown to play a role in development and cell lineage commitment in multiple contexts. For example, in Caenorhabditis elegans, the CHD4 homologue let-418 is required for the repression of germ line cell markers in differentiated cells (4). Similarly, in Arabidopsis thaliana, mutants of the CHD4 homologue PICKLE develop embryo-like characteristics after germination, indicating a role for CHD4 in the repression of embryonic genes (5, 6). Conditional CHD4 inactivation in the mouse has revealed important functions for this protein in both differentiation and homeostasis of hematopoietic stem cells (7, 8). Recently, however, a direct role for the NuRD complex in transcriptional activation has been demonstrated, with NuRD shown to be required for FOG-1-dependent activation of hematopoietic genes (9). NuRD has also been identified as a modulator of aging-associated chromatin defects (10) and implicated in DNA damage signaling and repair (11–14). Thus, the NuRD complex is likely to perform diverse functions centered on maintaining the balance between repression and activation of genes required for proliferation, differentiation, and homeostasis, as well as in DNA damage-response pathways. The NuRD complex is also strongly implicated in cancer, with the expression levels of a number of NuRD sub-

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4 The abbreviations used are: PTM, post-translational modification; NuRD, nucleosome remodeling and histone deacetylase; PHD, plant homeodomain; PDB, Protein Data Bank; r.m.s.d., root mean square deviation; HSQC, heteronuclear single quantum coherence.
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units, including MTA1/2 (15) and RbAp48 (16), elevated in numerous cancer cell lines and cancer tissues.

The 218-kDa CHD4 protein contains an ATP-dependent helicase domain and is one of the defining components of the NuRD complex. In addition to the ATPase domain, CHD4 contains two plant homedomain (PHDs) and two chromodomains (Fig. 1A). Chromodomains are emerging predominantly as methyl-lysine-binding domains (17–20), although the chromodomains of the Drosophila homologue of CHD4 (Mi-2) (21) and of MSL3 (22) have been shown to bind DNA.

The PHD is an ~50-residue module characterized by a conserved Cys4-His-Cys3 motif that coordinates two zinc ions in a “cross-brace” configuration, where each zinc ion is coordinated by alternate pairs of Cys/His ligands. The human genome contains ~150 PHDs, occurring in a wide variety of mostly nuclear proteins (23), and a subset of PHDs have been found to bind N-terminal histone tails, including the PHDs of bromodomain PHD finger transcription factor and ING2 (inhibitor of growth family member 2), which recognize H3K4me3 and thereby facilitate the interaction of bromodomain PHD finger transcription factor, ING2, and their associated corepressor complexes (NURF and Sin3, respectively) with chromatin (24–27). Since the initial discovery of the chromatin binding properties of several PHDs, it is becoming clear that PHDs recognize a range of different PTMs within H3. In a recent analysis of the 18 PHD fingers from Saccharomyces cerevisiae, 8 were found to recognize H3 methylated at Lys4 (H3K4me3), and 2 showed preference for methylation at Lys36 (28). Other PHDs from mammalian proteins recognize the methylation state of Lys9, including PHD2 from CHD4 and the PHDs from KDM5C and UHRF1 (29–31). To date, however, the best characterized interactions involve H3K4, with all currently available structures of PHD-histone complexes describing recognition of unmodified or methylated Lys4 in a distinct pocket on the surface of the PHD (with the exception of a tandem PHD finger from DPF3b, which recognizes several histone acetylation sites (32)). In contrast, we have previously shown that PHD2 of CHD4 is able to detect the modification state of both Lys4 and Lys9 of histone H3 (31). Here, we have used NMR spectroscopy to determine the molecular basis for the simultaneous recognition of Lys4 and Lys9, by solving the solution structure of CHD4-PHD2 in complex with H3(1–12) containing a K9me3 modification. Our structure reveals a unique arrangement in which the unmodified H3K4 side chain occupies the canonical binding pocket, whereas recognition of the K9me3 group is likely to be specified by a cation–π interaction with a surface phenylalanine ring. Additionally, we show that PHD1 of CHD4 also recognizes the N-terminal tail of H3, displaying similar affinities for H3 unmodified and trimethylated at Lys9, and perhaps a slight preference for unmodified Lys4 over K4me3. To our knowledge, this is the first example of a dual recognition system, in which two domains from a single protein bind to two separate histone tails. These data shed light on the biochemical function of CHD4 and the NuRD complex and expand our understanding of the functional diversity of the PHD in its role as a reader of chromatin modification state.

EXPERIMENTAL PROCEDURES

Sequence Analysis and Molecular Diagrams—Sequence analyses and alignments of DNA and proteins were carried out using ClustalW (33) and BioManager 3.0 (no longer available) followed by manual adjustment. Molecular diagrams were produced using MOLMOL (35) or PyMOL (36).

Cloning, Expression, and Purification—Constructs of PHD1(365–420), PHD2(446–501), and PHD12(364–506) from human CHD4 were cloned by PCR amplification from a K562 cDNA library and ligated into the pGEX-2P vector (a modified pGEX-2T vector that contains a human rhinovirus 3C protease cleavage site). Each construct was expressed in Escherichia coli BL21(DE3) cells and purified as described previously for PHD2 (37), with minor variations (including purification by size exclusion chromatography in place of anion exchange chromatography). The cleaved, purified proteins contained an additional five amino acids (GPLGS) derived from the human rhinovirus 3C protease cleavage site at the N terminus. The identity of each protein was confirmed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Western Blot Analysis—GST fusion CHD4-PHD1 was incubated with C-terminally biotinylated peptides (Upstate Biotechnology, Inc.) corresponding to the unmodified H3 (residues 1–21) and singly modified H3K4me1/3 (residues 1–21), H3K9me1/2/3 (residues 1–21), H3K27me1/2/3 (residues 21–44), and H3K36me1/2/3 (residues 21–44) histone tails in the presence of streptavidin-Sepharose beads (GE Healthcare) in binding buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% Nonidet P-40. The beads were collected via centrifugation and washed five times with the peptide binding buffer. Bound protein was detected by Western blot using anti-GST horseradish peroxidase (HRP)-conjugate monoclonal antibodies (GE Healthcare). Negative controls using GST fusion proteins in the absence of the peptides were run in parallel to ensure that the proteins did not bind to the streptavidin beads.

Combinatorial On-bead Screening Assay—A 5000-member PTM-randomized combinatorial peptide library based on the first 10 residues of the histone H3 N terminus was incubated first with the GST-tagged version of CHD4-PHD1; second with a GST-specific primary antibody; third with a biotinylated secondary antibody, and finally with streptavidin-conjugated alkaline phosphatase, catalyzing the turnover of 5-bromo-4-chloroindol-3-yl phosphate, which results in the formation of a turquoise precipitate on beads bearing sequences that bind to the target protein. The bead color intensity is proportional to affinity of the interaction (38). Peptides from individual beads were cleaved with cyanogen bromide and analyzed by MALDI-TOF mass spectrometry. PTM patterns were determined from the resulting mass ladders. Discrimination factors were obtained by dividing the frequency of each modification observed in the intensely blue beads by the frequency of each corresponding modification from a random group of 100 library members. Discrimination factors represent the likelihood of observing a particular modification in a protein screening experiment relative to random chance.
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We have previously shown that PHD2 of CHD4 is capable of recognizing H3 unmodified at Lys and methylated at Lys. To assess whether PHD1 could also act as a histone-reading module, we carried out pulldown experiments (Fig. 1). GST-tagged PHD1 was first incubated with biotinylated histone peptides corresponding to unmodified H3 (residues 1–21) and singly modified H3K4me1/3 (residues 1–21), and H3K9me1/2/3 (residues 1–21), H3K27me1/2/3 (residues 21–44), and H3K36me1/2/3 (residues 21–44) and then mixed with streptavidin-Sepharose beads. After collecting the beads by centrifugation, any protein retained on the beads was detected by Western blot analysis using an anti-GST antibody. The pulldowns demonstrated that PHD1 was able to recognize all peptides corresponding to H3 residues 1–21, with no binding observed to the H3 portion corresponding to residues 21–44.

To probe the binding affinity and specificity profile of PHD1 for methylation at Lys and Lys within H3, 15N HSQC spectra of uniformly 15N-labeled PHD1 were recorded in the presence of increasing concentrations of unmodified H3 (1–12), H3K4me3, or H3K9me3 (Fig. 1). Substantial chemical shift changes in the 15N HSQC spectra were observed for all three peptides, and the interactions were predominantly in fast exchange. To calculate the affinity of PHD1 for each peptide, binding curves were constructed from the 1H and 15N chemical shift changes of three different signals, and curves were fit to a 1:1 binding model by nonlinear least squares regression (Fig. 1, D and E). An additional parameter was included to correct for methylation at Lys4 and methylated at Lys9. To assess whether PHD1 could also act as a histone-reading module, we carried out pulldown experiments (Fig. 1). GST-tagged PHD1 was first incubated with biotinylated histone peptides corresponding to unmodified H3 (residues 1–21) and singly modified H3K4me1/3 (residues 1–21), and H3K9me1/2/3 (residues 1–21), H3K27me1/2/3 (residues 21–44), and H3K36me1/2/3 (residues 21–44) and then mixed with streptavidin-Sepharose beads. After collecting the beads by centrifugation, any protein retained on the beads was detected by Western blot analysis using an anti-GST antibody. The pulldowns demonstrated that PHD1 was able to recognize all peptides corresponding to H3 residues 1–21, with no binding observed to the H3 portion corresponding to residues 21–44.

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for errors in H3 peptide concentration (see under “Experimental Procedures”), and the resulting correction factor was found to be highly consistent between the three different signals monitored in each titration and fell within the expected range of error in peptide concentration determination. PHD1 was found to bind with similar affinity to H3(1–12) and H3K9me3, with $K_A$ values of $(3.1 \pm 0.6) \times 10^5$ M$^{-1}$ and $(2.6 \pm 0.5) \times 10^5$ M$^{-1}$, respectively. This contrasts with the specificity of PHD2, which displays a 20-fold increase in affinity upon trimethylation at Lys9 from $(5.6 \pm 0.2) \times 10^4$ M$^{-1}$ to $(1.6 \pm 0.3) \times 10^6$ M$^{-1}$ (31). Trimethylation at Lys4 reduced the affinity of PHD1 for H3 from $(3.1 \pm 0.6) \times 10^5$
m$^{-1}$ to $(1.1 \pm 0.2) \times 10^5$ m$^{-1}$, a smaller effect than was seen for PHD2. The affinities of PHD1 and PHD2 for H3 peptides fall within previously reported values observed between other PHDs and their target histone peptides, which range from $3 \times 10^4$ to $6 \times 10^6$ m$^{-1}$ (50, 53).

To determine whether other post-translational histone modifications influence the interaction of PHD1 with H3, the domain was screened against a library of modified H3(1–10) peptides using a colorimetric on-bead assay (Fig. 1F) (38, 54). The peptide library encompassed numerous possible modification states at position 2 (R, Rme1/2s/2a, Cit), 3 (T, Tph), 4 (K, Kme1/2/3, Kac), 6 (T, Tph), 8 (R, Rme1/2s/2a, Cit), 9 (K, Kme1/2/3, Kac), and 10 (S, Sph). 40 beads that indicated positive binding to PHD1 were selected, and the PTM state of the attached peptide was identified by MALDI-TOF mass spectrometry. Positional discrimination factors for each PTM were determined by dividing the observed frequency of that PTM within the PHD1-binding peptides by the frequency of occurrence of that PTM in a random sampling of 100 peptides. PHD1 showed preference for unmodified Lys$^4$ over methylated or acetylated Lys$^4$, in agreement with the $^{15}$N HSQC binding data presented above. The absence of phosphorylation at Thr$^3$, Thr$^6$, and Ser$^{10}$ in all detected peptides suggests that phosphorylation at these positions disrupts binding. In contrast, no clear preferences were observed for the modification state at Arg$^2$, Arg$^5$, or Lys$^7$.

Three-dimensional Structure of CHD4-PHD1—To investigate the mode of H3 binding by CHD4-PHD1, we first solved the solution structure of PHD1. Chemical shift assignments were made for more than 98% of commonly observed protons (including carbon-bound protons, backbone amides, and side chain N-H groups of Asn and Gln; assigned HSQC is provided in supplemental Fig. S1), and statistics for the 20 lowest energy water-refined structures are summarized in Table 1 (Ramachandran plots are provided in supplemental Fig. S2). In the final calculated structures, no angle restraints were violated by >5°, and only one distance restraint was violated by >0.5 Å, a 0.74 Å violation between Cys$^{396}$ Hβ2/3 and Val$^{375}$ Hα2. The structured regions of the 20 lowest energy water-refined structures overlaid with a backbone r.m.s.d. of 0.51 Å (Fig. 2A). The PHD1 structure has a similar global fold to the previously determined PHD2 structure (Fig. 2, B and C), and the well defined region stretches over a similar range of residues in each domain. An overlay of PHD1 and PHD2 gives an r.m.s.d. over structured backbone atoms of 1.80 Å. A similar overlay of PHD1 with the H3K4me3-binding PHD from ING4 (PDB code 2K1J (55)) gives a backbone r.m.s.d. of 2.1 Å.

H3-binding Site of CHD4-PHD1—The histone-binding surface of PHD1 was defined by calculating the weighted average chemical shift change (56) between the free and H3-bound PHD1 states for the N and HN frequencies of each signal in the $^{15}$N HSQC spectra (shown for the titration with H3K9me3 in Fig. 3A). A chemical shift change of >1 S.D. more than the mean was observed for backbone amides of five PHD1 residues: Gly$^{380}$, Ile$^{382}$, His$^{395}$, Glu$^{406}$, and Trp$^{409}$, suggesting that these residues are directly or indirectly involved in binding to the histone tail. All five residues are located on the same side of the PHD1 structure, indicating that this is the H3-binding surface (Fig. 3B). Similar patterns of chemical shift changes were observed for unmodified H3 and H3K4me3 (supplemental Fig. S3), indicating that the mode of

**TABLE 1**

Experimental restraints and structural statistics for the ensemble of the 20 lowest energy PHD1 structures

| Experimental restraints | Total distance restraints (unambiguous/ambiguous) | 972/16 |
|-------------------------|-----------------------------------------------|--------|
|                         | Intraresidue (i – i)                           | 309    |
|                         | Sequential (i | j | i – j = 1)                                 | 218    |
|                         | Medium range (2 ≤ | j | i – j ≤4)                                  | 154    |
|                         | Long range (i | j | i – j ≥5)                                  | 307    |
|                         | Total dihedral angle restraints                | 79°    |
|                         | φ                                                | 45°    |
|                         | ψ                                                | 25°    |
|                         | θ                                                | 9°     |

r.m.s.d. from mean structure

| Backbone atoms (residues 369–377, 381–398, 408–417) | 0.51 ± 0.11 Å |
| All heavy atoms (residues 369–377, 381–398, 408–417) | 0.95 ± 0.09 Å |

Quality control

| PROCHECK-NMR (52) statistics (residues 369–417) | 77.9 |
| Residues in most favored regions | 21.7% |
| Residues in generously allowed regions | 0.5% |
| Residues in disallowed regions | 0.0% |
| Mean deviations from ideal geometry | Bond lengths | 0.0045 ± 0.0004 Å |
| Bond angles | 0.53 ± 0.02° |
binding to these peptides is similar to H3K9me3 despite the different modifications. Comparison with the PHD2-binding surface (Fig. 3C) (31) shows that in both cases the significantly shifted residues map to the same side of the PHDs, and inspection of other PHD-H3 structures, such as the BHC80 PHD (Fig. 3D) (50), reveals that the H3 peptide lies on an equivalent binding surface in each case.

**Solution Structure of PHD2 in Complex with H3K9me3**—Having established that PHD1 and PHD2 share a similar mode of H3 binding, albeit with different power to discriminate between the methylation states of Lys4 and Lys9, we next sought to determine the molecular mechanism underlying the unusual preference of PHD2 for H3K9me3. We used standard heteronuclear solution NMR methods to determine the structure of PHD2 in complex with an unlabeled H3 peptide bearing a K9me3 modification. Chemical shift assignments were obtained for 99% of commonly observed protons in PHD2. By utilizing correlations from a $^{15}$N/$^{13}$C and $^{13}$C/$^{13}$C double half-filtered NOESY along with standard homonuclear spectra, near-complete assignments for the protons in H3K9me3 residues 1–11 was also achieved (the exceptions being Arg$^2$-HN and the side chain N-H moieties of arginine and lysine residues). In total, 123 intermolecular NOEs were observed between PHD2 and the H3 peptide; input restraints for structure calculations and statistics for the 20 lowest energy water-refined structures are summarized in Table 2. In the final structures, no distance or angle restraints were violated by more than 0.5 Å or 5°, respectively, and the struc-

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**TABLE 2**

| Experimental restraints | 20 lowest energy PHD2-H3K9me3 structures |
|-------------------------|-----------------------------------------|
| Total distance restraints (unambiguous/ambiguous) | 970/37 |
| Intermolecular | 123 |
| Intraresidue ($i$–$i$) | 180 |
| Sequential ($i$–$i$) = 1 | 210 |
| Medium range ($2 \leq |i|–|j| \leq 4$) | 129 |
| Long range ($|i|–|j| > 5$) | 350 |
| Total dihedral angle restraints | 82 |

r.m.s.d. from mean structure

| Backbone atoms (PHD2 residues 448–484, 487–495; H3 residues 1–9) | 0.35 $\pm$ 0.06 Å |
| All heavy atoms (PHD2 residues 448–484, 487–495; H3 residues 1–9) | 0.84 $\pm$ 0.13 Å |

Quality control

| PROCHECK-NMR statistics (PHD2 residues 448–495; H3 residues 1–9) | |
| Residues in most favored regions | 75.3% |
| Residues in allowed regions | 22.6% |
| Residues in generously allowed regions | 1.0% |
| Residues in disallowed regions | 1.1% |

For ideal geometry

| Bond lengths | 0.0049 $\pm$ 0.0002 Å |
| Bond angles | 0.68 $\pm$ 0.02° |
tures were overlaid with a backbone r.m.s.d. of 0.35 Å (Fig. 4, A and B). Surprisingly, despite the novel H3K9me3-binding preference of PHD2, the overall mode of recognition of H3 by PHD2 is similar to that of previously described PHD-H3 structures. The contact surface on PHD2 agrees closely with that predicted from chemical shift perturbation data (Fig. 4C) (31), and the peptide forms a third β-strand upon binding to PHD2 via the formation of hydrogen bonds between H3K9me3 residues Arg2, Lys4, and Ala7 and PHD2 residues Gly458, Leu461, Leu462, and Cys463 (Fig. 4, D and E). The N-terminal half of H3K9me3 lies in a shallow groove on the surface of PHD2, with clear binding pockets for the Ala1 and Thr3 side chains and with Lys4 housed in a shallow channel (Fig. 4C). In contrast, relatively little shape complementarity is observed for residues in the C-terminal half of the peptide.

An overlay of PHD2 in the free and H3K9me3-bound states over the well ordered regions of the free PHD2 (residues 449–456, 462–484, and 490–493 (37)) shows that the structures are very similar, with a backbone r.m.s.d. of 0.79 Å (Fig. 5A). The most notable change upon binding to H3K9me3 is an increase in the number of well ordered residues, with the greatest differences observed in regions of loop 1 and 3 (Fig. 5). Not surprisingly, both of these regions form part of the H3-binding interface; loop 1 constitutes the β-strand that lies adjacent to H3K9me3, and part of loop 3 is involved in contacting the H3 N terminus.

**FIGURE 4. Solution structure of PHD2 in complex with H3K9me3.** A, ensemble of the 20 lowest energy water-refined PHD2-H3K9me3 structures, overlaid over the well ordered backbone of PHD2 (residues 448–484 and 487–495) and H3 (residues 1–9). PHD2 (residues 446–497) is shown in blue, and H3K9me3 (residues 1–11) is shown in green. Zinc-ligating side chains of the lowest energy structure are shown in yellow, and the corresponding zinc ions are shown as brown spheres. B, ensemble of the structured portion of H3 (residues 1–9) from the 20 lowest energy water-refined PHD2-H3K9me3 structures. The backbone and side chains are displayed in line format. C, lowest energy structure of PHD2-H3K9me3, with PHD2 displayed in surface format and H3 displayed as green sticks. The H3-binding surface is highlighted in blue (31). D, lowest energy structure of PHD2-H3K9me3 displayed in ribbon format. PHD2 (residues 446–496) is shown in gray with β-sheet regions in cyan and the α-helical turn in red, and H3 (residues 1–9) is shown in green. E, backbone hydrogen bonds between PHD2 (gray) and H3 (green) are shown as dotted lines.
Specific residues within the H3K9me3 peptide are recognized by a combination of hydrogen bonding, hydrophobic contacts, and surface complementarity to PHD2. Similar to previously solved PHD-H3 structures, the N-terminal amine forms a hydrogen bond to the backbone carbonyl of Gly486 and also the backbone carbonyl of Pro484 in 8 of the 20 lowest energy structures (Fig. 6). This N-terminal recognition most likely helps to specify preference for Lys4 over lysines at other positions. The side chain of Ala1 is recognized by hydrophobic interactions, lying in a shallow pocket on the surface of PHD2 lined with Leu462, Ile483, and the backbone of Pro484 and Trp488. Similarly, the Thr3 methyl group is housed within a second hydrophobic binding pocket, lined by Glu460, Leu462, and Ile483 (Fig. 4C). In contrast, Arg2 appears to contribute little to the H3-binding specificity, with the side chain showing high variability between the 20 lowest energy calculated structures (Fig. 4B). Specific recognition of Lys4 in the unmodified state is achieved by hydrogen bonding between the ε-amino group of Lys4 and the backbone carbonyls of His488 and Met499 (Fig. 6A). The AIRE and BHC80 PHDs form an additional H-bond between the Lys4 side chain and an aspartate carboxyl group (50, 57, 58), and mutation of the equivalent glutamate side chain in PHD2 (Glu450) to alanine produces a 50-fold reduction in binding to H3 (31). In the PHD2-H3K9me3 structure, no hydrogen bond was observed between the Lys4 and Glu450 side chains; however, this could be a limitation of the data due to the lack of NMR-visible protons at the termini of these side chains. Additional Lys4 recognition is achieved by hydrophobic contacts between the Lys4 alkyl chain and the side chain of Leu461. This leucine residue is conserved in BHC80 and AIRE and lies in similar proximity to the Lys4 side chain in all three structures (Fig. 6, A and B). Finally, the His488 side chain in PHD2 occupies two alternate conformations in the 20 lowest energy structures. In 13/20 structures (e.g. Fig. 6A, top left panel), the ring is positioned near the Lys4 alkyl chain, and thus could potentially form a favorable C-H—π interaction with an alkyl proton of Lys4 (59). In the second conformation (7/20 structures), the His488 side chain points away from Lys4, similar to the conformation observed for the conserved His (His487) in the BHC80-PHD crystal structure (50). It has been proposed that the BHC80-PHD His487 β-protons in this conformation restrict the space available for the Lys4 side chain, sterically precluding the addition of methyl groups (50). Thus, CHD4-PHD2 His488 in either conformation could help to specify K4me0 binding. Recognition of Gln5 is achieved by hydrogen bonding between the Glu460 side chain carboxyl group and the Gln5 amide protons (Fig. 7A). The side chain methyl protons of Thr6 are recognized in a shallow hydrophobic indentation, which is lined with Gly458, Leu462, and Ile483 (Fig. 4C). In contrast, Arg8 makes no contacts with PHD2 and points away from the surface. The Arg8 ε- and γ-protons make hydrophobic contacts with Gly458 and Gly459 in a shallow groove, whereas the remainder of the Arg8 side chain displays considerable positional variability between the 20 lowest energy structures (Fig. 6A). The lack of recognition of the Arg2 and Arg8 side chains by PHD2 is consistent with the lack of preference for modification at either Arg observed in an on-bead H3 library screen (31). In addition, the absence of phosphorylation at Thr5 or Thr6 in all detected peptides is explained by the positioning of Thr5 and Thr6 within
shallow hydrophobic pockets on the PHD2 surface, leaving insufficient space for addition of a phosphate group.

Trimethylation of H3K9 was previously shown to produce a 20-fold increase in the affinity of PHD2 for H3 (31). In the PHD2-H3K9me3 structure, the side chain carboxyl group of Asp457 appears to hydrogen bond with the backbone amide of K9me3 (Fig. 7B). Additional NOEs were observed between the trimethylammonium protons of Lys9 and the ring protons of Phe451, and a cation-/H9266-interaction between these moieties could provide a basis for the observed preference for K9me3 over K9me0. Cation-π interactions are commonly observed in the recognition of methylated lysine side chains; all of the characterized PHD-H3K4me3 interactions display cation-π interactions between K4me3 and Trp and Tyr side chains of the PHD, and cation-π interactions involving Phe are also commonly observed in other proteins (60). Moreover, studies of cation-π interactions within simple β-hairpin peptides have demonstrated a significant enhancement of the interaction upon lysine methylation (61). In structures involving trimethylated lysine, the distance between the carbon atoms of the methyl groups and the center of the aromatic rings typically falls within the range of 3.1–4.1 Å (18, 25, 62, 63). In the structure of PHD2-H3K9me3, the carbon atoms of the methyl groups of Lys9 and the Phe51 aromatic ring are separated by a distance of ~5 Å, slightly outside the range typically observed. However, this likely reflects a limitation in the available distance restraints and consequent lack of resolution in the calculated structure. Furthermore, mutation of Phe51 to alanine reduced the binding of PHD2 to H3K9me3 by 8-fold (31), hence confirming the importance of the phenylalanine side chain for this interaction.

DISCUSSION

Conservation of the Histone Binding Ability of PHDs in CHD4-related Proteins—The NuRD complex has been purified from human (64–66), amphibian (67), and insect cells (68), and proteins with homology to individual NuRD components, including CHD4, have been identified in nematodes and plants (3). CHD4-PHD1 is conserved throughout the animal kingdom, and PHD2 is also conserved in plant homologues of CHD4 (supplemental Fig. S4A). Examination of the H3-interacting residues of PHD2 reveals that all are identical from humans through to Drosophila. The corresponding residues in PHD1 in addition to residues from the predicted H3-binding face of PHD1 are also identical throughout invertebrates, with only one exception (Ala283, which is a proline in Drosophila), sug-
gesting that the H3 binding ability of these domains is ancient and has been conserved through a long period of evolution. Interestingly, the same subset of residues is also mostly conserved in PHD1 from \textit{C. elegans}, although PHD2 diverges more markedly. The sequences of the single PHDs found in plant homologues of CHD4 also differ considerably from the human sequence, and a histone binding ability cannot consequently be assumed. Finally, although the linker between PHD1 and PHD2 is well conserved between mammals, amphibians, and bony fish, the \textit{Drosophila} and \textit{C. elegans} linker is less than half the length of the human linker. This could potentially affect the ability of the two domains to bind histone tails independently.

In mammals, CHD4 belongs to a family of nine CHD proteins. Members of the family are characterized by the presence of two chromodomains followed by an ATP-dependent helicase. PHDs feature only in CHD3 (Mi2α), CHD4, and CHD5, and a sequence comparison shows that both PHD1 and PHD2 are nearly identical between the three human proteins, with only one conservative change (F451Y in CHD3) among the residues predicted to contact H3 via their side chains (highlighted in gray in supplemental Fig. S4B). The linker between the PHDs varies slightly in length (25–30 residues), but in all cases it consists predominantly of negatively charged residues. It is therefore likely that the H3 binding ability and preferences established for the PHDs of CHD4 are conserved in CHD3 and CHD5.

The striking similarity between CHD3–5 suggests that the proteins perform overlapping functions. Indeed, although the NuRD complex predominantly utilizes CHD4 (66), CHD3 has been identified in NuRD purifications from some human cell lines (64, 65). Studies using conditional CHD4 knock-out mice have demonstrated a specific requirement for CHD4 at several stages during T-cell development (7, 8). However, an up-regulation of CHD3 in the knock-out thymocytes suggests that CHD3 could be partially compensating for CHD4 depletion (7). Both CHD3 and CHD4 are widely expressed, with CHD3 displaying similar mRNA expression patterns, albeit at lower levels compared with CHD4, across a variety of mouse neonatal tissues (69). It remains to be seen whether incorporation of either component into the NuRD complex follows functional or cell type-specific patterns. In contrast, CHD5 has been found to be preferentially expressed in the brain with moderate expression in the adrenal gland and no detectable expression in all other tissues examined (70). These data suggest that CHD5 could function as a third alternative NuRD complex component in neuronal tissues. Interestingly, CHD5 maps to a region of chromosome 1 that is often deleted in neuroblastomas (1p36), and the CHD5 protein has been specifically identified as a tumor suppressor that inhibits cell proliferation and promotes senescence (71). Assuming that CHD5 contributes to a NuRD-type complex, this finding would be consistent with a role for the NuRD complex in promoting differentiation by inactivating proliferation-associated genes. It is also notable that the existence of multiple CHD protein forms (homologous to human CHD3–5) has only been observed in vertebrates, with lower complexity invertebrates such as \textit{Drosophila melanogaster} containing only one version (Mi-2 in \textit{Drosophila}).

\textbf{FIGURE 7. Mode of H3 binding by PHD2. A,} hydrogen bonds between PHD2 (gray) and the N terminus and side chains of Lys\textsuperscript{4} and Gln\textsuperscript{5} of H3 (green) are shown as dotted lines. An alignment of the 20 lowest energy structures of H3 (residues 1–9) is shown below the stick diagram and in the same orientation, to give an indication of the degree of disorder in the H3 side chains. \textbf{B,} the putative cation-π interaction between K9me3 and Phe\textsuperscript{451} is indicated by an arrow. H3 is shown in green stick format, and PHD2 is displayed as a blue ribbon with side chains near to K9me3 shown as blue sticks. The dotted line indicates a hydrogen bonding interaction. An alignment of the 20 lowest energy structures is shown below the stick diagram, for the same portion and in the same orientation.
suggests that the evolution of additional forms might have been required for higher order tissue differentiation.

**Lys9 Recognition in Other PHD Complexes**—Of the two other PHD/H3K4me0 interactions for which structures are available, only AIRE-PHD1 shows any interaction with Lys9. Hydrogen bonds are observed between the side chains of Glu298 and Asp304 and the Lys9 side chain and backbone, respectively (58).

In this solution structure, the distance between the Glu298 donor group and the Lys9 side chain is only amenable to hydrogen bond formation in 7 of the 20 lowest energy structures, although affinity measurements indicate some preference for unmodified Lys9, with trimethylation or acetylation of Lys9 producing a 6- or 8-fold reduction in binding, respectively (58, 72). Interestingly, the K9me0-interacting residues in AIRE-PHD1 (Glu298 and Asp304) lie at the same sequence positions as the K9me3-interacting residues in CHD4-PHD2 (Phe451 and Asp457), shown as positions 6 and 12 and highlighted in blue in supplemental Fig. S5. Both CHD4-PHD1 and BHC80-PHD contain an aromatic residue at position 6, similar to CHD4-PHD2; however, the Asp at position 12, which forms a hydrogen bond with the Lys9 backbone, is changed to a Gin and Lys, respectively, both of which have a different hydrogen-bonding potential (supplemental Fig. S5). In contrast, neither residue (at position 6 or 12) is conserved in the H3K9me3-binding PHDs from KDM5C and UHRF1, suggesting that a different K9me3-binding mechanism might be utilized by these PHDs.

**Relevance of Multiple H3-binding Domains in Chromatin Recognition**—Many different domain types have been implicated in histone tail recognition, including PHDs, chromodomains, bromodomains, MBT domains, tudor domains, and WD40 repeats (reviewed in Ref. 73). The existence of several of these domains within the one protein, such as the two PHDs and two chromodomains of CHD4, is a common theme among chromatin-remodeling proteins. In addition, chromatin-remodeling complexes often contain multiple proteins that each contain one or more histone-binding domains. Understanding how these domains work in combination will be crucial to understanding how histone modifications affect the chromatin state.

Both PHD1 and PHD2 of CHD4 exhibit H3-binding properties, with PHD2 specifically recognizing unmodified Lys4 and trimethylated Lys9 and PHD1 showing a small preference for unmodified Lys4. This could permit binding of the two distinct H3 tails within a single nucleosome or even binding of H3 tails in adjacent nucleosomes. We are currently investigating how having the PHD fingers in tandem might affect their interactions with H3. The independent binding that has been observed for the CHD4 PHDs contrasts with the cooperative histone binding observed for other tandem domains, such as the tandem PHDs of DPF3b, which are adjacent in sequence and act as one functional unit to recognize H3 acetylated at Lys4 (32), or the tandem chromodomains of CHD1, which recognize H3K4me3 along a common groove (20), or the double tudor domains of JMJD2A that interdigitate to form two-hybrid, tudor domain-like lobes, one of which recognizes H3K4me3 (74). It is also notable that many of the PHDs for which a histone binding ability has been established are only one of several PHDs within their parent protein; however, the function of the additional PHDs within these proteins remains to be defined.

**Implications for the Function of the NuRD Complex**—The function of the NuRD complex is not well understood at a mechanistic level. Overall, the evidence points to a role in cell differentiation, through the repression of early developmental or cell proliferation genes, although NuRD has also been implicated in gene activation (9). The ability of PHD1 and PHD2 of CHD4 to bind H3 N-terminal tails unmodified at Lys9 and trimethylated at Lys9 begins to provide insight into the biochemical function of NuRD. Importantly, the specific preferences observed for these domains is also exhibited by the NuRD complex extracted from human cells, as shown by pulldown experiments using chemically synthesized histone peptides as bait. In experiments independently carried out by two groups, the NuRD complex from nuclear extract was found to bind to unmodified H3 and H3K9me3, but not to H3K4me3 or unmodified H4 (75, 76), consistent with the structural and binding data presented here.

Given the complicated and poorly understood interplay between active and repressed histone PTMs and chromatin-remodeling enzymes, it is difficult to speculate on the order in which chromatin-remodeling processes catalyzed by the NuRD complex occur. One possible sequence of events befitting the theme of NuRD as a transcriptional repressor could involve the recruitment of NuRD-relevant active genes that require deactivation, by direct binding to DNA-associated transcriptional corepressors, such as FOG-1 (34). Upon binding of the NuRD complex, the HDAC1 and -2 components could deacetylate nearby histone tails. Additional histone-modifying factors such as H3K4me3 demethylases and H3K9 methyltransferases might be co-recruited, either through binding to the NuRD complex, transcriptional corepressors, or by recognition of newly deacetylated regions, eventually allowing the binding of CHD4-PHD1 and -PHD2 to H3 tails bearing repressed marks. This histone-binding functionality of the PHDs could recruit the nucleosome remodeling ATPase of CHD4 to the required location, to convert the chromatin into a repressed conformation. It is also possible that the two PHDs could bind to H3 tails from distinct nucleosomes, thereby facilitating the compaction of chromatin.

A similar H3-binding scheme has been hypothesized for the Drosophila Polycomb protein, which is essential for maintaining repression of homeotic genes during development. Polycomb forms a dimer in solution, and this dimerization appears to be mediated by the H3K27me3-binding chromodomain, which crystallized in the form of a dimer bound to two H3K27me3 peptides (19). Because of the close juxtaposition of the H3-binding sites in the chromodomain dimer, it is unlikely that the two histone tails could come from the same nucleosome, and it is thought that simultaneous binding of two separate nucleosomes could effectively lock the nucleosomes into a more compact configuration. Further studies will be required to determine whether and how additional factors such as methyltransferases or demethylases are recruited and to establish the physical mechanism of chromatin compaction. Furthermore, the role of NuRD in transcriptional repression is most likely only one aspect to the function of this complex machine. Each
new piece of information adds to the NuRD complex puzzle and brings us closer to understanding the mechanisms of chromatin regulation in eukaryotes.

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