Structural Insights into Histone Demethylase NO66 in Interaction with Osteoblast-specific Transcription Factor Osterix and Gene Repression*

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Background: A novel JmjC domain-containing protein NO66 has been identified in the regulatory network of Osx.

Results: We show that the crystal structure of NO66 folds into a functional tetramer form.

Conclusion: Our data suggest that the interaction of NO66 with Osx depends on its tetrameric assembly.

Significance: We propose that homo-oligomerization of JmjC domain containing proteins may have significant physiological roles.

Osterix (Osx) is an osteoblast-specific transcriptional factor and is required for osteoblast differentiation and bone formation. A JmjC domain-containing protein NO66 was previously found to participate in regulation of Osx transcriptional activity and plays an important role in osteoblast differentiation through interaction with Osx. Here, we report the crystal structure of NO66 forming in a functional tetramer. A hinge domain links the N-terminal JmjC domain and C-terminal winged helix-turn-helix domain of NO66, and both domains are essential for tetrameric assembly. The oligomerization interface of NO66 interacts with a conserved fragment of Osx. We show that the hinge domain-dependent oligomerization of NO66 is essential for inhibition of Osx-dependent gene activation. Our findings suggest that homo-oligomerization of JmjC domain containing proteins may play a physiological role through interactions with other regulatory factors during gene expression.

Bone formation occurs through two distinct processes, endochondral ossification and intramembranous ossification. A cartilage intermediate is involved in the process of endochondral ossification, whereas in intramembranous ossification, bones form directly from differentiation of condensed mesenchyme (1). The differentiation of mesenchyme precursors into osteoblasts is regulated by osteoblast-specific transcription factors Runx2 and osterix (Osx), which play an essential role in osteoblast differentiation and bone formation (2, 3). Other factors such as histone deacetylases, Twist 1 and 2, ATF4, Nfatc, Msx, and Dlx, are also involved in the regulation of osteoblast differentiation (4–7). In Osx-null embryos, no expression of osteoblast-specific marker genes takes place, resulting in the arrest of differentiation from precursor cells to osteoblasts, and hence no bone is formed (8). Osx possibly acts with other factors to control the transcription of osteoblast-specific marker genes in vivo (9, 10).

NO66, a JmjC domain-containing protein, was previously identified as an Osx-interacting protein through proteomics and mass spectrometry approaches (11). NO66 is highly conserved in eukaryotes and was originally reported as a dual-locus protein present in both the nucleoplasm and nucleolus (12). Further analysis demonstrated that NO66 has histone demethylation activity toward methylated H3K4 and H3K36. NO66 interacts with Osx, mediates inhibition of Osx-target promoters, and henceforth, negatively regulates osteoblast differentiation (11). NO66 was also shown to associate with the PRC2 complex and remove the H3K36me3 mark that results in the silencing of mouse embryonic stem cell genes (13). Furthermore, a recent report indicated that NO66 catalyzes β-carbon histidine hydroxylation of the 60 S ribosomal protein Rpl8 (14). Regardless of how NO66 catalyzes the reactions, the detailed mechanism for interactions between NO66 and its partners remains unclear.

The abbreviations used are: Osx, osterix; NO66, nucleolar protein (66 kDa); JmjC, Jumonji C; ATF4, activating transcription factor 4; Nfatc, nuclear factor of activated T-cells; PRC2, polycomb repressive complex 2; c-hNO66, a fragment containing residues 183–641 of human NO66; Bsp, bone sialoprotein.

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The atomic coordinates and structure factors (code 4E4H) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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4 The abbreviations used are: Osx, osterix; NO66, nucleolar protein (66 kDa); JmjC, Jumonji C; ATF4, activating transcription factor 4; Nfatc, nuclear factor of activated T-cells; PRC2, polycomb repressive complex 2; c-hNO66, a fragment containing residues 183–641 of human NO66; Bsp, bone sialoprotein.
Here, we report the crystal structure of NO66 (residue 183–641, referred to as c-hNO66) at 2.28 Å resolution. We also mapped the NO66-binding sequence of Osx to a short fragment of conserved sequence and showed that the interaction depends on the hinge domain-dependent oligomerization interface of NO66. Further in vitro and in vivo analyses demonstrate that hinge domain-dependent oligomerization is essential for NO66 to interact with Osx and to inhibit Osx-dependent gene activation.

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Purification, and Crystallization**—DNA fragments encoding residues 183–641 (c-hNO66) of wild-type human NO66 or its mutants were amplified by PCR and ligated to the pET-28a (+) vector (Novagen) with an N-terminal fusion to SUMO protein. The c-hNO66 and the mutant variants were expressed and purified as described previously. Crystallization of c-hNO66 has also been described previously (15).

**Data Collection, Structure Determination, and Refinement**—The crystals of native and selenomethionine-labeled c-hNO66 were soaked in a cryoprotectant solution containing well solution and 20% glycerol for several seconds. Then, all of the crystals were flash-cooled in liquid nitrogen and used for x-ray diffraction data collection at 100 K at the synchrotron-radiation beamline BL17U1 at Shanghai Synchrotron Radiation Facility. Diffraction data were indexed, integrated, and scaled using the HKL-2000 program (16). The structure of c-hNO66 was solved by the single-wavelength anomalous dispersion method. The crystals of native and selenomethionine-labeled c-hNO66 were finally solved by the molecular replacement method. The data set collected from native c-hNO66 crys-

| TABLE 1
| Data collection and refinement statistics |
|-----------------------------------------|
| **Se-NO66** || **Native NO66** |
| Space group | P21,2,2 | P32 |
| a, b, c (Å) | 150.96, 82.52, 107.12 | 89.35, 89.35, 304.86 |
| α, β, γ | 90.0, 90.0, and 90° | 90.0, 90.0, and 120° |
| Resolution range (Å) | 50.00–2.75 (2.80–2.75) | 50.00–2.28 (2.33–2.28) |
| Wavelength (Å) | 0.9794 | 0.9999 |
| Unique reflections | 35,764 | 123,682 |
| I/σ(I) | 20.7 (2.1) | 11.0 (2.0) |
| Completeness (%) | 96.1 (91.3) | 98.9 (99.9) |
| Redundancy | 6.7 (4.9) | 3.3 (2.7) |
| Rmerge (%) | 9.9 (49.3) | 10.8 (55.1) |

* The values in parentheses refer to statistics in the highest shell.
* Rmerge = \( \sum_{hkl} \left| F_{\text{obs}}(hkl) - F_{\text{calc}}(hkl) \right| / \sum_{hkl} F_{\text{calc}}(hkl) \) where \( F_{\text{obs}}(hkl) \) is the intensity of the \( hkl \) measurement, and \( F_{\text{calc}}(hkl) \) is the mean intensity for that reflection.
* Rwork = \( \sum_{hkl} \left| F_{\text{obs}}(hkl) - F_{\text{calc}}(hkl) \right| / \sum_{hkl} F_{\text{obs}}(hkl) \) where \( F_{\text{obs}}(hkl) \) and \( F_{\text{calc}}(hkl) \) are the observed and calculated structure factor amplitudes, respectively.
* Rfree was calculated with 5.0% of the reflections in the test set.
* r.m.s.d., root mean square deviation; PDB, Protein Data Bank.

Hilosload 16/60 Superdex 200 column (GE Healthcare) pre-equilibrated with Buffer C and eluted using the same buffer. The molecular weight of each protein was estimated according to its elution volume.

**Construction of Expression Plasmids**—Wild type NO66 (183–641) and M1, M2, and M3A mutants were cloned in the mammalian expression vector pcDNA3.1A-myc-His (Invitrogen) by PCR followed by sequencing.

**Reporter Assays, Transfections, and Gene Expression**—For reporter assays, HEK293T cells were transfected using FuGENE 6 with 200 ng each of reporter constructs (5×-Gal-luciferase and Bsp-luciferase) and expression vectors pcDNA-NO66 (full-length), pcDNA-NO66 (183–641), pcDNA-NO66 (M1 mutant), pcDNA-NO66 (M2 mutant), pcDNA-NO66 (M3A mutant), and pTriEX-FLAG-HA-Osx. We also co-transfected the cells with 20 ng of plasmid pSV40/LacZ as control for transfection efficiency and to normalize the reporter activity.

Pre-osteoblast MC3T3 cells were plated in six-well plates and transfected with expression plasmids using FuGENE 6 for 48 h. Total RNA was isolated using TRIzol (Invitrogen) and treated with Turbo DNase (Ambion) followed by reverse transcription of RNA samples with random hexamer primers (Archive cDNA synthesis kit, Applied Biosystems). The cDNA equivalent to 25 ng of RNA was used in triplicate for real-time PCR analysis using a Taqman primer probe. Levels of RNA were normalized to those of hypoxanthine-guanine phosphoribosyltransferase. Gene-specific primer probes were obtained from Applied Biosystems.
RESULTS

Overall Structure of NO66—We determined the crystal structure of c-hNO66 by using a fragment lacking the first 182 residues. c-hNO66 folds into two distinct domains packing together through extensive interactions to form a globular core structure through which a hinge-like domain protrudes (Fig. 1A). The JmjC domain is located at the N-terminal half, which contains a β-jellyroll structure formed by seven β-strands (Figs. 1A and 2). The four β-strands interact with one layer of the β-jellyroll to extend the four-stranded anti-parallel β-sheets to form an eight-stranded anti-parallel β-sheet, with the seven helices located on the same side of the jellyroll (Fig. 1A). Next to the JmjC domain is the hinge domain composed of three long α-helices, linking the N- and C-terminal halves of the globular core structure. The C-terminal domain adopts a structure that loosely resembles the winged helix-turn-helix motif found in the structure of MccB (MATRICS consensus cognitive battery) protein (Fig. 1B). The hinge domain and C-terminal winged helix-turn-helix domain are connected by a linker comprising one α-helix and a short β-hairpin (Fig. 1A). The JmjC domain of NO66 adopts a non-canonical β-jellyroll fold composed of seven β-strands, in which the second β-strand of the canonical β-jellyroll folds into an unstructured loop (Fig. 1C).

Typically, the active site of a JmjC domain-containing histone demethylase is located in the center of the β-jellyroll fold and uses Fe²⁺ and α-ketoglutarate (α-KG) as co-factors to catalyze the demethylation reaction. In the active site, a highly conserved H-X-D/E-Xn-H motif provides three chemical groups to chelate the Fe²⁺ ion (23). Similar to other members in this family, the catalytic core structure of c-hNO66 has a double-stranded jellyroll fold. In the active site, three residues (His-334, Asp-342, and His-405) form a direct interaction to coordinate the Fe²⁺ ion (Fig. 1C). Although we tried to co-crystallize c-hNO66 with Fe²⁺ and α-ketoglutarate as well as soak the crystal of c-hNO66 in complex with Fe²⁺ in well solution containing α-ketoglutarate, no interpretable electron density corresponding to α-ketoglutarate was observed in the resultant c-hNO66 structure.

Tetrameric Assembly of NO66—Our initial experiments during purification of c-hNO66 using a size exclusion column indicated that c-hNO66 forms a tetramer in solution because of its elution into a single peak with an apparent molecular mass of 175 kDa (see Fig. 6B). The theoretical molecular mass of c-hNO66 is 54 kDa. Therefore, the oligomerization state of c-hNO66 in solution is most likely tetrameric. The crystal structure of c-hNO66 consistently reveals a tetramer formed by four molecules in one asymmetric unit (Fig. 3A). In the structure of the c-hNO66 tetramer, two adjacent subunits associate with each other through interaction of hinge domains to form a tight dimer, which further packs into a tetramer through the protruding β-hairpin motif (Fig. 3, B and C).

The hinge domains from two neighboring molecules contact with each other to form an extensive interaction interface designated as interface I. Each subunit has a large buried surface area of ~3260 Å², suggesting a strong interaction that might be present in solution (Fig. 3B). More specifically, helix α8 from one subunit runs antiparallel to helix α8’ from another (Fig. 4, A and C). Helices α9 and α9’ pack in the same way (Fig. 4B) and lay perpendicularly on the top of the helices α8 and α8’ (Fig. 4A). The interactions between these two pairs of α-helices consist primarily of hydrophobic interactions, including Val-485, Ala-486, Met-444, Pro-437, Leu-436, Gly-429, Thr-428, Phe-431, Leu-432, Ile-435, Leu-436, Ala-439, Ala-443, Leu-488, Phe-491, and Ala-492 from both subunits (Fig. 4, B–D). In addition, the other part of interface I is formed by a loop connecting α8–α9’ from one subunit and a groove from the other. The groove is formed by α2, α3, α7, α8, α10, β15, and the loops at one end of the JmjC domain. The loop is anchored in the grooves through extensive interactions involving salt-bridge interactions, hydrophobic interactions, and hydrogen bonds (Fig. 4E).

Interface II is mainly formed by a β-hairpin motif of one subunit protruding onto the α4’ and α5’ helices from the other...
subunit (Figs. 3C and 4F). Interface II has a buried surface area of only 1140 Å², which is three times smaller than that of interface I. In addition, residues Pro-535 and Trp-530 from the β-hairpin motif form hydrophobic interactions with Thr-244', Trp-287', and Tyr-290' on helices α4' and α5'. Hydrogen bonds are formed between the backbones of Ile-528 on the loop of the β-hairpin and Arg-252 on helix H4. Lys-629 and Glu-572 form two more hydrogen bonds with the backbones of Pro-278 and Arg-280 across the interface (Fig. 4F). Collectively, both hydrophobic interactions and hydrogen bonds are involved in the formation of interface II.

NO66 Binds to a Short N-terminal Fragment of Osx—The transcription activation domain of Osx (1–270) was previously shown to interact with full-length NO66 as well as c-hNO66 (11). To further define the region in Osx that binds to NO66, we performed an in vitro pulldown assay with Osx fragments fused to GST. Consistent with a previous report, we confirmed that the N-terminal region containing 175 amino acids of Osx is sufficient to bind c-hNO66. The C-terminal zinc finger DNA binding domain of Osx does not interact with c-hNO66 (Fig. 5A). We next used three N-terminal fragments of Osx overlapping one another to test their ability to interact with c-hNO66 (Fig. 5B). The result indicates that the NO66 interacting segment within Osx is located in the region spanning amino acid residues 118 to 163. Fig. 5C shows that a minimal fragment containing 16 amino acid residues (125 to 140) in the N-terminal region of Osx is sufficient to bind c-hNO66. Sequence alignment of Osx from various vertebrate species indicates that this segment is extremely conserved (Fig. 5D).
The Osx-binding Site of NO66 Is Located on Interface I—To gain further insights into the physiological significance of tetrameric NO66, we generated two mutants lacking each interaction interface, both which destabilize tetrameric assembly (Fig. 6A). In mutant M1, we replaced the hinge domain with six glycine residues, and in mutant M2, the /H9252/H9255-hairpin motif in the linker region was replaced by three glycine residues. Mutant M1 elutes at 43 kDa, which indicates it assembles as a monomer, and M2 elutes at 91 kDa, a dimer, when analyzed using size exclusion chromatography (Fig. 6B). Thus, no tetramer was observed in either mutant protein in solution. Deletion of the hinge interface (interface I) disrupts the entire tetramer assembly of NO66. Next, we examined the ability of mutant proteins M1 and M2 to interact with Osx using GST pulldown assays (Fig. 6C). The results indicate that both c-hNO66 and M2 interact with Osx. Notably, interaction of M2 with Osx was relatively lesser than that of c-hNO66. In contrast, no interaction between M1 and Osx was detected, suggesting that interface I in the NO66 tetramer is required for in vitro binding of NO66 to Osx. Because a short fragment of Osx is sufficient to bind NO66, the Osx-binding site of NO66 may only involve a shallow pocket. To further identify the Osx-binding site of NO66, we analyzed interface I and found two shallow grooves related by the pseudo-2-fold symmetry (Fig. 6D). Both of the grooves are formed by the helices /H9251/H9252 and /H9251/H9252 and loops connecting /H9251/H9252 from one subunit and the helices /H9251/H9252 and /H9251/ from the neighboring subunit (Fig. 6E). Furthermore, sequence alignment showed that the amino acid residues in this area are highly conserved, suggest-
ing that these regions might be involved in interaction with Osx (Fig. 2). To test this hypothesis, we generated seven point mutants of c-hNO66 (R221A, R225A, Y423A, F450A, R452A, P455A, and F477A) and tested their ability to interact with Osx(118–163), which was shown to interact with c-hNO66. Size exclusion chromatography of these mutants indicated their molecular weights corresponding to tetrameric assembly (data not shown). Our data indicate that although these mutants with a single point mutation have reduced interactions with Osx (Fig. 6F, upper panel), mutant M3A, with three point mutations (R221A, R225A, and Y423A) in c-hNO66, has almost lost its ability to interact with Osx (Fig. 6F, bottom panel). These results indicate that the hinge domain-mediated oligomerization of NO66 is essential for interaction with Osx and that interface I might serve as the binding site for Osx. Furthermore, the dynamic light scattering assay indicates that the molecular diameter of c-hNO66 seems quite similar either in the presence or absence of Osx(125–140) peptide, suggesting that interaction of Osx with c-hNO66 does not disrupt the tetramer assembly of NO66 (data not shown).

Oligomerization-dependent NO66-Osx Interaction Represses the Transcription of Osx Target Gene—To show that interface I of NO66 mediates interactions with Osx and that these interactions are required for repression of Osx-dependent activity, we tested their association in cells by co-immunoprecipitation. Consistent with the results of our in vitro pulldown assay, the in vivo association of Osx was observed only with c-hNO66, which retains an intact interface I, but not with interface I-lacking mutant M1 (Fig. 7A). These results indicate that interface I is necessary for the in vivo interaction of NO66 with Osx. Next, we examined the physiological roles of interface I of NO66 tetramers in the regulation of Osx-dependent gene expression. We used two reporter assays with the 5×GAL4-Luc reporter and osteoblast-specific Bsp-Luc, previously shown to be activated by Osx (11). Our results show that expression of NO66 or c-hNO66 is able to repress the Osx-mediated activity of both reporters (Fig. 7, B and C). In contrast, the mutant M1, which does not interact with Osx, is unable to repress the reporter activity (Fig. 7, B and C, left panel). Although M2 mutant is able to bind Osx in vitro to a lesser extent compared with wild type NO66, in contrast to our expectation and for unknown reasons, this mutant does not inhibit Osx-mediated reporter activity (Fig. 7B, right panel). In addition, the triple mutant M3A, which poorly binds to Osx in GST pulldown assays, is also unable to inhibit Osx-dependent activity (Fig. 7B, right panel).

Next, we performed quantitative RT-PCR for the levels of endogenous osteoblast-specific genes, including Bsp, Oc, and Akp, in preosteoblast MC3T3 cells transfected with c-hNO66 and M1 mutant. The repression ability of the M1 mutant is much weaker than c-hNO66 (Fig. 7D). Taken together, these results strongly suggest that interaction of the hinge domains of NO66 is required to build the binding site of Osx and to repress Osx-dependent gene expression.
DISCUSSION

Here, we report the crystal structure of NO66, a JmjC domain-containing protein, which shows that the fragment lacking the N-terminal 182 amino acid residues bears several unique structural features. The JmjC domain of NO66 adopt a non-canonical β-jellyroll fold composed of seven β-strands in which the second β-strand of the canonical β-jellyroll folds as an unstructured loop. However, it is still not clear whether this structural organization of the JmjC domain makes any contribution to the enzymatic activity or substrate specificity of NO66. However, it should be noted that the truncated NO66 (c-hNO66) also exhibits demethylase activity for H3K4me3 and H3K36me3 and is an active demethylase (data not shown).

It is unexpected to find that four c-hNO66 subunits are tethered together to form a tetramer mediated by the protruding hinge domain and the β-hairpin motif in the linker region. The previously reported structures indicate that JmjC domain-containing histone demethylases fold into compact globular shapes without any segment extended outside (23–25). In addition, the JmjC domain-containing hydroxylases, such as factor inhibiting hypoxia-inducible factor and TYW5 (tRNA yW-synthesizing enzyme 5), can form stable dimers through the interaction of C-terminal helices (26, 27). However, none of these enzymes form tetramers in solution or in crystals.

NO66 interacts with Osx and inhibits Osx-dependent promoter activity (11). We show here that NO66 is capable of binding Osx through the binding site on interface I (Fig. 6, D–F). Disruption of the tetrameric assembly of NO66 impedes its interaction with Osx both in vitro and in vivo (Figs. 6C and 7A), leading to derepression of Osx-dependent gene activation (Fig. 7, B–D). Overall, our data imply that the inhibitory function of NO66 on Osx-mediated gene activation depends on its tetrameric assembly as well as its interaction with Osx. Sequence alignment of the interacting domain of NO66 and Osx shows that the amino acid residues in NO66 participating in the formation of the Osx-binding groove and the 16 amino acid fragment in Osx are highly conserved in various species of vertebrates (Figs. 2 and 5D), suggesting a conserved role of NO66 in regulation of osteoblast differentiation and bone formation through interaction with Osx in vertebrates. Because NO66 was reported to be a histone demethylase or a histidyl hydroxylase (11, 13, 14), it is possible that interaction of NO66 with Osx or other proteins might cause hydroxylation or demethylation of these proteins and regulate their activities. Recently, NFATc1 was shown to be hydroxylated by Jmjd5, another JmjC domain-containing protein, and degraded through a ubiquitin-mediated pathway (29). Thus, interactions of NO66 with Osx, which is a master regulator of bone formation, might be physiologically significant in the regulation of Osx activity and osteoblast differentiation. Furthermore, our data indeed suggest that homo-oligomerization of JmjC domain-containing proteins plays important roles in the interaction with other regulatory factors and may have significant physiological roles.

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