Requirement of Nanog dimerization for stem cell self-renewal and pluripotency

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Pluripotency of embryonic stem (ES) cells is maintained by transcription factors that form a highly interconnected protein interaction network surrounding the homeobox protein Nanog. Enforced expression of Nanog in mouse ES (mES) cells promotes self-renewal and alleviates their requirement for leukemia inhibitory factor (LIF). Understanding molecular mechanisms by which Nanog functions should illuminate fundamental properties of stem cells and the process of cellular reprogramming. Previously, we showed that Nanog forms multiple protein complexes in mES cells. Here, we demonstrate that Nanog dimerizes through its C-terminal domain rather than the homeodomain. Dimerization is required for interaction with other pluripotency network proteins. We also show that enforced expression of the Nanog dimer, but not the monomer, functionally replaces wild-type Nanog to sustain LIF-independent self-renewal of ES cells. Our results demonstrate that Nanog–Nanog homodimerization is a critical aspect of its function promoting stem cell pluripotency.

embryonic stem cells | homeoprotein

Embryonic stem (ES) cells, derived from the inner cell mass of the early mouse embryo (1, 2), are distinguished by unlimited self-renewal potential and the capacity for multilineage differentiation. An understanding of the molecular underpinnings of these properties requires elucidation of regulatory networks operative in ES cells. Initial efforts in dissecting transcriptional (3–5) and protein interaction (6) networks of mouse and/or human ES cells form a foundation for further mechanistic studies.

Several transcription factors, notably the homeobox proteins Oct4 (7) and Nanog (8, 9), as well as the HMG box containing Sox2 protein (10), play fundamental roles in early development and stem cell pluripotency. These key factors act in combination to sustain pluripotency by activating ES cell critical factors (including themselves) and repressing differentiation-promoting genes. ES cells are sensitive to the dosage of Nanog (11) and Oct4 (12). Enforced expression of Nanog relieves ES cells from their leukemia inhibitory factor (LIF) requirement (9) and promotes transfer of pluripotency after cell fusion (13). In contrast, overexpression of Oct4 drives primitive endoderm differentiation (12), possibly because of direct repression of the Nanog promoter by excessive Oct4 (14). In addition, Sox2 stabilizes ES cells in a pluripotent state by maintaining the requisite level of Oct4 expression (15). Dosage sensitivity suggests that the ES cell state reflects a balance of multiple transcriptional inputs that are likely exerted through association and dissociation of multiprotein complexes. In specifying lineages, Oct4 and Cdx2 counteract each other's functions to shift the balance between trophectoderm and ICM fates (16), and Nanog and Gata6 antagonize each other to define epiblast and primitive endoderm lineages (17). The ES cell state, therefore, is likely to be maintained by the continuous and direct interplay of multiple nuclear factors, acting in cooperative and antagonistic modes.

The specific actions of transcriptional regulatory proteins are mediated through their selective association with other protein factors. Previously we showed that Nanog forms multiple protein complexes with apparent molecular sizes of ≈150 kDa to several

mega-Daltons (6). By protein microsequencing we demonstrated that Nanog associates with numerous other critical factors that, in aggregate, form a tight protein interaction network apparently dedicated to pluripotency (6). Here, we pursue how Nanog functions in this context.

As a divergent homeobox protein, Nanog is most closely related to mouse NK2 family members Nkx2.3 (8) and NKx2.5 (9). NKx2.5 has been studied in detail for its direct DNA contacts (18) and homodimerization via the homeodomain (HD) (19). HD proteins often form homodimeric and heterodimeric complexes through their HDs in specifying their transcriptional actions. In some cases, homodimerization may result in cooperative DNA-binding activity (20), whereas in other instances it may result in the inhibition of DNA-binding and/or transcriptional activity (21). Outside of its HD, Nanog shares little homology with NK2 proteins. Prior work suggests that the N-terminal domain (ND) and C-terminal domain (CD) of mouse Nanog possess transactivator function in conventional reporter assays (22), whereas the CD of human Nanog may be functionally dominant in transactivation (23). The CD is distinctive for a prominent tryptophan-rich (WR) subdomain that is highly conserved between mouse and human and acts as a strong transactivator in reporter assays (24).

To study how Nanog functions in the pluripotency network, and how it associates with other network proteins to regulate target gene expression, we have explored the structure–function relationship of the Nanog protein *in vivo* in mouse ES (mES) cells. We show that the Nanog polypeptide assembles in a homodimer that is mediated by the CD rather than the HD, and Nanog–Nanog homodimerization is necessary for its interaction with other critical factors in the pluripotency network. Finally, we provide functional evidence supporting a requirement of Nanog–Nanog dimerization in stem cell self-renewal and pluripotency.

Results

Nanog Forms Homodimers in Regulating Stem Cell Activity. To study how Nanog exerts transcriptional regulation on target gene expression, its HD–DNA contact was modeled after mouse NKx2.5, which predicted two possibilities: Nanog might act on DNA as a monomer and/or a dimer [supporting information (SI) Fig. S1]. These predicted structures presuppose that Nanog, like NKx2.5, homodimerizes via its HD (19). To ascertain whether Nanog, indeed, forms homodimers *in vivo*, we performed size exclusion chromatography of ES cell nuclear extracts to fractionate Nanog-containing protein complexes. Results reveal that, in addition to a broad range of Nanog multiprotein complexes as described (6), Nanog also forms a small complex with an apparent size of ≈70–80 kDa, approximating that of a
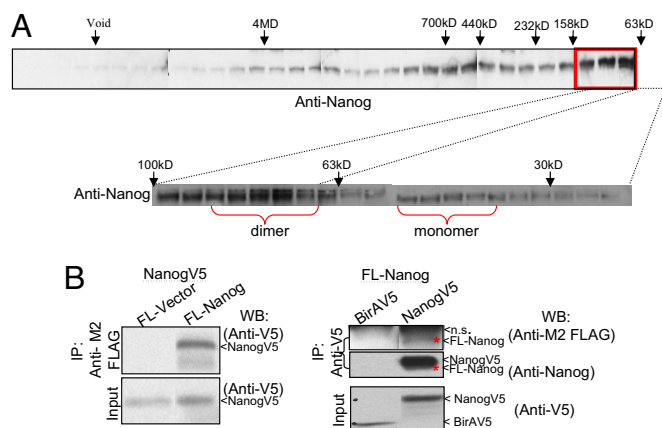
Fig. 1. Confirmation of Nanog–Nanog interaction (dimerization). (*A*) Western blot analysis of gel filtration fractions containing protein complexes using anti-Nanog antibody. (*Upper*) Fractions that may contain Nanog homodimers are indicated with a red rectangle. (*Lower*) A close-up examination of fractions containing potential Nanog dimers and monomers is also presented. (*B*) CoIP in heterologous 293T cells. 293T cells were transiently transfected with the constructs indicated, and coIP was performed in both directions (Fig. 1*B*) to minimize the potential interference of the affinity tags. The results show that Nanog polypeptides form dimers (Fig. 1B). A recent study also demonstrated self-association of the bacterially expressed recombinant Nanog proteins (26). Taken together, these data strongly argue that Nanog forms homodimers *in vivo* in mES cells.

dimer (Nanog monomer is 38 kDa) (Fig. 1*A Upper*). To further confirm this and distinguish the Nanog dimer from monomer *in vivo*, higher-resolution size chromatography of ES cell nuclear extracts was performed. The results revealed a greater abundance of putative Nanog dimers than monomers (Fig. 1*A Lower*). In addition, the presumed dimer fractions are predominant among all complexes (see the red rectangle in Fig. 1*A Upper*). Together, these suggest that the Nanog dimer may form the core of the Nanog protein complexes in mES cells.

Coimmunoprecipitation (coIP) experiments were performed to determine whether Nanog polypeptides form dimers. Two differently tagged Nanog proteins [Flag (FL)-Nanog and NanogV5] were introduced into heterologous 293T cells that do not themselves express Nanog, and coIP was performed in both directions (Fig. 1*B*) to minimize the potential interference of the affinity tags. The results show that Nanog polypeptides form dimers (Fig. 1B). A recent study also demonstrated self-association of the bacterially expressed recombinant Nanog proteins (26). Taken together, these data strongly argue that Nanog forms homodimers *in vivo* in mES cells.

Nanog Dimerizes via Its CD Rather than Through Its HD. To delineate the domains that mediate dimerization of Nanog, a series of truncated Nanog mutants, tagged with a V5his epitope, were constructed and tested for interaction with FL-tagged Nanog in 293T cells (Fig. 2*A*). Total lysates from cells expressing FL-Nanog and V5his-tagged mutants (A to K) and a BirA control (bacterial biotin ligase) were subjected to coIP, followed by Western blot analyses as detailed in *Materials and Methods*. The results show that only mutants (F-J) containing the CD interact with FL-Nanog (Fig. 2*B*). Thus, the CD, rather than the HD, mediates Nanog–Nanog homodimerization.

To further delineate the region within the CD required for Nanog dimerization, we generated a series of Flagbio (FLbio)-tagged mutants with point mutations in the CD1, WR, and CD2 subdomains (Fig. 2*C* and data not shown) and assessed their interaction with V5his-tagged wild-type Nanog. Using a similar coIP strategy followed by Western blot analyses, we found that

only a mutant bearing an alteration of 10 tryptophans (W) to alanines (A) within the WR domain (10WA) disrupted Nanog–Nanog interaction (compare lanes 1 and 3 in Fig. 2*D*). This finding identifies the WR subdomain within the CD as critical for Nanog homodimerization.

Nanog Dimerization Is Required for Interaction with Pluripotency Network Proteins. Our prior studies defined important roles in stem cell self-renewal and pluripotency for a number of newly identified critical ES cell factors, such as Sall4, Dax1, Zfp281, and Nac1 (6). To address the potential relevance of Nanog dimerization, we asked whether these and other critical factors within the ES cell pluripotency network (6) interact with the Nanog dimer.

We have already shown that Nanog dimers may constitute the core of Nanog protein complexes (Fig. 1). To confirm this, coIP studies were performed to test interaction of Sall4, Zfp281, Zfp198, Dax1, Nac1, and Oct4 with dimeric Nanog and monomeric Nanog^{10WA} (Fig. 3). The results show that Sall4, Zfp281, Zfp198 (Fig. 3*A*) and Dax1 (Fig. 3*B*) only interact with FLbioNanog (a dimerizing-competent version of the Nanog protein), but not FLbioNanog^{10WA} (a nondimerizing version of the protein). Nac1, a BTB-POZ domain protein, preferentially associates with dimeric FLbioNanog, and to a much less extent with monomeric FLbioNanog^{10WA} (Fig. 3*C*). Oct4, a critical factor for ES cell identity, also preferentially interacts with dimeric FLbioNanog (Fig. 3*D*; note the input of monomeric FLbioNanog^{10WA} is more than dimeric FLbioNanog and yet the fraction of dimeric FLbioNanog immunoprecipitated by Oct4 was much higher). These results suggest that Nanog dimers likely constitute a core for Nanog-containing complexes.

Forced Nanog Dimer and Monomer Maintain DNA Binding Capacity. To address whether Nanog homodimerization is necessary and/or sufficient for stem cell pluripotency, we asked whether a forced Nanog dimer and/or monomer can replace endogenous Nanog in ES cells. Our approach is based on a now classical strategy used to assess the role of MyoD and E47 heterodimerization in myogenesis (27). In this method, two transcription factors are tethered together by using the 22-aa flexible polypeptide [GT(GGGS)$_4$GGGT]. As intramolecular interactions are greatly favored over intermolecular interactions, forced dimers predominate.

Following the same strategy, we constructed two Nanog mutants. By joining two units of wild-type Nanog (N) with the same flexible polypeptide linker, a forced Nanog dimer (NN) was generated (NN; Fig. 4*A Upper*). A Nanog monomer mutant (NNH) was constructed by joining a wild-type Nanog unit (N) with another Nanog mutant lacking the dimerization-mediating CD domain (NH; Fig. 4*A Lower*). The NH mutant itself acts solely as a truncated monomer without any dominant negative effects when overexpressed in ES cells (see Fig. S2). This finding ensures the monomeric properties of the NNH mutant driven by the intramolecular interaction between the wild-type Nanog unit and the NH mutant (see *Discussion*).

To validate the DNA-binding capacity of the tethered Nanog mutants, we did EMSAs using COS extracts of mutant-expressing cells and probes containing a Nanog consensus binding site (8). The proximal promoters of both *Cdx2* and *Gata6* contain Nanog consensus binding sites and were used for EMSA. The results (Fig. 4*B*) show that both the tethered Nanog dimer (NN) and monomer (NNH) bind to the Nanog-binding site in the *Cdx2* (Fig. 4*B*, lanes 4 and 6) and the *Gata6* (Fig. 4*B*, lanes 10 and 12) promoter DNAs. The binding specificity was further confirmed by supershift of the binding bands by anti-Nanog antibody (Fig. 4*B*, lanes 5, 7, 11, and 13). Binding of wild-type Nanog protein to the *Cdx2* promoter sequence, as both a dimer and a monomer, served as a positive control (Fig. 4*B*, lanes 8 and 9).
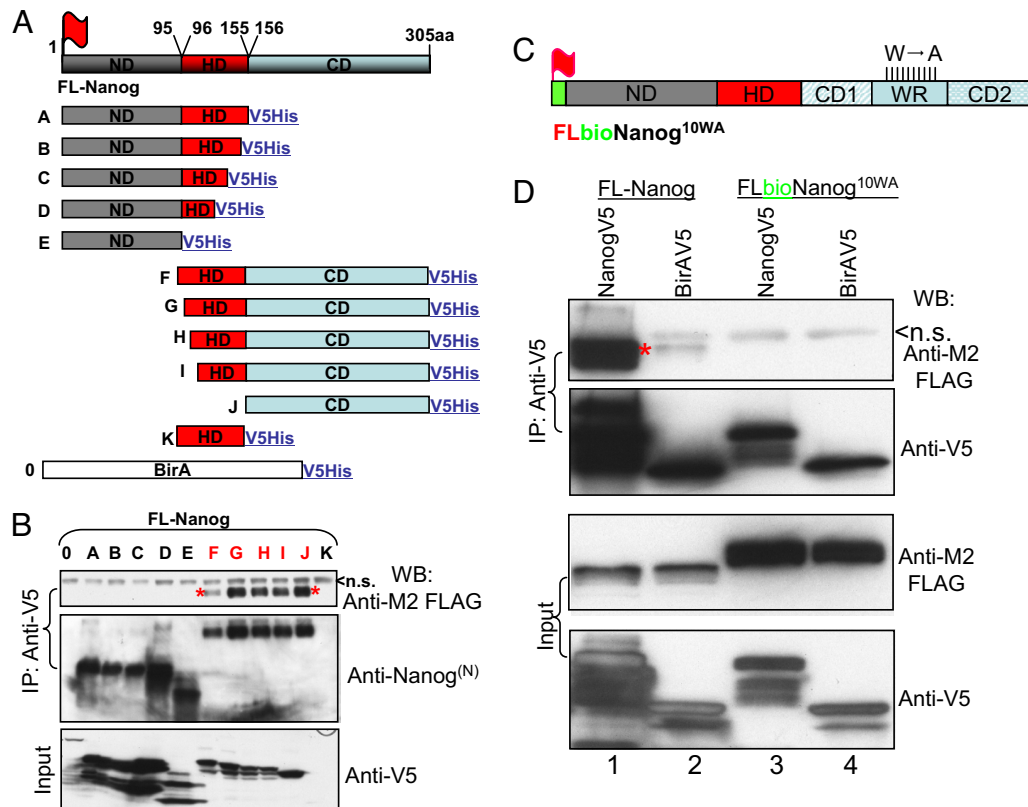
Fig. 2. The CD of Nanog is required for Nanog homodimerization. (*A*) Depiction of the FL-tagged full-length Nanog (FL-Nanog) and truncated Nanog mutants A to K; ''0'' is the BirAV5his construct used as a negative control. (*B*) CoIP of FL-Nanog and truncated Nanog mutants shown in *A*. <n.s. indicates a nonspecific signal; * denotes the specific FL-Nanog signal. CoIP was performed as described in Fig. 1. The anti-Nanog[(N)] antibody was raised against the N terminus of Nanog (Santa Cruz Biotechnology) and used to detect the truncated mutants A to E and FL-Nanog coimmunoprecipitated by the mutants F-J. The construct K is small and has migrated off the gel but it has been confirmed properly expressed (data not shown). (*C*) Depiction of FL and biotin (FLbio) dually tagged full-length Nanog[10WA] mutant. The vertical lines indicate alteration of the 10 tryptophans (W) to alanines (A). Individual Nanog subdomains (ND, HD, CD1, WR, and CD2) are also indicated. (*D*) CoIP of wild-type NanogV5his with FL-Nanog shown in *A* and FLbio-Nanog[10WA] mutant shown in *C*. BirAV5his was used as a negative control for CoIP. <n.s. indicates a nonspecific signal (in lanes 1 and 2) that could also overlap with FLbioNanog[10WA] signals (in lanes 3 and 4). Note the differential migration between FL-Nanog and FLbioNanog[10WA] (compare lanes 1 and 2 with lanes 3 and 4 in the third panel). * indicates the FL-Nanog signal. The residual FL-Nanog signal in lane 2 is presumably the background binding or overflow from lane 1. Note the data in lanes 1 and 2 further confirm the data in Fig. 1*B Right*.

Untransfected and vector-transfected COS cell extracts were used as negative controls to identify background binding signals (Fig. 4*B*, lanes 1–3). Our results indicate that the tethered Nanog proteins retain intrinsic DNA binding capacity, a prerequisite for subsequent testing of their biological activities.

Forced Nanog Dimer, but Not Monomer, Maintains LIF-Independent Self-Renewal. To test the function of tethered Nanog dimers and monomers for self-renewal and pluripotency, we asked whether enforced expression of the dimer and/or monomer is able to functionally replace endogenous Nanog in ES cells. To this end, an episomal overexpression system (9) was used to achieve clonal, ectopic overexpression of the NN and NNH mutants after their introduction into the E14T ES cells by pPyCAGIZ vectors (see *Materials and Methods*).

Two complementary strategies were used to study the consequences of overexpression of the tethered Nanog dimer and monomer mutants in E14T cells. First, a qualitative assessment of cells expressing the mutants was performed in the presence and absence of LIF after serial passages. As shown in Fig. 5 *A* and *B*, after 8 days of serial splitting and passage, Nanog dimer (NN) expressing cells grown in the absence of LIF retain ES cell morphology and stem cell-specific alkaline phosphatase (AP) staining (Fig. 5*A Upper*). The stem cell characteristics of these cells were further supported by Western blot analysis, which

shows that endogenous Nanog expression is maintained in dimer expressing cells in the absence of LIF (Fig. 5*B Upper*). In contrast, when grown in the absence of LIF, monomer (NNH)-expressing cells lose typical compact ES cell morphology and differentiate, which is confirmed by negative AP staining (Fig. 5*A Lower*). Moreover, expression of the endogenous Nanog protein was lost or dramatically reduced (Fig. 5*B Lower*).

A second strategy used a colony formation assay to provide a quantitative analysis of mutant expressing cells for LIF-independent self-renewal. A limited number of mutant expressing cells were cultured at clonal density in the presence and absence of LIF for 6 days followed by AP staining, and differentially stained colonies were enumerated (Fig. 5*C*). When cells were grown in the presence of LIF, i.e., on top of endogenous Nanog expression, overexpression of both Nanog dimer (NN) and monomer (NNH) increases uniformly undifferentiated colony formation (and thus enhances self-renewal) (Fig. 5*D*); however, upon LIF withdrawal, and consequent depletion of endogenous Nanog, the Nanog dimer, but not monomer, sustains self-renewal of the cells to a level approximately that of wild-type E14T cells grown in the presence of LIF (Fig. 5*D*). These data mimic the colony formation assay of ES cells with enforced expression of wild-type Nanog protein (9). Therefore, our findings argue that the Nanog dimer is the active form of
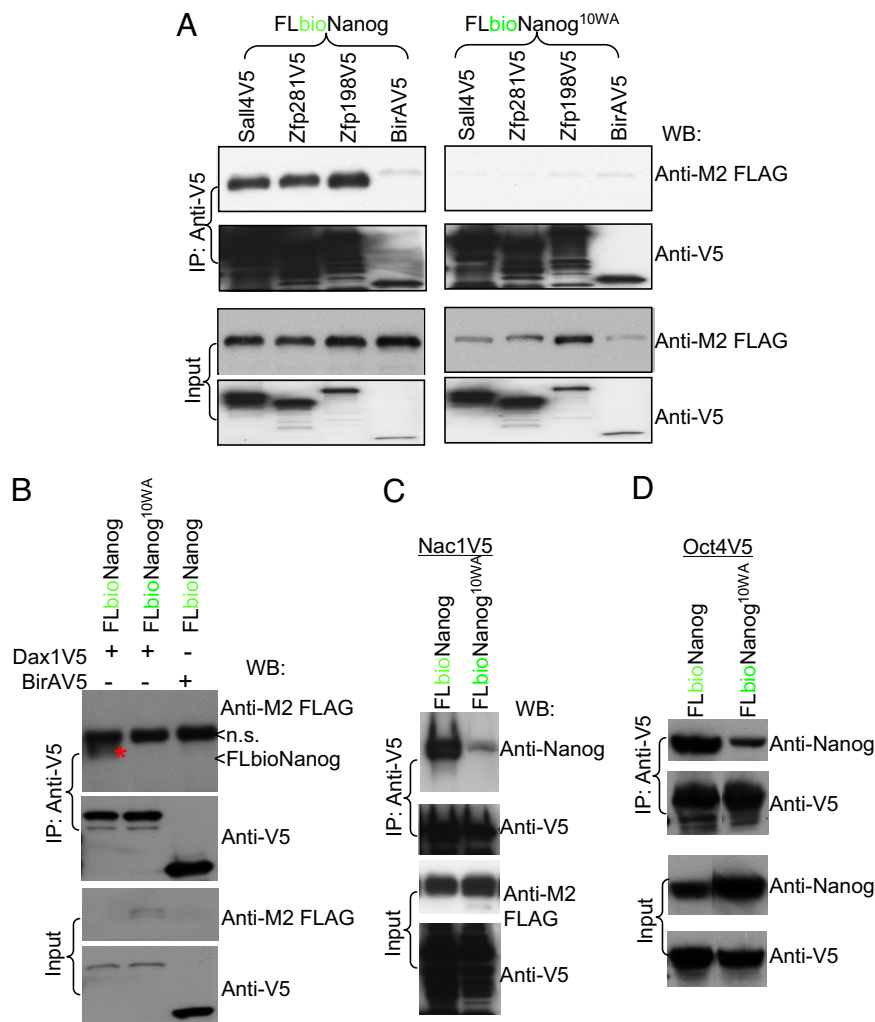
Fig. 3. Nanog homodimerization is required for its interaction with other pluripotency factors. (*A*) CoIP of V5his-tagged pluripotency network proteins Sall4, Zfp281, Zfp198, and BirA (control) with dimeric FLbioNanog and monomeric FLbioNanog[10WA] mutants. (*B*) CoIP of V5his-tagged Dax1 and BirA (control) with dimeric FLbioNanog and monomeric FLbioNanog[10WA] mutants. <n.s. indicates a nonspecific signal, and * indicates the FLbioNanog signal. Note the much lower input level of FLbioNanog than FLbioNanog[10WA] and yet a higher IP signal (*) of FLbioNanog than FLbioNanog[10WA]. (*C*) CoIP of V5his-tagged Nac1 with dimeric FLbioNanog and monomeric FLbioNanog[10WA] mutants. (*D*) CoIP of V5his-tagged Oct4 with dimeric FLbioNanog and monomeric FLbioNanog[10WA] mutants.

Nanog protein that serves to replace wild-type Nanog for LIF-independent self-renewal upon overexpression.

Discussion

In this study we have explored how the Nanog protein acts within the pluripotency network (6). Previously, we focused on Nanog-containing protein complexes rather than on the Nanog protein itself. By performing refined size exclusion chromatography, we have now shown that Nanog–Nanog homodimers constitute a major fraction of Nanog protein complexes in ES cells (Fig. 1*A*). We confirmed the direct Nanog–Nanog interaction in heterologous cells (Fig. 1*B*) and delineated the dimerization domain in the Nanog protein (Fig. 2). Moreover, we demonstrated that Nanog homodimerization is required for interaction with a number of critical factors in the network (Fig. 3). Finally, we showed that enforced expression of a Nanog dimer, but not monomer, functionally replaces endogenous Nanog to sustain LIF-independent self-renewal of ES cells (Figs. 4 and 5). Our study confirms and extends the established role of Nanog in stem cell pluripotency and provides insights into the mechanism of Nanog transcriptional regulation for target gene expression in stem cell pluripotency.

As a unique variant HD protein, Nanog bears no obvious relationship with previously characterized proteins, save for modest similarity of its HD with that of the NK2 family of homeoproteins (9). However, unlike NKx2.5, the HD of Nanog is not required for homodimerization (Fig. 2). Besides its presumed role in direct DNA contact, the HD of Nanog has been shown to be required for nuclear localization of the human homolog (28) and interaction with another ES cell critical factor Sall4 in mES cells (29). Quite unexpectedly, we found that the CD of Nanog, particularly the WR subdomain, mediates homodimerization (Fig. 2). The CD has been reported to contain transactivation activity in reporter assays (22, 24), although the *in vivo* function of this domain remained largely uncharacterized. Our study indicates that an important role of the CD is to mediate Nanog–Nanog homodimerization.

The functional significance of Nanog homodimerization is suggested by association of a number of pluripotency network proteins with Nanog dimers, as opposed to monomers (Fig. 3). This observation is consistent with the notion that, on average, homodimers have twice as many interaction partners as nonself-interacting proteins in protein–protein interaction networks (30). Although we stress the relevance of Nanog dimers in
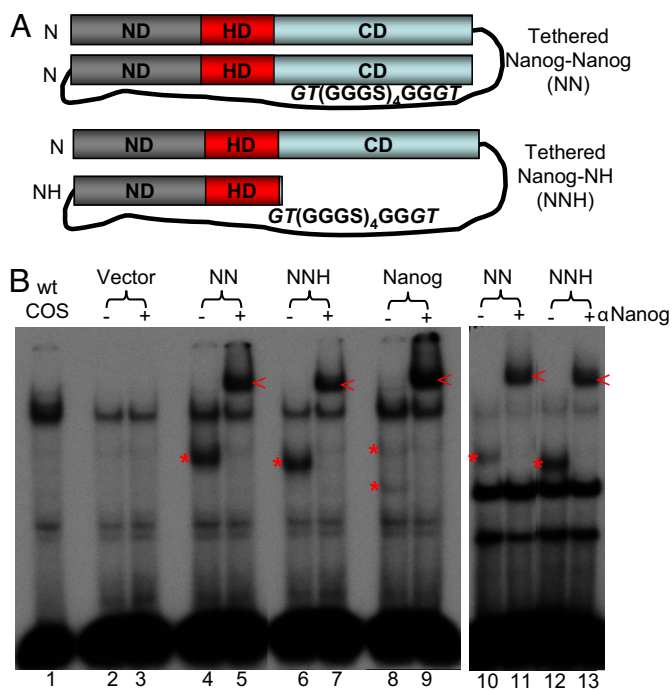
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Fig. 4. Tethered nanog mutants maintain DNA-binding capacity (*A*) Schematic depiction of the tethered Nanog dimer (NN) and monomer (NNH) mutants. The 22-aa flexible polypeptide linker is shown. (*B*) EMSA of mutants' binding to the Cdx2 (lanes 1–9) and the Gata6 (lanes 10–13) promoter DNA sequences. ∗ indicates specific binding signals, and <denotes the supershift bands by anti-Nanog. Wild-type and vector-transfected COS extracts were used as negative controls.

Fig. 5. Nanog dimer, but not monomer, maintains endogenous nanog expression and sustains LIF-independent self-renewal of ES cells. (*A*) Serial passage of ES cells expressing the tethered Nanog dimer (NN; *Upper*) and monomer (NNH; *Lower*) in the presence (*Left*) and absence (*Right*) of LIF. Cells were serially split and passaged for 8 days followed by AP staining and phase-contrast photography. (*B*) The tethered Nanog dimer (NN), but not monomer (NNH), maintains endogenous Nanog expression. Total lysates from two representative clones of each mutant were used for Western blot analysis with anti-Nanog antibody. β-Actin was used as loading control. (*C*) Schematic depiction of the strategy for colony formation assays. (*D*) Quantitative presentation of the colony formation assay in parental E14T cells and stable transfectants expressing the Nanog dimer (NN) and monomer (NNH). Cells were plated at clonal density in the presence and absence of LIF (1,000 units/ml). After 6 days of culture, colonies stained for AP were scored into three categories (uniformly undifferentiated, partially differentiated, and fully differentiated) as indicated on the right.

regulating stem cell activity, we cannot formally exclude a possible role of Nanog monomers in target gene regulation, particularly in light of enhanced self-renewal of monomer (NNH)-expressing cells in the presence of LIF (Fig. 5*D*). Two possible explanations may account for enhanced self-renewal by the Nanog monomeric mutant (NNH). First, the Nanog monomer and dimer might regulate distinct sets of target genes for stem cell self-renewal. In this case, an increase of monomers in the presence of steady-state levels of Nanog dimers and monomers would presumably enhance self-renewal. Alternatively, enhanced self-renewal might result from an increase in dimer formation upon overexpression of NNH in cells grown in the presence of LIF. In this case, an intermolecular, functional Nanog dimer might be formed between wild-type Nanog and the NNH mutant in the presence of steady-state levels of wild-type Nanog. This possibility is supported by *in vitro* coIP data showing that NNH can still interact with Nanog (data not shown). However, such intermolecular dimer formation was not favored upon LIF withdrawal and subsequent depletion of endogenous Nanog.

Interpretation of the functional data relies on the authenticity of the mutants generated by the tethering strategy (27) in relation to the endogenous protein. Although direct protein structure data are lacking, the strategy has been successfully applied for studies on the heterodimerization of myogenic transcription factors MyoD-E47 (27) and the heterodimerization of hematopoietic transcription factor NF-E2 subunits p18–p45 (31). We have carefully addressed the relevance of the mutants to the functional data by ensuring their intact intrinsic DNA-binding capacity (Fig. 4) and using two complementary strategies to provide biological readouts (Fig. 5). In addition, we have noted that the NH-truncated mutant used to construct the tethered, monomeric Nanog (NNH) is inactive in ES cells (see
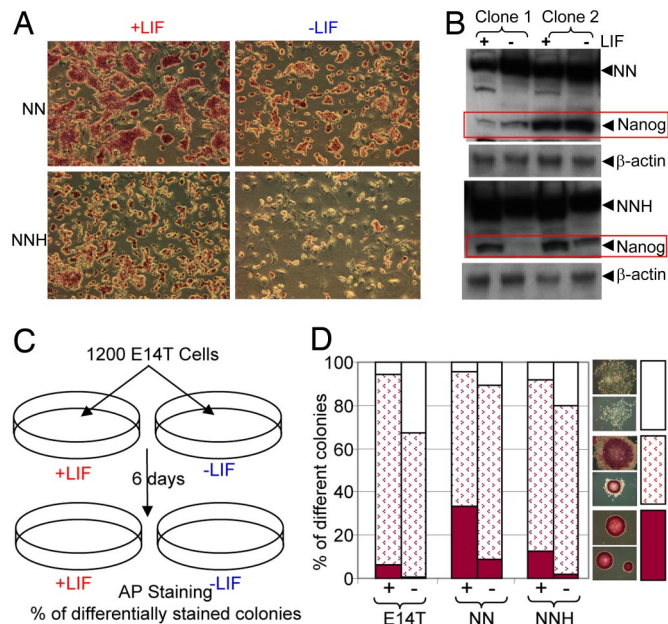
Fig. S2). This observation ensures that the monomeric version of the Nanog protein (NNH; Fig. 4*A*) is free of any dominant negative effect and serves as a better monomer mutant than Nanog[10WA] itself (which may have additional effects resulting from the sequence change). Interestingly, it has been reported that the same NH mutant behaves in a dominant negative fashion in F9 embryonic carcinoma cells (32). This finding may be explained by the much weaker pluripotency network in F9 cells than that in ES cells, such that a subtle imbalance between the endogenous Nanog and the NH mutant (when overexpressed) drives cells toward the primitive endodermal lineage. In contrast, ES cells that express high levels of Nanog and possess a robust network are resistant to effects of the NH mutant.

The importance of the Nanog protein for pluripotency is underscored in factor-based reprogramming experiments (reviewed in ref. 25) that demonstrate the utility of selection for Nanog expression (33–35) and direct participation of Nanog (36) for the generation of high-quality induced pluripotency stem cells. In addition, Nanog has been shown to facilitate transfer of pluripotency after cell fusion (13). These data suggest that Nanog may act as a master transcriptional organizer that entrains the hierarchy of pluripotent gene expression after erasure of the differentiated epigenome. How Nanog functions in this process is not well understood. In this study, we provided one aspect of Nanog transcriptional regulation, i.e., homodimerization, which is necessary for its function in promoting stem cell pluripotency.

Materials and Methods

Cell Culture, Total Lysate and Nuclear Extract Preparation, and Size Exclusion Chromatography (Gel Filtration). ES cell lines (J1 and E14T) were maintained on gelatin-coated plates without feeders as described (25). 293T and COS cells were maintained in DMEM (low glucose) supplemented with 10% FCS and 50 units/ml penicillin/streptomycin. These cells were split every other day to maintain ≈50–70% confluence for optimal transfection.

CoIP and Western Blot Analysis. CoIP in 293T cells and Western blot analyses using total lysates or nuclear extracts were performed as described (25).

Plasmid Construction. Truncated Nanog mutants were constructed by using a PCR strategy. Briefly, PCRs were performed with the primers in the 5′ and 3′ ends of Nanog cDNA and the primers with progressive deletion of the HD from 3′ to 5′ (mutants A to E) and 5′ to 3′ (mutants F to J), respectively. The mutant K was constructed by using the primers spanning the HD. The FLbioNanog^{10WA} mutant was constructed by using a site-directed mutagenesis (Stratagene) kit, the template plasmid FLbioNanog as described (6), and two primers containing the alterations of 10 tryptophan residues to alanine. The tethered Nanog dimer and monomer mutants were constructed with three subcloning steps. First, a wild-type unit of Nanog was joined 5′ terminally to the full-length 22-aa polypeptide linker by annealing Nanog cDNA and the polypeptide linker containing overlapping the Nanog 3′ end cDNA sequence followed by PCR with the two outmost primers. The resulting PCR product containing a XhoI site before the 5′ end of Nanog cDNA and a BamHI site in the polypeptide linker was then cloned into XhoI and BamHI sites of the pBluescript SK vector; second, either a wild-type unit or truncated NH unit of Nanog was joined 3′ terminally to the full-length 22-aa polypeptide linker by annealing Nanog cDNA and the polypeptide linker containing overlapping Nanog 5′ end cDNA sequence followed by PCR with the two outmost primers. The resulting PCR product containing a BamHI site in the polypeptide linker and a NotI site after the 3′ end of Nanog cDNA was then cloned into BamHI and NotI sites of the pBluescript SK subclone from step 1; third, the XhoI and NotI fragment from

the subclone in step 2 was released and cloned into pPyCAGIZ vectors previously digested with XhoI and NotI.

All PCR products described above were subcloned into pCRII-TOPO vector (Invitrogen) for sequence verification (performed by the Mental Retardation Research Center at Children's Hospital Boston) followed by cloning into the respective expression vectors (pEF1V5his from Invitrogen; pPyCAGIZ vectors kindly provided by Ian Chambers, University of Edinburgh, Edinburgh). Sequences for oligonucleotides used in this study are available on request.

EMSA. The sense and antisense oligonucleotides for probes were synthesized with a 5′ single nucleotide ''G'' overhang for labeling with ^{32}P-dCTP (Amersham) by Klenow enzyme (New England Biolabs) fill-in reaction. The probe sequences are as follows: *Cdx2* probe sense, 5′-gCTGTTTTAAACTAATTTAATGCTTTA-ATATTTATTTTA-3′; *Cdx2* antisense, 5′-gTAAAATAAATATTAAAGCATTAAATT-AGTTTAAAACAG-3′; *Gata6* sense, 5′-gAGAATAAAGTAAAAAGGTTAATGGCT-GAGGGTGGGCCTCA-3′, antisense, 5′-gTGAGGCCCACCCTCAGCCATTAA-CCTTTTTACTTTATTCT-3′. The sense and antisense oligonucleotides were annealed before being labeled with Klenow enzyme and ^{32}P-dCTP. EMSA was performed as described (37) .

Serial Passage, Colony Formation Assays, and ES Cell Growth Assay. For serial passage, ES cells were grown in the presence (1,000 units/ml) and absence of LIF, split every other day to maintain 50–80% confluence. After 8 days of serial splitting and passage, cells were subjected to AP staining (Sigma) per the manufacturer's instruction. Colony formation assays were performed as described (9), except that 1,200 cells were grown on a 10-cm plate, and ES cell growth assay were performed as described (8).

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