Role for Med12 in Regulation of Nanog and Nanog Target Genes

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Nanog, Oct4, and Sox2 form the core of a transcription factor network that maintains embryonic stem cells in the pluripotent state in both humans and mice. These critical factors have been implicated as both positive and negative regulators of transcription, varying by promoter and differentiation state of the cell. The Mediator complex, a ubiquitous conserved complex of ~30 subunits, facilitates transcription by coordinating RNA polymerase II binding to target promoters via gene-specific activators and can be divided into several functional subcomplexes. Med12 is part of a subcomplex of four proteins associated with the core Mediator complex and has been found to function both in repressing and activating transcription when recruited to target promoters. We identified an interaction between Med12 and Nanog and present evidence of involvement of Med12 in regulation of Nanog function. Gene expression analysis of embryonic stem (ES) cell pluripotency is governed by a complex transcription network, the core of which includes the stem cell-specific factors Nanog, Oct4, and Sox2 (1, 2). In addition to promoting their own expression, these factors are required for the trans expression of a number of additional ES cell-specific factors (1, 2). Nanog, Oct4, and Sox2 are also bound to the promoters of cell lineage-specific genes, which are maintained in a repressed state in ES cells until differentiation is induced and which become derepressed at carefully defined stages of embryonic development. Small interfering RNA (siRNA)-mediated knockdown of Nanog or Oct4 initiates expression from these repressed targets, suggesting that these core pluripotency factors actively participate in gene repression (1). This notion is supported by the observation that many developmentally repressed genes that are occupied by Nanog, Oct4, and Sox2 are also occupied by PRC2 (Polycomb repressive complex 2), which includes the histone methyltransferase Suz12 (3).

Maintenance of pluripotency by Nanog, Oct4, and Sox2 is likely a bimodal mechanism, requiring both activation and repression of target genes. Similar dual mechanisms are seen in a number of developmental signaling pathways. Gli2, a downstream effector of the Sonic Hedgehog signaling pathway, contains both positive and negative transcriptional regulatory domains (4). In addition, the transcriptional co-activator CBP (cAMP-responsive element-binding protein-binding protein)/p300 has been shown to both positively and negatively regulate the Wnt signaling pathway (5). Thus far, the means by which Nanog, Oct4, and Sox2 exert their positive and negative influences on transcriptional programs remains unclear. Recently, it was shown that CBP/p300 is recruited to sequences bound by clusters of transcription factors that include Nanog, Oct4, and Sox2 and resemble transcription enhanceosomes (6). This finding may provide a clue to the mechanism by which Nanog, Oct4, and Sox2 can function in an activator context.

In an effort to further understand the mechanisms regulating ES cell pluripotency, we utilized the yeast two-hybrid screening method to identify interacting partners of Nanog, Oct4, and Sox2. One intriguing hit from a screen using Nanog as bait was a subunit of the Mediator complex, Med12. The Mediator complex is a large multisubunit complex that links gene-specific transcriptional activators with the RNA polymerase II (RNA-PII) general transcription machinery (for review, see Ref. 7). It consists of a highly conserved core of 20–25 subunits and also associates with other distinct stable subcomplexes. One such subcomplex is composed of Med12 (TRAP230), Med13 (TRAP240), Cdk8 (SRB10), and CycC (SRB11) (for review, see Ref. 8). This subcomplex is involved in regulating distinct gene expression programs during development. In Drosophila, the homologs of Med12 and Med13, Kohtalo and Blind Spot, play a critical role in eye development (9). In addition, the homolog of Med12 acts as a co-activator for Sox9-dependent neural crest development in zebrafish (10) and is required for asymmetric
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cell division in *Caenorhabditis elegans* (11). Recent work has also identified a role for Med12 in endoderm development in zebrafish (12). The involvement of Med12 in embryonic development suggests that Med12 could also play a role in ES cell regulation.

Although the core Mediator complex is thought to be universally required at all RNAPII promoters, the subcomplexes that associate with it may confer distinct regulatory functions to a subset of promoters depending on their interaction with specific activators. In addition, there is evidence that the Med12-Med13-Cdk8-CycC subcomplex may be involved in both positive and negative transcriptional regulation of promoters to which this subcomplex is targeted (9, 13–16). The Med12 subcomplex has been thought to prohibit the recruitment of RNAPII by the Mediator complex (17); however, Med12 positively regulates Wnt-responsive genes via interaction with β-catenin (18, 19). The proposed ability of the Med12 subcomplex to both positively and negatively regulate gene expression makes it a potential regulatory cofactor by which Nanog may exert its dual regulation.

In addition to confirming the interaction between Med12 and Nanog, we sought to determine whether Med12 is essential for Nanog signaling and maintenance of ES cell pluripotency. siRNA-mediated knockdown of Med12 had a similar effect on Nanog target promoters as knockdown of Nanog, suggesting that Med12 may play a unique role within the Nanog transcription network. Moreover, we found that Med12 and Nanog co-occupied many Nanog target promoters in ES cells and that they dissociated from these promoters upon differentiation. Collectively, our data suggest that Med12 and Nanog function together to regulate Nanog target gene promoters.

**EXPERIMENTAL PROCEDURES**

**ES Cell Culture**—Pluripotent murine ES cells (J1 or 129/B6) were maintained on gelatin-coated plates in Knockout™ Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 15% fetal bovine serum, 10 mM HEPES (Invitrogen), 100 U/ml penicillin/streptomycin (Invitrogen), 1 mM L-glutamine (Invitrogen), 100 μM β-mercaptoethanol (Sigma). Cells were grown in a humidified incubator at 37 °C and 5% CO2 and were routinely passaged at 1:3. 129/B6 cells expressing full-length Nanog and Med12, whole cell lysates were prepared in 150 mM NaCl, 100 mM Tris (pH 8.0), and 0.1% Nonidet P-40. No cross-linking agent was added to the 293T lysates. 150–200 μg of nuclear ES cell lysate or 293T lysate was incubated overnight with 8 μg of either anti-Med12 antibody (A300-774A, Bethyl Laboratories) or a rabbit IgG control antibody (R&D Systems) at 4 °C. Antibodies were precipitated with protein G-agarose beads (Invitrogen), and proteins were eluted using 1 × lithium dodecyl sulfate sample buffer (Invitrogen) for analysis by Western blotting.

**Western Analysis**—For Western analysis, antibodies to Stat3 (sc-7179, Santa Cruz Biotechnology), Stat3 phospho-Tyr705 (ab30646, Abcam), and α-tubulin (CP06, Calbiochem) were used at 1:5000 dilution. Antibodies to Nanog (AB5731, Chemicon), Med12 (A300-774A, Bethyl Laboratories), Oct4 (ab19857, Abcam), and Sox2 (AB5603, Chemicon) were used at 1:2000 dilution. Antibodies to histone H3 (07-690, Upstate) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-32233, Santa Cruz Biotechnology) were used at 1:16,000 dilution.

**RNA Interference**—A mixture of four siRNA duplexes (SMARTpool, Dharmacon) targeting Med12, Nanog, Oct4, and Sox2, or single siRNA control duplexes targeting lamin or GL3 luciferase (Dharmacon) were diluted in Opti-MEM I (Invitrogen) to a working concentration of 600 nM. The transfection reagent DharmaFECT 4 (Dharmacon) was separately diluted in Opti-MEM I to 7–14 μl/ml. Equal volumes of these working reagents were combined and added to gelatin-coated tissue culture plates (250 μl/well of a 12-well plate). Reagents were incubated for 30 min at room temperature to allow siRNA-lipid complexes to form. During this time, cells were trypsinized and resuspended to 40,000 cells/ml in normal ES cell medium. 1.2 ml of this cell suspension was added to the transfection reagents. siRNAs were at a final concentration of 50 nM, and transfection reagents were at 0.59–1.17 μl/ml. Plates were incubated at 37 °C and 5% CO2 for 24–96 h, and cells were pelleted and lysed for RNA extraction or Western analysis.

**Quantitative Real-time PCR**—Total ES cell RNA was purified with the RNAeasy miniprep kit (Qiagen Inc.), DNase-treated (RQ1, Promega), and reverse-transcribed (iScript, Bio-Rad).
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Quantitative PCRs (qPCRs) were performed using 20 ng of cDNA, 1× TaqMan gene expression assay, and 1× TaqMan Fast Universal Mastermix (Applied Biosystems) in an ABI7500 fast real-time PCR system according to the manufacturer’s instructions. TaqMan gene expression assays were purchased for Med12 (Mm00804032_m1), Oct4 (Mm00658129_gH), Sox2 (Mm00488369_s1), REST (Mm00832686_m1), Dkk1 (Mm00438422_m1), FoxH1 (Mm00514851_m1), Trp53 (Mm0041964_g1), Cdx2 (Mm00432449_m1), Cldn4 (Mm00515514_s1), Bmp4 (Mm00432087_m1), Nefl (Mm01315666_m1), GAPDH (Mm99999915_g1), Pgk1 (Mm00435617_m1), and Stat3 (Mm00456961_m1). Upon initiation of these studies, a TaqMan assay for Nanog was unavailable, so a custom TaqMan assay was created: forward primer, 5’-TCTCGGCTTCTCTCTGAA-3’; reverse primer, 5’-CAGGACTTGAAGCTTTTGTGTTGG-3’; and reporter sequence, 5’-CAGCCTGTATTCTTCT-3’. Results were subsequently confirmed with Nanog TaqMan assay Mm02384862_g1. Average threshold values (C_T) from two to three PCRs were determined for target genes, and these values were normalized to average GAPDH values (ΔΔC_T). Changes in gene expression were calculated as -fold change compared with control samples using the comparative ΔΔC_T method. To determine the significance of differences between cells treated with target siRNAs and lamin siRNA, GraphPad Prism was used to conduct a one-way analysis of variance, followed by post-hoc Tukey’s test. The p value used for significance in Fig. 2 is <0.001, with n = 2; that for Fig. 3 is <0.001, with n = 2; and that for Fig. 4 is <0.05, with n = 3 except where not significant, which is noted in the figure.

Microarray Analysis—Affymetrix Mouse Genome 430 2.0 expression arrays were probed with cDNA synthesized from total RNA isolated from ES cells treated for 96 h with siRNA oligonucleotides as described above. Data analysis was done using Bioconductor Version 2.3. Data QC of the CEL files was performed through box plots, MA plots, RNA degradation plots, and visual inspection of the pseudo-image. Data normalization was done using the MAS5 algorithm, and one-way analysis of variance analysis was done on predefined sample groups, with n = 3. The heat map was generated using z-scores across all samples. For the heat maps, genes whose expression was changed ≥2.0-fold relative to lamin siRNA-treated cells were sorted in order of fold-change.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation (ChIP) was performed using 2 × 10⁷ ES cells as described in the on-line protocol provided by Millipore. Purified DNA was amplified with primers spanning promoter regions as indicated using SYBR PCR Mastermix (Applied Biosystems). Relative enrichment was calculated as 2^(-ΔΔC_T(control ChIP) - ΔC_T(experimental ChIP)), where ΔC_T is equal to the C_T(immunoprecipitated sample) - C_T(input). For normalization to the Pgk1 promoter, the relative enrichment for each triPLICATE sample was divided by the relative enrichment for the Pgk1 promoter. Graphs represent the average of three triPLICATE reactions, with error bars marking S.D. Anti-Nanog antibody was from Cosmo Bio Co. (REC-RCAB0002P-F). We designed primers for qPCR based on genomic locations published as regions where Oct4, Nanog, or Sox2 was bound (1). The primers for the Pgk1 promoter (which does not contain any Oct4/Nanog/Sox2 consensus sequences) overlap the transcription start site and produce a 225-bp amplicon. The primers for the Bmp4 promoter overlap the Nanog-binding site at –5.5 kb from the transcription start site and produce a 250-bp amplicon. The primers for the Neff promoter overlap the Nanog-binding site at –17 kb from the transcription start site and produce a 250-bp amplicon. The primers for the Nanog promoter overlap the Oct4/Nanog/Sox2 consensus site located at –0.2 kb from the transcription start site as well as 39 bp of the first exon and produce a 289-bp amplicon. The primers for the REST promoter overlap the Oct4/Nanog/Sox2 consensus site located at –3.1 kb from the transcription start site and produce a 250-bp amplicon. The primers for the Dkk1 promoter overlap the Oct4/Nanog/Sox2 consensus site at –4.4 kb from the transcription start site and produce a 250-bp amplicon. The primers were designed for qPCR based on genomic locations published as regions where Oct4, Nanog, or Sox2 was bound (1).

RESULTS

Med12 Interacts with Nanog—To identify proteins that interact with the core pluripotency factors, we performed yeast two-hybrid screens against a murine ES cell library using murine Nanog, Oct4, and Sox2 as bait in separate screens. As full-length Nanog and Sox2 self-activated, N- and C-terminal portions of the proteins were used for the screen. Among the interactors identified using Nanog and Sox2 as bait was Med12, a conserved component of the Mediator complex. Interaction of Med12 with Nanog and Sox2 mapped to the C-terminal domains of Nanog and Sox2, corresponding to previously mapped activation domains (20, 21). We confirmed the interaction between Nanog and Med12 in ES cells by co-immunoprecipitation, with Nanog protein co-precipitating with anti-Med12 antibody (Fig. 1A). This interaction was detectable only after cross-linking with DSP, indicating that the interaction may be transient or too unstable to survive the immunoprecipitation procedure. The cross-linking did not seem to increase nonspecific interactions, as histone H3, which was present in the lysate at levels similar to Nanog, did not co-precipitate with anti-Med12 antibody. To further validate this interaction, we expressed full-length Nanog and Med12 by transient transfections in 293T cells. Nanog co-precipitated with anti-Med12 antibody, and DSP was not required to detect this interaction (Fig. 1B). This result also indicates that no additional ES cell proteins, such as Sox2 and Oct4, are required to mediate the interaction between Nanog and Med12. However, we were unable to confirm an interaction between Med12 and Sox2 in ES cells through co-immunoprecipitation.
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Med12 Is Required for Nanog Expression and ES Cell Pluripotency—Given the role of Med12 as part of a transcriptional cofactor subcomplex, we asked whether it functions in regulating the expression of Nanog or Nanog target genes. To do this, we first utilized siRNA-mediated knockdown to reduce levels of Med12 in murine ES cells. 24 h after siRNA treatment of 129/B6 or J1 ES cells, Med12 transcript levels were reduced by 80–90% relative to a lamin siRNA control (data not shown), and these levels were maintained through 96 h as determined by qPCR (Fig. 2A). Med12 protein was also reduced by at least 80% at 96 h (Fig. 2B). qPCR analysis of Nanog transcript levels in J1 and 129/B6 cells revealed that Med12 knockdown reduced Nanog transcripts by 55–65% at 96 h (Fig. 2A). Nanog protein levels were also similarly reduced upon knockdown of Med12 (Fig. 2B), indicating that Med12 is required for Nanog expression in ES cells. Knockdown of Med12 also caused the cells to undergo differentiation, as shown by the increase in markers of primitive ectoderm (Fgf5) and mesoderm (Hand1) (Fig. 2C). The expression patterns and levels of these lineage markers closely mimic those induced by Nanog siRNA and show less similarity to Sox2 siRNA knockdown, indicating that the major role for Med12 in ES cell pluripotency may be the maintenance of Nanog and/or Nanog target genes.

To determine whether Med12 has a role in pluripotency outside of maintaining Nanog levels, we knocked down Med12 expression in cells that express Nanog from a doxycycline-inducible system. In the presence of doxycycline, the levels of Nanog protein were maintained in the absence of LIF (Fig. 2D), and this was sufficient to rescue the expression of the pluripotency factors Oct4, Sox2, and Rex1 from LIF withdrawal (Fig. 2E). Although the milder reduction of Nanog protein levels resulting from Med12 knockdown was also rescued by doxycycline, the corresponding reduction of pluripotency factors was unaffected. This suggests that Med12 has a role in addition to maintaining Nanog expression and may be required for Nanog activity at its target genes.

Med12 Regulates Nanog Independently of Stat3 Signaling—Although direct transcriptional regulation of Nanog expression by Med12 would be the most likely mechanism, we could not discount the possibility that Med12 functions through a more peripheral pathway. To gain further insight into the mechanism by which Med12 regulates Nanog, we sought to examine signaling pathways critical to Nanog expression. The LIF signaling pathway, known to play a critical role in maintaining ES cell pluripotency in murine ES cells, facilitates Stat3 phosphorylation upon interaction with the activated LIF receptor/gp130 heterodimer (22, 23). This leads to activated Stat3, which dimerizes and localizes to the nucleus (22), where it binds to the Nanog enhancer and critically regulates Nanog gene expression (24). The Wnt/β-catenin signaling pathway is also critical for Nanog expression and ES cell pluripotency, by cooperating with the LIF pathway to up-regulate Stat3 (25). As Med12 interacts directly with β-catenin to up-regulate targets of Wnt/β-catenin signaling (18), we sought to determine whether the effect of Med12 knockdown on Nanog expression is the result of Stat3 down-regulation. 129/B6 or J1 ES cells were grown in the absence (Fig. 3A) or presence (Fig. 3B) of 125 nM RA and analyzed for Stat3 expression following knockdown of Med12, Nanog, or Sox2. We used lamin control siRNA in the absence of LIF as a positive control for reduced Stat3 signaling. Knockdown of Med12 or Nanog in the absence of RA reduced Stat3 transcript levels by <30%, whereas in the presence of RA, Stat3 levels were actually elevated. In contrast, removal of LIF from the culture medium reduced Stat3 expression by >90%. Next, we assessed by Western analysis whether Stat3 protein levels or activation (phospho-Tyr705) was decreased under the same conditions (Fig. 3C). There was little variation in Stat3 protein expression or phosphorylation following knockdown of Med12 or Nanog, whereas both total and phospho-Tyr705 Stat3 protein levels were drastically reduced upon LIF withdrawal. Thus, the reduction of Nanog levels following knockdown of Med12 is not due to impaired Stat3 signaling.

Med12 Regulates Nanog Target Promoters but Not Oct4/Sox2 Target Promoters—Nanog positively regulates its own transcription and controls expression of its target genes via binding to a specific DNA recognition sequence (1). These Nanog targets comprise both actively transcribed and repressed genes in ES cells (1, 2). The distribution of active versus repressed Nanog targets tightly correlates with whether those genes function in ES cell maintenance, in which case they are likely to be actively transcribed, or in lineage-specific differentiation pathways, in which case they are more likely to be repressed. This suggests that Nanog can function as either an activator or a repressor, depending on the context. We wanted to determine how Med12 might play a role in the transcriptional activities of Nanog, so we chose six different classes of target genes and
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A, lamin or Med12 siRNA was transfected into 129/B6 or J1 ES cells, and samples were harvested after 96 h for qPCR analysis of Med12 and Nanog expression. Gene expression is indicated relative to lamin (control) siRNA-treated cells. B, shown are the results from Western blot (WB) analysis of Med12 and Nanog protein levels in 129/B6 and J1 ES cells following siRNA-mediated knockdown of Med12 as described for A. Protein levels were normalized to tubulin and are indicated relative to lamin (control) siRNA-treated cells. C, shown are the results from qPCR analysis of ES cell pluripotency and differentiation markers upon knockdown of Med12. D, shown are the results from Western analysis of Nanog protein levels. D, shown are the results from qPCR analysis of ES cell pluripotency and differentiation markers upon knockdown of Med12. E, shown are the results from qPCR analysis of ES cell pluripotency and differentiation markers upon knockdown of Med12 or withdrawal of LIF. Gene expression is indicated relative to lamin (control) siRNA-treated cells. The + doxycycline rescue of LIF withdrawal is significant (p < 0.001), whereas the + doxycycline rescue of Med12 knockdown (KD) is not significant.

 examined the effect of knocking down Nanog, Oct4, Sox2, and Med12 on the expression of these different groups of genes.

The first class of genes we examined was Nanog target genes that are positively regulated by all three core ES cell factors, Nanog, Oct4, and Sox2. We examined Oct4, Nanog, and Sox2 themselves, as well as REST (1), and predicted that siRNA-mediated knockdown of any of the positive regulatory factors would cause a corresponding decrease in these transcript levels. The knockdown efficiency for each direct siRNA target was at least 80% (Fig. 4A). As expected, knockdown of Oct4, independently of Nanog. To test this possibility, we examined genes that are positively or negatively regulated by Oct4 and Sox2 but are independent of Nanog regulation (1). We chose four genes: FoxH1 and Trp53, known positive targets of Oct4/Sox2 but not Nanog, and Cdx2 and Cldn4, known negative targets of Oct4/Sox2 but not Nanog. As predicted, knockdown of either Oct4 or Sox2 resulted in a corresponding decrease in FoxH1 and Trp53 transcripts (Fig. 4C) and an increase in Cdx2 and Cldn4 transcripts (Fig. 4D). By contrast, knockdown of Nanog or Med12 resulted in minimal...
changes in the expression level of any of these genes, again supporting a unique role for Med12 in gene regulation by Nanog.

We also asked whether knockdown of Med12 would have any effect on Nanog targets that lack Oct4/Sox2-binding sites. For this, we chose two target genes: Bmp4, which is positively regulated by Nanog, and Nefl, which is a negative target of Nanog (1). Knockdown of Nanog caused a modest (~35%) reduction in Bmp4 transcript levels (Fig. 4E), as did knockdown of Med12 and Sox2, whereas knockdown of Oct4 resulted in increased Bmp4 transcripts. Nefl expression increased upon knockdown of Nanog (Fig. 4F), consistent with it being a negative target of Nanog; however, Med12 knockdown decreased Nefl expression. It is possible that at the Nefl promoter, Med12 and Nanog function in a manner independent of each other and that Nanog represses Nefl independently of Med12. Nonetheless, the gene expression data suggest overall a linked role of Nanog and Med12 in regulating Nanog targets, whereas Oct4/Sox2 targets that are not regulated by Nanog appear to be regulated independently of Med12.
Promoters in Proliferating ES Cells—Because the Nanog
transcriptional cofactor, we asked whether Nanog and Med12 co-occupy the promoters of Nanog target genes under proliferating conditions and whether they are lost as these genes become activated or silenced upon differentiation. To efficiently differentiate murine ES cells into a neuro-epithelial lineage (27), LIF was withdrawn from the medium, and 5 
M RA was added. After 5 days, differentiation of ES cells approached 100%, as assessed by morphological changes and the disappearance of factors critical to ES cell maintenance, such as Nanog, Oct4, and Sox2 (Fig. 6A) (23). By contrast, Med12 expression increased ~3.5-fold upon differentiation, and this correlated with an increase in protein abundance (Fig. 6A). We next performed ChIP on extracts from ES cells cultured under proliferation or differentiation culture conditions for 1 and 5 days to assess Nanog and Med12 association at various Nanog target promoters (Fig. 6, B–G). As a control, we performed ChIP on the Pgk1 promoter, which lacks binding sites for Nanog, Oct4, and Sox2 (Fig. 6B) and whose expression remains constant upon differentiation (Fig. 6A). We normalized the enrichment of Med12 and Nanog at target promoters to the relative levels at the Pgk1 promoter. Remarkably, for every ChIP primer set tested on Nanog target promoters, both Nanog and Med12 were found to be highly enriched under proliferation conditions and followed a time-dependent decrease in abundance following induction of differentiation independent of whether the promoters are positive targets (Bmp4, Nanog, and REST) or negative targets (Nefl and Dkk1) of Nanog. Thus, Med12 and Nanog are present at Nanog target promoters in proliferating ES cells and dissociate together upon differentiation, and this further supports the model in which Nanog and Med12 co-regulate Nanog target gene expression.

**DISCUSSION**

Although recent evidence supports both positive and negative transcriptional regulation by Nanog in the maintenance of ES cell pluripotency, no direct mechanism for this dual role has yet been revealed. The data presented here provide the first functional link between Nanog and Med12 at Nanog target promoters and extend the role of Med12 in developmental processes to ES cell regulation. We confirmed biochemically the interaction between Med12 and Nanog identified in the yeast two-hybrid screen, although the cross-linking agent DSP was required to reliably detect the interaction in ES cells. This suggests that the interaction between these factors may be transient, consistent with the previously proposed model for the Med12 subcomplex, whereby it may be released upon recruitment of the Mediator core complex to a target promoter and initiation of transcription (7). A similar stabilization of protein interaction between Nanog and Oct4 has also been described previously, despite evidence for widespread association of Nanog and Oct4 by genome-wide ChIP techniques (28). It is likely that the inability to detect Med12 in Nanog immunoprecipitates is due to the low Med12 expression relative to Nanog in ES cells, and this may also explain why Med12 was not previously identified as part of the Nanog protein interaction network (29). Alternatively, the anti-Nanog antibody that was used for the immunoprecipitations may disrupt the Nanog-Med12 interaction, as this could also explain the lack of Med12 co-precipitation with anti-Nanog antibody seen in 293T cells.

**Microarray Analysis Reveals Substantial Overlap in Nanog and Med12 Target Genes**—Given the striking pattern of similarities in Nanog target gene activity following knockdown of either Nanog or Med12, we performed a genome-wide expression microarray analysis following knockdown of Nanog or med12 (Fig. 5 and supplemental Table S1). We used Affymetrix mouse genome arrays representing ~39,000 murine genes and probed each array with total cDNA from lamin, Med12, or Nanog siRNA-treated cells. Microarray analysis confirmed our results from individual qPCR analyses (Fig. 4) and revealed additional Med12 regulation of Nanog targets not previously examined, such as Eomes (a Nanog negatively regulated target) and Lef1 (a Nanog positively regulated target). Clustering of genes with a >2-fold change in expression relative to lamin knockdown shows similar global changes in gene expression following Nanog or Med12 knockdown (Fig. 5A). Analysis revealed that 43% of all genes whose expression was changed by 2-fold or greater in Nanog siRNA-treated cells also overlapped with those from Med12 siRNA-treated cells (Fig. 5B). In contrast, only 25% of genes with a >2-fold change following Med12 knockdown overlapped with those following Nanog knockdown (Fig. 5B), illustrating additional roles for Med12 independent of Nanog, consistent with its broader role as a transcriptional cofactor.

**Med12 Is Bound to the Nanog Promoter and Nanog Target Promoters in Proliferating ES Cells**—Because the Nanog-Med12 interaction data, the strong correlation of gene expression upon knockdown of either nanog or Med12, and the lack of pluripotency factor rescue by constitutive Nanog expression in Med12 knockdown cells support a role for Med12 as a Nanog

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**FIGURE 5. Overlapping changes in global gene expression following Nanog or Med12 knockdown.** Cells were transfected with siRNA against lamin, Med12, or Nanog for 96 h in triplicate wells, and total cDNA was used to probe the arrays. A, heat map showing clustering of genes whose -fold change (FC) was >2.0 following knockdown of Nanog or Med12. Genes were sorted by -fold change relative to lamin. B, Venn diagrams showing overlap of Med12 and Nanog target genes. Upper, genes whose expression was increased >2-fold following knockdown; lower, genes whose expression was decreased >2.0-fold following knockdown.

**A**

**B**

Positive Overlap (FC>2.0)

Nanog

Med12

313

363

705

Negative Overlap (FC>2.0)

Nanog

Med12

516

279

1161
The 293T cell results also indicate that no additional ES cell-specific proteins (such as Sox2 and Oct4) are required for the Nanog-Med12 interaction. To further illustrate a direct interaction between Nanog and Med12, we attempted pulldowns using recombinant Nanog and deletion fragments of Med12 from *in vitro* transcription-translation reactions. No interaction was seen between Nanog and full-length Med12 fragments, indicating that full-length Med12 may be required for this interaction. Regardless, the co-occupancy of Nanog target promoters and the synchronized release of both Nanog and Med12 during differentiation suggest that these proteins function together, further supporting the interaction results.

Knockdown of Med12 in ES cells reduced Nanog transcript and protein levels. Med12 knockdown was also sufficient to induce differentiation into primitive ectoderm and mesoderm, resulting in lineage marker expression that resembled Nanog knockdown but was distinct from Sox2 knockdown or LIF withdrawal. This suggests that the dominant function of Med12 in ES cells is to maintain Nanog expression and/or support Nanog target gene regulation. This is strengthened by the observation that genes with Nanog bound at their promoters are affected by knockdown of Med12, whereas Oct4/Sox2 target genes are largely unaffected by Med12 knockdown. We extended this observation with microarray analysis of gene expression changes following either Nanog or Med12 knockdown. This analysis corroborated our qPCR analysis of Nanog target genes and also revealed additional Nanog targets not previously examined by qPCR analysis, such as Lef1 and Eomes (1), whose expression is similarly affected by knockdown of either Med12 or Nanog. On a global scale, upon Nanog knockdown, 53% of the Nanog-repressed targets and 35% of the Nanog-activated targets responded similarly to knockdown of either Med12 or Nanog. In contrast, only 33% of Med12-repressed targets and 19% of Med12-stimulated targets overlap with Nanog, illustrating additional Nanog-independent roles for Med12. The data also clearly demonstrate that knockdown of Med12 is not merely interfering with global transcription by disruption of a general transcription requirement and that there is a level of...
cooperation between Med12 and Nanog that helps refine which genes are expressed in ES cells under different cellular contexts, the timing of which is critical at early stages of development.

As it may be postulated that the effect of Med12 knockdown on transcription of Nanog targets is merely the indirect consequence of reducing Nanog protein levels, we utilized cells expressing Nanog from the doxycycline-inducible system. Maintenance of Nanog expression was insufficient to rescue expression of pluripotency factors from Med12 knockdown, suggesting a direct role for Med12 in the regulation of Nanog target genes.

Under proliferating culture conditions, Med12 and Nanog were found to co-occupy Nanog target genes, and their presence at each target promoter was independent of whether the promoter is negatively or positively regulated by Nanog. As cells underwent differentiation, the levels of Nanog and Med12 at each target promoter decreased in a time-dependent manner. Although it is possible that reduced Nanog levels at target promoters following differentiation is merely a reflection of the decrease in global Nanog expression, this cannot explain the concurrent loss of Med12 at these promoters, as global Med12 levels increased by ~3.5-fold in differentiated cells. Rather, Med12 is removed from Nanog target promoters during differentiation, and we postulate that its removal is tied to the loss of Nanog from these promoters. This co-occupation further supports a cofactor role for Med12 in Nanog target genes.

Like Nanog, previous reports have demonstrated both positive and negative regulatory roles for the Med12-containing Mediator subcomplex. For example, it has been shown by electron microscopy that the Med12-Med13-Cdk8-CycC subcomplex occludes Mediator interaction with RNAPII (17), consistent with a role for this subcomplex in transcriptional repression. By contrast, Med12 has been shown to be recruited to β-catenin-responsive promoters in a β-catenin-dependent manner to activate transcription in response to Wnt signaling (18). As knockdown of Med12 results in both up-regulation and down-regulation of gene expression, we propose that Med12 mediates the dual role of gene regulation by Nanog. However, other possibilities exist, such as Med12 is required for the expression of additional downstream gene regulators. In addition, although we showed that reduction of Nanog levels by Med12 knockdown is not the result of perturbation of activated Stat3, this does not eliminate the possibility that knockdown of Med12 affects cross-talk between other pathways regulating Nanog.

Both Nanog and Med12 have roles in development, and mutations in Med12 have been linked to a number of diseases, including Lujan and Opitz-Kaveggia syndromes (30, 31). These X-linked mental retardation diseases are also associated with craniofacial and digestive system disorders. Recent findings suggest that Med12 has a role in endoderm development (12) and that this may be the cause of these disease phenotypes. It would be interesting to determine whether these Med12 mutations would inhibit the Nanog-Med12 interaction and the recruitment of Med12 to Nanog target genes, possibly identifying an underlying mechanism in these developmental disorders. Further studies in model organisms may shed light on these questions.

An additional subunit of the Med12-Med13-Cdk8-CycC subcomplex, Cdk8, was found at Nanog target promoters along with Nanog and Med12, and it also dissociated upon 5 days of differentiation (data not shown), suggesting that the entire subcomplex is present at these promoters. siRNA-mediated knockdown of Cdk8 gave similar but milder phenotypes as Med12 knockdown (data not shown), also supporting a role for the entire subcomplex in Med12-mediated gene expression. However, these results were difficult to interpret due to siRNA-induced cell death, possibly due to additional roles of the Cdk8-CycC complex. Further studies will be needed to determine whether components of other Mediator subcomplexes are present at Nanog target genes in ES cells. It will also be interesting to determine whether other Mediator subcomplexes are involved in Oct4 or Sox2 signaling and whether they further demonstrate the bimodal mechanism of transcriptional activation and repression common to both Nanog and Med12.

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