A major challenge in chromatin biology is to understand the mechanisms by which chromatin is remodelled into active or inactive states as required during development and cell differentiation. One complex implicated in these processes is the Nucleosome Remodelling and histone Deacetylase (NuRD) complex, which contains both histone deacetylase and nucleosome remodelling 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 remodelling 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 methylated at K9. Here, we determine the solution structure of PHD2 in complex with H3K9me3, revealing the molecular basis of histone recognition including a cation–π recognition mechanism for methylated K9. Additionally we demonstrate that the first PHD finger also exhibits binding to the N-terminus of H3, and we establish the histone-binding surface of this domain. This is the first instance where a 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 remodelling.

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 recognised by effector proteins and complexes that regulate how the underlying genetic information is used (1). The complicated and intertwined processes of tagging the histone tails, recognising the tags, remodelling chromatin into active (open) or repressed (compacted) states and removing the tags requires the coordination of multiple protein functions.

The Nucleosome Remodelling and histone Deacetylase (NuRD) complex is unique among nucleosome remodelling complexes in that it couples histone deacetylase activity with nucleosome remodelling 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 (for example, reviewed in (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 germline cell markers in differentiated cells (4). Similarly, in *Arabidopsis thaliana*, mutants of the CHD4 homologue PICKLE develop embryonic-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 haematopoietic 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 haematopoietic genes (9). NuRD has also been identified as a modulator of aging-associated chromatin defects (10), and implicated in DNA damage signalling 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 subunits, 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 homeodomains (PHDs) and two chromodomains (Figure 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 a ~50-residue module characterised by a conserved Cys4HisCys3 motif that coordinates two zinc ions in a ‘crossbrace’ 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 BPTF (bromodomain PHD finger transcription factor) and ING2 (inhibitor of growth family member 2) which recognise H3K4me3 and thereby facilitate the interaction of BPTF, 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 recognise a range of different PTMs within H3. In a recent analysis of the 18 PHD fingers from *S. cerevisiae*, eight were found to recognise H3 methylated at K4 (H3K4me3), and two showed preference for methylation at K36 (28). Other PHDs from mammalian proteins recognise the methylation state of K9, 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 K4 in a distinct pocket on the surface of the PHD (with the exception of a tandem PHD finger from DPF3b which recognises several histone acetylation sites (32)). In contrast, we have previously shown that PHD2 of CHD4 is able to detect the modification state of both K4 and K9 of histone H3 (31). Here, we have used NMR spectroscopy to determine the molecular basis for the simultaneous recognition of K4 and K9, 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 sidechain 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 recognises the N-terminal tail of H3, displaying similar affinities for H3 unmodified and trimethylated at K9, and perhaps a slight preference for unmodified K4 over K4me3.
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 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 (34) 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 (HRV) 3C protease cleavage site). Each construct was expressed in E. 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 HRV 3C protease cleavage site at the N-terminus. The identity of each protein was confirmed using MALDI-TOF (matrix-assisted laser desorption/ionisation time-of-flight) mass spectrometry.

Western blot analysis. GST-fusion CHD4-PHD1 was incubated with C-terminally biotinylated peptides (Upstate Biotechnology) corresponding to the unmodified H3 (residues 1–21) and singly modified H3K4me1/2/3 (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 HRP (horseradish peroxidase)-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 (post-translational modification)-randomised 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, catalysing the turnover of BCIP (5-bromo-4-chloroindol-3-yl phosphate), which results in formation of a turquoise precipitate on beads bearing sequences that bind to the target protein. The bead colour intensity is proportional to affinity of the interaction (38). Peptides from individual beads were cleaved with cyanogen bromide and analysed 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.

\[^{15}\text{N}}\text{-HSQC titrations.}\] All \[^{15}\text{N}}\text{-HSQC}\) spectra were recorded at 298 K on a 600 MHz Bruker Avance spectrometer equipped with a TCI cryoprobe. PHD constructs and H3 peptides were dialysed into buffer containing 10 mM sodium phosphate (pH 7.0), 5 mM NaCl, and 1 mM DTT, using Micro DispoDIALYZERS\(^\text{TM}\) (100-Da MWCO, Harvard Apparatus, Holliston, MA, U.S.A.)
for the H3 peptides. For H3(1–12) peptides containing no aromatic residues, concentrations were determined by absorbance at 215 and 225 nm as described previously (39). H3(1-12) peptides (2–5 mM; synthesised by the Peptide Core Facility, University of Colorado Denver, CO, U.S.A) were incrementally titrated into solutions containing 15N-labelled PHD constructs (25–50 μM). Association constants were determined from the chemical shift changes of individual resonances by non-linear least-squares regression analysis using a 1:1 binding model as described previously (40). An additional parameter in the form of a peptide concentration multiplication factor was included to correct for errors in peptide concentration determination. Use of this factor resulted in high convergence and improved χ² values.

NMR resonance assignment. All spectra were recorded at 298 K on 600 or 800 MHz Bruker Avance spectrometers equipped with TCI cryoprobes. For PHD2:H3K9me3 assignment and structure determination, PHD2 and peptide were prepared as described above. For PHD1 assignment and structure determination, PHD1 was dialysed into 10 mM sodium phosphate, pH 7.5, 50 mM NaCl and 1 mM DTT. Protein concentrations were typically 0.3–1.6 mM. H, 15N and 13C assignments of free PHD1 and H3K9me3-bound PHD2 were obtained from HNCA, CBCA(CO)NH, HNCACB, HNHA, HBHA(CO)NH, HNCO, HN(CA)CO, C(C)(CO)NH-TOCSY, (HB)CB(CGCD)HD, (HB)CB(CGCDCE)HE, H(C)CH-TOCSY and HCC(CO)HN-TOCSY spectra, with the last two listed spectra recorded on samples in D2O buffer. NMR data were processed using Topspin (Bruker) and analysed with Sparky (41). 1H assignments of unlabelled, PHD2-bound H3K9me3 peptide (ARTKQTRK6STG synthesised by the Peptide Core Facility, University of Colorado Denver, CO, U.S.A. or ARTKQTRK6STGGY purchased from Peptide 2.0, Chantilly, VA, U.S.A.) were obtained from 15N/13C-double-half-filtered-NOESY, 2D-COSY and 2D-TOCSY spectra as well as a 13C/13C-double half filtered-NOESY acquired on a sample in D2O buffer.

Data analysis for structure determination. PHI (φ) angle restraints were obtained by analysis of HNHA spectra (42) and CHI (χ₁) angle restraints for PHD1 were obtained by analysis of an HNHB and short mixing time (50 ms) TOCSY and NOESY spectra (43). Additional dihedral angles were calculated using TALOS (44) and TALOS+ (45), and only angles predicted to be reliable by both programs were used as restraints. For the H3K9me3 peptide, additional negative φ angle restraints were included for residues for which the intraresidue Hα-HN NOE was clearly weaker than the NOE between Hα and the HN of the following residue (46).

For PHD1 structure calculations, all distance restraints were derived from integration of a 2D NOESY acquired on an unlabelled PHD1 sample. For calculation of the PHD2:H3K9me3 structure, distance restraints were obtained from 2D-NOESY, 15N-NOESY and 15N/13C-double-half-filtered-NOESY spectra, as well as 13C-NOESY and 13C/13C-double-half-filtered-NOESY spectra acquired on samples in D2O buffer. For NOESY experiments, PHD2 and H3K9me3 were present at a 1:1 molar ratio, or up to a 5% molar excess of H3K9me3 (as judged by inspection of 15N-HSQC titration data).

Structure calculations. The molecular-viewing programs MOLMOL (35) and PyMOL (36) were used to analyse calculated structures throughout the structure determination process. Initial structure refinement was carried out using CYANA 2.1 (47), and final calculations were performed using ARIA 1.2 (48) for PHD1. For PHD2:H3K9me3, final calculations were performed using ARIA 2.2 (49) with upper distance limits for intermolecular NOEs calibrated using the CALIBA module of CYANA 2.1 (for PHD2:H3K9me3). Trimethylated lysine was added to the library file of CYANA 2.1 and MOLMOL, and to numerous defining files of ARIA 2.2 (topallhdg5.3.pro, parallhdg5.3.pro, PseudoAtom.py, atomnames.xml and
The coordination geometry for each zinc ion in PHD1 and PHD2 was defined to be consistent with high resolution (<1.8 Å) crystal structures of PHD domains (e.g. PDB codes 2PUY (50) and 3KQI (51)), using bond (2.3 Å for Zn–S and 2 Å for Zn–Nδ1) and angle (S–Zn–S at 109°, C–S–Zn at 107°, S–Zn–Nδ1 at 107° and C–Nδ1–Zn at 125°) constraints. An initial cycle of 100 structures was followed by six cycles of 20 structures and a final cycle of 1000 calculated structures. The 50 lowest energy structures were subjected to water refinement, and the 20 lowest energy water-refined structures analysed by PROCHECK-NMR (52).

RESULTS

CHD4-PHD1 recognises histone H3. We have previously shown that PHD2 of CHD4 is capable of recognising H3 unmodified at K4 and methylated at K9. In order to assess whether PHD1 could also act as a histone-reading module, we carried out pulldown experiments (Figure 1B). 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), 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 recognise 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 K4 and K9 within H3, 15N-HSQC spectra of uniformly 15N-labelled PHD1 were recorded in the presence of increasing concentrations of unmodified H3(1–12), H3K4me3 or H3K9me3 (Figure 1C). 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 non-linear least-squares regression (Figure 1D and E). An additional parameter was included to correct for errors in H3 peptide concentration (see Methods), 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 ± 0.6) × 10^5 M⁻¹ and (2.6 ± 0.5) × 10^5 M⁻¹, respectively. This contrasts with the specificity of PHD2, which displays a 20-fold increase in affinity upon trimethylation at K9 from (5.6 ± 0.2) × 10^6 M⁻¹ to (1.6 ± 0.3) × 10^6 M⁻¹ (31). Trimethylation at K4 reduced the affinity of PHD1 for H3 from (3.1 ± 0.6) × 10^5 M⁻¹ to (1.1 ± 0.2) × 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 × 10^4 to 6 × 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 (Figure 1F; (38,54)). The peptide library encompassed numerous possible modification states at position 2 (R, Rme1/2s/2a, Cit), 3 (T, Thr), 4 (K, Kme1/2/3, Kac), 6 (T, Thr), 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 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 K4 over methylated or acetylated K4, in agreement with the $^{15}$N-HSQC binding data presented above. The absence of phosphorylation at T3, T6 and S10 in all detected peptides suggests that phosphorylation at these positions disrupts binding. In contrast, no clear preferences were observed for the modification state at R2, R8 or K9.

**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 sidechain N-H groups of Asn and Gln; assigned HSQC is provided in Figure S1) and statistics for the 20 lowest energy water-refined structures are summarised in Table 1 (Ramachandran plots are provided in Figure S2). In the final calculated structures, no angle restraints were violated by $\geq 5^\circ$, and only one distance restraint was violated by $>0.5$ Å: a 0.74 Å violation between C396 Hβ2/3 and V375 HN. The structured regions of the 20 lowest-energy water-refined structures overlayed with a backbone RMSD of 0.51 Å (Figure 2A). The PHD1 structure has a similar fold to the previously determined PHD2 structure (Figure 2B and C) and the well-defined portion stretches over a similar range of residues in each domain. An overlay of PHD1 and PHD2 gives an RMSD 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 RMSD of 2.1 Å.

The 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 Figure 3A). A chemical shift change of more than one SD from the mean was observed for backbone amides of five PHD1 residues: G380, I382, H393, E406 and W409, 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 (Figure 3B). Similar patterns of chemical shift changes were observed for unmodified H3 and H3K4me3 (Figure S3), indicating that the mode of binding to these peptides is similar to H3K9me3 despite the different modifications. Comparison with the PHD2 binding surface (Figure 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 (Figure 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 K4 and K9, 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 unlabelled H3 peptide bearing a K9me3 modification. Chemical shift assignments were obtained for 99% of commonly observed protons in PHD2. By utilising 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 R2-HN and the sidechain 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 summarised in Table 2. In the final structures, no distance or angle restraints were violated by more than 0.5 Å or $5^\circ$, respectively, and the structures overlayed with a backbone RMSD of 0.35 Å (Figure 4A 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 (Figure 4C; (31)), and the peptide forms a third $\beta$-strand upon binding to PHD2 via the formation of hydrogen bonds between H3K9me3 residues R2, K4 and A7 and PHD2 residues G458, L461, L462 and C463 (Figure 4D and E). The N-terminal half of H3K9me3 lies in a shallow groove on the surface of PHD2, with clear binding pockets for the A1 and T3 sidechains and with K4 housed in a shallow channel (Figure 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, 490–493; (37)) shows that the structures are very similar, with a backbone RMSD of 0.79 Å (Figure 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 (Figure 5). Not surprisingly, both of these regions form part of the H3-binding interface: loop 1 constitutes the $\beta$-strand that lies adjacent to H3K9me3, and part of loop 3 is involved in contacting the H3 N-terminus.

Specific residues within the H3K9me3 peptide are recognised 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 G486 and also the backbone carbonyl of P484 in eight of the 20 lowest-energy structures (Figure 6A). This N-terminal recognition most likely helps to specify preference for K4 over lysines at other positions. The sidechain of A1 is recognised by hydrophobic interactions, lying in a shallow pocket on the surface of PHD2 lined with L462, I483 and the backbone of P484 and W488. Similarly, the T3 methyl group is housed within a second hydrophobic binding pocket, lined by E460, L462 and I483 (Figure 4C). In contrast, R2 appears to contribute little to the H3-binding specificitiy, with the sidechain showing high variability between the 20 lowest-energy calculated structures (Figure 4B).

Specific recognition of K4 in the unmodified state is achieved by hydrogen bonding between the $\varepsilon$-amino group of K4 and the backbone carbonyls of H448 and M449 (Figure 6A). The AIRE and BHC80 PHDs form an additional H-bond between the K4 sidechain and an aspartate carboxyl group (50,57,58), and mutation of the equivalent glutamate sidechain in PHD2 (E450) to alanine produces a 50-fold reduction in binding to H3 (31). In the PHD2:H3K9me3 structure no hydrogen bond is observed between the K4 and E450 sidechains; however this could be a limitation of the data due to the lack of NMR-visible protons at the termini of these sidechains. Additional K4 recognition is achieved by hydrophobic contacts between the K4 alkyl chain and the sidechain of L461. This leucine residue is conserved in BHC80 and AIRE and lies in similar proximity to the K4 sidechain in all three structures (Figure 6A and B). Finally, the H448 sidechain in PHD2 occupies two alternate conformations in the 20 lowest-energy structures. In 13/20 structures (e.g. Figure 6A, top left), the ring is positioned near to the K4 alkyl chain, and thus could potentially form a favourable C-H—$\pi$ interaction with an alkyl proton of K4 (59). In the second conformation (7/20 structures), the H448 sidechain points away from K4, similar to the conformation observed for the conserved His (H487) in the BHC80-PHD crystal structure (50). It has been proposed that the BHC80-PHD H487 $\beta$-protons in this conformation restrict the space available for the K4 sidechain, sterically precluding the addition of methyl groups (50). Thus, CHD4-PHD2 H448 in either conformation could help to specify K4me0 binding.

Recognition of Q5 is achieved by hydrogen bonding between the E460 sidechain carboxyl group and the Q5 $\varepsilon$-amide protons (Figure 7A). The sidechain methyl protons of T6 are
recognised in a shallow hydrophobic indentation, which is lined with G458, G459, the backbone of E450 and the sidechain of L461. In contrast, the A7 sidechain makes no contacts with PHD2 and points away from the surface. The R8 \( \beta \) and \( \gamma \)-protons make hydrophobic contacts with G458 and G459 in a shallow groove, while the remainder of the R8 sidechain displays considerable positional variability between the 20 lowest-energy structures (Figure 6A). The lack of recognition of the R2 and R8 sidechains 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 T3 or T6 in all detected peptides is explained by the positioning of T3 and T6 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 sidechain carboxyl group of D457 appears to hydrogen bond with the backbone amide of K9me3 (Figure 7B). Additional NOEs were observed between the trimethylammonium protons of K9 and the ring protons of F451, and a cation–\( \pi \) interaction between these moieties could provide a basis for the observed preference for K9me3 over K9me0. Cation–\( \pi \) interactions are commonly observed in the recognition of methylated lysine sidechains: all of the characterised PHD:H3K4me3 interactions display cation–\( \pi \) interactions between K4me3 and Trp and Tyr sidechains of the PHD, and cation–\( \pi \) interactions involving Phe are also commonly observed in other proteins (60). Moreover, studies of cation–\( \pi \) interactions within simple \( \beta \)-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 of K9 and the F451 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 F451 to alanine reduced the binding of PHD2 to H3K9me3 by eight-fold (31), hence confirming the importance of the phenylalanine sidechain 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 (Figure 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 (A483, which is a proline in Drosophila), suggesting 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 *C. elegans*, whilst 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, while the linker between PHD1 and PHD2 is well conserved between mammals, amphibians and bony fish, the *Drosophila* and *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 characterised 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) amongst the residues predicted to contact H3 via their sidechains (highlighted in grey in Figure S4B). The linker between the PHDs varies slightly in length (25–30 residues), but in all cases 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, 4 and 5 suggests that the proteins perform overlapping functions. Indeed, while the NuRD complex predominantly utilises CHD4 (66), CHD3 has been identified in NuRD purifications from some human cell lines (64,65). Studies using conditional CHD4-knockout mice have demonstrated a specific requirement for CHD4 at several stages during T-cell development (7,8). However, an upregulation of CHD3 in the knockout 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 to 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 tumour 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, 4 and 5) has only been observed in vertebrates, with lower complexity invertebrates such as Drosophila melanogaster containing only one version (Mi-2 in Drosophila). This suggests that the evolution of additional forms might have been required for higher-order tissue differentiation.

**K9 recognition in other PHD complexes.** Of the two other PHD:H3K4me0 interactions for which structures are available, only AIRE-PHD1 shows any interaction with K9. Hydrogen bonds are observed between the sidechains of E298 and D304 and the K9 sidechain and backbone, respectively (58). In this solution structure, the distance between the E298 donor group and the K9 sidechain is only amenable to hydrogen bond formation in 7 of the 20 lowest-energy structures, although affinity measurements indicate some preference for unmodified K9, with trimethylation or acetylation of K9 producing a six- or eight-fold reduction in binding, respectively (58,72). Interestingly, the K9me0-interacting residues in AIRE-PHD1 (E298 and D304) lie at the same sequence positions as the K9me3-interacting residues in CHD4-PHD2 (F451 and D457), shown as positions 6 and 12 and highlighted in blue in Figure 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 K9 backbone is changed to a Gln and Lys, respectively, both of which have a different hydrogen-bonding potential (Figure S5). In contrast, neither residue (at position 6 or 12) is conserved in the K9me3-binding PHDs from KDM5C and UHRF1, suggesting that a different K9me3-binding mechanism might be utilised by these PHDs.
The 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 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 remodelling proteins. In addition, chromatin remodelling 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 recognising unmodified K4 and trimethylated K9, and PHD1 showing a small preference for unmodified K4. 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 recognise H3 acetylated at K14 (32), or the tandem chromodomains of CHD1, which recognise 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 recognises 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 K4 and trimethylated at K9 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-synthesised 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 remodelling enzymes, it is difficult to speculate on the order in which chromatin remodelling processes catalysed 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 (77). 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 remodelling ATPase of CHD4 to the required location, in order 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 hypothesised for the Drosophila Polycomb protein, which is essential for maintaining repression of homeotic genes during development. Polycomb forms a dimer in solution, and this dimerisation appears to be mediated by the H3K27me3-binding chromodomain, which crystallised in the form of a dimer bound to two H3K27me3 peptides (19). Due to 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 if 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.

ACKNOWLEDGEMENTS

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REFERENCES

**FIGURE LEGENDS**

Figure 1. PHD1 binds N-terminal histone H3 peptides. (A) The domain structure of human CHD4. (B) Pulldowns using PHD1 and a range of different H3 peptides. The H3 portion to which each peptide corresponds is indicated below the blot, and the methylation state of the modified lysine is shown above each lane. GST-PHD1 was detected using an anti-GST antibody. (C) Portions of 15N-HSQC spectra of PHD1 at a starting concentration of 25 or 50 μM, recorded at increasing concentrations of the indicated peptides. The molar ratio of PHD1 to H3 peptide is indicated in the coloured key for each spectrum. (D) Example binding curves mapping the 15N chemical shift change for one resonance per titration (the amide nitrogen of T387), with curves fitted to a one-to-one binding model by non-linear least-squares regression. (E) Binding affinities averaged from at least three different amide resonances, with the 1H and 15N dimensions analysed separately. Error bars show ± 1 SD from the mean. (F) Screen of a PTM-randomised H3 peptide library for binding to PHD1. Each observed PTM is represented by different bars at each H3 residue position, with the height corresponding to the discrimination factor calculated for that PTM. The discrimination factor equals the ratio of the frequency of a single PTM within the PHD-binding peptides (30–40) to the frequency of that PTM at the same residue position in a random peptide library sampling (50 peptides). PTM abbreviations are as follows: Lys mono-, di- or trimethylation (Kme1, Kme2, Kme3); Arg monomethylation, symmetrical or asymmetrical dimethylation (Rme1, Rme2s, Rme2a).

Figure 2. Solution structure of PHD1. (A) Ensemble of the 20 lowest energy PHD1 structures (residues 365–420). The zinc ion and zinc-ligating sidechains from the lowest energy structure are shown in red and yellow, respectively. (B) The lowest energy PHD1 structure shown in ribbon format, with zinc-ligating side chains displayed as described in (A). (C) The lowest energy PHD2 structure (residues 446–501; PDB code 1MM2 (37)) depicted in the same format and orientation as PHD1 in (B).

Figure 3. NMR analysis of the PHD1:H3 interaction. (A) The difference in chemical shift between free and H3K9me3-bound PHD1 for the amide moiety of each residue is shown. Residues that shift more than one SD from the mean (indicated by the dotted line) are highlighted in blue. (B) The H3-binding surface of CHD4-PHD1 (residues 367–417). (C) The H3-binding surface of CHD4-PHD2 (residues 447–496) (31,37). (D) The complex between BHC80-PHD (residues 487–534, PDB code 2PUY, ref. (50)) and unmodified H3 (residues 1–9 shown in stick representation) (50). Structures in (B), (C) and (D) are displayed in the same orientation.

Figure 4. Solution structure of PHD2 in complex with H3K9me3. (A) Ensemble of the 20 lowest-energy water-refined PHD2:H3K9me3 structures, overlayed over the well-ordered backbone of PHD2 (residues 448–484, 487–495) and H3 (residues 1–9). PHD2 (residues 446–497) is shown in blue and H3K9me3 (residues 1–11) shown in red. Zinc-ligating sidechains 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 sidechains are displayed in line format. (C) The 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) The lowest-energy structure of PHD2:H3K9me3 displayed in ribbon format. PHD2 (residues 446–496) is shown in grey 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 (grey) and H3 (green) are shown as dotted lines.
Figure 5. Comparison of free PHD2 with PHD2 in complex with H3K9me3. (A) An overlay of the 20 lowest-energy structures of free PHD2 shown in blue (37) and the PHD2:H3K9me3 complex, in which PHD2 (residues 446–496) is shown in red and H3K9me3 (residues 1–11) is shown in green. (B) Sequence of PHD2 (residues 446–497) showing regions of relative disorder in the free PHD2 structure (top sequence) and the PHD2:H3K9me3 complex (bottom sequence). Disordered portions in the loop regions are shown in yellow and flexible termini are shown in grey. Residues that form the β-strand adjacent to the H3 peptide are underlined, and zinc-ligating residues are shown with asterisks.

Figure 6. Comparison of K4 recognition in different PHD:H3 structures. (A) Portions of the structures of CHD4-PHD2:H3K4me0K9me3, AIRE-PHD1:H3K4me0 (PDB code 2KE1, 58), BHC80-PHD:H3K4me0 (PDB code 2PUY, 50) and ING2-PHD:H3K4me3 (PDB code 2G6Q, 25) are shown, highlighting the modes of K4 recognition. PHDs are displayed as grey ribbons with K4-interacting residues shown as sticks, and the H3 peptides are displayed as orange sticks. PHD residues that form hydrogen bonds to the K4 ε-amino group are additionally labelled according to whether the sidechain (sc) or backbone (bb) is involved. (B) A sequence alignment of the four PHDs from part A, highlighting residues involved in the recognition of unmodified or trimethylated K4 in yellow and green, respectively. SwissProt accession codes are as follows: CHD4 (Q14839), AIRE (O43918), BHC80 (Q96BD5) and ING2 (Q9H160).

Figure 7. Mode of H3 binding by PHD2. (A) Hydrogen bonds between PHD2 (grey) and the N-terminus and sidechains of K4 and Q5 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 sidechains. (B) H3 is shown in green stick format, and PHD2 is displayed as a blue ribbon with sidechains 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.
### Table 1. Experimental restraints and structural statistics for the ensemble of the 20 lowest energy PHD1 structures.

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Table 2 Experimental restraints and structural statistics for the ensemble of the 20 lowest-energy PHD2:H3K9me3 structures.

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AIRE-PHD1 + H3K4me0

BHC80-PHD + H3K4me0

ING2-PHD + H3K4me3

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* * *
The plant homeodomain (PHD) fingers of CHD4 are histone H3-binding modules with preference for unmodified H3K4 and methylated H3K9
Robyn E. Mansfield, Catherine A. Musselman, Ann H. Kwan, Adam L. Garske, Foteini Davrazou, John M. Denu, Tatiana G. Kutateladze and Joel P. Mackay

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