Published in final edited form as:
Mol Cell Endocrinol. 2011 January 30; 332(1-2): 116–124. doi:10.1016/j.mce.2010.10.003.

LRH-1 AND NANOG REGULATE DAX1 TRANSCRIPTION IN MOUSE EMBRYONIC STEM CELLS

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Abstract

Dax1, an atypical orphan nuclear receptor expressed in steroidogenic tissues, has recently been shown to be expressed in mouse embryonic stem (mES) cells and is required for pluripotency. While the mechanisms of transcriptional regulation of Dax1 in steroidogenic organs have been well characterized, those in mES cells have not. Here we report that only 500 bp of the Dax1 gene promoter sequence are needed to drive expression in mES cells. In steroidogenic tissues, NR5A1 (Sf1) binds to nuclear receptor binding sites within this sequence to regulate Dax1 expression. In mES cells, while NR5A1 (Sf1) is not expressed, NR5A2 (LRH-1) expression is robust. Luciferase assays, EMSA and overexpression/knockdown studies demonstrate that LRH-1 binds the −128 site and regulates Dax1 in mES cells. Predicated on recent work indicating that Nanog binds to the Dax1 intron, we have used chromatin immunoprecipitation experiments (ChIP) to define an intronic site that is bound by Nanog. Overexpression and knockdown of Nanog in mES cells result in alteration of Dax1 expression, and luciferase assays reveal that this sequence can enhance transcription of a Dax1 reporter construct. These data indicate that LRH-1 and Nanog cooperate to regulate Dax1 expression in mES cells.

1. Introduction

Mouse embryonic stem (mES) cells are the cells derived from the inner cell mass of a blastocyst that give rise to all the differentiated tissues of an organism. The molecular mechanisms by which these cells maintain their undifferentiated state have been studied extensively, and it has become clear that several transcription factors including STAT3, Nanog, Oct4 and Sox2 are critical proteins in a network that maintains pluripotency (reviewed in (1)). As each of these factors is absolutely necessary for self renewal and pluripotency in mES cells, multiple potentially redundant mechanisms are predicted to contribute to their expression. For example, expression of Oct4 has been shown to be regulated by at least eight factors, including Oct4 itself (reviewed in (1)). Defining the transcriptional mechanisms that control expression of these factors is essential for our understanding of the biology of pluripotency of mES cells.

Dax1 is a nuclear receptor recently found to be involved in mES cell biology. Dax1 is expressed in the steroidogenic organs of the adult animal where it functions as both a

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transcriptional repressor and activator to maintain steroidogenic homeostasis (reviewed in (2)) (3). Recently Dax1 was found to be expressed in mES cells, and it was shown that knockdown of Dax1 in mES cells results in differentiation (4–5). Moreover, whole genome binding studies in mES cells have shown that Dax1 is bound to thousands of sites throughout the genome (6). Furthermore, Sun *et al* have shown that Dax1 binds directly to Oct4 in mES cells to prevent Oct4 activation of genes (7). Thus, Dax1 plays a significant role in mES cell biology that remains to be fully elucidated.

In steroidogenic cells, the mechanisms that control Dax1 transcription have been well characterized. Steroidogenic factor 1 (Sf1) has been shown to bind to two sites within the proximal promoter of Dax1 (8–10). The Wnt pathway transcriptional mediator, β-catenin, in complex with Sf1 activates Dax1 transcription, and indeed Wnt4 knockout mice have reduced expression of Dax1 in the female gonad (11). Finally, we have shown that the adrenal glucocorticoids, through a glucocorticoid receptor (GR)/ Sf1 complex also stimulate Dax1 expression in adrenocortical cells (12).

In mES cells, STAT3 and Oct4 regulate Dax1 expression through a STAT3 site at −158 in the proximal promoter, and an Oct4/Sox2 dual site in the intron at +2054/+2063 (13). However, mutations in these sites do not result in complete loss of promoter activity, indicating that other sites are important in Dax1 regulation. While the Wnt/β-catenin pathway has also been shown to regulate Dax1 expression in mES cells, the importance of this mechanism for Dax1 expression has not been clarified (14). Here we report that a site in the Dax1 proximal promoter is critical for regulation of Dax1 expression, as mutation of this site results in complete loss of expression. We show that this site is bound and regulated by LRH-1. Additionally, based on previous data that indicated Nanog binding to the Dax1 intron, we have characterized a novel Nanog binding site that cooperates with the LRH-1 binding site to mediate Dax1 expression in mES cells (15). This study positions Dax1 centrally in the protein network controlling mES cell pluripotency and predicts additional layers of regulatory control that remain to be determined.

2. Materials and methods

2.1 Cell Culture and Transfection

D3 mouse embryonic stem (ES) cells (kind gift from Dr. K. Sue O'Shea, University of Michigan) were cultured on 0.1% gelatin-coated substrates in ES medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% ES-tested fetal bovine serum (FBS) (Hyclone), 10^{-4} M β-mercaptoethanol (Sigma), 0.224 μg/ml L-glutamine (Gibco), 1.33 μg/ml HEPES (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 1,000 U/ml LIF (Chemicon). HEK 293 cells were maintained in DMEM with 10% BS (Gibco) and penicillin-streptomycin. All cells are incubated at 37°C under a humidified atmosphere of 5% CO_2. Transient transfection was carried out with Lipofectamine 2000 (Invitrogen) at a ratio of 3 μl:1 μg DNA.

Plasmids—The following plasmids have been previously described: pGL3 Basic, −500-Dax1-Luc, −700-Dax1-Luc, −900-Dax1-Luc, −500m128-Dax1-Luc, −500m80-Dax1-Luc (−500-Dax1-Luc, −700-Dax1-Luc, −900-Dax1-Luc were referred to previously as pGL3Basic-mDx(−500), pGL3Basic-mDx(−700) and pGL3Basic-mDx(−900), respectively) (12). pcDNA3.1 LRH-1 was a generous gift from Dr. William Rainey (Medical College of Georgia). pGIPZ LRH-1 and pGIPZ Scramble were obtained from the University of Michigan shRNA Core Facility. pEpi Nanog and Nanog shRNA plasmids were generous gifts from Dr. K. Sue O'Shea (University of Michigan). −500/intron, −500m128/intron were created as follows: A 347 bp portion of the Dax1 intron containing a putative Nanog binding site was amplified by PCR with the following primers: 5′-gaggatgctgatgctgtcttaatc-3′ and 5′-

ccttccttcctgtctgttcg-3′ and the PCR fragment subjected to TA cloning into pCRII using the Dual Promoter TA Cloning Kit (Invitrogen) to create pCRII/intron. The fragment was then excised using KpnI/XhoI, ends subjected to blunting by Quick Blunting Kit (NEB) and inserted into the downstream insertion site of pGL3 −500-Dax1-Luc or pGL3 −500m128-Dax1-Luc after digestion with BamHI/SalI and blunting. For the Nanog binding site mutant constructs (−500/intron m2770 and −500m128/intron m2770), mutagenesis was performed on pCRII/intron with Quick Change Mutagenesis Kit (Stratagene) using the following primers: 5′ aaattttgtaaaggaagtaagaaaacgtatatcattgcctaagcaaatctgcttgaaagttgcttttgagtcat 3′ and 5′ atgactcaaaagcaactttcaagcagatttgcttaggcaatgatatacgttttcttacttccctttacaaaattt 3′, which mutates the core motif CATT to TGCC (15). The fragment was then inserted into pGL3 −500-Dax1-Luc or pGL3 −500m128-Dax1-Luc as described above. Constructs were confirmed by sequencing analysis by the University of Michigan DNA Sequencing Core. Recombinant DNA work followed the NIH Guidelines for Research Involving Recombinant DNA Molecules.

2.2 Luciferase assays

D3 or HEK293 cells were plated at a density of 1×10^5 or 5×10^4 cells per well, respectively, in 24-well plates. Twenty-four hours after plating cells were transiently transfected and harvested 48 hours post transfection (unless otherwise noted). Cell lysates were assayed for luciferase activity using Dual Luciferase Assay (Promega) with an injector luminometer. Assays were normalized by transfection of pRL-TK (Promega).

2.3 Electrophoretic Mobility Shift Assays (EMSA)

In vitro transcribed/translated (IVTT) LRH-1 was prepared from pcDNA3.1 LRH-1 using the TnT Coupled Reticulocyte Lysate System (Promega). Nuclear extracts were prepared as previously described (16). Oligonucleotide probes are listed in Table 1. To label oligos, 0.5 μl of 200 ng/μl oligo was incubated with 1.5 μl γ32P ATP (10 uCi/μl), and 0.5 μl T4 Polynucleotide Kinase (NEB) for 1 hr. Labeled oligos were purified using the QIAquick Nucleotide Removal Kit (Qiagen), eluted with 100 μl elution buffer, and sense and antisense oligos annealed by heating to 95°C for 10 min and then cooling to room temperature. IVTT protein (1–2 μl of 50 μl reaction) or 5 μg of nuclear extract were incubated with 1 μl of labeled oligos at room temperature for 30 min in EMSA buffer containing 1 μg of poly(dI-dC) and 0.1 μl 100× BSA (10 mg/ml) in 50mM Tris HCl (pH 8.0), 750 mM KCl, 2.5 mM EDTA, 0.5% Triton-X 100, 62.5% glycerol and 1 mM DTT. Bands were resolved on non-denaturing 5% polyacrylamide gel. Detection was performed by exposure to film overnight at −80 degrees.

2.4 Gene Expression Studies

Overexpression experiments were carried out as follows: 2.5×10^5 cells were plated into 6 well plates, and twenty-four hours later transfected with 3 μg DNA. Forty-eight hours later cells were harvested, RNA isolated, cDNA synthesized and quantitative PCR (QPCR) carried out as described previously (12). Knockdown experiments were carried out as follows: cells were plated into 10 cm plates, and twenty-four hours later transfected with 10 μg DNA. Twenty-four hours later cells were harvested and sorted by flow cytometry for GFP expression from the GFP cassette in the shRNA plasmids. Cells were immediately harvested for RNA, and analyzed by QPCR using primer pairs listed in Table 2.

2.5 Western Blotting

Overexpression experiments were carried out as described above, and then D3 cells were harvested and cellular protein collected by lysis in a buffer containing 40 mM HEPES, 120 mM sodium chloride, 10 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1

mM EDTA, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, 1% Triton X-100 buffer and protease inhibitor cocktail (Sigma), followed by 1 hr rotation at 4°C. Soluble protein was collected from centrifuged total lysates and quantified by Bradford assay (BioRad). Protein lysates were resolved on a 10% SDS-PAGE and transferred to nitrocellulose membrane by standard procedures. Proteins were detected using anti-Dax1 antibody (1:1000, R&D), anti-Nanog antibody (1:1000, CosmoBio), anti-LRH-1 antibody (1:200, Santa Cruz) or anti-β-actin antibody (1:5000, Sigma), followed by blotting with goat anti-mouse HRP (Pierce) or rabbit anti-goat HRP (Thermo Scientific) and detection was performed using Super Signal West Dura Extended Duration Substrate (Pierce). For knockdown experiments, transfection and sorting were carried out as described above and then proteins harvested and Western blotting performed as described above.

2.6 Chromatin Immunoprecipitation (ChIP)

ChIP assays and analysis (except where additional analysis is noted) were carried out as previously described (16). Nuclear extracts were immunoprecipitated with 1 μg anti-Nanog antibody (Cosmo Bio), anti-LRH-1 antibody (kind gift from Dr. Austin Cooney, Baylor College of Medicine) or normal rabbit serum. Primer pairs used for ChIP assays are listed in Table 2. Results shown are representative and from independent experiments, quantitated by QPCR or visualized after PCR by agarose gel.

Statistics—Statistical analyses were performed by Anova and/or Student's t-test. p values are defined in the Figure Legends.

3. RESULTS

3.1 LRH-1 upregulates Dax1 promoter activity in mES cells

Dax1 is highly expressed in mES cells, but data on the mechanisms that regulate its transcription are limited. To determine the minimal length of the Dax1 promoter required for expression in mES cells, different lengths of Dax1 promoter sequence were tested for the ability to activate a reporter construct. Our results indicate that 500 base pairs (bp) of the promoter directly upstream of the transcriptional start site were sufficient for high expression, approximately 6.5 fold higher than control empty pGL3 (Figure 1A). Additional lengths of promoter to 3000 bp did not result in increased activity (data not shown). Previous work had shown the nuclear receptor Sf1 bound to two sites within this region to induce Dax1 expression in steroidogenic tissues, at −80 bp and −128 bp from the Dax1 ATG (8–10). However, Sf1 mRNA and protein are not detectable in mES cells (data not shown). On the other hand, the Sf1 NR5A family member, LRH-1, was recently shown to be expressed in mES cells and play a critical role in their maintenance (17). As LRH-1 has been shown to bind to and regulate Sf1 response elements (RE) in other promoters, we hypothesized that in mES cells LRH-1 may directly activate the Dax1 promoter (18). In HEK293 cells, which do not express LRH-1, Sf1 or Dax1, luciferase assays showed that LRH-1 can upregulate the −500 Dax1-Luciferase reporter almost 160 fold (Figure 1B). To show that LRH-1 upregulates the −500 Dax1-Luciferase in mES cells, and to determine the RE responsible for this action, we carried out similar reporter studies in mES cells with this wild type reporter and the corresponding constructs with the Sf1-REs mutated: −500m80 Dax1-Luc and −500m128 Dax1-Luc. Co-transfection of LRH-1 with the −500 Dax1-Luc induced expression almost 2 fold, even in the presence of endogenously expressed LRH-1 in the cells (Figure 1C). Mutation of the −80 RE had little effect on activity, whereas mutation of the −128 RE caused a 5 fold decrease in expression of the reporter, and this mutation prevented LRH-1 mediated upregulation of the expression. Together, these data support a mechanism by which LRH-1 regulates Dax1 promoter activity through the −128 RE in mES cells.

3.2 LRH-1 binds to the −128 RE in the Dax1 promoter in vitro

To demonstrate that LRH-1 binds directly to the Dax1 promoter, we performed electrophoretic mobility shift assays (EMSA) using *in vitro* transcribed and translated (IVTT) LRH-1. This approach assures that any observed signal reflects LRH-1 protein specifically binding to the probe. Adding increasing amounts of IVTT LRH-1 resulted in increased binding to the wildtype −128 RE probe (−128-LRH-1 RE wt), while mutation in the Sf1 consensus sequence caused complete loss of the binding in one band and near abolishment of the other (−128-LRH-1 RE mut) (Figure 1D) (9). Additionally, to further show that LRH-1 specifically binds to this promoter site, we performed supershift with anti-LRH-1 antibody (Figure 1E). Finally, to show that endogenous LRH-1 in mES cells can bind to this site, we performed EMSA with nuclear extracts from mES cells. With wildtype probe, there are two clear bands, similar to those with IVTT LRH-1 (Figure 1F, lane 1). When nuclear extract was incubated with mutant probe, there is a reduction in one band and complete loss of the other (Figure 1F, lane 2). These data indicate that LRH-1 protein binds to the −128 RE in the Dax1 promoter *in vitro.*

3.3 LRH-1 binds to the −128 RE in the Dax1 promoter in vivo

To show binding of endogenous LRH-1 to the Dax1 promoter in mES cells, we performed chromatin immunoprecipitation (ChIP) assays. Immunoprecipitation with anti-LRH-1 antibody, as normalized to normal serum immunoprecipitation control, resulted in significant enrichment of the −128 Dax1 promoter site over a control genomic site (Figure 1G). These data indicate that LRH-1 binds to the Dax1 promoter in mES cells.

3.4 LRH-1 overexpression and knockdown can alter endogenous Dax1 mRNA and protein levels

Our data suggest that LRH-1 upregulates Dax1 expression through binding to the −128 RE. Therefore we hypothesized that alteration of LRH-1 levels should change endogenous Dax1 mRNA levels. To test this hypothesis, we carried out LRH-1 overexpression and knockdown studies. Transient overexpression of LRH-1 in mES cells caused a statistically significant 2-fold increase in Dax1 mRNA levels as assessed by QPCR, despite high expression of endogenous LRH-1 (Figure 2). To ensure that overexpression is increasing levels of LRH-1 and not preventing differentiation and thus indirectly affecting Dax1 levels, we performed alkaline phosphatase (AP) staining and found similar percentages of AP positive cells in the empty vector and LRH-1 transfected cultures (data not shown). Next we wanted to examine the direct transcriptional effects of LRH-1 knockdown independent of secondary effects due to differentiation. Because prolonged loss of LRH-1 causes differentiation of mES cells (17), to show that LRH-1 knockdown causes direct downregulation of Dax1, we employed the following strategy: mES cells were transfected with a vector containing an shRNA against LRH-1 and a GFP cassette, and only twenty-four hours later harvested and sorted by flow cytometry for GFP positive cells. This procedure results in a significant knockdown of LRH-1 (approximately 70 percent, data not shown) without observed effects of differentiation. Specifically, we analyzed expression of trophectoderm markers that would be expected to increase with LRH-1 knockdown-mediated differentiation. We found that there was no increase in Fgfr2 or Cdx2 with the short-term knockdown (Figure 2B). Dax1 mRNA levels were reduced by almost 50 percent in the LRH-1 knockdown cells (Figure 2). Additionally, overexpression and knockdown of LRH-1 results in increased and decreased Dax1 protein levels, respectively (Figure 2C and 2D). These data reveal that overexpression and knockdown of LRH-1 results in upregulation and downregulation of endogenous Dax1 levels, respectively. Taken together with the luciferase assay, EMSA experiments and ChIP these data demonstrate that LRH-1 upregulates Dax1 transcription in mES cells, and that this action is mediated through the −128 RE.

3.5 Nanog binds to a novel Dax1 intronic site in mES cells

Prior published reports utilizing whole genome ChIP paired-end ditag (PET) data indicated a Nanog binding site within the Dax1 intron, but this observation has not been validated or characterized further (15). Thus, we carried out experiments aimed at determining the exact binding site of Nanog in the Dax1 intron and its contribution to Dax1 transcription in mES cells. We examined the Dax1 intron for putative Nanog binding sites as defined by the motif determined by ChIP-PET and identified five possible sites using the core CATT site. Using ChIP assays with primers flanking these putative sites, we found a significant enrichment over control when using the primers flanking the +2770 site (from the Dax1 start site) (Figure 3A). To validate binding to this site, we performed ChIP using the +2770 site, Dax1 proximal promoter and control primers, and found that immunoprecipitation with anti-Nanog significantly enriched for the +2770 site but not for the promoter or control sites (Figure 3B). To further prove that the +2770 site is a bona fide Nanog binding site, we performed EMSA with mES cell nuclear extracts. Using nuclear extracts, we show that wildtype hot probe binds and can be specifically competed by wildtype cold probe (Figure 3C). Mutating the hot probe with a mutation that specifically loses Nanog binding (19) causes loss of binding, and competition of the hot wildtype probe with cold mutant does not result in loss of binding (Figure 3C).

3.6 Nanog overexpression and knockdown alter endogenous Dax1 mRNA and protein levels

To determine whether Nanog could alter endogenous levels of Dax1, we overexpressed and knocked down Nanog and examined Dax1 mRNA levels. When Nanog was transiently overexpressed in mES cells, despite high endogenous levels of both Nanog and Dax1, a 2.5-fold increase in Dax1 mRNA was observed by QPCR (Figure 4). To ensure that overexpression is increasing levels of Nanog and not preventing differentiation and thus indirectly affecting Dax1 levels, we performed alkaline phosphatase (AP) staining and found similar percentages of AP positive cells in the empty vector and LRH-1 transfected cultures (data not shown). As knockdown of Nanog will also cause differentiation, a similar approach to the LRH-1 knockdown studies was used (19). By examining changes only 24 hours after transfection, observed effects are predicted to be a direct result of Nanog knockdown rather than secondary effects of mES cell differentiation. Accordingly, when Nanog was knocked down more than 80 percent (data not shown), Dax1 mRNA levels were reduced by almost 30 percent (Figure 4). To ensure that differentiation has not occurred, we analyzed expression of endoderm markers that would be expected to increase with Nanog knockdown-mediated differentiation. We found that there was no increase in COUP-TF1 or COUP-TF2 with the short-term knockdown (Figure 4B). Additionally, overexpression and knockdown of Nanog results in increased and decreased Dax1 protein levels, respectively (Figure 4C and 4D). These data combined with the ChIP data indicate that Nanog binds to the Dax1 intron at +2770 and upregulates Dax1 transcription in mES cells.

3.7 LRH-1 and Nanog are both required for maximal Dax1 transcription in mES cells

Based on the above data, we hypothesized that LRH-1 and Nanog cooperate to activate Dax1 transcription through the proximal promoter and intron, respectively. Therefore we constructed a reporter plasmid that would utilize both of these mechanisms. The reporter plasmid contains the 500 base pairs of proximal promoter upstream of the luciferase start site and a 347 base pair region of the intron that includes the Nanog +2770 binding site downstream of the luciferase sequence, thereby mimicking the intact genomic structure of Dax1. Additionally, mutations of the −128 LRH-1 site and of the +2770 Nanog site were introduced either separately or in combination. A schematic of the reporter plasmids used is shown in Figure 5A. To determine whether the Nanog binding intronic region enhances the LRH-1 mediated activation of the promoter, we performed luciferase assays with the −500

Dax1-Luc, Empty/intron, and the −500/intron reporters (Figure 5B). The −500/intron reporter displayed higher luciferase activity than −500 Dax1-Luc or Empty/intron reporters, suggesting that the intronic region can enhance the LRH-1 mediated transcriptional activity from the promoter.

To formally interrogate the contribution of each of these sites to Dax1 transcription, we performed luciferase assays with the −500/intron reporter with the −128 LRH-1 site, the +2770 Nanog site, or both sites mutated. The Nanog mutation was designed by examination of the binding motif elucidated previously, and we have tested the mutation to confirm loss of binding by EMSA assays (Figure 3C) (15). While mutation of the intronic Nanog site in the context of the intact LRH site (−500/intron m2770) did not decrease promoter activity when compared to the −500/intron construct (intact LRH and Nanog site), mutation of both of these sites together completely abrogated transcription of the reporter compared to the −500m128/intron construct (mutant LRH site, intact Nanog site) (Figure 5D).

Finally, in order to show that LRH-1 and Nanog together regulate Dax1 expression, we performed double knockdown of LRH-1 and Nanog and found that double knockdown reduced Dax1 mRNA by about three-fold (Figure 5E), a greater reduction than by knockdown of LRH-1 or Nanog separately. In addition, knockdown of LRH-1 and Nanog reduced Dax1 protein (Figure 5F). These data together demonstrate that LRH-1 and Nanog are both required for Dax1 transcriptional regulation.

4. DISCUSSION

In this study, we have identified two important mechanisms of regulation of Dax1 transcription in mES cells. First, LRH-1 binds to the −128 nuclear receptor binding element and upregulates expression of Dax1. Secondly, Nanog binds within the Dax1 intron at a site +2770 from the transcription start and enhances LRH-1 dependent transcription of Dax1.

We have previously studied the Dax1 proximal promoter as the site of Dax1 transcription regulation by the nuclear receptor Sf1 in steroidogenic cells. We were surprised to find that the −128 Sf1 consensus binding site appeared to be driving Dax1 expression in mES cells despite the fact that Sf1 is not expressed in these cells. LRH-1 and Sf1 can bind identical sites within gene promoters, specifically in the adrenal and ovary (18,20). Interestingly, a recent study of pluripotent stem cells demonstrated that LRH-1, expressed only in mES cells, and Sf1, expressed only in embryonal carcinoma (EC) cells, activate Oct4 expression in the cells in which they are expressed through the same promoter site (17). As Dax1 is expressed in both mES and EC cells (unpublished observation), we would hypothesize that Sf1 rather than LRH-1 may regulate Dax1 expression in EC cells.

In luciferase assays, mutation of the −128 nuclear receptor site abrogated all expression of Dax1 in mES cells, suggesting that this site is necessary for Dax1 expression. The previously reported Stat3 binding site within the Dax1 proximal promoter, located −158 from the transcriptional start site, is very close to the LRH-1 binding site (13). When the −128 binding site was mutated, complete loss of reporter expression was observed (Figure 1C). These data suggest that in this *in vitro* system, the Stat3 site is not required for Dax1 expression.

The studies presented here on the intronic regulation of Dax1 by Nanog are reminiscent of the previous report that Oct4 binds to and regulates a dual Oct4-Sox2 site within the Dax1 intron at +2054/+2063 from the transcriptional start site (13). While the Nanog site is some distance from this one, reports that Oct4 and Nanog form a protein complex lends support to a possible Oct4-Sox2-Nanog complex that regulates expression of Dax1 (21). Indeed, it has been shown previously that Oct4-Sox2 and Nanog co-occupy many target genes (22).

Further examinations of similar complexes on the Dax1 gene are warranted. However, it is worth noting that the −500/intron construct does not contain the Oct4-Sox2 binding sequence, but can still enhance transcription by the −500 bp promoter. This suggests that Nanog can bind to the Dax1 intron and activate transcription independently of Oct4-Sox2.

In experiments detailed in this report, the Dax1 intronic region conferred enhanced transcription from the proximal promoter. However, mutation of the Nanog site alone in the context of the intact LRH-1 site (−500/intron m2770) did not cause a loss of promoter activity compared to the −500/intron construct (intact LRH-1 site, intact Nanog site). This puzzling result may suggest that the Nanog site is most utilized only in concert with the LRH-1 site, perhaps suggesting a protein-protein interaction between Nanog and LRH-1. However, co-immunoprecipitation experiments to elucidate an interaction between these proteins have been unsuccessful (data not shown).

Nanog is specifically expressed in ES cells and EC cells (19); data here show that Nanog regulates Dax1 in mES cells, but we have also observed regulation of Dax1 in EC cells by Nanog (data not shown). However, as Nanog is not expressed in adult tissues in which Dax1 is expressed, this mechanism is likely not utilized after embryogenesis (19). On the other hand, LRH-1 is expressed in the ovary, perhaps in the cells in which Dax1 is expressed (23). This leaves open the possibility that LRH-1 regulates Dax1 expression in cells other than ES cells, specifically ovarian cells.

Regulation of expression of mES cell factors that are necessary for maintenance of pluripotency is critical, as loss of regulation of these factors has been shown to lead to differentiation. For example, Oct4 mRNA levels must be kept within a tight range of expression in order to maintain pluripotency of mES cells. Several layers of redundancy are predicted to ensure such appropriate temporal and quantitative expression of these factors. Emerging studies indicate that Dax1 is a key mediator of mES cell pluripotency (24). As its name implies, Dax1 (Dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on X chromosome gene 1) has dose-dependent effects. Indeed, this has been shown on the scale of the whole organism, where duplication of Dax1 causes sex reversal, and at the molecular level, where different levels of Dax1 expression determine whether Dax1 acts as a transcriptional repressor or activator (3,25). Accordingly, Dax1 may have dose specific effects in mES cells, requiring exquisite control of its expression level. This may account for the multiple mechanisms that appear to regulate its expression.

Acknowledgments

We would like to thank Joanne Heaton and Michelle Wood for critical reading of the manuscript. V.R.K. was supported in part by grant T32 HD07048 to the Training Program in Reproductive Sciences at the University of Michigan. Work was supported by NIDDK NIH DK062027 (G.D.H.) and in part by a grant to Dr. Colin Jefcoate (NIDDK NIH DK074819).

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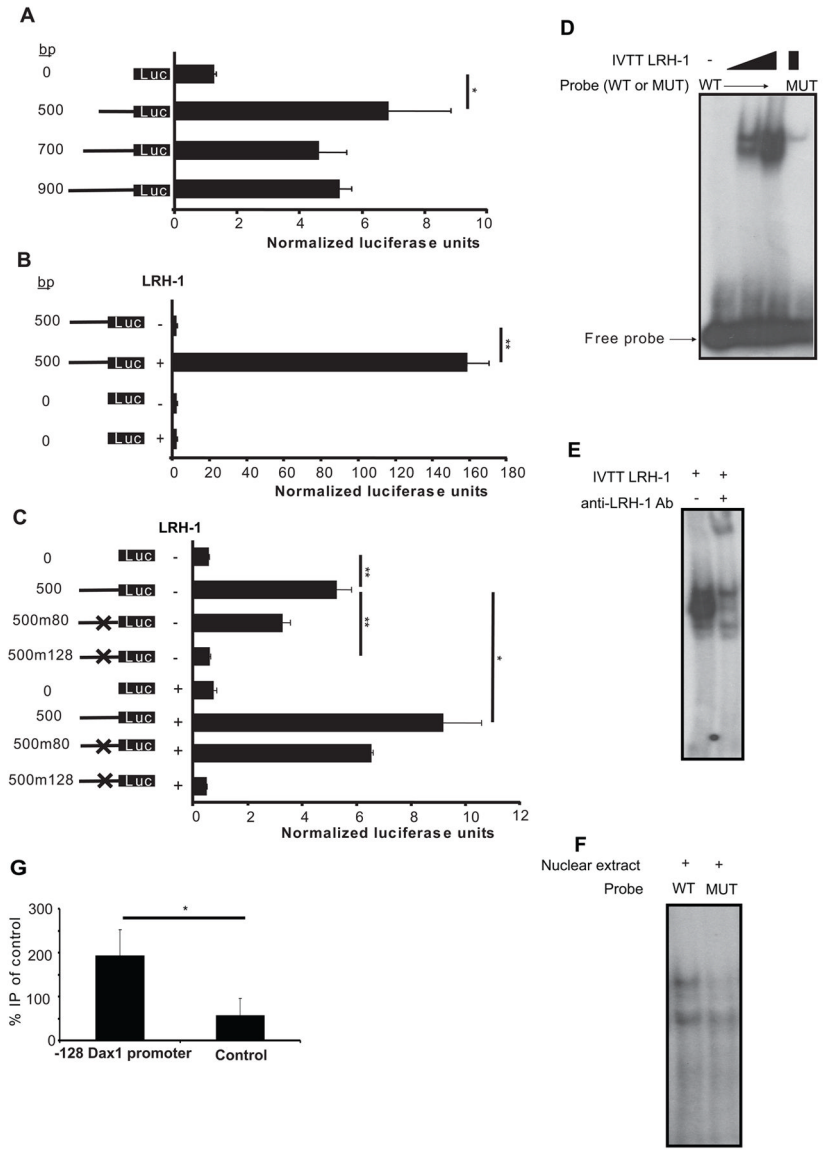

Figure 1. LRH-1 regulates Dax1 expression in mES cells through the −128-LRH-1 response element

(A) mES cells were transfected with 200 ng of luciferase reporter vectors containing various lengths of Dax1 promoter, as indicated by number of base pairs (bp). Luciferase assay was performed on lysates 48 hr hour post-transfection and values were normalized to Renilla luciferase internal control. The data are presented as fold over empty vector control. *$p<0.05$

(B) HEK293 cells were transfected with 200 ng empty pGL3 basic or −500 Dax1-Luc and co-transfected with 200 ng pcDNA3.1 LRH-1 or empty vector. Luciferase assays were carried out as above. **$p<0.005$

(C) mES cells were transfected with 200 ng empty pGL3, −500 Dax1-Luc, or −500 Dax1-Luc with the −80 or −128 site mutated, and co-transfected with 200 ng LRH-1 or empty vector. Luciferase assays were carried out as above.

D) Increasing amounts of *in vitro* transcribed/translated (IVTT) LRH-1 was subjected to electrophoretic mobility shift assay with γ-^{32}P-ATP labeled oligonucleotides correlating to

the −128 LRH-1 site or the mutated version as indicated (See Table 1). 0–0.5 μl of a 50 μl IVTT reaction was used in each reaction.
(E) EMSA in which IVTT LRH-1 (0.5 μl) was incubated without or with anti-LRH-1 antibody (2 μl) for supershift of the complex and the wildtype −128 Dax1 promoter probe.
(F) EMSA was performed with mES cell nuclear extract (10 μg), which was incubated with wildtype or mutant −128 Dax1 promoter probe.
(G) Chromatin immunoprecipitation was performed in mES cells with anti-LRH-1 antibody or normal serum and QPCR performed with primers flanking the −128 Dax1 promoter site or a control site as described in methods. Percent input was determined as described in Methods and percent immunoprecipitation over control calculated by dividing percent input with anti-LRH-1 by percent input with normal serum.

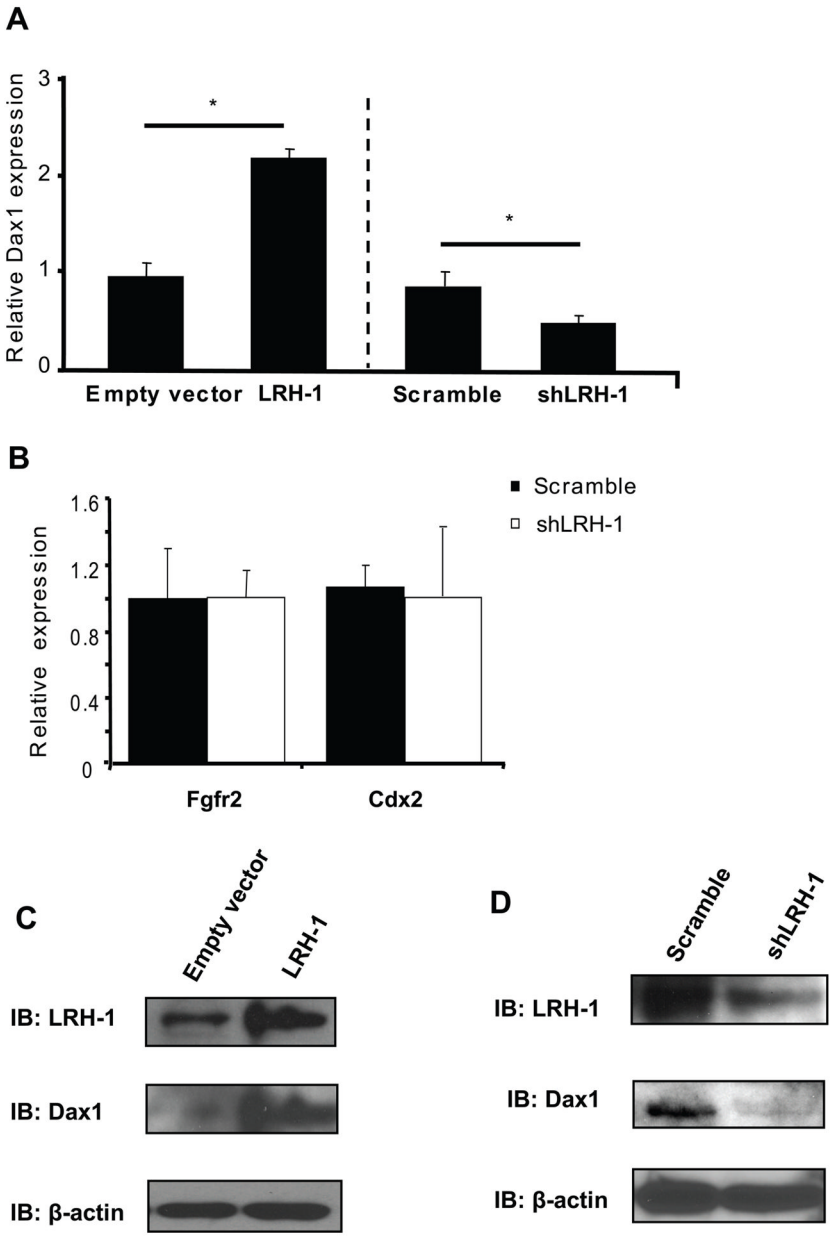

Figure 2. Overexpression and knockdown of LRH-1 alters endogenous levels of Dax1

(A) For overexpression, mES cells in a 6 well plate were transiently transfected with 2 μg empty vector or pcDNA3.1 LRH-1 and 48 hours later harvested for RNA. cDNA was synthesized and QPCR was carried out. Dax1 values were normalized to GAPDH and data presented as fold over empty vector control.

For knockdown, mES cells in a 10 cm plate were transfected with 10 μg of a vector containing a GFP cassette and either shRNA against LRH-1 or scrambled control. Twenty-four hours after transfection, cells were sorted, GFP positive cells harvested and RNA isolated. cDNA synthesis and QPCR were carried out. *$p<0.05$

(B) QPCR was performed on cDNA from (A) for expression of differentiation markers Fgfr2 and Cdx2 with LRH-1 knockdown. Data was analyzed as described in (A).

(C) Overexpression of LRH-1 was performed as described in (A) and cells lysed for protein and Western blotting performed. Immunoblotting was performed with anti-LRH-1, anti-Dax1 and anti-β-actin antibodies as described in methods.
(D) Knockdown of LRH-1 was performed as described in (B), cells lysed for protein and Western blotting performed as described.

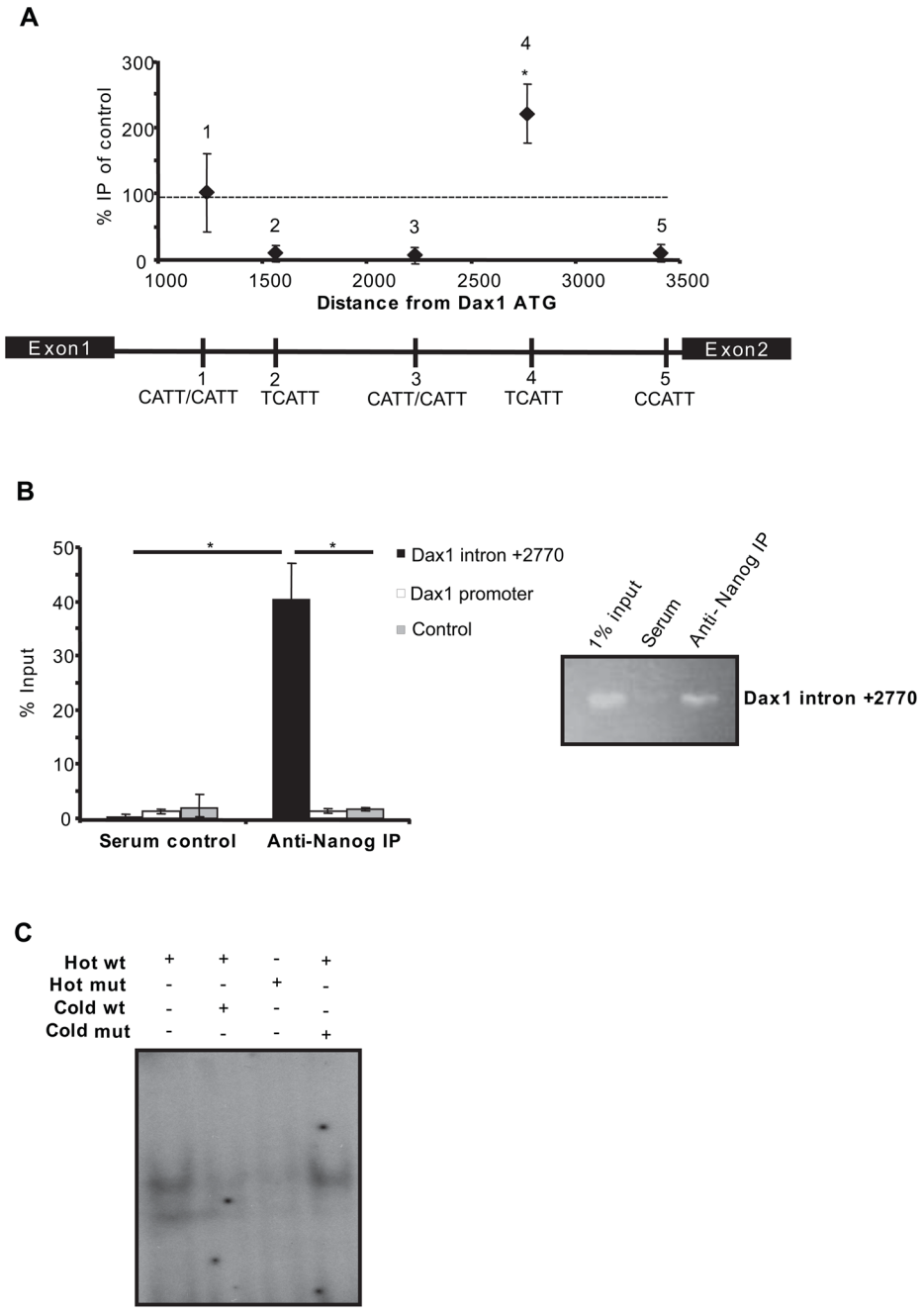

Figure 3. Nanog binds to the Dax1 intron at +2770

(A) Primer sets flanking putative Nanog binding sites shown schematically (bottom) were designed, and ChIP experiments were performed on mES cells with anti-Nanog antibody or serum control as described in Methods. Percent input was determined as described in Methods and percent immunoprecipitation over control calculated by dividing percent input with anti-Nanog by percent input with normal serum. Baseline (dotted line) indicates the enrichment of a control primer set. *$p<0.0006$.

(B) Further ChIP analysis was performed in mES cells with the Dax1 intron +2770, Dax1 proximal promoter, and control primer sets. Results are shown from independent

experiments quantitated by either QPCR (left) or PCR and agarose gel analysis (right). *p<0.008

(C) Nuclear extracts from mES cells were used in an EMSA assay with labeled probes to the +2770 Nanog binding site from the Dax1 intron. Addition of cold wildtype or cold mutant probes is indicated with plus signs.

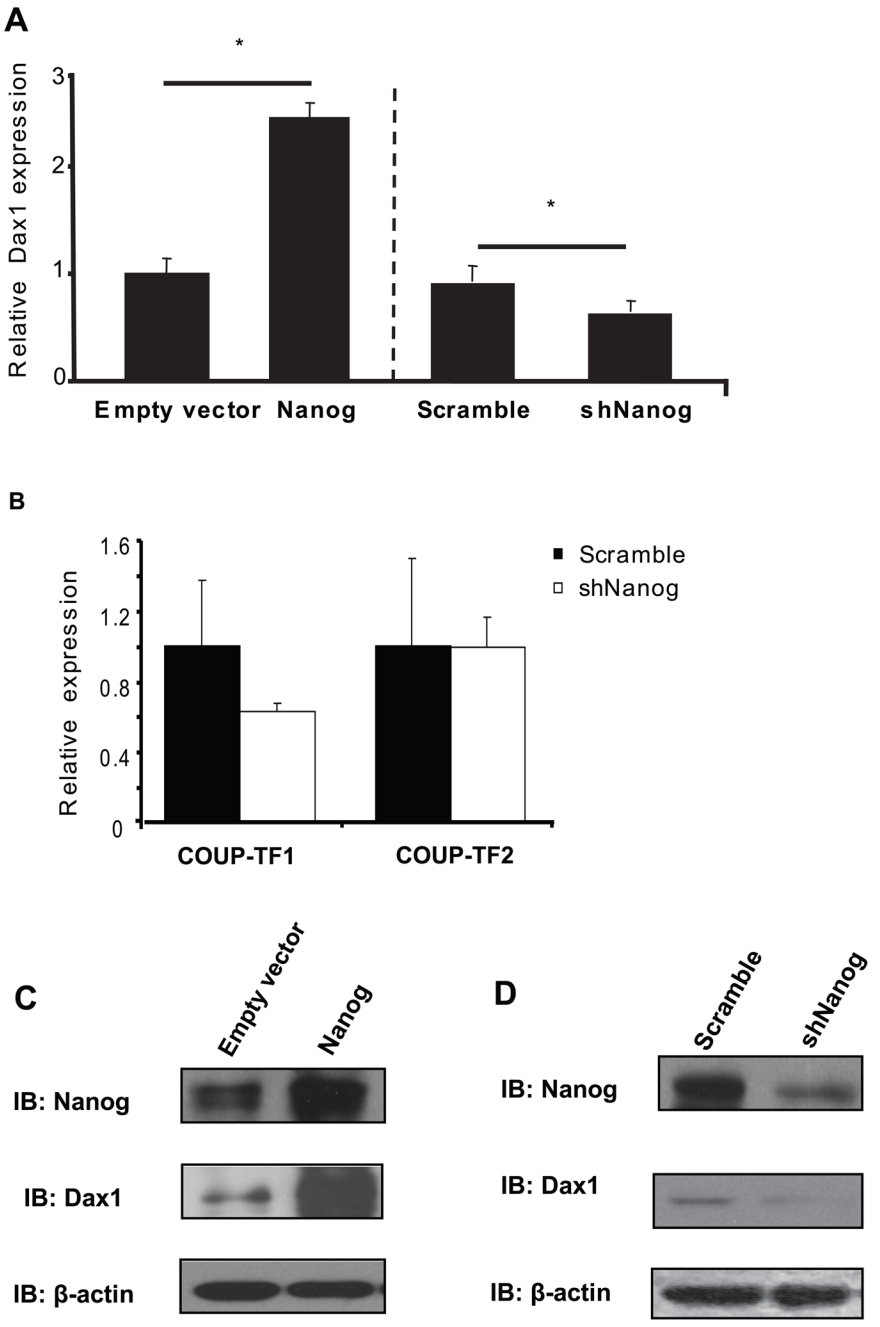

Figure 4. Nanog alters endogenous levels of Dax1

(A) For overexpression, mES cells in a 6 well plate were transiently transfected with 2 μg empty vector or pEpi Nanog and 48 hours later harvested for RNA. cDNA was synthesized and QPCR was carried out. Dax1 values were normalized to GAPDH and data presented as fold over empty vector control.

For knockdown, mES cells were transfected in a 10 cm plate with 10 μg of a vector containing shRNA against Nanog or scrambled control and a GFP expression cassette. Twenty-four hours after transfection, cells were sorted and GFP positive cells were harvested for RNA. cDNA synthesis and QPCR were carried out. Dax1 values were normalized to GAPDH and data presented as fold over scramble control. *$p<0.005$

(B) QPCR was performed on cDNA from (A) for expression of differentiation markers COUP-TF1 and COUP-TF2 with Nanog knockdown. Data was analyzed as described in (A).
(C) Overexpression of Nanog was performed as described in (A) and cells lysed for protein and Western blotting performed. Immunoblotting was performed with anti-Nanog, anti-Dax1 and anti-β-actin antibodies as described in methods.
(D) Knockdown of LRH-1 was performed as described in (B), cells lysed for protein and Western blotting performed as described.

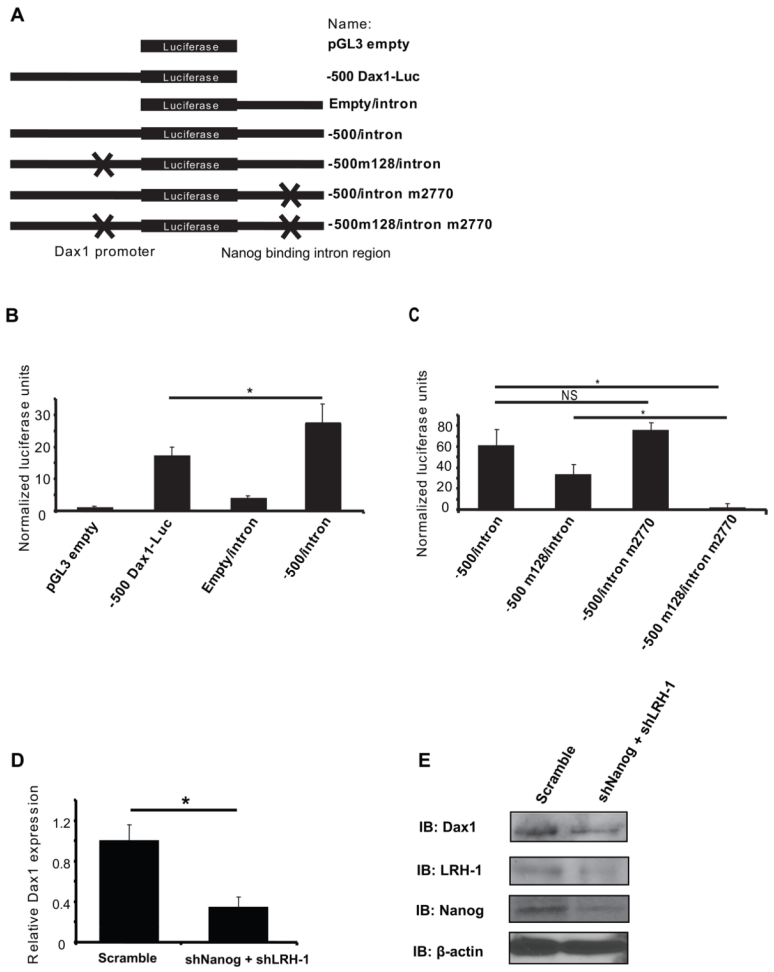

Figure 5. LRH-1 and Nanog co-regulate Dax1 transcription

(A) Schematic representation of luciferase reporters.

(B) mES cells were transfected with 200 ng of pGL3 empty or luciferase reporter vectors containing either 500 bp of the Dax1 promoter, the intronic region, or both the promoter and intron. Luciferase assay was performed on lysates 48 hr hour post-transfection and values were normalized to Renilla luciferase internal control. The data are presented as fold over empty vector control. *$p<0.005$

(C) mES cells were transfected with 200 ng of luciferase reporter vectors containing 500 bp of the Dax1 promoter and the intronic region, with or without the −128 LRH-1 site and/or +2770 Nanog site mutated. Luciferase assays were carried out as above. Data are presented as fold over −500 Dax m128/intron m2770.

(D) mES cells were transfected in a 10 cm plate with 5 μg each of vectors containing shRNA against Nanog and LRH-1 or scrambled control and a GFP expression cassette. Twenty-four hours after transfection, cells were sorted and GFP positive cells were harvested for RNA. cDNA synthesis and QPCR were carried out. Dax1 values were normalized to GAPDH and data presented as fold over scramble control.

(E) Knockdown of Nanog and LRH-1 was performed as described in (E), cells lysed for protein and Western blotting performed as described. Immunoblots were performed with anti-LRH-1, anti-Nanog, anti-Dax1 and anti-β-actin antibodies as described in methods.

Table 1

EMSA oligos

	Sense	**Antisense**
−128-LRH-1 RE wt	gctttcgaGGTCAtGGccacacac	gtgtgtggCCaTGACCtcgaaagc
−128-LRH-1 RE mut	gctttcgaTTCCAtTTccacacac	gtgtgtggAAaTGGAAtcgaaagc
Dax1 intron +2770 wt	cgtatatcaTCATTtaagcaaat	atttgcttaAATGAtgatatacg
Dax1 intron +2770 mut	cgtatatcaTTGCCtaagcaaat	atttgcttaGGCAAtgatatacg

Table 2

QPCR and ChIP primers

	Forward	Reverse
−128 Dax1 promoter	caggtagaggcaggaggggtggag	ggccgccgcttgggacttattta
Dax1 QPCR	accgtgctctttaacccaga	ccggatgtgctcagtaagg
LRH-1 QPCR	tcctggttactggagaacacg	ttgttgaacgcgacttctgt
GAPDH QPCR	aatgtgtccgtcgtggatct	cccagctctccccatacata
Fgfr2 QPCR	agcatcctcatcccaagatgcctt	tgatgaatgtgtaacccgggcaga
Cdx2 QPCR	tgtaaatgccagagccaacctgga	agatcagtgactcgaacagcagca
Nanog QPCR	ttcttgcttacaagggtctgc	agaggaagggcgaggaga
COUP-TF1 QPCR	caaagccatcgtgctattca	cctgcaggctttcgatgt
COUP-TF2 QPCR	cctcaaagtgggcatgagac	tgggtaggctgggtaggag
Dax1 intron +1179/1231 ChIP	ttcaggtcagaaaagcacca	gctcacccatttgaccactt
Dax1 intron +1560 ChIP	ctgctacagaagtttctgtcttc	ctgtttctcagatgacctca
Dax1 intron +2233/2244 ChIP	atctaaagccttatacaggacaaag	ggtattctcattcatactgt
Dax1 intron +2770 ChIP	gaggatgctgatgctgtcttaatc	ccttccttcctgtctgttcg
Dax1 intron +3407 ChIP	gtgtttgtgcacactttag	tttatttggcggggttaatg
Dax1 promoter ChIP	caggtagaggcaggaggggtggag	ggccgccgcttgggacttattta
Control ChIP	agagggtcaaggatggaatgatt	cagtgtgctccctcccacc