Identification and Characterization of Nardilysin as a Novel Dimethyl H3K4 Binding Protein Involved in Transcriptional Regulation

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*Running title: NRDc as a novel H3K4me2 binding protein

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Background: Very few proteins are known to read histone methylation in a state-specific manner and NRDc is not known for a role in transcriptional regulation.

Results: NRDc binds specifically H3K4me2 and has a role in transcriptional regulation.

Conclusion: NRDc is a novel H3K4me2-binding protein.

Significance: NRDc is the first identified protein that reads specifically H3K4me2.

SUMMARY

Histone methylation on lysine residues is believed to function primarily as docking sites to recruit specific proteins termed as histone code “readers” or “effectors”. Each lysine residue can be mono-, di and tri-methylated and different methylation states can have different effect on chromatin function. While an increasing number of proteins have been identified and characterized as specific effectors for methylated histones, very few of the proteins are known to recognize a particular state of methylation. In this study, we identified nardilysin (NRDc), a member of M16 family metalloendopeptidases, as a novel dimethyl-H3K4 (H3K4me2) binding protein. Among three methylated states, NRDc binds preferentially H3K4me2 both in vitro and in vivo. Biochemical purification demonstrated that NRDc interacts with the NCoR/SMRT corepressor complex. We identified target genes repressed by NRDc through microarray. We showed that NRDc is physically associated with and recruits the NCoR complex to some of the repressed genes and this association correlates with binding of H3K4me2. Thus, our study has identified a novel H3K4me2 binding protein and revealed a role of NRDc in...
transcriptional regulation.

In eukaryotic cells, DNA is packaged with histones in the form of chromatin. Among various histone modifications identified, histone lysine methylation functions in virtually all chromatin-based biological processes including transcriptional regulation and DNA damage repair (1-3). To date, at least seven different lysine residues in various histones have been identified as functionally relevant sites of methylation (K4, K9, K27, K36 and K79 of H3, K20 of H4 and K26 of H1) (4). Importantly, each lysine residue can be mono-, di-, or tri-methylated. These different states of methylation could be generated by progressive methylation catalyzed by the same or different histone methyltransferases that differ in methylation processivity and the balancing effect of opposing demethylases (5,6). Numerous studies have revealed that different methylation states on the same lysine residue exhibit distinct global and gene-specific distribution patterns (7-9). For example, H3K4me1, me2 and me3 are differently distributed along a transcribed gene, with H3K4me3 mainly found in the promoter around the transcriptional start site, H3K4me2 peaks within the coding region and H3K4me1 enriched predominantly at the 3' end. While H3K4me3 has been strongly associated with active transcription, H3K4me2 has also been implicated in transcription repression (9,10). Thus, the biological effects of histone methylation are not only determined by the specific sites of methylation but also influenced by the methylation states.

Mechanistically, histone methylation is believed to function at least in part as docking sites to recruit (or repel) specific chromatin-associated proteins called “reader” or “effector” proteins (3,11). The effector proteins are therefore critical for interpreting the methylation codes and thus mediate the biological effect of histone methylation. If different methylation states can specify different functional consequences, one would expect the existence of effector proteins that read methylated histones in a methylation state-dependent manner. Significant effort has so far been directed toward the identification and characterization of the effectors of site-specific methylated histones including K4, K9, K27 in H3 and K20 in H4 (4,12). To date, although an increasing number of proteins with royal family domains have been identified and characterized as methylated histone binding proteins, very few act in a methylation-state-specific manner. For example, currently identified methylated H3K4 binding proteins include CHD1 (13), BPTF (14,15), ING2 (16,17), TAF3 (18), PHF2 and PHF8 (19-21), all of which recognize both H3K4me2 and H3K4me3 and do this through either a chromodomain (CHD1) or PHD domain (BPTF, ING2, TAF3, PHF2 and PHF8). In fact, PELP1 is the only protein that has been reported to bind specifically di-methylated H3K4 and H3K9 but not mono- and tri-methylated H3K4 and H3K9 (22). A glutamic acid rich region within the C-terminus of the protein was shown to be required for this di-methylated histone binding specificity.

Nardilysin (also called N-arginine dibasic convertase or NRDC), is a member of M16 family metalloendopeptidases that cleaves substrates at paired basic residues (23,24). It was also identified as a receptor for heparin-binding epidermal growth factor-like growth factor (HB-EGF) and shown to facilitate HB-EGF ectodomain shedding (25,26). More recently, NRDC is shown to regulate axonal maturation and myelination and is required for antigen processing in cytotoxic T lymphocytes (CTL) (27,28). Majority of NRDC-/- mice die within 48h after birth and exhibit growth retardation (27), suggesting a pleiotropic role.
for NRDC. In this study, we identified NRDC as a novel H3K4me2-binding protein and present evidence that NRDC has a role in transcriptional regulation.

EXPERIMENTAL PROCEDURES

Plasmids and antibodies- The plasmids pCDNA3-NRDC-V5 and pCDNA3-NRDC(E>A)-V5 expressing both wild type and peptidase deficient mutant have been reported (25,26). This NRDC (aa1-1100) corresponds to amino acids 50-1150 of BC008775 in Genbank. The △AD, △48-89 and △171-260 deletion of NRDC expression constructs were generated by PCR-based site-directed mutagenesis using pCDNA3-NRDC-V5 plasmid. Other deletions of NRDC were generated by PCR based cloning with pSG5-HA vector for in vitro synthesis of proteins or pET3a for expression as a 6xHis-NRDC(aa1-621) fusion protein. To express Flag-tagged NRDC full-length in mNRDC -/- MEF cells, the full-length NRDC was cloned into the retroviral vector pOZ-Flag. All plasmid constructs were verified by DNA sequencing. Antibodies against MTA1, NCoR, TBL1 and HDAC3 were as reported previously (29). Antibodies against H3K4me1, H3K4me2, H3K4me3, H3K9me2 and H3K9me3 were purchased from Abcam, and anti-Flag M2 and actin antibodies from Sigma and V5 tag antibody from Invitrogen. Anti-NRDC antibody for purification of NRDC complex(es) and anti-PHF2 antibody were purchased from Nexus Biological. Anti-PHF2 antibody was as reported previously (19).

In vitro binding of histone peptides with HeLa nuclear extracts, 35S-methionine-labeled proteins and recombinant proteins- Nuclear extracts were prepared from HeLa cells by the protocol of Dignam (30) and precleared with streptavidin-coated agarose beads. The various K4 and K9 methylated histone H3 tail peptides contain the H3 amino acids 1-21 followed by a GGK linker sequence and a C-terminal biotin. The K27 peptides have aa18-36 sequence of H3 plus a GGK linker sequence and a C-terminal biotin. All peptides were synthesized and purified by Scilight Biotechnology LLC (Beijing, China). The peptide based pulldowns were performed essentially as described (31). In brief, approximately 1 µg of histone tail peptides were first immobilized onto 10 µl streptavidin-coated agarose beads, and after washing to remove the unbound peptide, they were incubated with approximately 250 µg of HeLa cell nuclear extracts in 100 ul reaction with binding buffer (20 mM HEPES [pH 7.9], 150 mM KCl, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 10% glycerol, 0.1% NP-40, proteinase inhibitors) for 3 h at 4°C. Unbound proteins were removed by washing the beads with washing buffer (20 mM HEPES [pH 7.9], 150 mM KCl, 1 mM DTT, 1 mM PMSF, 0.1% NP-40, proteinase inhibitors) 4 times for 5 min each. The proteins that remained bound to the peptides were separated by SDS-PAGE followed by western blotting analysis.

For characterizing methylated H3K4 binding activity of NRDC using in vitro translated proteins, various 35S-methionine labeled NRDC mutants and full-length NRDC were generated with the TNT coupled reticulocyte lysate system (Promega). The reactions were diluted 1:10 with the binding buffer and subjected to binding assays as above except that the binding of the proteins was revealed by autoradiography.

For determining direct binding of histone peptides by full-length NRDC, the 293T cells were transiently transfected with pSG5-Flag-NRDC and two days after transfection the cells were harvested and processed for purification of Flag-NRDC using anti-Flag M2 beads essentially as described. For
testing the binding of 6xHis-NRDc(1-621), this protein was expressed and purified from E.coli. The binding of the purified proteins to various H3 peptides were essentially as described above except the presence of 0.2 mg/ml BSA in the binding buffer and the washing buffer containing 300 mM NaCl. The binding of Flag-NRDc was revealed by Western blot analysis using anti-Flag antibody, whereas the binding of 6xHis-NRDc(1-621) was revealed by Coomassie blue staining. For in vitro competition assay, about 2 μg of 6xHis-NRDc(1-621) was incubated with 0.5 μg of immobilized H3K4me2 peptide in the presence of 0, 5, 10 and 15 μg of H3, H3K4me1, H3K4me2 and H3K4me3 peptides without biotin respectively. After extensive washing, the binding of 6xHis-NRDc(1-621) was revealed by Coomassie blue staining.

**Generation of stable cell lines, immunostaining and Immunoprecipitation**- The stable cell line mMEF -/- Flag-NRDc was established by retrovirus infection followed by affinity selection of IL-2 receptor surface marker as described (32). Immunofluorescent staining for exogenous and endogenous NRDc was carried out with V5-tag antibody and NRDc antibody in HeLa and NIH3T3 cells. Immunoprecipitation with HeLa nuclear extracts and whole cell extracts were essentially as described (33).

**Microarray and RT-PCR**- RNA microarray analysis was carried out on Illumina BeadChip according to the manufacturer’s instructions. qPCR analyses were carried out with SYBR Premix Ex Taq (TaKaLa) and the PCR primers are shown in Table S1A.

**Chromatin immunoprecipitation (ChIP) and Re-ChIP assay**- ChIP assays were performed essentially as described (29) and the primers for ChIP assays are shown in Table S1B. For re-ChIP analyzing the association of NRDc with NCoR, HDAC3 and mono-, di- and tri-methylated H3K4, the first ChIP was carried out using anti-Flag antibody. The re-ChIP were then performed essentially as described using antibodies as indicated (34).

**RESULTS**

**Identification of NRDc as a H3K4me2-binding protein.** We reported recently an effort to identify H3K4me2 or H3K9me2 effector proteins from HeLa nuclear extracts using an unbiased pull-down assay (35). NRDc was identified among the proteins that bound to H3K4me2 peptide by LC-MS/MS mass spectrometry analysis (data not shown). As a member of metalloendopeptidases, NRDc is not known for any nuclear function. To investigate the histone binding specificity, we carried out pull-down assays with HeLa nuclear extracts and various synthetic H3 peptides followed by Western blot analysis. Consistent with previous reports (19,35,36), PHF2 was found to bind both H3K4me2/3 peptides and exhibited a stronger binding for H3K4me3 peptide. However, Western blot analysis using an NRDc specific antibody revealed that NRDc bound only to the H3K4me2 peptide but not H3K4me3 and other H3 peptides (Fig. 1A). As additional controls for binding specificity, the same pull-down samples were analyzed for binding of ICBP90/UHRF1 and MTA1 that are known for binding of H3K9me2/3 and unmodified H3, respectively. The results in Fig. 1A showed that UHRF1 bound specifically to the H3K9me2 and H3K9me3 peptides, as reported previously (31). In agreement with previous reports (37,38), MTA1, a subunit of Mi-2/NuRD complex, was found to bind unmodified H3 but not H3K4me1/2/3 peptides. Interestingly, MTA1 was found to also bind H3K9me1 and H3K9me2 peptides but not the H3K9me3 peptide. As MTA1 binds unmodified H3 peptide, a plausible explanation is that the binding of H3 peptide by Mi-2/NuRD complex is not influenced by H3K9me1/2 methylation but
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NRDc binds directly H3K4me2. To address if NRDc binds to H3K4me2 directly or indirectly, we first synthesized 35S-Met labeled NRDc proteins using in vitro coupled transcription and translation system and tested its binding to H3K4me2 by pull-down assay. As shown in Fig. 1B, this in vitro synthesized NRDc exhibited strongest binding of H3K4me2, although a weaker binding of unmodified H3 peptide was also observed. In multiple experiments, we consistently observed a binding specificity in the order of H3K4me2>H3>H3K4me1/3 for in vitro synthesized NRDc (see also Fig. 2A). To test further if NRDc binds H3K4me2 directly, we expressed and purified a Flag-NRDc from 293T cells by immune-affinity purification using anti-Flag M2 agarose beads. The resulting purified Flag-NRDc was essentially a simple band when analyzed by Coomassie blue staining (Fig. 1C, left panel). When subjected to in vitro binding assay, this highly purified Flag-NRDc also exhibited a preferential binding of the H3K4me2 peptide over unmodified H3 in the order of H3K4me2>H3>H3K4me1/3 (Fig. 1C, left panel). Furthermore, we expressed and purified NRDc aa1-621 as 6xHis fusion proteins from bacteria and subjected it to in vitro peptide pulldown assay. The results in Fig. 1D showed that this recombinant protein exhibited a H3K4me2 binding specificity. Finally, we tested the H3K4me2-binding specificity of NRDc by a competition assay. The purified 6xHis-NRDc(1-621) proteins were incubated with immobilized biotin-H3K4me2 peptides in the presence of increasing amount (0, 5, 10, and 15 μg respectively) of H3, H3K4me1, H3K4me2 and H3K4me3 peptides and the binding of 6xHis-NRDc(1-621) was revealed by commassie blue staining. A representative result in Fig. 1E showed that the binding of 6xHis-NRDc(1-621) to immobilized H3K4me2 peptide was blocked only efficiently by free H3K4me2 but not other peptides. The quantitative results for two independent competition experiments were shown in Fig. 1F. Together these results provide evidence that NRDc binds preferentially and directly H3K4me2 in vitro.

NRDc may mainly be a nuclear protein. NRDc was shown previously as a secreted and cytoplasmic protein and was not known for nuclear function. The presence of NRDc in HeLa nuclear extracts and its preferential binding of H3K4me2 imply a nuclear function for NRDc. To analyze the subcellular localization of NRDc, we first transfected HeLa and NIH3T3 cells with a V5-tagged NRDc expression construct and examined the subcellular localization of V5-NRDc by immunostaining. The representative results in Fig. 1G showed that, although V5-NRDc could also be detected in the cytoplasm, it was enriched in the nucleus in both HeLa and NIH3T3 cells. Furthermore, by using a NRDc monoclonal antibody that is capable of detecting transfected V5-NRDc (Fig. 1H top panel), we found the endogenous NRDc in NIH3T3 cells was also resided mainly in the nucleus (Fig. 1H, lower panel). Together these data provide evidence that NRDc may mainly be a nuclear protein.

The H3K4me2-binding activity resides in the N-terminal region of NRDc. NRDc is an evolutionarily conserved protein with an acidic stretch in the N-terminus, an enzyme active domain and two enzyme inactive domains which are known for holding the substrate in enzyme reaction (24) (Fig. 2A). However, NRDc contains no sequence resembling to any royal family domain such as chromodomain, MBT and Tudor and PHD finger that have been classified as the signature domains for
recognition of methylated histones (4,39). To identify the region(s) required for binding of H3K4me2, a series of NRDc deletions were constructed, expressed as 35S-Met labeled proteins and subjected to peptide pulldown assays. The results in Fig. 2A showed that mutants with gradual deletion of enzyme inactive and active domains (NRDc1-621, 1-445 and 1-260) all bound preferentially the H3K4me2 peptide. However, further deletion up to 171aa abolished the H3K4me2-binding activity. On the other hand, deletion of N-terminal amino acids 1-148 also abolished the H3K3me2-binding activity, as no binding was observed for NRDc 149-621 construct. Together these data indicate that the major determinant for H3K4me2 specificity is located within aa1-260 of NRDc.

Within aa1-260 NRDc contains a stretch of glutamic and aspartic acid rich region (aa93-148). Given that PELP1 was shown to bind H3K4me2 and H3K9me2 through a glutamic acid rich region (22), we generated a NRDc mutant (NRDcΔAD) with internal deletion of the acidic region. Interestingly, pulldown assay (Fig. 2A) showed that this mutant retained the H3K4me2-binding specificity, indicating that, unlike the case of PELP1, the acidic stretch of NRDc is not required for binding of H3K4me2. We also tested if its peptidase activity is involved in the binding of H3K4me2. The result showed that a peptidase-deficient NRDc E186A mutant (NRDcE>A) bound H3K4me2 as the wild-type NRDc.

Through sequence alignment of NRDc proteins from multiple organisms, we noticed the existence of an evolutionarily conserved region spanning human NRDc aa48-89 (Fig. 2B). We thus made a mutant with internal deletion of this region (NRDcΔ48-89). Pulldown analysis in Fig. 2A showed that, while this mutant retained a weak binding for unmodified H3 peptide, it lost its preferential binding activity for H3K4me2 (now binds equally to H3 and H3K4me2 peptides), indicating this region is required for the observed preferential binding of H3K4me2 by NRDc. Furthermore, as binding of methylated histones often involves aromatic amino acid residues in the effector proteins (17,40,41), we also tested if two conserved tyrosine residues, Y64/Y66, within the region of aa48-89 play any role in binding of H3K4me2. The result in Fig. 2A, however, showed that these two tyrosine residues are not required for binding of H3K4me2, as the NRDc mutant that converted two tyrosine residues to alanines (NRDc YY-AA) retained the H3K4me2-binding specificity (Fig. 2A).

To substantiate the peptide pulldown results derived from in vitro translated proteins, we also expressed various NRDc mutants in 293T cells via transient transfection and analyzed their H3K4me2-binding activity in the form of whole cell extracts. In agreement with the results in Fig. 2A, NRDc1-260 and NRDc1-621 expressed in 293T cells bound preferentially the H3K4me2 peptide. Similarly, NRDc1-171 and NRDc149-621 expressed in 293T cells failed to bind H3K4me2 peptide, confirming that the aa1-171 of NRDc is essential, but not sufficient for binding of H3K4me2. In addition, deletion of either aa48-89 or aa171-260 abolished the binding of H3K4me2 by NRDc, further supporting the importance of these two regions for binding of H3K4me2.

While endogenous NRDc in HeLa nuclear extracts and exogenous NRDc in 293T whole cell extracts bound virtually exclusively to H3K4me2 but not other H3 peptides under our assay conditions (Fig. 1A and Fig. 2C), it is noteworthy that a weak binding for unmodified H3 peptide was consistently observed for in vitro translated NRDc proteins and Flag-NRDc purified from 293T cells, indicating that NRDc may also bind unmodified H3. However, disregard the sources of NRDc proteins, we
observed that NRDc displayed a robust preference for H3K4me2 over the H3K4me1 and H3K4me3 peptides in all pulldown assays. Together these results indicate that NRDc is capable of distinguishing H3K4me2 from H3K4me1 and H3K4me3 and binds selectively H3K4me2.

NRDc associates with the NCoR/SMRT corepressor complexes. To investigate the potential nuclear function of NRDc, we wished to identify the nuclear proteins that associate with NRDc. Toward this end, we carried out immuno-affinity purification of NRDc from HeLa nuclear extracts using a NRDc-specific antibody. MS analysis of the purified proteins revealed the presence of abundant NRDc and three subunits of NCoR/SMRT complex: SMRT, NCoR and HDAC3 (Fig. 3A). NCoR and SMRT are two highly related corepressor proteins that have important and broad function in transcriptional repression. These two proteins have been shown to form large protein complexes containing core subunits HDAC3, GPS2, and two highly related proteins TBL1 and TBLR1 (33,42,43). The interaction between NRDc and NCoR complex was confirmed by subsequent reciprocal immunoprecipitation experiments using HeLa nuclear extracts and antibodies against NRDc and NCoR (Fig. 3B). This experiment indicated that only a small fraction of NRDc in the HeLa nuclear extracts was associated with NCoR, as only a small fraction of NRDc was co-precipitated with NCoR. In the contrary, a significant amount of TBL1 was coprecipitated with NCoR, in agreement with TBL1 being a core subunit of the NCoR complex.

To test if NRDc directly interacts with NCoR, we coexpressed V5-NRDc and Myc-NCoR in HeLa cells and performed co-immunoprecipitation assays using antibodies against V5 and Myc tags. The results in Fig. 3C showed that NRDc did not appear to interact directly with NCoR, as no co-immunoprecipitation was detected between V5-NRDc and Myc-NCoR. Similarly, we coexpressed V5-NRDc with Flag-HDAC3 and Flag-TBL1 and detected a reciprocal co-immunoprecipitation between V5-NRDc and Flag-HDAC3 but not Flag-TBL1 (Fig. 3D). In support, a fusion protein of maltose-binding protein (MBP) and HDAC3 but not MBP itself was able to bind NRDc in pulldown assay in vitro (Fig. 3E), whereas GST-TBL1 failed to pulldown V5-NRDc (Fig. 3F). Together these data reveal a physical association between NRDc and NCoR/SMRT complex that is likely mediated through a direct interaction between NRDc and HDAC3. Additional proteins including MAD1L1 and COBLL1 were found to be copurified with NRDc (Fig. 3A), but their association with NRDc remains to be tested.

NRDc has a role in transcriptional repression. With the findings that NRDc binds H3K4me2, localizes mainly in the nucleus and physically associates with SMRT/NCoR corepressor complexes, we sought to investigate the potential role of NRDc in transcriptional regulation. Toward this end, we first wished to identify the potential NRDc-target genes through gene expression profiling analysis of mouse embryonic fibroblast (MEF) cells derived from the wild type and NRDc null mice (mNRDc -/-) using Illumina MouseRef-8 v2.0 BeadChip. A total of 75 genes were up-regulated and 60 genes were down-regulated for more than 2 folds in mNRDc -/- MEF cells (Fig. 4A), suggesting that NRDc might regulate a small subset of genes. The gene ontology analysis revealed that the genes repressed by NRDc are relatively enriched in the skeletal system development, gland development and collagen catabolic process (Fig. 4B), although the biological relevance of this observation remains to be determined.

To examine if the observed gene expression...
changes are a result of the loss of NRDC, we made stable rescued pool of cells by expressing human Flag-NRDC in the mNRDC-/- MEFs by retroviral transduction (-/-FL) (Fig. 4C). By qPCR analysis we found that expression of human NRDC restored the transcriptional repression for all eight genes tested (Fig. 4D). Interestingly, analysis of several down-regulated genes by qPCR revealed they were not consistently rescued by reexpression of NRDC (data not shown), suggesting they may not be directly regulated by NRDC. Together the microarray and rescue studies support identified a subset of genes regulated by NRDC and support a role of NRDC in transcriptional repression.

**NRDC associates with repressed target genes and recruit NCoR complex.** To test if NRDC binds to chromatin and regulate transcription directly, we next attempted to map the NRDC binding regions for the genes that were repressed by NRDC. By designing one amplicom in every 0.5 kb within the regions 2.5 kb upstream and 0.5 kb downstream of the transcriptional start sites and performing chromatin immunoprecipitation (ChIP) analysis using Flag-NRDC rescued cells, we were able to identify two Flag-NRDC associated regions for each of the Efna5, Rgs4, Nrbp2 and Bst2 genes as indicated in Fig. 5A out of ten genes tested (data not shown, see 5B). The two Flag-NRDC positive regions identified for each of the four genes most likely represent independent NRDC-binding sites and are not due to DNA physical linkage (Supplementary Fig. S1). The identification of these NRDC associated regions allowed us to determine whether the association of NRDC would lead to recruitment of NCoR complex. Semi-quantitative ChIP analysis of mNRDC-/- MEFs and mNRDC-/- MEFs expressing Flag-NRDC revealed a Flag-NRDC-dependent association of NCoR at all Flag-NRDC binding sites except the E1 region in the Efna5 gene (Fig. 5B). Although a weak signal of HDAC3 was detected at the R1 and N1 region in the mNRDC-/- MEFs, increased HDAC3 association was also observed at these regions in the mNRDC-/- MEFs expressing Flag-NRDC. These ChIP results were further verified by quantitative PCR analysis as shown in Supplementary Fig. S2. Together these results indicate a role of NRDC in targeting NCoR complex to genes repressed by NRDC.

Consistent with a role in transcriptional repression, the binding of Flag-NRDC is in general associated with reduced levels of histone H3 acetylation and H3K4me3 (Fig. 5B). However, the binding of NRDC does not appear to consistently alter the levels of H3K4me1 and H3K4me2 in its associated regions (Fig. 5B). These changes in histone modifications were supported by quantitative ChIP data in Supplementary Fig. S2. We suggest that the reduced level of H3 acetylation is likely a result of recruitment of NCoR/HADC3 complex.

**Sequential ChIP analysis demonstrates that NRDC binds H3K4me2 in chromatin and associates with the NCoR complex.** The mapping of NRDC-binding sites in four of the target genes allowed us to test if the H3K4me2-binding activity of NRDC plays a role in NRDC chromatin association. If NRDC associates with chromatin through its ability to bind H3K4me2, we would expect NRDC to be enriched with chromatin containing H3K4me2 but not H3K4me1 and H3K4me3. We addressed this issue by sequential ChIP assay (re-ChIP). The first ChIP was carried out with anti-Flag antibody to enrich Flag-NRDC associated chromatin. The re-ChIPs were performed with antibodies against H3K4me1, H3K4me2 and H3K4me3. The results in Fig. 5C show that H3K4me2 but not H3K4me1 and H3K4me3 was significantly enriched in re-ChIP assays, thus demonstrating that NRDC was indeed
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associated with the chromatin with H3K4me2. In addition, NCoR and HDAC3 were also enriched in re-ChIP, indicating these proteins co-occupied chromatin with NRDc. The semi-quantitative ChIP results in Fig. 5C were further supported by quantitative PCR results in Supplementary Fig. S2.

DISCUSSION

The effectors for methylated histones identified so far in general bind either preferentially higher methylation state (me2 and me3) or lower methylation state (me1 and me2) (4). These effectors contain one or more royal superfamily structural domains such as Chromo, Tudor and MBT or PHD finger. Structural studies indicates that, for effectors that bind higher lysine methylation, the methylammonium group of the methyl-lysine, which carries a diffuse positive charge, interacts with the partially negative charged aromatic rings through cation-π interactions and to a lesser extent by hydrophobic contacts (16,17,40). For effectors that bind lower state of methylation such L3MBTL1, the aromatic cages are smaller and bury within a deep protein cleft. The limited dimensions of the binding pockets would restrict the access of the larger trimethyl-lysine residues (41,44). In this study we identified NRDc as a novel H3K4me2 binding effector. Several lines of evidence support a specific binding of H3K4me2 by NRDc. First, NRDc in HeLa nuclear extracts exhibited an exclusive binding activity toward H3K4me2 (Fig. 1). Second, in vitro synthesized and bacteria expressed NRDc also exhibited a strong binding preference of H3K4me2, indicating that NRDc has an intrinsic binding activity for H3K4me2. Third, ChIP and re-ChIP experiments demonstrate that NRDc associates with chromatin with H3K4me2 but not H3K4me1 and H3K4me3 (Fig. 5). As NRDc binds only the H3K4me2 and differs from CHD1, ING2, PHF2 and PHF8 that bind both H3K4me2/3 and PELP1 that recognizes both H3K4me2 and H3K9me2 (22), to our knowledge, NRDc is the first identified mammalian protein that exhibits a unique H3K4me2-binding specificity.

Currently the mechanism by which NRDc binds H3K4me2 is not known. Within the region of NRDc aa1-260 that primarily determines the H3K4me2-binding specificity, it does not appear to have a royal superfamily structure domain. Consistent with this idea, mutations of two conserved Tyr residues within the region required for binding of H3K4me2 has no effect on binding, suggesting that the binding of H3K4me2 by NRDc may not require an aromatic cage commonly used for binding of methyl-lysine by royal family domains (4). Although NRDc contains a glutamic rich region that has been implicated in binding of H3K4me2 and H3K9me2 by PELP1, deletion of this region also has no effect on binding of H3K4me2 by NRDc. Thus, exactly how NRDc is able to bind H3K4me2 awaits for answers from structural study of NRDc. It is worth noting that, although NRDc is a member of M16 family metalloendopeptidases that cleaves substrates at paired basic residues, we could not detect any cleavage activity toward core histones and various synthetic histone peptides (data not shown).

NRDc has been reported to function as a specific receptor for HB-EGF and facilitate HB-EGF ectodomain shedding (25,26). Recent studies indicate that NRDc is required for postnatal survival and growth, regulates axonal maturation and myelination, and is required for antigen processing in cytotoxic T lymphocytes (CTL) (27,28). Prior to our study, however, NRDc is not known for a direct transcriptional function. In support of a potential nuclear function, ectopic expressed and endogenous NRDc proteins in HeLa and NIH3T3 locate primarily in the nucleus (Fig. 1G and 1H). Biochemical purification of NRDc revealed a
physical association of NRDC with the SMRT/NCoR corepressor complexes. This interaction is supported by the observed Flag-NRDC-dependent recruitment of NCoR and HDAC3 to the endogenous NRDC target genes in ChIP and re-ChIP assays (Fig. 5). However, NRDC is not a stoichiometric component of the SMRT/NCoR complexes, as only a small fraction of NCoR complex appears to contain NRDC (Fig. 3B). Thus, it is likely that NRDC represses transcription through recruitment of SMRT/NCoR complexes.

Taking together, our study identifies NRDC as a novel H3K4me2 binding protein and supports a working model in Fig. 5D that NRDC has a role in transcriptional regulation. NRDC can bind to its target genes such as Efna5, Rgs4 and Nrbp2 through its H3K4me2 binding activity. The subsequent recruitment of NCoR complex by NRDC is likely to result in histone deacetylation and transcriptional repression. In this regard, our study also provides a mechanism for H3K4me2-mediated transcriptional repression.

REFERENCES

NRDc as a novel H3K4me2 binding protein


NRDc as a novel H3K4me2 binding protein

development 14(9), 1048-1057


FOOTNOTES
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FIGURE LEGENDS

FIGURE 1. NRDc binds specifically the H3K4me2 peptide. A. NRDc in HeLa nuclear extracts binds only to the H3K4me2 peptide. Pull-down assays were performed with HeLa nuclear extracts and H3 tail peptides as indicated. The bound proteins were analyzed by Western blotting using antibodies as indicated. B. In vitro synthesized NRDc also binds preferentially H3K4me2. 35S-methionine labeled NRDc was synthesized in vitro using transcription-translation coupled reaction. The bound NRDc proteins were resolved by SDS-PAGE and revealed by autoradiography. C. Flag-NRDc was expressed in 293T cells and purified by anti-Flag M2 Affinity beads. The eluted Flag-NRDc was examined by SDS-PAGE and revealed by Coomassie blue stain. The resulting protein was subjected to peptide pulldown analysis and revealed by Western blot analysis using anti-Flag antibody. D. His-tagged NRDc 1-621 was expressed in bacteria, purified by Ni-NTA agarose beads and subjected to pulldown assay. The binding of purified protein was revealed by Coomassie blue staining. E. The binding of 2 μg of purified 6xHis-NRDc 1-621 to immobilized H3K4me2 peptide was performed in the presence of increasing amount (0, 5, 10, and 15 μg respectively) of free H3, H3K4me1, H3K4me2 and H3K4me3 peptides. All these peptides are aa1-21 of H3 and without C-terminal biotin. F. The results of two independent competition experiments in E were quantified and presented. G. Ectopic expressed V5-NRDc resides mainly in the nucleus. HeLa and NIH3T3 cells were transfected with V5-tagged NRDc construct and immunostained with anti-V5 antibody. Nuclei were stained with Hoechst 33342. H. Endogenous NRDc in NIH3T3 cells were immunostained with a NRDc monoclonal antibody that is capable of detecting ectopic expressed V5-NRDc. The nuclei were stained by Hoechst 33342.

FIGURE 2. Characterization of NRDc H3K4me2 binding activity. A. Mapping the NRDc region required for binding of H3K4me2 using in vitro translated 35S-methionine labeled proteins and peptide pulldown. Left panel, schematic illustration of NRDc deletion and point mutation mutants. NRDcΔAD, NRDc with deletion of glutamic acid rich region of aa93-148; NRDcΔ48-89, NRDc with deletion of aa48-89; NRDc E>A, NRDc E186A enzymatic activity deficient mutant; NRDc YY-AA, NRDc with mutation of Y64/Y66 to alanines. Right panel, the results of pulldown assays revealed by autoradiography. B. The region of amino acids 48-89 of NRDc is conserved in evolution.
NRDc as a novel H3K4me2 binding protein

Alignment is made for sequences corresponding to human NRDc amino acids 48-89 from different species in the order from top to bottom as listed. The yellow color marks the amino acids identical to human NRDc and blue marks the amino acids that are similar. The two conserved tyrosines are marked by “*” in red. C. Pulldown assays with 293T expressed NRDc and mutants. The binding of NRDc and mutants was detected by Western blot analysis using either anti-V5 or anti-HA antibody as indicated.

FIGURE 3. NRDc interacts with NCoR/SMRT complex. A. Summary of mass spectrometry results of immuno-affinity purified NRDc complex(es). The number of peptide hits identified for each protein was shown. B. Reciprocal immunoprecipitation confirms interaction between NRDc and NCoR complex. HeLa nuclear extracts were immunoprecipitated using anti-NRDc and NCoR antibodies, and analyzed by immunoblot using anti-NCoR, NRDc and TBL1 antibodies. C. NRDc did not appear to interact directly with NCoR. The 293T cells were transfected with plasmids encoding V5-NRDc and Myc-NCoR and the interaction between NRDc and NCoR was examined by reciprocal immunoprecipitation assay. D. NRDc co-immunoprecipitated with HDAC3 but not TBL1. The 293T cells were transfected with plasmids encoding V5-NRDc and Flag-HDAC3 or V5-NRDc and Flag-TBL1 and the interaction was examined by reciprocal immunoprecipitation assay. E. NRDc bound to immobilized MBP-HDAC3. MBP and MBP-HDAC3 expressed in bacteria were purified and immobilized to resin and then incubated with V5-NRDc expressed in 293T cells. The binding of V5-NRDc was detected by Western blot analysis. F. NRDc did not bind to GST-TBL1 in pulldown assay. The experiment was performed essentially as described in E except GST and GST-TBL1 were used.

FIGURE 4. NRDc has a transcriptional repression function. A. Heatmap data of the microarray analysis for mNRDc +/+ and -/- MEF cells. The genes with more than 2 fold changes in expression are listed. The fold difference was calculated by log_{10}. The numbers on the color key represent the gene expression changes. Red represents up-regulated and green represents down-regulated genes in mNRDc-/- MEF cells. B. The gene ontology analysis showing the relative enrichment of the NRDc-repressed genes in different biological processes. C. Establishment of mNRDc-/- MEF cells stably expressing Flag-tagged full-length human NRDc. Actin serves as the loading control. D. Q-PCR analysis of the mRNA levels of eight NRDc repressed genes in the mNRDc +/+ , -/-, and +/- MEF cells expressing Flag-NRDc. Note expression of Flag-NRDc restored the transcriptional repression of these genes.

FIGURE 5. Transcriptional repression by NRDc correlates with its association with H3K4me2-enriched chromatin and recruitment of NCoR complex. A. Schematic representation of the target genes in which the binding sites for Flag-NRDc were identified by ChIP. Red box marks the first exon and black arrow indicates the transcription start site. The regions positive for binding of Flag-NRDc are E1 and E2 for Efna5, R1 and R2 for Rgs4, N1 and N2 for Nrbp2 and B1 and B2 for Bst2 genes. B. ChIP assays reveal the binding of Flag-NRDc correlates with the recruitment of NCoR complex and reduced levels of H3 acetylation and H3K4me3. C. Re-ChIP analysis demonstrates that the NRDc-associated chromatin is enriched of H3K4me2 and NCoR complex. re-ChIP assays were performed with first ChIP against Flag-NRDc and second ChIP with antibodies.
against NCoR, HDAC3, H3K4me1, H3K4me2, and H3K4me3 as indicated. For simplicity, only the data for E2 of Efna5, R1 and R2 of Rgs4 and N1 and N2 of Nrbp2 were shown. D. The working model for how NRDc mediates transcriptional repression. NRDc can bind to at least some of its target genes via its H3K4me2 binding activity. The chromatin associated NRDc recruits NCoR complex, which presumably helps to repress transcription through histone deacetylation via associated HDAC3.
**Li et al., Figure 1**

(A) Western blot (WB) analysis showing the expression levels of various proteins, including α-NRDc, α-PHF2, α-UHRF1, and α-MTA1, in different samples.

(B) Autoradiography of a sample labeled with 35S-NRDc. The sample is analyzed using 15% input, Mock, H3, H3 K4me1, H3 K4me2, and H3 K4me3.

(C) Coomassie Blue Staining of Flag-NRDc samples with 10% input, H3, H3 K4me1, H3 K4me2, and H3 K4me3.

(D) Coomassie Blue Staining of His-NRDc (1-621) samples with 20% input, Mock, H3, H3 K4me1, H3 K4me2, and H3 K4me3.

(E) Comassie Blue Staining of H3K4me2 + His-NRDc (1-621) with competitor peptides including H3, H3 K4me1, H3 K4me2, and H3 K4me3.

(F) Graph showing the relative binding activity of H3, H3 K4me1, H3 K4me2, and H3 K4me3 with increasing concentrations (0-20 μg) of the competitor peptide.

(G) Immunofluorescence images of V5-NRDc and α-V5 in HeLa and NIH3T3 cells.

(H) Immunofluorescence images of α-NRDc and endogenous V5-NRDc in NIH3T3 cells.
### Protein Score Table

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### Figures

**Figure 3A**
- Protein Score Table

**Figure 3B**
- Immunoblot analysis of NCoR, NRDc, and TBL1

**Figure 3C**
- Immunoblot analysis of Myc-NCoR

**Figure 3D**
- Coomassie Staining of V5-NRDc Flag-HDAC3 and V5-NRDc Flag-TBL1

**Figure 3E**
- Immunoblot analysis of MBP and MBP-HDAC3

**Figure 3F**
- Immunoblot analysis of GST and GST-TBL1

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Li et al., Figure 3
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Identification and Characteriation of Nardilysin as a Novel Dimethyl H3K4 Binding Protein Involved in Transcriptional Regulation
Jing Li, Mingyue Chu, Shanshan Wang, Doug Chan, Shankang Qi, Meng Wu, Zhongliang Zhou, Jiwen Li, Eiichiro Nishi, Jun Qin and Jiemin Wong

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