The Stem Cell Pluripotency Factor Nanog Activates Transcription With Two Unusually Potent Subdomains at its C-terminus

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Abstract:

Embryonic stem cells are pluripotent progenitors for virtually all cell types in our body, thus, possess unlimited therapeutic potentials for regenerative medicine. Nanog, an NK-2 type homeodomain gene, has been proposed to play a key role in maintaining stem cell pluripotency presumably by regulating the expression of genes critical to stem cell renewal and differentiation. Here, we provide the evidence that nanog behaves as a transcription activator with two unusually strong activation domains embedded in its C-terminus. First, we identified these two transactivators by employing the Gal4-DBD fusion and reporter system and named them as WR and CD2. While CD2 contains no obvious structural motif, the WR or W-repeat contains 10 pentapeptide repeats starting with a Trp in each unit. Substitution of Trp with Ala in each repeat completely abolished its activity while mutations at the conserved Ser, Gln and Asn had relatively minor or no effect on WR activity. We then validated the activities of WR and CD2 in nanog by constructing a reporter plasmid bearing 5 nanog binding sites. Deletion of both WR and CD2 from nanog completely eliminated its transactivation function. Paradoxically, while the removal of CD2 reduced nanog activity by ~30-70%, the removal of WR not only did not diminish but actually enhanced its activity by ~50-100% depending on the cell lines analyzed. These data suggest that either WR or CD2 is sufficient for nanog to function as a transactivator.
Introduction

Embryonic stem cells hold the key to regenerative medicine which offers an alternative to classic treatments based primarily on operations or drugs (1-4). The regenerative potential of stem cells may provide the “fountain of youth” for our ever aging population and has sparked tremendous interest both socially and scientifically in recent years. Yet, conceptual as well as technical hurdles recognized in recent years may set back any realistic application of stem cell biology and regenerative medicine for decades if not centuries. One such hurdle is how to maintain stem cells in pluripotent states in vitro and trigger their differentiation towards a specific lineage suitable for transplantation (1-4). Investigation into the pluripotency of stem cells should provide the necessary tools to both control and utilize the regenerative potential of embryonic stem cells for therapeutical purposes (1).

Our understanding of stem cell pluripotency has been focused on a couple of transcription factors that regulate both positively and negatively distinct sets of genes for pluripotency and differentiation of stem cells. Oct4 is the first such factor and has been studied extensively over the past decade(1,3,5). Interestingly, a novel homeodomain protein, Nanog, was also demonstrated to play a key role in maintaining pluripotency for mouse embryonic stem cells (6,7), consistent with the notion that the pluripotency of embryonic stem cells is regulated at the transcription level by transcription factors (1,3,5). In addition, factors such as STAT3 have also been implicated in stem cell pluripotency.
(5,8,9). However, neither STAT3 nor Oct-4 is sufficient to maintain stem cell pluripotency (10,11), suggesting that additional factors such as Nanog may play a more important role for ES cell self renewal (6,7,11).

The mechanism through which Nanog regulates stem cell pluripotency remains entirely unknown. Based on the differences in gene expression between wild type and nanog null cells, it has been proposed that Nanog regulates pluripotency mainly as a transcription repressor for downstream genes such as gata4 or gata6 (6,7). However, Nanog may function more broadly than originally proposed. First, Nanog appears to function in parallel with STAT3 and be sufficient for maintaining stem cell pluripotency without gp130/STAT3 activation (6,7). Secondly, Nanog not only inhibits the differentiation of stem cells into endoderm, but also actively maintains pluripotency, in contrast to Oct-4’s role as a blocker of differentiation of ICM and ES cells into trophectoderm (1,5-7,10,12). Consequently, Nanog has been proposed as the missing determinant of pluripotency for inner cell mass (ICM) and embryonic stem (ES) cells (7). Since differentiation and self-renewal are likely to be regulated through the expression of mutually exclusive genes, Nanog may assume a bi-functional role to repress those genes important for differentiation and activate the ones necessary for self renewal (13).

Nanog is a multi-domain protein with a well conserved Nk-2 homeodomain (6,7,13,14). By fusing various domains of nanog with the DNA binding
domain of the yeast transcription factor Gal4, we have previously identified two transactivators in mouse Nanog—the N-terminal transactivation domain or ND, and the C-terminal transactivation domain or CD (13). We assume that the signature 60 residue HD should be able to bind DNA and interact with other proteins as demonstrated for Oct-4 (1,13). The ND contains 95 residues rich in Ser and Thr and acidic residues found in typical transactivators (6,7,13,15). The CD is 150 residues long with no apparent transactivation motifs (13). It remains unclear if nanog is a transcription activator by itself and which domain is required for its transactivation activity. Here we demonstrate for the first time that nanog can transactivate a reporter plasmid bearing its cognate binding site. Furthermore, we have dissected two unusually strong transactivation domains in its C-terminus that are required for its transactivation activity, thus, may contribute to the expression of downstream genes critical for maintaining stem cell pluripotency.
Materials and Methods

Cell Lines and Plasmids: HEK 293 cells, P19 cells, and NIH3T3 cells were cultured in DMEM (Invitrogen, CA) supplemented with 10% FBS (Hyclone, UT) and antibiotics (penicillin and streptomycin, 100μg/ml) as described (13). Mouse embryonic stem cells or ESCs were maintained on MEFs in ESC medium, which contains DMEM supplemented with 20% FBS, non-essential amino-acids (100mM), 0.55mM 2-mercaptoethanol (Invitogen, CA). To remove MEFs, cells were collected by trypsinization and plated on 3.5cm dish for 30min. Non-adherent cells, mostly ESCs, were replaced on gelatin coated 24 well plate (Corning, MI) and grown in ESC medium supplemented with LIF (1000u/ml). The expression plasmids pCR3.1-NanogF and pCR3.1-Gal4DBD were prepared as described (13). pCR3.1-Gal4-CD1, pCR3.1-Gal4-WR, pCR3.1-Gal4-CD2, pCR3.1-Gal4-C1WR and pCR3.1-Gal4-WRC2 were generated by inserting a PCR fragment encoding Nanog C1 domain (aa 156-197), W-repeat domain (aa 194-247), C2 domain (aa:244-305), C1WR domain (aa 156-247) or WRC2 domain (aa 194-305) to the downstream EcoRV site of pCR3.1-Gal4DBD respectively as described (13). Oligonucleotides encoding (W/A)*5, (W/A)*10, S/A, Q/A or N/A were chemically synthesized and inserted to the downstream EcoRV site of pCR3.1-Gal4 DBD after being 5’-end phosphoryrared using T4 kinase and annealed as shown in Fig. 3. Deletions for Nanog N1, N3 and N4 were prepared by inserting a PCR fragment encoding aa 1-197(N1), aa 1-247(N3) and aa 1-197/248-305(N4) to
the EcoRV site of a modified pCR3.1II. For the nanog reporter plasmids, oligonucleotides containing the nanog binding site and a SalI restriction site were chemically synthesized (sense: 5’ tcgacacctgcgatattaagtaagctgc 3’, antisense: 5’ tcgacttaagtacttaatcggcgaaggggtg 3’). After being 5’-end phosphorylated by T4 kinase and ATP, these oligonucleotides were annealed and ligated to the SalI site of p37tk-luciferase. Positive clones were randomly picked and the copy number of the inserted nanog binding site were determined by sequencing. Oligonucleotides bearing mutations for the nanog binding site were also made and inserted into the p37tk-Luc reporter in a similar fashion as a negative control. All plasmids generated were confirmed by sequencing.

**Transfections, Western Blotting and Reporter Assay:** For western blotting analysis, HEK293T cells cultured in 12 well tissue culture plates were transfected by calcium phosphate co-precipitation method with expression plasmids (1 ug each) as described (13). Cells were harvested 48h after transfection. Western blotting analysis were performed as described (13,16). For reporter assay, cells seeded in 24 wells plate were transiently transfected with reporters such as p5G-e1b-luciferase or p5N-tk-luciferase (0.1ug each) and effector plasmids (0.5ug) using LipofectAmine (Invitrogen, CA) according to the manufacture’s instruction. pCMV-Renilla (0.005ug per transfection, Promega, WI) was co-transfected into each well as an internal reference and the DNA concentrations for all transfections were normalized to equal amounts.
by adding pCR3.1 empty vector. 36 hours later, cells were washed by PBS and lysed in 70ul of 1x PLB buffer (Promega, WI). Luciferase activity was measured using the Dual-luciferase Reporter Assay System (Promega, CA) and TD2020 Luminometer (Turner Design, CA) as described (17). Each transfection was carried out in duplicate and repeated at least twice. For ESCs transfection, MEFs were removed as described above, the ESCs seeded on gelatin coated 24 wells tissue culture plate and transfected with plasmids in the same manner as described above using Lipofectamine 2000 (Invitrogen, CA).
Results

The C-terminus of Nanog encodes two unusually potent transactivators:

Although nanog was originally proposed to function primarily to repress the expression of genes such as gata4 and gata6, we have generated preliminary evidence that nanog contains transactivation domains by fusing domains both N and C-terminal to the conserved homeodomain to the DNA binding domain of Gal4 and demonstrating that both fusions are able to transactivate a luciferase reporter construct bearing 5 copies of the Gal4 binding sites (13). Interestingly, the C-terminal domain or CD of Nanog possesses transactivation activity at least 6 times as active as that of its N-terminal domain or ND (13).

The most prominent feature of the CD in mouse Nanog is the presence of 10 pentapeptide repeats starting with a tryptophan or W residue, thus, named W-repeat or WR (6,7,13) (Fig.1A and 1B). We reasoned that the WR may contribute to the unusually high activity for the CD and subsequently divided the entire CD into CD1\textsuperscript{156-197}, WR\textsuperscript{198-247} and CD2\textsuperscript{248-305} as illustrated in Fig. 1A. We fused these three subdomains with the Gal4 DNA binding domain individually or in combinations as shown in Fig. 1B. Upon transfection into HEK293 cells, these fusion constructs expressed proteins of expected size (Fig. 1C, lanes 2-7). To evaluate their transactivation potentials, these constructs in three doses were co-transfected with the reporter plasmid, p5G-e1b-luciferase, and their activities were quantified as described (17) and presented in Fig. 1D. These results confirmed our initial reasoning that the
WR is indeed a functional structure. First, the WR itself is a potent transactivator capable of activating the reporter in a dose dependent fashion up to ~900 fold (Fig. 1D, lanes 8-10). Surprisingly, CD2, which is downstream of WR, encodes an even stronger transactivator that activates the report up to ~3000 fold, apparently stronger than the viral VP16, arguably the best transactivator known so far (Fig. 1D, lanes 11-13 vs 20-22). The CD1, on the other hand, appears to be inactive, like the Gal4 DBD expressed alone (Fig. 1D, lanes 2-7). Interestingly, the C1W and WC2 combinations yielded activity lower than either WR or CD2 (Fig. 1D, lanes 14-19). Nonetheless, these results revealed two potent transactivators embedded in the C-terminus of Nanog.

Both WR and CD2 are active both in pluripotent and non-pluripotent cells: The cells used in Fig. 1D are HEK293 cells which were derived from human embryonic kidney and are not known to be pluripotent. To assess the activities of both WR and CD2 in pluripotent cells, we co-transfected these constructs with the reporter plasmid into mouse embryonic stem cells, P19 germ tumor cells and mouse NIH3T3 cells and the results were obtained and shown in Fig. 2. Both WR and CD2 can mediate the trans-activation of the reporter gene in all three cell lines including pluripotent cells (ES cells and P19 cells) and non-pluripotent cells (NIH3T3 cells), confirming their strong and universal activities for these two domains as observed in different cells. Nevertheless, both constructs are much more active in mouse ES cells than
P19 and NIH3T3 cells (~300 vs 50 vs 20 for WR and ~1600 vs 300 vs 200 for CD2) (Fig. 2, panels A, B, and C). As observed in HEK293 cells, the C1W and WC2 combinations are not active as WR or CD2 alone, suggesting that C1 may negatively regulate WR and WR in turn may negatively impact CD2 function as well.

The Tryptophan Residues are Required for Transactivation Activity of the WR: The defining feature of the C-terminal domain of nanog is the WR which contains 10 pentapeptide repeats starting with the W or tryptophan residue. To test the role of the W residues in the function of WR, we mutated half or all of the 10 Ws into As as shown in Fig. 3A. The mutant proteins migrated slightly higher than the wild type proteins perhaps reflecting changes in the shapes of the mutant molecule (Fig. 3B). Both mutants lost almost all of its transactivation activity in all three cell lines tested (Fig. 3C, 3D and 3E, lanes 4 and 5 vs 3). It is of interest to note that the mutant (W/A)x5 has some residual activity (Fig. 3C, 3D and 3E, lanes 4 vs 2), suggesting that the remaining 5 Ws can help maintain part of the WR structure, thus, preserving part of its transcription activity. Nevertheless, these data demonstrate that the Ws in the WR are required for its transactivation activity. In addition to W residues, there are several potentially important residues such as the Ser residues which may be phosphorylated, Asn or N and Gln or Q repeated alternately in every two repeats (Fig. 3A). These residues may also play important roles in WR function. To test these possibilities, we mutated S, N or Q into A in this domain.
and also fused them to Gal4 DBD as illustrated in Fig. 3A. Each mutation construct produced the same protein size as wild type WR did as shown in Fig3B. The data from the reporter gene assay in three different cell lines demonstrated that virtually all three mutants have the same transactivation activities as the wild type WR (Fig. 3 C, D, and E, lanes 6, 7, 8 vs 3). Taken together, these data suggest that the W residues play a vital role in maintaining the activity of the WR domain, while other conserved residues do not.

**Nanog transactivates a reporter bearing Nanog binding sites:** So far, we have demonstrated the transactivation potential of Nanog through the DNA binding sites of Gal4. To prove that Nanog is a transactivator by itself, we designed a reporter construct as shown in Fig. 4B. First, we synthesized two Nanog binding sites, one wild type (1N) and one mutant (1Nmu), based on the consensus binding site obtained by the SELEX procedure as reported by Mitsui et al (7). We then confirmed that the wild type binding site can bind to Nanog protein in a gel shift assay as shown in Fig. 4A (lane 3). The binding is specific because the mutant did not bind and unlabeled binding site can compete for binding from the P$^{32}$ labeled binding site (Fig. 4A, lanes 4 and 5 vs 3), while the unlabeled mutant did not (data not shown). The binding sites were cloned into a reporter containing the minimal TK promoter (17) and obtained multiple reporter constructs bearing 2, 4 and 5 copies for the wild type site (p2N, p4N and p5N) and 3 copies for the mutant site (p3Nm) as shown in Fig. 4B. To see if Nanog can transactivate these reporters specifically, we
co-transfected Nanog with these reporters into P19 cells and present the results in Fig. 4C. As expected, Nanog activated the reporters bearing wild type binding sites, but failed to do so towards the reporter bearing 3 mutant nanog sites (Fig. 4C, lanes 6, 8, 10 vs 4). Interestingly, the copy numbers did not appear to influence the activity of transactivation for nanog significantly (Fig. 4C, lanes 6 vs 8 vs 10). These results demonstrated that Nanog is a transcription activator capable of activating reporters bearing its binding sites.

**Either the WR or CD2 is required for Nanog to function as a transcription activator:** To test the role of WR or CD2 in mediating transactivation in the context of native Nanog protein, we constructed deletion mutants lacking both subdomains (N1), and either of them (N3 and N4) as shown in Fig. 5B. All these constructs expressed proteins with the expected sizes when transfected into HEK293 cells as shown in Fig. 5C (lanes 2, 3, 4). To evaluate the transcription activity of these deletion mutants, we transected them with either pTK or p5N (Fig. 5A) into P19 cells. As shown in Fig. 5D, N1 is consistently negative in HEK293, NIH3T3, P19 and F9 cells (lanes 3 vs 2), suggesting that the WR and CD2 are required for nanog to function as a transactivator. On the other hand, N3 can activate the reporter, but less efficiently at ~30% in HEK293 cells, 60% in NIH3T3 cells, ~50% in both P19 and F9 cells compared to full length nanog (Fig. 4D, lanes 4 vs 2). These data would suggest that CD2 is required for nanog to express its full activity and WR is sufficient to maintain ~30-70% of its activity. Surprisingly, N4 appears to have better activity than
not only N3 (Fig. 5D, lanes 5 vs 4), but also the wild type Nanog (Fig. 5D, lanes 5 vs 2), suggesting that CD2 alone is sufficient to maintain potent activity in the absence of WR which in fact may interfere with the activity of CD2 in the native configuration. These findings are consistent with the results obtained with the WC2 combination in Fig. 1D. Nevertheless, these data also validate the finding in Fig. 1D that CD2 is more potent activator than WR. Among the 4 different cell lines tested, P19 is the best (70 folds), followed by F9 (50 folds), then HEK293 (15 folds) and NIH3T3 (10 folds) in supporting nanog function. Since both P19 and F9 are considered pluripotent while HEK293 and NIH3T3 are not, these differences in activity for nanog may reflect a degree of cell type specificity towards pluripotent cells. Thus, this nanog reporter should be a useful tool in future analysis of nanog function. Taken together, both WR and CD2 should play important roles in Nanog mediated transactivation.
Conclusion

We report here that Nanog is a transcription activator capable of transactivating a reporter plasmid bearing its cognate binding sites. Furthermore, we also demonstrated that Nanog employs dual activation domains, i.e., WR and CD2, at its C-terminus to mediate transcription activation. Therefore, these findings provide a mechanistic understanding of Nanog as a stem cell pluripotency factor. The multiple and redundant nature of transactivation domains in Nanog, like the ones in Oct4, would pose considerable challenge in our future investigations into to their precise roles in maintaining stem cell pluripotency (1,5). However, a better understanding of nanog and other similar transcription factors may help us design effective tools to control stem cell pluripotency pharmacologically and achieve therapeutically favorable end points for many degenerative diseases in the near future.

Nanog as a transcription activator: OCT4 is the first homeodomain protein known to regulate stem cell pluripotency by both repressing and activating distinct sets of downstream genes (1,18). However, when Nanog was discovered, it was proposed that it should act as a transcription repressor for proteins such as gata4 and gata6 (6,7). However, it has also been noted that Nanog differs from OCT4 in that Nanog can positively maintain the status of pluripotency while OCT4 can not (6,7,11), perhaps, by activating genes critical for the maintenance of pluripotency. Therefore, our findings in this report provide direct evidence for this possibility, i.e., Nanog being capable of
activating genes bearing its cognate binding sites. We are currently searching for promoters bearing the cognate binding sites and characterizing the effect of Nanog towards these promoters.

**WR as a novel transactivator:** The W-repeat or WR was recognized as a structural feature in Nanog, yet without any function prescribed (6,7). Here, we provide the first evidence proving that it functions as a potent transactivator. Remarkably, its activity is dependent on the W residues as shown in Fig. 3. Database searches failed to identify similar motifs in other proteins so far. Therefore, it might have been evolved specifically in Nanog to activate genes in stem cell self renewal and pluripotency. Although WR functions well in multiple cell types, WR exhibits higher activity in mouse ES cells than P19 and NIH3T3 cells (Fig. 2). Structurally, we demonstrated that the tryptophan residues are absolutely required, perhaps, to maintain a unique structure for WR, most likely of a repetitive nature. One may argue that the unique structure of WR may represent a specific regulatory pathway for Nanog to interact with the transcription machinery which regulates the expression of genes critical to stem cell pluripotency. However, deletion of WR from native nanog appears to suggest that it is not required for nanog to exert robust activity (Fig. 5D). Instead, the N4 mutant without WR actually activates the reporter more strongly than the wild type molecule (Fig. 5D), consistent with the observation in Fig. 1 that the combination of WR and CD2 in the Gal4-based system also has lower activity than CD2 alone. It is possible that
the close association between WR and CD2 may hinder their interactions with any potential co-activator or the general transcription machinery. Alternatively, simultaneous engagement of the transcription apparatus by WR and CD2 reduces the efficiency of transcription. Further studies are required to clarify these two scenarios.

**The redundancy of WR and CD2:** While WR appears to lower the activity of CD2 as discussed above, it is clear that both are strong activators by themselves, thus, redundant at the C-terminus of nanog. One may argue that WR and CD2 may be able to substitute each other functionally, at least partially, as demonstrated in Fig. 5. Given the structural difference, we propose that WR and CD2 regulate transcription activation through distinct pathways. At the present, we have little evidence to support this idea. We have initiated a yeast-based strategy to identify binding partners for WR and CD2. Should different binding partners been identified, we would be able to define distinct pathways that Nanog regulate gene activation. Alternatively, we should be able to define the downstream genes using N3 or N4 as activators in a DNA chip based survey of downstream genes in either ES or P19 cells. Nevertheless, further studies are required to sort out the mechanism through which different domains of Nanog regulate the expression of genes critical to stem cell pluripotency.
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The abbreviations used are: ESC, embryonic stem cells; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; WR, W-repeat or tryptophan repeat; CD1, c-terminal domain 1; CD2, c-terminal domain 2; DBD, DNA binding domain.
Fig.1: Transactivation activities associated with the C-terminal Domain of Nanog:  

**A)** Division of the Nanog CD based on the W-repeat domain (underlined) into three subdomains.  

**B)** Schematic illustrations of fusion constructs between Gal4 DNA binding domain and the various sub-domains of Nanog CD, CD1, WR and CD2. The Flag tag was located at the N terminal of the Gal4 DBD for protein detection.  

**C)** Western blotting analysis of fusion constructs. HEK293 cells were transfected with control vector(lane1), Gal4 DNA binding domain (lane2), Gal4-CD1(lane3), Gal4-WR(lane5), Gal4-CD2(lane6), Gal4-C1W (lane7), Gal4-WC2(lane8) as described (13). Cell lysates were fractioned, blotted, and probed with anti-FLAG antibody. All constructs express proteins of the expected sizes.  

**D)** Transcription activities of fusion constructs. The reporter gene, p5xGal4-e1b-luciferase(0.1ug) were co-transfected with control vector (lane 1, v), Gal4 DBD (lane 2), Gal4-CD1 (lane 3), Gal4-WR (lane 4), Gal4-CD2 (lane 5), Gal4-C1W (lane 6), Gal4-WC2 (lane 7) and Gal4-VP16 (lane 8) with increasing doses (0.25ug,0.5ug,1ug respectively from left to right) into HEK 293 cells as described in “Materials and Methods”. Renilla plasmids (0.005ug) were co-transfected in each well as internal references. The luciferase activities for each transfection were assayed 36 hours post transfection using Dual-reporter assay systems (Promega, WI). The results were the average of two independent transfection experiments and the error bars indicated the standard deviation in duplicate assays.
Fig. 2: The WR and CD2 function in both pluripotent and non-pluripotent cells. The reporter p5Gal-e1b-luc plasmids (0.1ug each) were co-transfected with control vector (V), Gal4DBD (G), Gal4-CD1 (CD1), Gal4-WR (WR), Gal4-CD2 (CD2), Gal4-C1W (C1W), Gal4-WC2 (WC2) at two doses (0.5ug on the left, 1ug on the right) into mouse ES as indicated in (A), P19 (B) and NIH3T3 (C) cells respectively. Transfection efficiency were normalized by co-transfection of Renilla plasmid (0.005ug each well). Each transfection were carried out in duplicate and repeated at least two times. The luciferase activities were measured and analyzed as described (13).

Fig. 3: The WR is a potent transactivator. A: Schematic illustrations of the WR domain, mutants (W/A)x5 and (W/A)x10, S/A, N/A, and Q/A depicting the changes of residues. B: Western blot analysis of the constructs from A. HEK293T cells were transfected with control vector (lane 1), or plasmids carrying Gal4 DNA binding domain (lane 2), Gal4-WR (lane 3), Gal4-(W/A)x5 (lane 4), Gal4-(W/A)x10 (lane 5), Gal4-S/A (lane 6), Gal4-N/A (lane 7), Gal4-Q/A (lane 8). The cell lysates were probed with anti-Flag antibody and developed as described (13). C: Transcription activities for W-repeat and derivatives as listed in A in HEK293 cells. The same constructs (0.5 ug per construct) were co-transfected with p5xGal4-e1b-luc reporter (0.1 ug per transfection) and an internal reference in duplicate into HEK293T cells and the activities were measured and analyzed as described (13). D and E: The same transfections as described in C were carried out in P19 (D) and NIH3T3
Fig. 4: Nanog mediates transactivation through its cognate binding site.

A. EMSA of oligos containing Nanog consensus binding site (1N) or mutant binding site (1Nmu) with nuclear extracts (NE) of 293T cells transfected with NanF. NE of 293T cells transfected with NanF were incubated with double stranded 1N or 1Nmu labeled with $^{32}$P as indicated and analyzed by 5% non-denature polyacrylvide gel followed by autoradiography. End-labeled 1N were incubated with no NE or NE of 293T cells transfected with control vector as control (lanes 1 and 2). Specific binding was analyzed by competition with an approximately 100 fold excess of unlabeled 1N as indicated (lane 5). B. Construction of reporters containing Nanog binding sites. Annealed oligos containing Nanog binding site or mutant binding site were inserted upstream of a luciferase gene under the control of a minimal TK promoter. The copy numbers of the inserted binding site were determined by sequencing. C. Nanog can transactivate the reporter gene containing the wild type Nanog binding site, but not its mutant. 0.2ug reporters containing 2 copies(p2N), 4copies(p4N), and 5 copies(p5N) of Nanog binding site or 3 copies mutant binding site (p3Nmu) were co-transfected with the control vector (CK) or 0.5 ug of NanogF (N) plasmids as indicated to P19 cells in 24 well plate. Internal Reference and luciferase assay were as described in Fig. 1.

Fig. 5: Nanog mediates transactivation through WR and CD2. A:
Schematic illustration of Nanog reporters used in this study, pTK and p5N as described in Fig. 4.; B: Schematic illustration of deletion mutants of Nanog. A FLAG was fused to the C terminal of each deletion for protein detection as described in Materials and Methods; C: Western blot analysis of constructs of Nanog deletions in B. HEK 293T cells transfected with control vector (lane 1), N1 (lane 2), N3 (lane 3), N4 (lane 4), and NanogF (lane 5) were lysed and analyzed by western blotting using anti-flag antibody as described (13). D: Transcriptional activity of Nanog deletion in HEK293 cells, NIH3T3 cells, p19 and F9 cells. 0.2 ug of p5N plasmid was co-transfected with 0.5 ug of control vector (lane 1) or NanogF (lane 2), N1 (lane 3), N3 (lane 4), N4 (lane 5) as indicated in 24 well plates. The luciferase activities were determined and presented as described in Fig. 4.
Reference

1. Pan, G. J., Chang, Z. Y., Scholer, H. R., and Pei, D. (2002) *Cell Res* 12, 321-329

2. Audet, J. (2004) *Expert Opin Biol Ther* 4, 631-644

3. Constantinescu, S. (2003) *J Cell Mol Med* 7, 103-112

4. Koike, N., Fukumura, D., Gralla, O., Au, P., Schechner, J. S., and Jain, R. K. (2004) *Nature* 428, 138-139

5. Pesce, M., and Scholer, H. R. (2001) *Stem Cells* 19, 271-278

6. Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003) *Cell* 113, 643-655

7. Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003) *Cell* 113, 631-642

8. Niwa, H., Burdon, T., Chambers, I., and Smith, A. (1998) *Genes Dev* 12, 2048-2060

9. Guo, Y., Costa, R., Ramsey, H., Starnes, T., Vance, G., Robertson, K., Kelley, M., Reinbold, R., Scholer, H., and Hromas, R. (2002) *Proc Natl Acad Sci U S A* 99, 3663-3667

10. Niwa, H., Miyazaki, J., and Smith, A. G. (2000) *Nat Genet* 24, 372-376

11. Cavaleri, F., and Scholer, H. R. (2003) *Cell* 113, 551-552

12. Pesce, M., and Scholer, H. R. (2000) *Mol Reprod Dev* 55, 452-457

13. Pan, G. J., and Pei, D. Q. (2003) *Cell Res* 13, 499-502
14. Hart, A. H., Hartley, L., Ibrahim, M., and Robb, L. (2004) *Dev Dyn* **230**, 187-198

15. Pei, D. Q., and Shih, C. H. (1991) *Mol Cell Biol* **11**, 1480-1487

16. Pei, D. (1999) *Cell Res* **9**, 291-303

17. Pan, G., Qin, B., Liu, N., Scholer, H. R., and Pei, D. (2004) *J Biol Chem* **279**, 37013-37020

18. Niwa, H., Masui, S., Chambers, I., Smith, A. G., and Miyazaki, J. (2002) *Mol Cell Biol* **22**, 1526-1536
Fig. 1

A 156  KNQWL KTSNGLIQKG SAPVEYPSIH CSYPQGYLVN ASGSLSMWGS
201 QTWTNPTWSS QTWTNPTWNN QTWTNPTWSS QAWTAQSWNG QPWNAAPLHN
251 FGEDFLQFYV QLQQNFSASD LEVNLEATRE SHAHFSTPQA LEFLNYSVT
301 PPGEI

B nanog
Gal4-CD  
Gal4-CD1  
Gal4-WR  
Gal4-CD2  
Gal4-C1W  
Gal4-WC2  

CD

D

MW (kDa)

62  
33  
25  

1 2 3 4 5 6 7

1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21 22

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Fig. 2

A  ES cells  B  P19 cells  C  NIH3T3 cells
Fig. 3

A

WR   WGSQTWTPTWSSQTWNPTWNQTWWNQTWSPWSSQAQSWNGQPWNAAP
(W/A)x5  AGSQTWTPASSQTWNPTANNQTWNPASSQAQSWNGQPWNAAP
(W/A)x10  AGSQTATNPASSQTATNPANNQATNPASSQAATAQSWNGQPWNAAP
S/A   WGAQTWNPTWAATWNPTWNQTWTPWAQATWPWNGQPWNAAP
N/A   WGSQTWTAPTWSQTWNTPWTAPTWNQTWTAPTWSSQAWTASWNGQPWNAAP
Q/A   WGSATWNPWSSATWNPTWNNATWNPTWNNATWNPTWSSAWTASWNGQPWNAAP

Fig. 3 Pan et al

B

MW (kDa)  CK        Gal4        WR        (W/A)x5        (W/A)x10        S/A        N/A        Q/A
          33          25          -          -          -          -          -

C

HEK293 cells

Fold Activity vs. Treatment

D

P19 cells

Fold Activity vs. Treatment

E

NIH3T3 cells

Fold Activity vs. Treatment
Fig. 4

A

B

C

Fig. 4

A

B

C
Fig: 5

Pan et al

A

pTK

Tk

Luciferase

Nanog Binding Sites

p5N

Tk

Luciferase

B

nanog

N1

N3

N4

C

MW (kDa) CK N1 N3 N4 F

D

HEK293T

NIH3T3

P19

F9

fold activity

fold activity
The stem cell pluripotency factor nanog activates transcription with two unusually potent subdomains at its C-terminus
Guangjin Pan and Duanqing Pei

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