REGULATION AND ROLE OF DAX1 IN MOUSE EMBRYONIC STEM CELLS

by

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ABSTRACT

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Embryonic stem cells (ESCs) are derived from the early embryo and give rise to all cells of an organism. Harnessing the power of ESCs promises to open a new era of regenerative medicine. To achieve this goal, we must first understand the basic biology of ESCs and the mechanisms that maintain self renewal and pluripotency, the hallmarks of ESCs.

Many factors have been shown to be critical for mouse ESC (mESC) biology, including Dax1, an atypical nuclear receptor. Dax1 was originally thought to be expressed only in steroidogenic tissues. However, recent work has shown that Dax1 is one of the top twenty mRNAs enriched in mESCs, that Dax1 participates in the critical ES cell network by forming protein complexes with many important ES cell factors and

that Dax1 binds to over 1700 sites within the genome. These data suggest a significant role for Dax1 in the transcriptome of mESCs.

To investigate the role of Dax1 in mESC biology, we began by examining mechanisms of transcriptional regulation of Dax1. In steroidogenic tissues, Dax1 expression is regulated by the nuclear receptor, Sf1. However, Sf1 is not expressed in mESCs; on the other hand, LRH-1, a close family member, is expressed. Our data show that LRH-1 binds to the Dax1 proximal promoter and regulates Dax1 expression. Additionally, we identified an intronic site that is bound by Nanog and contributes to Dax1 regulation.

Because in steroidogenic cells Dax1 binds to Sf1 protein to modulate Sf1 target genes, we hypothesized that Dax1 may also bind to LRH-1. We found that Dax1 and LRH-1 interact in mESCs, and that Dax1 localizes to the LRH-1 binding site on the promoter of Oct4, a critical ESC factor. Furthermore, Dax1 and LRH-1 co-activate Oct4 expression in order to maintain proper levels of Oct4. In addition, our data show that Dax1 binds to a functional RNA, called SRA, which acts as a scaffold for co-activator recruitment. Knock down of SRA attenuates Dax1 co-activation of Oct4.

These results establish an important role for Dax1 in mESC biology and enhance our knowledge of the mechanisms by which mESCs maintain pluripotency.

CHAPTER 1

INTRODUCTION

Dax1—Historical Perspective

Dax1, <u>D</u>osage-Sensitive Sex Reversal-<u>A</u>drenal Hypoplasia Congenita Critical Region on the <u>X</u>-Chromosome, is a fascinating atypical nuclear receptor which was cloned in 1994 as the gene responsible for adrenal hypoplasia congenita (AHC) in humans (1-2). There are two forms of AHC: autosomal recessive miniature and X-linked cytomegalic. Mutations in Dax1 result in X-linked cytomegalic AHC, characterized by adrenal insufficiency, which is lethal if left untreated. The adrenal glands are the main modulators of the mammalian stress response via secretion of glucocorticoids (cortisol in humans or corticosterone in mice), catecholamines and mineralocorticoids. Patients with mutations in Dax1 also suffer from hypogonadotropic hypogonadism (HHG), a condition characterized by low or absent function of the testes or ovaries. Gain of function of Dax1 also causes human conditions, as duplication of Dax1 has been shown to cause male to female sex reversal (3). These observations of human syndromes were the first clues to the role that Dax1 plays in steroidogenesis and sex determination.

Dax1 is categorized as nuclear receptor NR0B1. The nuclear receptor superfamily includes proteins that exhibit three conserved domains: a transactivation site (AF1), a

DNA binding domain (DBD) with two canonical zinc fingers and a hinge domain. Classification is based on homology within the DBD, and Dax1 was placed into the most primitive phylogenetic group NR0B because of the atypical structure of Dax1 (Figure 1.1). Though it harbors a putative ligand binding domain (LBD), it lacks the classical DBD, having only an alternative DNA binding motif. No known ligand exists for Dax1, making Dax1 an orphan nuclear receptor. Additionally, Dax1 has three repeats of an LXXLL motif in its N-terminus, a domain shown to be involved in protein-protein interactions with nuclear receptors. The Dax1 gene is made up of two exons, with exon one encoding the atypical DBD and part of the LBD, and exon two coding for the rest of the LBD (Figures 1.2 and 1.3) (4).

Postnatal expression of Dax1 is restricted to the adrenal gland, testis, ovary, pituitary and hypothalamus, consistent with its roles in AHC, HHG and sex determination. It was observed that expression of Dax1 colocalized with that of the nuclear receptor steroidogenic factor 1 (Sf1, also NR5A1), the transcription factor responsible for expression of steroidogenic genes (5). This observation led to the discovery that Sf1 regulates Dax1 expression through sites within the Dax1 proximal promoter (6-9). More recently, Sf1 has been shown to regulate Dax1 expression by complexing with either the glucocorticoid receptor in the presence of glucocorticoids or the Wnt transcriptional effector β-catenin (10-11). Finally, androgens can downregulate

Dax1 expression in the adrenal, which leads to sexually dimorphic Dax1 expression in the adrenal cortex (12).

Dax1 expression in the mouse is first seen during embryogenesis, at E10.5 when cells of the urogential ridge begin to specify (13). The cells of the urogenital ridge give rise to the adrenogonadal primordial (AGP), which gives rise to the adrenal cortex and ovary or testis. Dax1 expression persists in the AGP, and then by adulthood when organ development is complete, Dax1 expression is maintained in the adrenal cortex and ovary, but lessens in the testis.

Dax1 as a transcriptional repressor

Studies on the structural domains of Dax1 identified a transcriptional repression domain in the C-terminus (14). Further work showed that Dax1 interacts with Sf1 and inhibits transactivation of Sf1 target genes (15-16). Consistent with this role, Dax1 has been shown to inhibit steroidogenesis and expression of many steroidogenic genes (17-18). *In vivo*, the repressive action of Dax1 on Sf1 was verified by comparison of haploinsufficient Sf1 mice to Sf1 haploinsufficient- Dax1 knockout mice. As predicted, the loss of Dax1 rescued the adrenal hypoplasia and increased adrenal responsiveness to pituitary signals, namely adrenocorticotropic hormone (ACTH) (19). The Dax1 knockout mouse will be discussed further in another section.

Dax1 has been shown to interact with many other nuclear receptors besides Sf1, including, but not limited to, the estrogen receptor, AR, progesterone receptor, Nurr77, liver receptor homolog 1 (LRH-1), SHP and retinoic acid receptor (1,16,20-25). When Dax1 binds to these proteins, it inhibits transactivation of their target genes, likely by

recruitment of corepressors or physical obstruction of coactivators. Indeed, Dax1 has been shown to interact with many corepressors, including N-CoR, SMRT and Alien (20,23,26). Though it is not clear that a single domain of Dax1 is always required for interaction with other nuclear receptors or corepressors, it is worth noting that the large majority of Dax1 mutations identified in patients with AHC fall within the LBD, and *in vitro* studies have shown that these mutations result in loss of transcriptional silencing ability (15,27). However, loss of the ability to interact with these factors is not the only mechanism by which AHC-Dax1 mutations prevent inhibition of transcription. It has also been shown that many of these mutations result in either misfolded or cytoplasmic localization of Dax1, preventing its effects on transcription (28-29).

In addition to interaction with corepressors, Dax1 has been shown to inhibit transcriptional activity through other mechanisms. First, Dax1 has been shown to bind to the androgen receptor and tether it in the cytoplasm to prevent transcriptional activation in the nucleus (22). Second, Dax1 was shown to bind to DNA hairpin structures within the steroid acute regulatory protein (StAR) promoter and thereby block gene transcription (17). Thus, Dax1 plays significant roles in the regulation of steroidogenesis through a variety of mechanisms of transcriptional repression and interaction with many other factors. A decade ago, these repressive functions were the defined set of mechanisms in which Dax1 participated. More recently, however, our understanding of the multifaceted roles of Dax1 has increased, expanding our knowledge of the role of Dax1 significantly.

Dax1—Increasing complexity a decade later

One of the first studies that began to broaden our knowledge of Dax1 alternate roles reported that an alternatively spliced isoform of Dax1 exists, at least in human cells (30-31). The isoform, named Dax1α, is encoded by exon 1 and a previously unrecognized exon within the intron that only appears upon use of a cryptic splice site. It has subsequently been shown that this isoform is highly expressed in many tissues, including the adrenal gland, brain, kidney, ovary and testis. Interestingly, this isoform can not repress transactivation by Sf1, but it can antagonize Dax1. No function of Dax1α has been elucidated and the existence of this isoform in mouse cells has not yet been shown.

Dax1 as a transcriptional activator

AHC is characterized by adrenal cortex hypoplasia. Interestingly, the Sf1 knockout mouse also has adrenal hypoplasia, in fact not developing functional adrenal glands at all (32-33). These results are paradoxical if Dax1 is strictly a repressor of Sf1 function. If this were the case, you would expect loss of Dax1 to have the opposite effect as loss of Sf1. While comparing mouse models to humans may be a caveat in this logic, one possibility is that Dax1 can also act as a transcriptional coactivator for Sf1. Indeed, when testis development was studied in the Sf1 haploinsufficient-Dax1 null mouse, expression of fetal Leydig cell markers Cyp17 and Cyp11a1 and the Sertoli cell product desert hedgehog was lower than in the Sf1 haploinsufficient mouse, suggesting that perhaps Dax1 functions to activate expression of these factors (34). Recently, our group in collaboration with the Koenig Lab investigated this possibility; we found that in certain contexts and at high concentrations Dax1 can act as a coactivator for Sf1 (35).

We found that Dax1 and Sf1 interact with a functional RNA called steroid receptor RNA activator (SRA). SRA has been shown to complex with several nuclear receptors and act as a scaffold for the recruitment of coactivators (36-38). This publication demonstrated that Dax1 also associated with TIF2, a coactivator in the p160 family of proteins. In adrenocortical cell lines, we found that at high concentrations Dax1 upregulated expression of StAR and the melanocortin 2 receptor, and that SRA knockdown abrogated this effect. Thus, transcriptional regulation by Dax1 is not as straightforward as once thought and investigation into the mechanism by which Dax1 can function as a transcriptional coactivator will allow us to understand more fully the roles of Dax1.

Though Sf1 has been shown to regulate Dax1 expression in steroidogenic organs, and the expression of Dax1 is virtually undetectable in the Sf1 null mouse, there have been reports of Dax1 positive Sf1 negative cells in the ovary (7,39). Additionally, these studies identified Sf1 positive/Dax1 negative cells in the ovary. These observations support additional roles beyond transcriptional repression by Dax1 that have not yet been elucidated.

Dax1 knockout mouse

A Dax1 null mouse model was generated in order to study the effects of loss of Dax1 on adrenal and gonadal development (40). A traditional knockout approach of replacing the first exon of the gene with a neomycin cassette failed to generate undifferentiated embryonic stem (ES) cells, requiring the use of an alternate strategy. As such, the researchers used the Cre-Lox system, flanking the second exon of the gene with Flox sites. In cell culture, however, excision with Cre-recombinase again resulted in

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differentiation of the ES cells. Instead, a mouse line with the Flox sites flanking the second exon was created and crossed to a CMV-Cre mouse line in order to delete the second exon *in vivo*, which was successful. While loss of Dax1 had no effect on ovarian development, Dax1 loss resulted in disruption of spermatogenesis with degeneration of the testicular germinal epithelium. Studies on the adrenal gland of the Dax1 null mouse have shown that there is a failure of normal regression of the fetal adrenal gland, and a decrease in ACTH to corticosterone ratio, suggesting hyperdifferentiation of the adrenal gland (19). This observation, which is in contrast to human Dax1 mutations causing adrenal insufficiency, may be explained by species differences in the gene, by timing of disease onset or due to problems with the method of knockout of the Dax1 gene in the mouse.

Dax1 in mouse ES cells

While the study of the Dax1 null mouse answered some questions and raised others regarding the *in vivo* role of Dax1, these studies were the first to observe the expression of Dax1 in mouse ES cells. In fact, this was the first demonstration of Dax1 expression before embryogenesis of the steroidogenic organs. Interestingly, a much later report listed Dax1 as one of the top twenty enriched mRNAs in mES cells (41). Before we examine what is known about Dax1 in mES cells, we will review ES cell biology.

ES cells are pluripotent cells derived from the early embryo which can give rise to the three germ layers in the body, ectoderm, mesoderm and endoderm (Figure 1.4). In mouse development, the fertilized zygote progresses through a series of cleavage divisions (for review, see (42)). After this, the morula, which exists on approximately

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embryonic day 2.5 (E2.5), contains sixteen cells that are beginning to segregate into those which will give rise to embryonic structures and those which will give rise to extra-embryonic structures. At the blastocyst stage, with about thirty-two cells at E3.5, this segregation is largely complete. The fluid-filled structure is comprised of two layers. The outer layer of extra-embryonic cells now begins to form the trophoblast, the support cells of the embryo. The inner cells of the blastocyst make up the inner cell mass (ICM), which give rise to the epiblast cells that will give rise to all the tissues of the body. ES cell lines are derived from the ICM and maintain the ability to differentiate into the three germ layers. mES cell lines were first generated almost thirty years ago, and since then techniques for maintaining and manipulating these cells have expanded enormously (for review, see (43)).

In order to form an embryo, the early cells of the embryo must be able to proliferate and maintain their pluripotency until the late blastocyst stage. Using mES cells as a model of the ICM *in vitro*, investigators have elucidated much of the transcriptional networks which regulate this proliferation and maintenance of pluripotency. Several transcription factors have been shown to be critical in this network including Oct4, Sox2, Nanog, Klf4, and Stat3. The relevant details of these pluripotency markers will be discussed further here.

One of the key reagents to maintaining undifferentiated mES cells in culture (without a feeder layer of fibroblasts) is leukemia inhibitory factor (LIF). LIF is a soluble cytokine that signals through the gp130 family of receptors to regulate activity of the signal transduction and activation of transcription (STAT) family of factors. Studies on the role of LIF in mES cells showed that it promotes activity of STAT3, which helps

inhibit expression of differentiation genes and activate c-myc, a protein involved in proliferation of mES cells (44-45). However, it is thought that STAT3 plays a more accessory role in the maintenance of mES cells.

In the absence of LIF, overexpression of the homeodomain factor Nanog has been shown to be able to maintain self renewal and pluripotency (41,46). Nanog null embryos fail to form epiblast cells and only form primitive endoderm. Under various culture conditions, Nanog has been shown to block endodermal, mesodermal and neuronal differentiation (44,47). Nanog expression has been shown to be regulated by Sox2, Oct4 and Klf4, and Nanog in turn regulates Sox2 and Oct4 expression (48-50). Thus, Nanog plays a key role in maintaining pluripotency.

The POU family transcription factor, Oct4, is perhaps the most critical ES cell regulator (51). Oct4 null embryos fail to form an ICM, and blastocysts contain only trophoblast cells. This is at least in part due to Oct4 transcriptional repression of the trophoblast factor Cdx2 (52). Oct4 expression is necessary for pluripotency, but Oct4 expression must also not exceed two-fold overexpression, as this causes differentiation to endoderm-like cells (53). Thus, a specific Oct4 concentration serves as a gatekeeper of pluripotency. As such, Oct4 expression in mES cells is carefully regulated by at least eight factors, including itself, Sox2, LRH-1, Nanog and Klf4 (48-50). Oct4 in turn regulates expression of the other pluripotency factors, including Nanog and Sox2.

Sox2, a member of the Sox (SRY-related HMG box) gene family, is also required for pluripotency, as Sox2 null embryos fail at a similar time as either Nanog or Oct4 null embryos (54). Studies have shown that Oct4 and Sox2 bind to dual binding sites throughout the genome and cooperate to regulate expression of many genes (55). Sox2

expression is regulated by Nanog, Oct4, Klf4 and itself, and Sox2 regulates expression of each of these as well as c-myc. However, the main mechanism of maintenance of pluripotency by Sox2 is regulation of Oct4 (56). Finally, the last major factor is the Kruppel-like factor 4, Klf4. While not required for the maintenance of the undifferentiated state, Klf4 has been shown to cooperate with Oct4 and Sox2 to activate important mES cell factor expression (57). These include regulation of Oct4, Sox2, Nanog and c-myc.

As evidenced by their transcriptional targets mentioned, these four factors (Nanog, Oct4, Sox2 and Klf4) make up a tight transcriptional network where each factor regulates itself and each other. This serves to ensure proper expression of each factor with several layers of backup mechanisms. These critical factors thus make up the core circuitry which is absolutely required for mES cell pluripotency (see Figure 1.5 and for review, (58)).   Moreover, recent studies have shown that exogenous expression of Oct4, c-myc, Klf4 and Sox2 can "reprogram" somatic cells to an ES cell-like state, known as induced pluripotent stem (iPS) cells (59).

Though study on Dax1 in mES cells is limited, the reports that have been published make it clear that Dax1 is critical for mES cell pluripotency. Dax1 was shown to be expressed specifically in undifferentiated mES cells and cells of both the ICM and trophoblast, and its expression in mES cells decreases when the cells are forced to differentiate by a variety of mechanisms (60). Multiple reports show that loss of Dax1 results in differentiation of mES cells. First, knockdown of Dax1 with siRNA resulted in clear morphological differentiation of mES cells and an upregulation of endoderm markers (61). Conditional deletion of Dax1 in mES cells also resulted in spontaneous

differentiation. A more thorough examination of Dax1 knockdown was performed and showed differentiation to a mixture of all three germ layers (62).

Multiple transcription factors have been shown to regulate Dax1 expression in mES cells. First, it was reported that STAT3 and Oct4 promote Dax1 transcription, and the sites through which these transcription factors regulate Dax1 were identified (63). STAT3 was shown to bind to and regulate Dax1 transcription through a proximal promoter site 158 base pairs upstream of the Dax1 start site. Oct4 was shown to bind to a compound Oct4-Sox2 site within the intron of Dax1, at 2053 base pairs downstream of the Dax1 start site. These results clearly place Dax1 in the core transcriptional network in mES cells in terms of its transcriptional regulation. However, it is not clear whether these were the only factors that regulated Dax1 expression, especially since most critical mES cell factors have many layers of transcriptional regulation in order to ensure their maintained expression. Indeed, another report showed preliminary data indicating that the Wnt/β-catenin pathway also regulates Dax1 expression in mES cells (62). However, the mechanism was not characterized to indicate whether β-catenin regulates Dax1 directly or in complex with another factor, similar to the β-catenin/Sf1 complex which regulates Dax1 expression in the gonad (10).

While there have been multiple studies aimed at understanding regulation of Dax1 expression, very few studies have identified specific mechanisms in which Dax1 participates in mES cells. Hints to the role of Dax1 have been published in systems biology papers examining protein-protein complexes and whole genome binding sites. Using affinity purification with biotinylated-mES cell factors and mass spectrometry, Dax1 was shown to be in complex with many proteins including Nanog itself (64).

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Further showing that Dax1 plays a significant role in mES cell biology, a study in which biotinylated-mES cell factors were used for ChIP-microarrays demonstrated that Dax1 is localized to 1754 genome sites (55). Comparison of Dax1 enriched sites with those of Nanog, Sox2, Nac1, Oct4, Klf4 and Zfp281 demonstrated that all of these factors showed significant similarity in their binding sites, and sites with at least four of these factors were more likely to be within the locus of a gene that is expressed in mES cells. This study clearly indicates that Dax1 plays a significant role in the gene expression network of mES cells that is necessary for maintenance of pluripotency and self renewal.

In addition to characterizing the multi-lineage differentiation that occurs with Dax1 knockdown, gene expression microarrays were performed at 24 and 48 hours following Dax1 knockdown in order to determine what lineages Dax1 controls (62). The study showed upregulation of many genes involved in muscle, heart (mesoderm) and neural (ectoderm) differentiation in addition to cell proliferation and apoptosis genes. The majority of genes which were altered in the microarray analysis were upregulated with knockdown (suggesting transcriptional repression by Dax1), but 10% of the genes were downregulated. As there is more evidence of Dax1 acting as a transcriptional repressor, these results are perhaps not surprising. However, the ChIP-microarray study mentioned above, which placed Dax1 more often on gene targets which are actively expressed in mES cells, is at odds with this observation (55). Clearly, much more study is needed to fully understand Dax1 transcriptional regulation in these cells.

A recent report showed that Dax1 binds directly to Oct4 in a protein-protein complex and Dax1 thereby inhibits Oct4 transactivation of target genes (65). The complex is mediated by the POU domain of Oct4 and a portion of the LBD of Dax1.

Furthermore, overexpression or knockdown of Dax1 results in downregulation or upregulation of Oct4 target gene luciferase reporters, respectively. It was also demonstrated by ChIP assays that the amount of Oct4 bound to its target promoters decreased with overexpression of Dax1. Finally, the group showed that overexpression of Dax1 resulted in differentiation of mES cells to a trophectoderm lineage. While this result seems surprising because the knockdown of Dax1 causes differentiation, this phenomenon has been observed before with Oct4 expression. However, differentiation to trophectoderm implies a downregulation of Oct4, which was observed in this experiment but not in those where Dax1 was overexpressed and ChIP performed (53). However, Dax1 regulation of Oct4 expression complicates the interpretation of these data. The exact details of the relationship between Dax1 and Oct4 remain to be determined.

Other nuclear receptors have been shown to be required for mES cell pluripotency, specifically, ERRβ and LRH-1. ERRβ is expressed in a subset of extra-embryonic cells; therefore, the ERRβ knockout mouse is characterized by impaired trophoblast cell differentiation (66-67). ERRβ regulates many genes in mES cells and knockdown of ERRβ in these cells results in differentiation (68). LRH-1 on the other hand is expressed in the ICM. LRH-1 upregulates Oct4 expression in mES cells, and LRH-1 null epiblast cells differentiate and embryos die between E6.5 and 9.5 in a strain dependent manner (69-70).

Sf1 has been shown to be expressed in embryonal carcinoma cells, but data regarding Sf1 expression in mES cells is not convincing, as a single study showed that only after repeated PCR amplification of cDNA from mES cells, could a signal be achieved (60,69). Indeed, our data show that neither Sf1 mRNA nor protein is present in
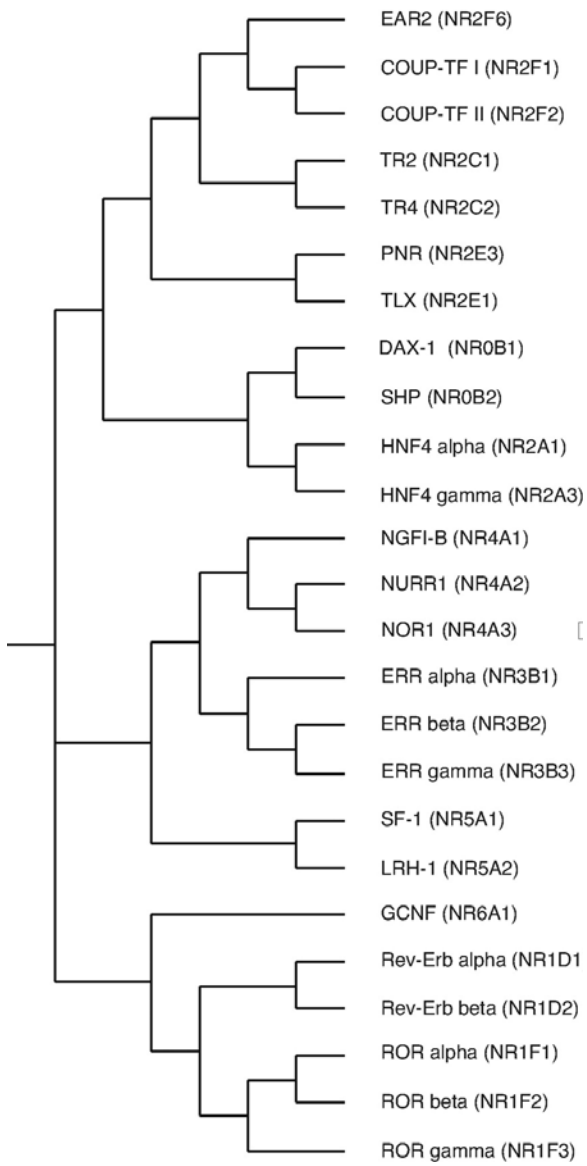
mES cells. However, Sf1 is expressed in the ICM of embryos, so it may have a role in the early embryo. Nonetheless, the lack of Sf1 expression in mES cells raises many questions about the regulation and role of Dax1 in mES cells, as Sf1 has been shown to be the main regulator of Dax1 in other tissues. While some mechanisms have been shown to regulate Dax1 expression in mES cells, there are likely more factors which activate Dax1 transcription. Though these data have begun to reveal how Dax1 expression is regulated in mES cells, there are likely more mechanisms which regulate its transcription. Specifically, two mechanisms are worthy of attention. First, though Sf1 is not expressed in mES cells, its closest nuclear receptor family member LRH-1 is expressed, and it has been extensively demonstrated that LRH-1 binds to similar and many of the same promoters as Sf1 (71). Thus, whether LRH-1 binds to the Dax1 proximal promoter and regulates expression remains to be determined.

In addition, a chromatin immunoprecipitation (ChIP) study on the genome wide binding targets of Nanog identified Dax1 intron DNA which was bound by Nanog, suggesting that Nanog may regulate Dax1 expression; however, the site was not characterized or validated (49). As it has been shown that genes significantly expressed in mES cells typically have at least four transcription factors bound to their promoters, it would not be surprising that Dax1 is regulated by several transcription factors (55). As mentioned, Oct4 has been shown to have at least eight factors which regulate its expression. Thus, there may be many different mechanisms that serve to ensure proper Dax1 expression. Chapter 2 of this thesis will show that both LRH-1 and Nanog regulate Dax1 expression in mES cells through sites within the proximal promoter and intron, respectively.

Because mES cells and blastocysts do not generate steroids (which would require Sf1 expression), Dax1 must participate in processes other than steroidogenesis. In steroidogenic cells, Sf1 regulates Dax1 transcription and then Sf1 and Dax1 participate in a protein-protein complex whereby Dax1 regulates Sf1 target gene activity, either by coactivation or corepression. As we demonstrate that LRH-1 regulates Dax1 expression in mES cells, we hypothesized that Dax1 and LRH-1 would form a protein-protein complex and Dax1 would regulate LRH-1 target genes. Chapter 3 of this thesis will demonstrate that in mES cells Dax1 binds to LRH-1 and the functional RNA SRA to co-activate Oct4 expression.

This thesis, titled "Regulation and Role of Dax1 in mES Cells", contributes significantly to our understanding of an important regulator of stem cell biology. Further work on Dax1 will elucidate the mechanisms that regulate the self renewal and pluripotency of mES cells, which is necessary for developing therapeutics using ES cells.

Figure 1.1 The Orphan Nuclear Receptor Families. Note the NR0 family which

contains Dax1 and the other member SHP, and the NR5A family, including Sf1 and

LRH-1.   Modified from (72).

EAR2 (NR2F6)

COUP-TF I (NR2F1)

COUP-TF II (NR2F2)

TR2 (NR2C1)

TR4 (NR2C2)

PNR (NR2E3)

TLX (NR2E1)

DAX-1 (NR0B1)

SHP (NR0B2)

HNF4 alpha (NR2A1)

HNF4 gamma (NR2A3)

NGFI-B (NR4A1)

NURR1 (NR4A2)

NOR1 (NR4A3)

ERR alpha (NR3B1)

ERR beta (NR3B2)

ERR gamma (NR3B3)

SF-1 (NR5A1)

LRH-1 (NR5A2)

GCNF (NR6A1)

Rev-Erb alpha (NR1D1

Rev-Erb beta (NR1D2)

ROR alpha (NR1F1)

ROR beta (NR1F2)

ROR gamma (NR1F3)

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Figure 1.2 Dax1 mouse genomic locus and gene structure. The Dax1 gene is located on the X-chromosome. The Dax1 gene is comprised of two exons and one intron. Exon 1 encodes the DNA binding-like domain and part of the ligand binding domain and exon 2 encodes the rest of the ligand binding domain. From (73).
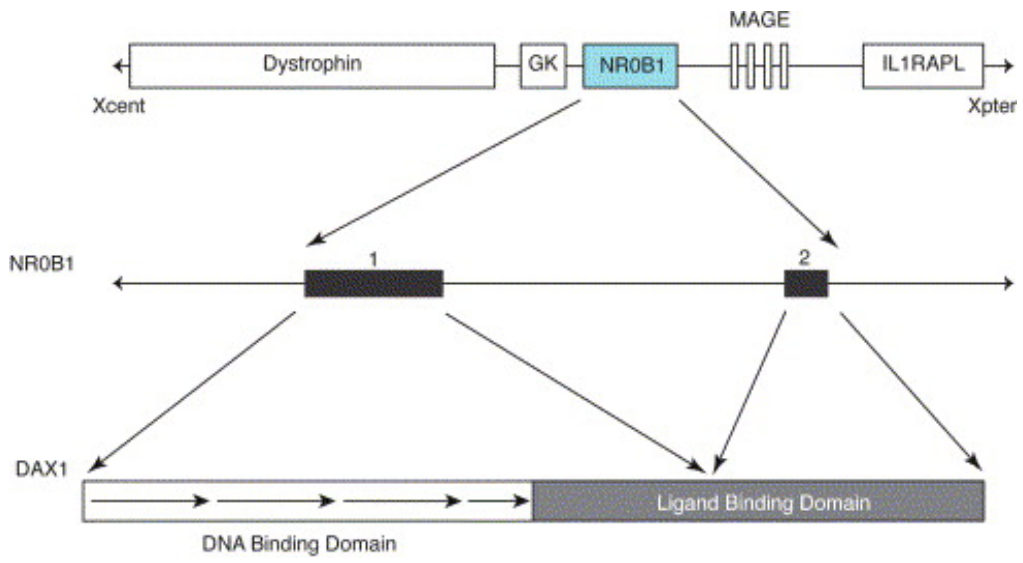
Figure 1.3 Comparison of the domains of prototypical nuclear receptors (NRs) (A) with Dax1 (B). The "Modulator" domain of NRs can contain a constitutive activation domain. The DNA-binding domain (DBD) contains two zinc fingers for binding to targets genes. The hinge region allows bending between the DBD and the ligand binding domain (LBD), which mediates ligand binding, dimerization and nuclear localization. Dax1 contains a ligand binding like domain and the classical AF-2 transactivation domain, but no other conserved NR domains. From (74).

A

A/B   C   D   E   AF-2

Modulator   DBD   Hinge   LBD

B

R   E   AF-2

Repetitive Domain (NTD)   LBD-like (CTD)

→ 65-70 amino acid repeat motif
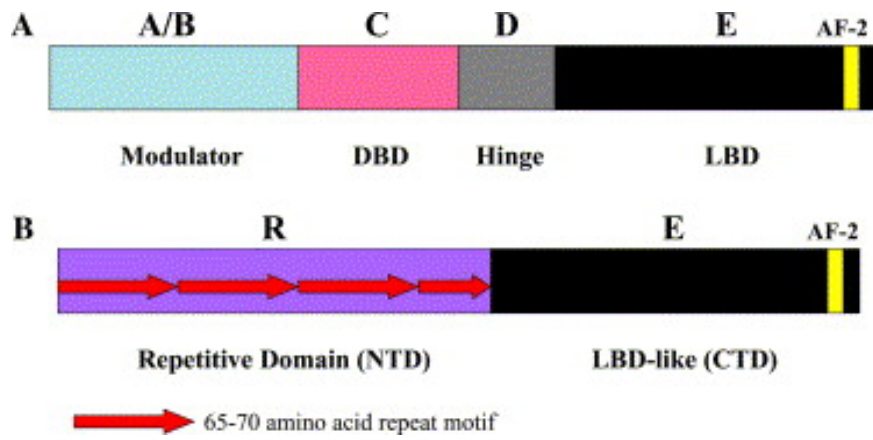
Figure 1.4 Stem Cell Heirarchy. The zygote undergoes a series of cleavage divisions, eventually forming pre-implantation embryos termed the morula and the blastocyst. ICM cells of the blastocyst form ES cell lines, which can give rise to the three germ layers. The ICM cells give rise to all tissues of the body. From (43).
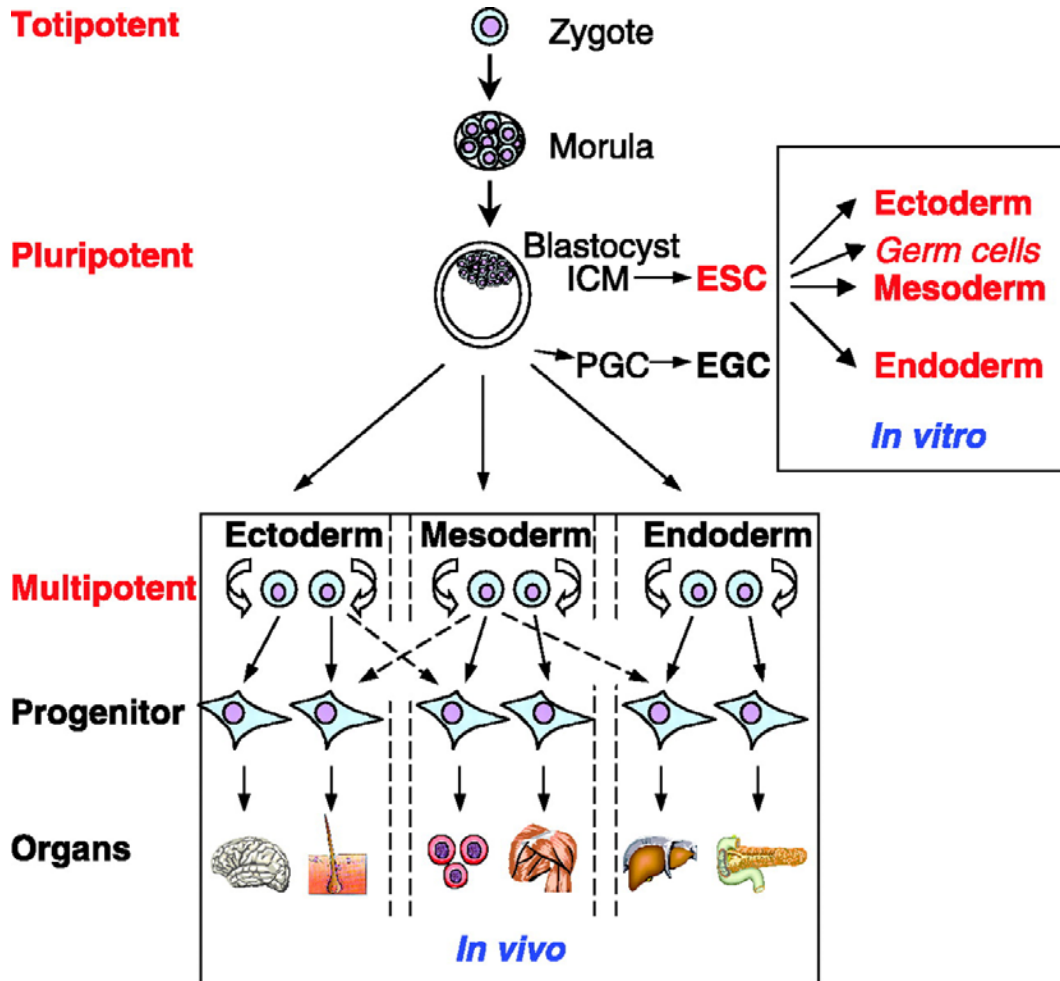
Figure 1.5 The Core Transcriptional Network of mES cells. Oct4, Nanog, Sox2, Klf4

and Myc are proposed to be the core transcription factors which regulate the

transcriptome in mES cells. Arrows indicate direct transcriptional regulation. From (58).
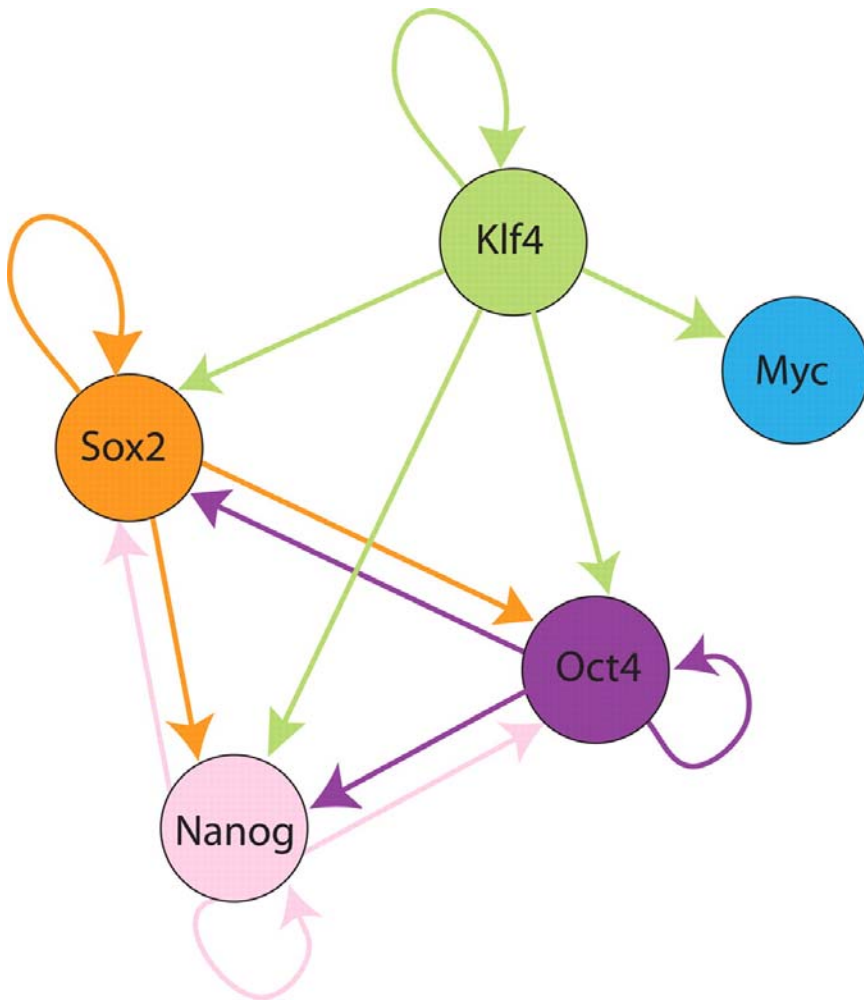
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CHAPTER 2

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

ABSTRACT

Dax1, an atypical orphan nuclear receptor expressed in steroidogenic tissues has recently been shown to be expressed abundantly in mouse embryonic stem (mES) cells where it is required for pluripotency. While the mechanisms of transcriptional regulation of Dax1 in steroidogenic organs have been well studied, those in mES cells have not. Here we report that the proximal 500 base pairs of the Dax1 gene promoter sequence are sufficient to drive expression of Dax1 in mES cells. In the adrenal cortex, NR5A1 (Sf1) binds to two NR5A nuclear receptor binding sites within this sequence to regulate Dax1 expression. In mES cells, while NR5A1 (steroidogenic factor 1, Sf1) is not expressed, NR5A2 (liver receptor homolog 1, LRH-1) expression is robust. Luciferase assays, EMSA, as well as overexpression and knockdown studies demonstrate that LRH-1, through binding to these sites, 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 contains a Nanog

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binding motif bound by Nanog. Overexpression and knockdown of Nanog in mES cells result in alteration of Dax1 expression, and luciferase assays reveal that the addition of 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 through the proximal promoter and an intronic site, respectively.

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 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,

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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.

MATERIALS AND METHODS

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 37ºC 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). The

plasmids -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' atgactcaaaagcaactttcaagcagatttgcttaggcaatgatatacgttttcttacttcctttacaaaattt 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.

Luciferase assays- D3 or HEK293 cells were plated at a density of 1 x 10^5 or 5 x 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).

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 95ºC 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 100x 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.

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.

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 4ºC. Soluble protein was collected from centrifuged total lysates

(13,000 rpm for 10 min) and quantified by Bradford assay (BioRad). Protein lysates (15-

25 µg) 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) (1:10,000) 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.

Chromatin Immunoprecipitation (ChIP)- ChIP assays 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.

RESULTS

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 did not result in increased activity (data not shown). Previous work had shown that the nuclear receptor Sf1 bound to two sites within this region to induce Dax1 expression in steroidogenic tissues (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.

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

To determine if 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 eliminated binding (-128-LRH-1 RE mut) (Figure 1D) (9). These data indicate that LRH-1 protein binds to the -128 RE in the Dax1 promoter *in vitro.*

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

To determine whether endogenous LRH-1 binds to the Dax1 promoter in mES cells, we performed chromatin immunoprecipitation (ChIP) assays. Immunoprecipitation with anti-LRH-1 antibody but not normal serum results in enrichment of the -128 Dax1 promoter by QPCR (Figure 1E). These data indicate that LRH-1 binds to the Dax1 promoter in mES cells.

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 more than 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 mainly through the -128 RE.

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).

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.

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 tested the

mutation to confirm loss of binding by EMSA assays (Figure 5C) (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).

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 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

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of promoter activity compared to the -500/intron construct (intact LRH-1 site, intact Nanog site). This puzzling result may suggest a competition between LRH-1 and Nanog, such that when Nanog can no longer be bound, LRH-1 can activate the promoter response elements more strongly.

Nanog is specifically expressed in ES cells and EC cells; 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. On the other hand, LRH-1 is expressed in the ovary, perhaps in the cells in which Dax1 is expressed. 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 (23). 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 (3,24). 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.

ACKNOWLEDGEMENTS

Figure 2.1. **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). 0-2 µl of a 50 µl IVTT reaction was used in each reaction.

(E) 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 2.1
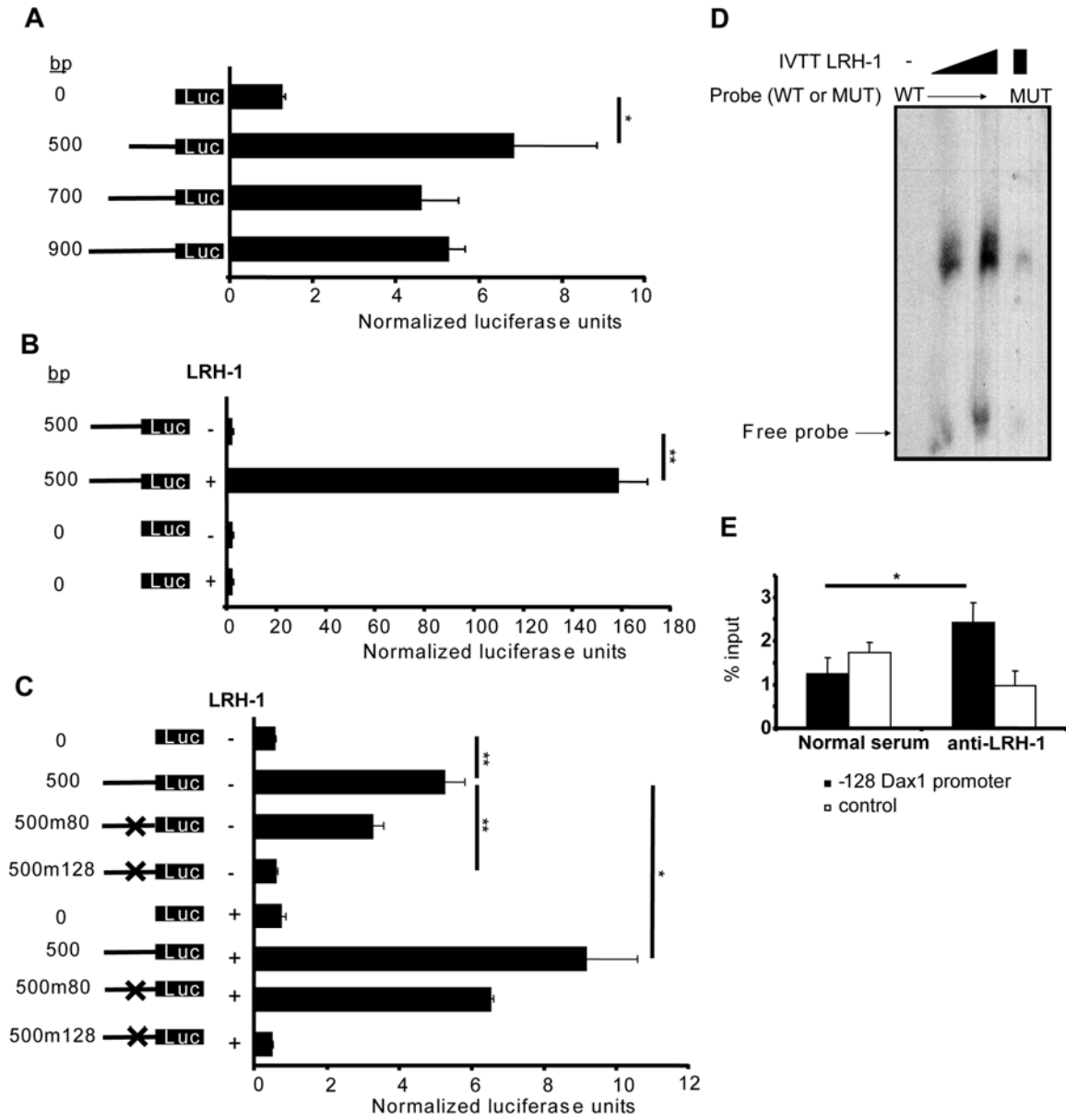
Figure 2.2. **Overexpression and knockdown of LRH-1 alters endogenous levels of Dax1.**

(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.
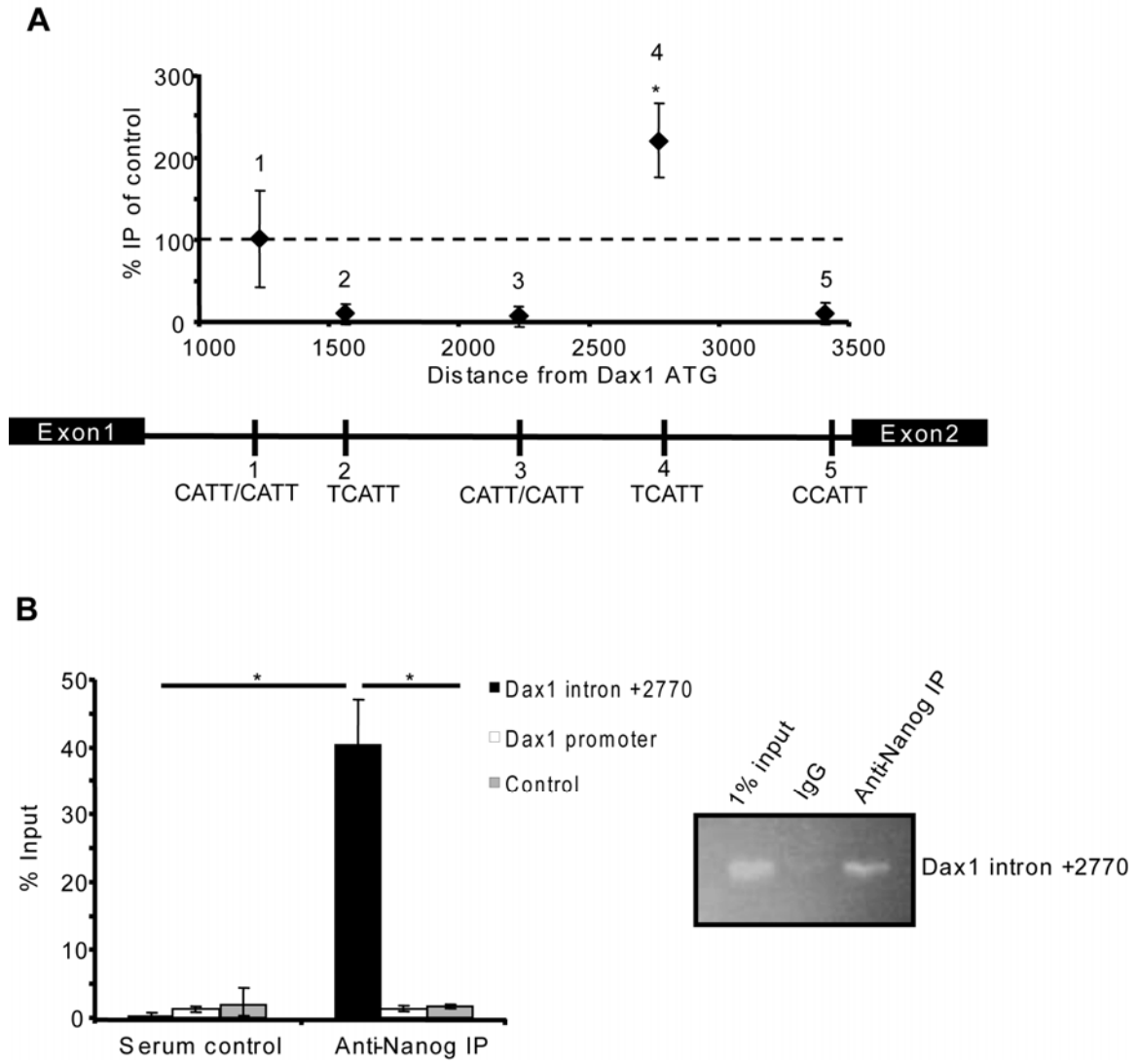
Figure 2.2
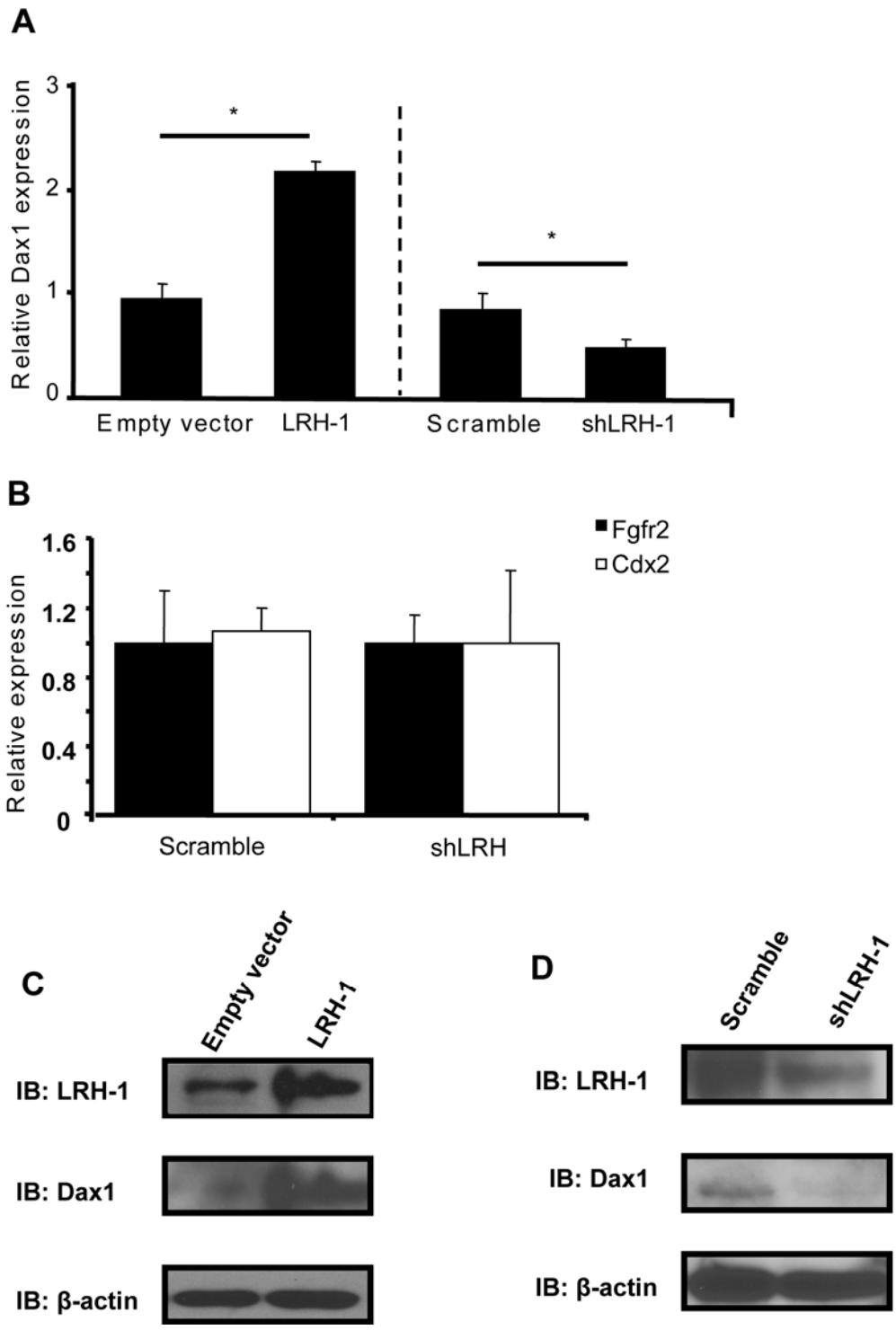
Figure 2.3. **Nanog binds to the Dax1 intron at +2770.**

(A) Primer sets flanking putative Nanog binding sites shown schematically (bottom) were designed, and chromatin immunoprecipitation (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. *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

Figure 2.3

Figure 2.4. **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 2.4
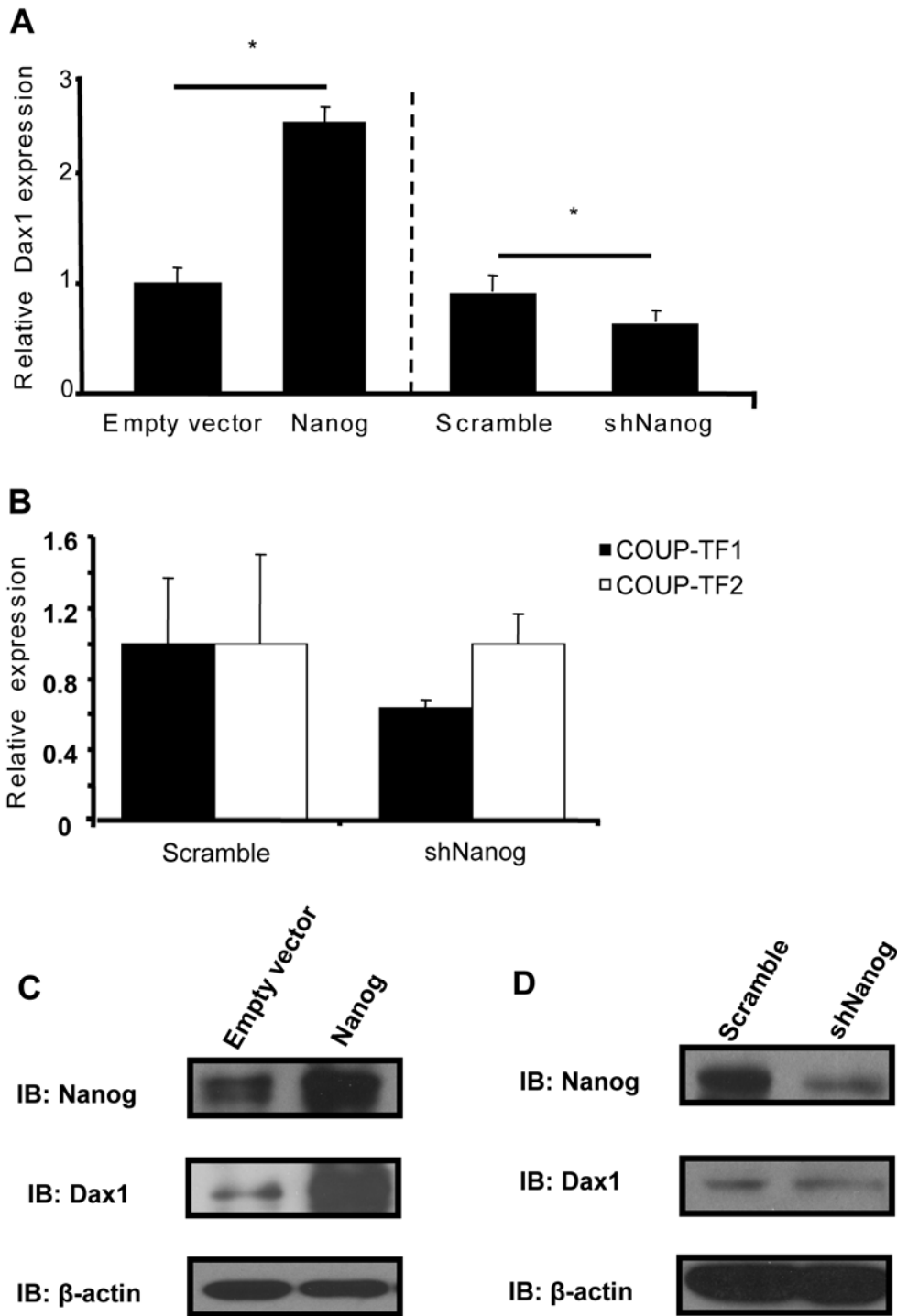
Figure 2.5. **LRH-1 and Nanog co-regulate Dax1 transcription.**

(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) 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.

(D) 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.

Figure 2.5
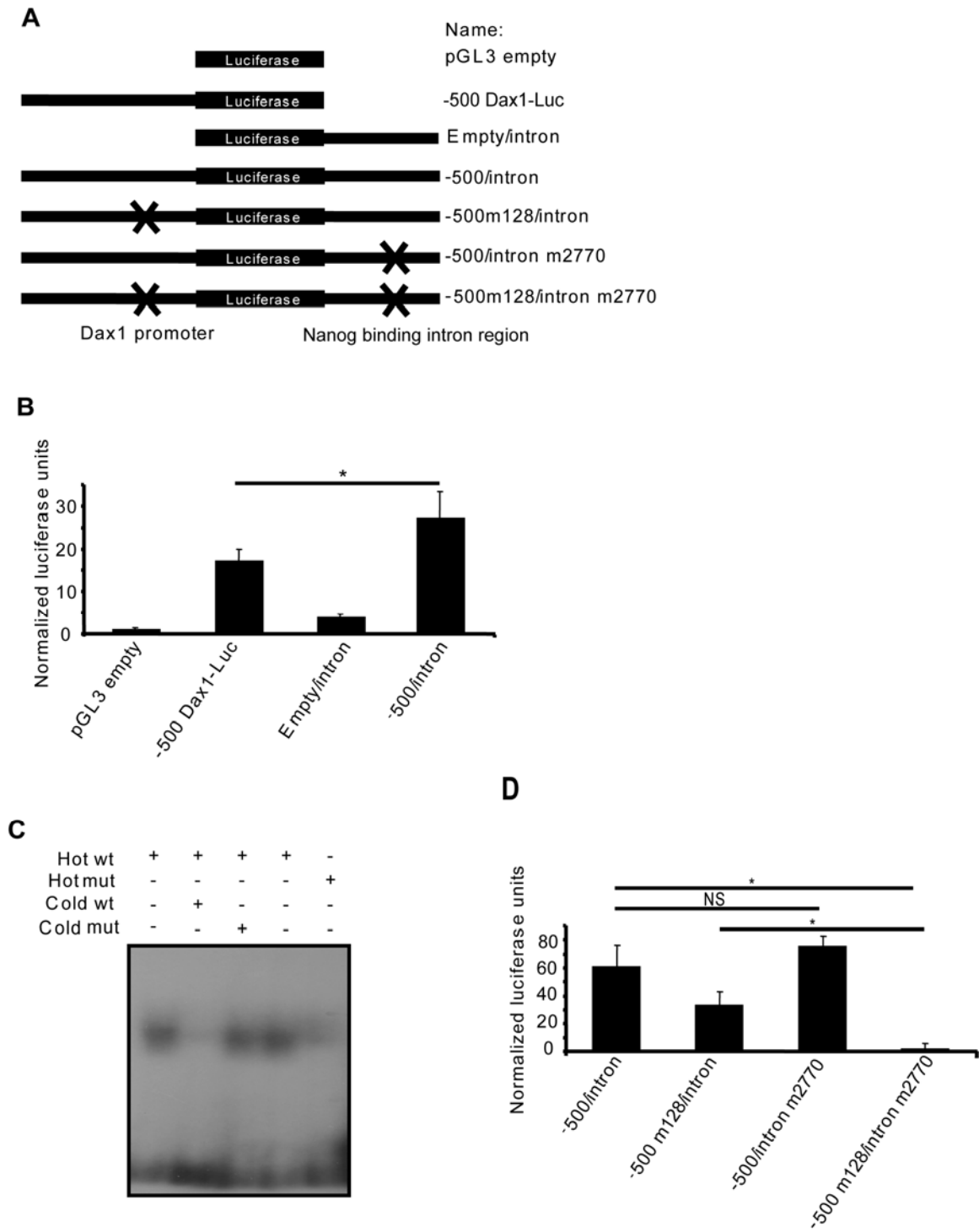
Table 2.1 EMSA oligos

	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 2.2 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

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CHAPTER 3

DAX1 UPREGULATES OCT4 EXPRESSION IN MOUSE EMBRYONIC STEM CELLS VIA LRH-1 AND SRA

ABSTRACT

Dax1 is an atypical orphan nuclear receptor that has recently been shown to play a significant role in the maintenance of mouse embryonic stem (mES) cell pluripotency. Here we explore the role of Dax1 in mES cells and describe a potential mechanism by which Dax1 maintains pluripotency. In steroidogenic cells Dax1 protein interacts with the NR5A nuclear receptor steroidogenic factor 1 (Sf1) to inhibit transcription of target genes. In mES cells, liver receptor homolog 1 (LRH-1), the other NR5A family member, is expressed and LRH-1 has been shown to interact with Dax1. We demonstrate by co-immunoprecipitation experiments that Dax1 is, indeed, able to form a complex with LRH-1 in mES cells. As Dax1 was historically characterized as an inhibitor of Sf1 mediated transcriptional activation, we hypothesized that Dax1 would similarly inhibit LRH-1 action in mES cells. Therefore, we examined the effect of Dax1 on the LRH-1 mediated activation of the critical ES cell factor Oct4. While chromatin

immunoprecipitation localized Dax1 to the Oct4 promoter at the LRH-1 binding site, luciferase assays together with Dax1 overexpression and knockdown experiments revealed that rather than repress, Dax1 accentuated LRH-1 mediated activation of the Oct4 gene. Similar to our previously published studies that defined the RNA co-activator Steroid Receptor RNA activator (SRA) as the critical mediator of Dax1 co-activation function, Dax1 augmentation of LRH-1 mediated Oct4 activation is dependent upon SRA. Thus, our results indicate that Dax1 plays an important role in the maintenance of pluripotency in mES cells through transcriptional activation of Oct4.

INTRODUCTION

Self renewal and pluripotency are essential properties that allow ES or ICM cells to form all cell types of an organism. In the case of mouse ES (mES) cells, the network circuitry that maintains the undifferentiated state is complex and involves several transcription factors, including Oct4, Nanog, Sox2 and STAT3 (for review, see (1)). The regulation of expression of these factors is marked by a high degree of redundancy, likely due to their critical roles in maintaining pluripotency. For example, Oct4 levels in mES cells must remain within an exquisitely tight window, as only two fold increase in expression of Oct4 results in endoderm/mesoderm differentiation (2). Indeed, there have been reports that at least eight factors, including Oct4 itself, regulate the proximal Oct4 promoter (reviewed in (1)).

Dax1 is an atypical orphan nuclear receptor that was cloned more than a decade ago as the gene mutated in patients with X-linked congenital adrenal hypoplasia (3). Since that time, Dax1 has been defined as a transcriptional repressor that binds the

nuclear receptor steroidogenic factor 1 (Sf1) to inhibit Sf1 dependent transcription of steroidogenic genes (reviewed in {}). Recently, our understanding of the roles of Dax1 has expanded enormously. The observation that Dax1 is expressed in mES cells suggests that Dax1 participates in cellular processes in addition to the inhibition of steroidogenesis (4-5). Indeed, knockdown of Dax1 results in differentiation of mES cells to multiple lineages, showing that Dax1 is required for the maintenance of pluripotency (5-6). Additionally, microarray and whole genome binding studies in mES cells have shown that Dax1 binds to and regulates expression of thousands of genes (6-7). These reports illustrate that Dax1 plays a significant, but largely undefined role in the maintenance of pluripotency of mES cells.

Although an early study suggested that Dax1 can bind to a hairpin loop structure in the promoter of the steroidogenic acute regulatory protein (StAR), minimal evidence exists for direct DNA binding by Dax1 (8). Therefore, we began to explore a role for Dax1 in mES cells by hypothesizing a protein-protein interaction to provide clues to its targets. Indeed, a recent publication showed that Dax1 could bind directly to the Oct4 protein and inhibit its activation of target genes, indicating one way in which Dax1 regulates gene transcription in mES cells (9). In steroidogenic cells, Dax1 interacts with Sf1 protein and through this interaction alters target gene activation (10-11). Sf1 is not expressed in mES cells, but LRH-1, the closest nuclear receptor family member, is expressed in these cells, and furthermore is required for expression of the critical ES cell factor Oct4 (12). In this report we show that Dax1 and LRH-1 physically interact in mES cells, and that Dax1 localizes to the Oct4 promoter at the LRH-1 binding site. However, rather than repress Oct4 activation, we found that Dax1 co-activates LRH-1 mediated

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Oct4 transcriptional activation. Our recent work has shown that the interaction between

Dax1 and a novel RNA activator Steroid Receptor RNA activator (SRA) allows Dax1 to

act as a context dependent activator (11,13). We now demonstrate that Dax1 interacts

with SRA in mES cells, and that SRA is required for the co-activation of Oct4 by Dax1.

Our results identify a specific mechanism for Dax1 dependent Oct4 expression and define

a potential mechanism by which Dax1 serves to maintain pluripotency in mES cells.

MATERIALS AND METHODS

Cell culture and Transfection- The D3 line of mouse embryonic stem (ES) cells

(a kind gift from K. Sue O'Shea) was 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 (Hyclone), 10^{-4}M β-

mercaptoethanol (Sigma), 0.224 µg/ml L-glutamine (Gibco), 1.33 µg/ml HEPES (Gibco),

100 units penicillin, 100 µg/ml streptomycin and 1,000 units/ml LIF (Chemicon). F9

embryonal carcinoma cells were maintained on 0.1% gelatin-coated substrates in DMEM

with 10% bovine serum (Gibco) and penicillin-streptomycin. All cells were grown at

37ºC in a humidified atmosphere of 5% CO_2. Transient transfection was performed with

Lipofectamine 2000 at a ratio of 3 µl:1 µg DNA (D3 cells) or 7 µl:1 µg DNA (F9 cells).

Plasmids- pcDNA3 Dax1, pHA-Dax1 (previously named pHA Dax1-VC), pDax-

1-Myc and pCMV-3tag-4A have been previously described (11). pGL3-Oct4 (hereafter

called Oct4-Luc) was cloned by insertion of a 1141 bp PCR amplified Oct4 promoter

fragment into the KpnI/XhoI sites of pGL3 Basic. Primers used are as follows:

5'CCGGGTACCCCCATGGCTGGACACCTGGCTTCA 3' and 5' CCGCTCGAGACCCCAAAACTTCAGGTTCTCTTGTCT 3'. A mutated Oct4-Luc construct (Oct4-Luc mPP) was generated using Quik Change Mutagenesis Kit (Stratagene) and the following primers: 5' ggggccagaggtcaaacctagagggtgggatt 3' and 5' aatcccaccctctaggtttgacctctggcccc 3'. shRNA expression constructs (Open Biosystems pGIPZ shDax1, pGIPZ scramble) were obtained from the University of Michigan shRNA Core Facility (http://fgc.lsi.umich.edu/). pcDNA LRH-1 was a generous gift from Dr. William Rainey (Medical College of Georgia). pSuperior shSRA and pSuperior scramble were used previously as described (11).

Immunocytochemistry and Alkaline Phosphatase Staining- For immunocytochemistry, D3 cells were washed with phosphate buffered saline (PBS) and then fixed for 1 minute with 1:1 acetone:methanol. Cells were washed and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. After a second wash with PBS, cells were blocked and stained with anti-HA mouse IgG-Alexa Fluor 488 antibody at 1:1000 (Invitrogen) in buffer with 1% ovalbumin and 1% BSA for 2 hours at room temperature. After washing 3x5 minutes in PBS, the cells were stained with anti-Oct4 antibody at 1:100 (Santa Cruz) at 4° overnight. Cells were washed and then stained with rabbit anti-goat biotinylated secondary antibody (Pierce, 1:250) for two hours at room temperature followed by incubation for one hour at room temperature with streptavidin- Texas Red (Sigma, 1:400). Cells were counter-stained with DAPI. For alkaline phosphatase (AP) staining, cells were washed with PBS and then fixed for 2 minutes with 4% formalin in PBS. Cells are then equilibrated in AP buffer for 5 minutes (100 mM Tris-HCl pH 9.5,

100 mM NaCl and 10 mM $MgCl_2$). After equilibration, AP staining was performed using 90 µl NBT/BCIP (Roche) in 10 ml AP buffer for 10 minutes. Images were captured using Leica Stereo or Inverted microscopes in the Microscopy and Image-analysis Laboratory (MIL) at the University of Michigan Department of Cell & Developmental Biology. Images were subjected to colony counting using Metamorph software (Molecular Devices).

Luciferase assays- D3 or F9 cells were plated at a density of 1 x 10^5 cells per well in 24-well plates. Twenty-four hours after plating cells were transiently transfected with luciferase reporter constructs as noted in each figure, and harvested 48 hours post transfection (except where noted). Cell lysates were assayed for luciferase activity using Dual Luciferase Assay (Promega) with an injector luminometer. Luciferase activity was normalized by transfection of pRL-TK Renilla luciferase (Promega).

Gene Overexpression and Knockdown Assays- Overexpression experiments were carried out as follows: 2.5 x 10^5 cells were plated in 6 well plates, and twenty four hours later transfected with 2-3 µg DNA. After forty eight hours cells were harvested, RNA isolated, cDNA synthesized and quantitative PCR carried out as described previously (14). To analyze for upregulation of protein, 3 x 10^6 cells were plated in 10 cm plates and and twenty four hours later transfected with 10 µg pcDNA3 Dax1 or empty vector. Forty eight hours later cells were harvested as described under Western Blotting procedures.

The method of stable knockdown of endogenous SRA in mouse ES cells was modified from previously described methods (11). The short hairpin RNA (shRNA) construct targeting mouse SRA and the scramble-sequence shRNA control are as described (11). The SRA and control shRNAs were expressed from the retroviral vector pSuperior.retro.puro (OligoEngine). The retroviruses were grown in and harvested from phoenix cells (kindly provided by G. Bommer, University of Michigan) and then used to infect mES cells three times with 8-12 hour intervals. The infected cells were selected with 1 µg /ml of puromycin. The SRA-silencing effects of shRNAs were confirmed by QPCR using mouse SRA-specific primers (11).

For transient knockdown studies, experiments were carried out as follows: cells were plated in 10 cm plates, and twenty four hours later transfected with 10 µg pGIPZ shDax1 or scramble control. Twenty four hours after transfection cells were harvested and sorted by flow cytometry (using a FACSDiva) for expression from the GFP cassette in the shRNA plasmids. Cells were immediately harvested for RNA isolation and QPCR carried out using primer pairs listed in Table 1.

Co-Immunoprecipitation (Co-IP) and Western Blotting- D3 cells in 10 cm plates 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 4ºC. 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-Oct4 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 co-IP, lysates were cleared by incubation with Protein A agarose beads (Invitrogen) and subsequent washing. Immunoprecipitations were performed on 100 μg protein using 3 μg anti-LRH-1 (Santa Cruz) or normal serum and 40 μl Protein A agarose followed by stringent washing with lysis buffer. Bound proteins were resolved by SDS-PAGE and immunoblot performed as described above.

Chromatin Immunoprecipitation (ChIP)- ChIP assays were performed on mES cells as previously described (15). 2 μg anti-Dax antibody (Santa Cruz) was used per immunoprecipitation.   Results shown are representative and from independent experiments, quantitated by QPCR or visualized by PCR and agarose gel electrophoresis. Primer pairs used for ChIP assays are listed in Table 2.

RNA-Immunoprecipitation (RNA-IP)- For the immunoprecipitation of Dax1 to evaluate enrichment of SRA, mouse ES cells were transiently transfected with pDax1-Myc or empty pCMV-3tag-4A vector. The subsequent immunoprecipitation procedures were modified from previously described methods (11). Briefly, cells were washed with PBS and cross-linked with 0.1% formaldehyde for 10 min at room temperature. After the addition of 0.25 M glycine for 5 min, cells were harvested and lysed in RIPA buffer

containing protease inhibitor cocktail tablet (Roche), and 40 U/μl RNasin (Promega) and sonicated (11,16). Following immunoprecipitation with anti-Myc antibody (Immunology Consultants Laboratory), reversal of cross-linking and DNase I treatment were performed as described previously (11). RNA was then isolated by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation containing glycogen (Ambion), and total amount used for cDNA synthesis. QPCR was carried out using 2 μl of the resulting cDNA and primers that amplify mouse SRA. Data were normalized to housekeeping gene expression.

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

RESULTS

Dax1 and LRH-1 interact in mES cells

Dax1 is an atypical nuclear receptor with a well conserved ligand binding domain, but no conserved DNA binding domain (3). Although there is a report suggesting that Dax1 can bind specific DNA structures within a promoter, the predominant known role of Dax1 has been to interact with the nuclear receptor steroidogenic factor 1 (Sf1) to inhibit transactivation of target genes (8,10,17-19). Though Sf1 is not expressed in mES cells, the closest nuclear receptor family member liver receptor homolog 1 (LRH-1) is highly expressed. Because Sf1 and LRH-1 share similar protein structures, we hypothesized that LRH-1 and Dax1 may interact in mES cells. Indeed, the crystal structure of a LRH-1/ Dax1 protein complex has recently been published (20). To

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determine if this interaction occurs in mES cells, we performed co-immunoprecipitation (co-IP) experiments. When we immunoprecipitated with anti-LRH-1 antibody, immunoblotting with an anti-Dax1 antibody shows enrichment of Dax1 protein over control immunoprecipitation with normal serum (Figure 1A). These results demonstrate that LRH-1 and Dax1 are present in a protein-protein complex in mES cells.

Dax1 co-activates LRH-1 mediated Oct4 activation

Because Dax1 is known to inhibit Sf1 mediated activation of transcription, we hypothesized that Dax1 similarly inhibits LRH-1 activity. LRH-1 has been shown to activate expression of Oct4 (2,12). As such, we predicted that Dax1 would regulate LRH-1 mediated activation of the Oct4 promoter in order to maintain the appropriate expression level of Oct4 in mES cells. To investigate this possibility, we carried out luciferase reporter assays utilizing a cell line that lacks endogenous LRH-1, F9 embryonal carcinoma cells. When we transfected an Oct4-luciferase reporter along with the LRH-1 expression vector, we saw the expected LRH-1 dependent increase in reporter expression (Figure 1B). However, when we co-transfected increasing amounts of pcDNA3 Dax1, we found that Oct4 promoter activity increased above the level with LRH-1 alone, indicating that Dax1 coactivated LRH-1 mediated Oct4 promoter activity.

To confirm that Dax1 activated Oct4 promoter activity, we carried out luciferase assays in mES cells. When we transfected cells with the luciferase reporter driven by the Oct4 promoter and co-transfected a Dax1 expression vector, we observed a modest but statistically significant increase in Oct4 promoter activity (Figure 1C). Conversely, we transfected the Oct4-luciferase reporter and co-transfected a vector that codes for an

shRNA against Dax1, and harvested cells for luciferase assay only 24 hours after transfection in order to examine direct effects of Dax1 knockdown and not differentiation. Knockdown of endogenous Dax1 inhibited Oct4 promoter activity when compared to scramble control (Figure 1C). These data confirm that Dax1 acts as an activator of Oct4 promoter activity in mES cells.

Dax1 overexpression does not drive differentiation

A recent publication demonstrated that stable overexpression of Dax1 caused differentiation of mES cells (6,9). Because our experiments utilize transient Dax1 overexpression, we wanted to determine whether differentiation was occurring, as this could complicate the interpretation of our results. Therefore, we overexpressed an HA-tagged Dax1 and examined Oct4 expression by immunocytochemistry as a measure of pluripotency. Cells stained with an anti-HA antibody (green) colocalize with those stained with the anti-Oct4 antibody (red), indicating that HA-Dax1 overexpressing cells remain undifferentiated (Figure 2A). Additionally, to determine whether overexpression of Dax1 changes the number of undifferentiated cell colonies, we overexpressed Dax1, performed alkaline phosphatase staining and counted the number of positive colonies as compared to empty vector control. Dramatic overexpression of Dax1 did not change the number of undifferentiated colonies (Figure 2B, 2C). In three separate experiments, the range of Dax1 overexpression was 75- to 500-fold. However, none of these resulted in decreased colony numbers.

Dax1 activates the Oct4 promoter though the LRH-1 proximal promoter site

Based on our data that Dax1 activates Oct4 promoter activity and interacts with LRH-1, we investigated whether Dax1 is localized to the Oct4 promoter at a previously characterized site of LRH-1 binding (12). To this end, we performed chromatin immunoprecipitation (ChIP) assays that detect protein bound to DNA either directly or through a complex. Immunoprecipitation with anti-Dax1 antibody, and not with normal serum, enriched for the LRH-1 site within the proximal promoter of Oct4 (PP) (Figure 3A) (12). These data together with the lack of enrichment of sequence in the proximal enhancer (PE) or control sites are consistent with Dax1 localization to the Oct4 promoter at the LRH-1 binding site within the proximal promoter. These results were observed by both quantitative PCR (QPCR) and PCR followed by agarose gel (Figure 3A, right).

The binding of Dax1 to the Oct4 promoter at the LRH-1 PP binding site suggested that the activation of the Oct4 promoter by Dax1 could be mediated by the LRH-1 PP site. To test this hypothesis, an Oct4 promoter-luciferase construct harboring a previously characterized mutation in the LRH-1 PP site (12) was tested in luciferase assays in mES cells. When mES cells were transfected with the wildtype reporter along with either empty vector or pcDNA3 Dax1, a Dax1 dependent upregulation of the reporter was observed, but when cells were transfected with the mutated reporter (Oct4-Luc mPP) there was no increase in promoter activity upon overexpression of Dax1 (Figure 3B). These data indicate that Dax1 upregulates Oct4 promoter activity through interaction with the LRH-1 PP site.

Dax1 overexpression and knockdown result in changes in Oct4 mRNA and protein

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To examine the effect of Dax1 expression on endogenous Oct4 levels, mES cells were transiently transfected with pcDNA3 Dax1 and 48 hours later harvested for RNA, and gene expression studies were performed. QPCR revealed that overexpression of Dax1 resulted in modest, but statistically significant, upregulation of Oct4 mRNA levels (Figure 4A). To examine direct changes in Oct4 mRNA levels with Dax1 knockdown, as opposed to secondary effects due to differentiation, mES cells were transfected with a vector containing an shRNA against Dax1 or scrambled and a GFP cassette, and sorted by FACSDiva flow cytometry for GFP positive cells 24 hours after transfection. Prior to any potential differentiation phenotype, a greater than 50 percent loss in Oct4 levels was observed (Figure 4A). These data indicate overexpression and knockdown of Dax1 result in upregulation and downregulation of Oct4 mRNA, respectively.

To determine whether Oct4 protein levels are altered with the mRNA levels, mES cells were transiently transfected with pcDNA3 Dax1 and 48 hours later harvested for protein. Western blot analysis determined that when Dax1 is overexpressed, Oct4 protein levels are concomitantly increased (Figure 4B). Conversely, to examine Oct4 protein changes with Dax1 knockdown, mES cells were transfected with a vector containing an shRNA against Dax1 or scrambled and a GFP cassette, and sorted by FACSDiva flow cytometry for GFP positive cells 24 hours after transfection. Western blot analysis determined that when Dax1 is knocked down, Oct4 protein levels are likewise decreased (Figure 4C). These data show that Dax1 overexpression and knockdown result in an increase or decrease in Oct4 protein in mES cells, respectively.

Dax1 interacts with SRA in mES cells

The Steroid Receptor RNA activator (SRA), an RNA that interacts with nuclear receptors and forms a scaffold for a p160 family co-activator complex to activate target gene transcription, has recently been shown to bind directly to Dax1 to facilitate Dax1 co-activator (and not co-repressor) function (11,13). Therefore, we hypothesized that SRA may mediate the ability of Dax1 to activate Oct4 expression in mES cells. RNA-IP experiments using a myc tagged Dax1 revealed that immunoprecipitation with anti-myc antibody significantly enriched for SRA in myc-Dax1 transfected mES cell lysates over empty myc vector transfected (Figure 5A).

Loss of SRA results in attenuation of the Dax1 effect on Oct4

Having determined that Dax1 interacts with SRA in mES cells, we set out to determine whether SRA mediates the Dax1- enhanced activation of Oct4. Therefore, a mES cell line with stable shRNA mediated knock down of SRA was generated. An approximately 60 percent knockdown of SRA compared to the scramble control was achieved (Figure 5B). To determine if SRA was responsible for the Dax1 activating effect on the Oct4 promoter, we carried out luciferase assays in the SRA knockdown or scramble mES cells. When scramble control cells were transfected with the luciferase reporter driven by the Oct4 promoter and co-transfected with pcDNA3 Dax1, a statistically significant increase in reporter activity was observed. In SRA knockdown cells, in contrast, overexpression of Dax1 did not induce reporter activity (Figure 5C). Similarly, when we overexpress Dax1 in SRA knockdown and scramble control cells, the SRA knockdown cells have at least two-fold less upregulation of endogenous Oct4 as

determined by QPCR (Figure 5D). These data indicate that SRA is necessary for the ability of Dax1 to co-activate Oct4 transcription.

DISCUSSION

While evidence that Dax1 plays a significant role in the maintenance of pluripotency in mES cells is abundant, few studies have examined a specific mechanism of Dax1 action in these cells. In this study, we elucidate a mechanism by which Dax1 upregulates Oct4 levels; this pathway maybe important in the maintenance of Oct4 levels within the 'window of pluripotency'. Our data show that Dax1 interacts with LRH-1 and is localized to the Oct4 promoter at the site of LRH-1 binding in the proximal promoter. It is through this site that Dax1 exerts its activating effects. Dax1 is typically known as a corepressor; however, we found that Dax1 co-activates Oct4 transcription mediated by LRH-1. We recently reported that interaction of Dax1 with the novel RNA activator, SRA, in steroidogenic cells resulted in the ability of Dax1 to act as an activator in certain contexts in a dose-dependent manner (11). In the current study, we demonstrate that Dax1 interacts with SRA in mES cells, and stable knockdown of SRA results in loss of the ability of Dax1 to co-activate Oct4. Thus, we conclude that Dax1 through interaction with LRH-1 and SRA on the Oct4 proximal promoter upregulates Oct4 expression. This is likely an important mechanism by which Dax1 maintains pluripotency in mES cells.

A previous study reported that Dax1 overexpression caused mES cell differentiation (9). Since high levels of Oct4 cause differentiation and our study shows that Dax1 overexpression results in upregulation of Oct4, we carefully evaluated the mES

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cells for any differentiation phenotype, but did not observe this in our system. Through ICC, we observed colocalization of HA-Dax1 and Oct4, proving that Dax1 does not drive differentiation of mES cells, as assessed by the pluripotency marker Oct4. Additionally, we did not see a decrease in AP-positive colonies with overexpression of Dax1. Though some HA-Dax1 positive colonies did not colocalize with Oct4 expression, this does not prove that Dax1 forced differentiation. This indicates that changes in Oct4 levels were not merely reflective of a change in differentiation, an important potential interpretation of these studies. In a transient transfection experiment, previously differentiated cells can also be transfected. Additionally, many Oct4 negative cells are present that do not colocalize with HA-Dax1 expression. Perhaps these results are at odds with the previous study because experiments described herein were not performed in stable Dax1 overexpressing lines, and utilized traditional as opposed to episomal plasmids. However, the well characterized dose dependence of Dax1 on transcription, or length of time expressed, may also play a role in the different results observed in these two studies. In humans, duplication of Dax1 is associated with gonadal sex reversal, and our recent work indicates that different concentrations of Dax1 determine whether Dax1 acts as a transcriptional repressor or activator (11,21). Thus, our finding that Dax1 overexpression does not cause differentiation but does upregulate Oct4 levels could be specific to the timing and concentration of Dax1 used in our experiments.

Our results demonstrate that Dax1 interacts with both LRH-1 and SRA in mES cells. As this interaction is not necessarily restricted to the Oct4 promoter, it is likely that Dax1 in complex with these factors controls many other gene promoters. It is interesting to hypothesize that Dax1 may act on many different promoters with varied protein or

RNA partners, each resulting in a context specific activation or repression. These data constitute the first example of the role of SRA in mES cells. Whether SRA may bind to LRH-1 as well, in the absence of Dax1, remains an open question.

Oct4 expression levels must be kept within a tight window to maintain pluripotency: a less than two-fold increase causes differentiation into a mixture of primitive endoderm and mesoderm lineages, and a decrease in Oct4 levels causes dedifferentiation to trophectoderm (2). Our experiments show that Dax1 overexpression or knockdown results in subtle changes in Oct4 mRNA and protein levels. Specifically, in luciferase reporter and gene expression studies, Oct4-Luc and Oct4 mRNA levels never change more than two-fold. A confounding variable in these overexpression experiments is that endogenous Dax1 levels in mES cells are high, with Dax1 being one of the top twenty mRNAs enriched in mES cells (22). Thus, overexpression of Dax1 may have only minor effects if Dax1 is not limiting. However, we suggest that these subtle changes in Oct4 mRNA are anticipated due to the tight requirements for Oct4 expression. Additionally, with the numerous levels of transcriptional regulation that control Oct4 levels, as experimental manipulation elevates Oct4 levels, other compensatory mechanisms may attempt to downregulate its expression to maintain pluripotency.

Although we have achieved high levels of overexpression of Dax1, we have not observed differentiation (as mentioned above). Sun et al, however, found that stable Dax1 overexpressing mES cells differentiated into trophectodermal lineage, expressing the markers Cdx2 and Rhox6 (9). This is consistent with a downregulation of Oct4, which they also observed. Their study showed that Dax1 inhibits Oct4 mediated

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transcription; as Oct4 has been shown to upregulate its own transcription, it was suggested that Dax1 may downregulate Oct4 through interaction with Oct4 on its own promoter. Additionally, previous studies have shown an interaction of Dax1 with Nanog, which has also been shown to activate Oct4 expression (23-24). Thus, we cannot rule out the possibility that Dax1 acts on other sites of the Oct4 promoter through interactions with other proteins or complexes. However, it is worth noting that our data indicate that the overall net effect of Dax1 on Oct4 expression appears to be activation.

In a separate report, Oct4 was also shown to participate in the regulation of Dax1 expression in mES cells through a site within the Dax1 intron (25). Together these data allow speculation that there may be a positive feedback loop in which higher levels of Dax1 induced by Oct4 may serve to upregulate Oct4 expression; however, because Dax1 can also inhibit the transcriptional activity of Oct4, this could serve two functions. First, this could provoke negative feedback to keep levels of Oct4 in check via its auto-induction, and second, Dax1 would inhibit Oct4 activity thereby preventing high Oct4 levels from causing differentiation. These dual mechanisms would serve to rapidly regulate the actions of Oct4 in the cell.

Previous work characterizing the effects of Dax1 knockdown in mES cells showed that after 24 hours of knockdown, 90% of altered genes in a microarray were upregulated, indicating that Dax1 likely repressed these genes (6). Thus, 10% of these genes that changed were downregulated, suggesting a role for Dax1 as a coactivator on some promoters. Additionally, the report demonstrated that in a luciferase reporter assay using a construct containing domains of Dax1 fused to the yeast Gal4 DNA binding domain that this construct repressed some artificial promoters, but failed to repress

others, showing the context specificity of the actions of Dax1. However, it was stated that Oct4 levels did not change in their experiment. Interestingly, supplemental data from an additional publication are consistent with the data shown here that knockdown of Dax1 results in a downregulation of endogenous Oct4 levels (23).

While many studies have examined Dax1 in the context of genomic experiments, characterizing the overall importance of Dax1 in mES cells, ours is only the second study to show a specific mechanism by which Dax1 regulates gene expression. Though it is important to appreciate the importance of Dax1 in these cells by the number of genomic sites it is localized to and the numerous protein interactions it participates in, understanding the details of the mechanisms is critical for full understanding of the role of Dax1 in mES cell biology. Thus, this study, showing Dax1 co-activation of Oct4 through interaction with LRH-1 and SRA, is an important contribution to the understanding of the role of Dax1 in mES cells.

ACKNOWLEDGEMENTS

Figure 3.1. **Dax1 and LRH-1 cooperate to upregulate Oct4 in mES cells.**

(A) Dax1 and LRH-1 interact in mES cells.

mES cell lysates were immunoprecipitated with anti-LRH-1 antibody and Protein A agarose beads. Bound proteins were subjected to SDS-PAGE and immunoblotting was performed with anti-Dax1 antibody as described in methods.
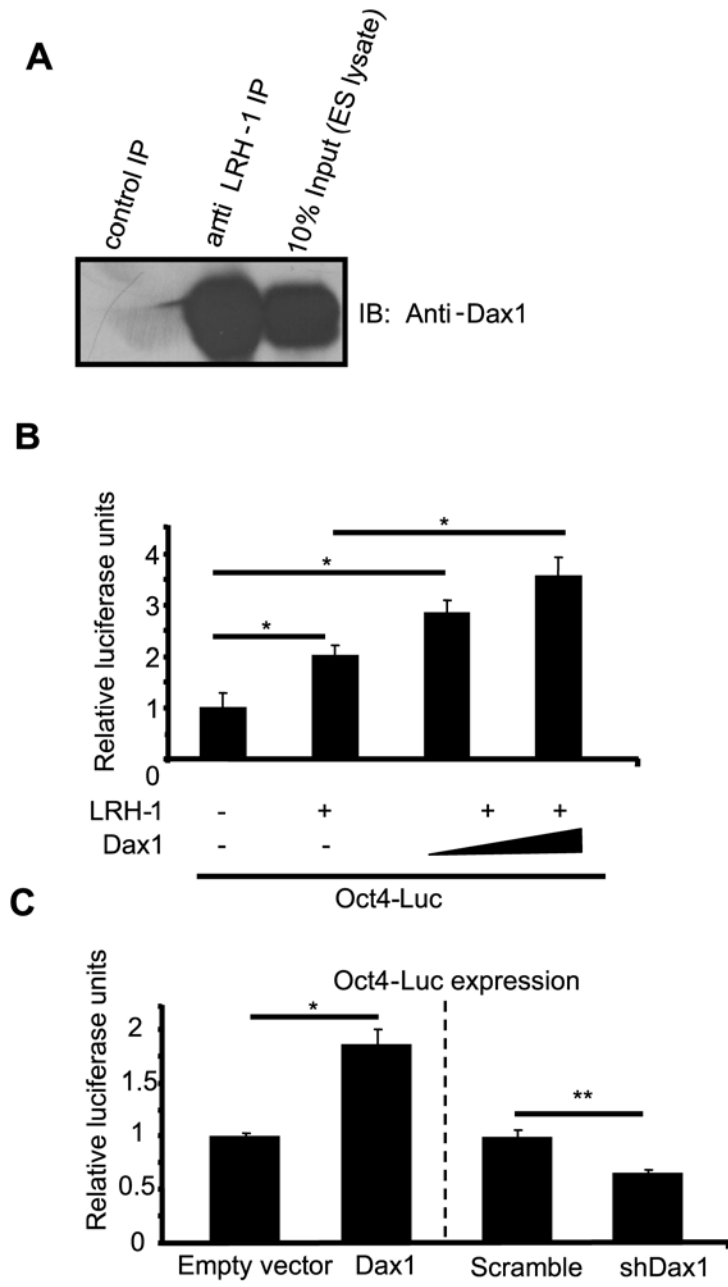

(B) Dax1 upregulates LRH-1 mediated Oct4-Luc activation in F9 cells.

F9 embryonal carcinoma cells in 24 well plates were transfected with 200 ng Oct4-Luc, and co-transfected with 200 ng empty vector, pcDNA3.1 LRH-1, and/or empty vector or 150-300 ng Dax1. Luciferase assays were carried out on lysates 48 hr hour post-transfection and values normalized to Renilla luciferase internal control. Data are presented as relative to empty vector control. $*p<0.03$, $**p<0.002$

(C) Overexpression or knockdown of Dax1 in mES cells alters Oct4-Luc reporter expression.

mES cells in 24 well plates were transfected with 200 ng Oct4-Luc and co-transfected with 200 ng empty vector, pcDNA3 Dax1, scramble or shDax1 vector. For Dax1 and empty vector transfection, cells were harvested 48 hr post-transfection and lysates subjected to luciferase assay. shDax1 and scramble transfected cells were harvested 24 hr post-transfection and luciferase assay carried out as described above. Data are presented as relative to empty vector and scramble controls.

Figure 3.1

A

control IP
anti LRH-1 IP
10% Input (ES lysate)

IB: Anti-Dax1

B

Relative luciferase units

| LRH-1 | – | + | | + | + |
| Dax1 | – | – | | | |

Oct4-Luc

C

Relative luciferase units

Oct4-Luc expression

Empty vector   Dax1   Scramble   shDax1

**Figure 3.2.** **Transient overexpression of Dax1 does not drive differentiation.**

(A) mES cells in 24 well plates were transfected with 1 µg pHA-Dax1 and forty eight hours later immunocytochemistry for HA-Dax1 and Oct4 performed as described in methods. Inset shows overlay of Oct4 staining and DAPI counterstain to demonstrate nuclear staining of Oct4.

(B) mES cells in 24 well plates were transfected with 1 µg empty vector or HA-Dax1. Forty eight hours later cells were fixed and alkaline phosphatase staining peformed as described in methods. Alkaline phosphate positive colonies per well (not shown) were counted using Metamorph software, and the average of three experiments is shown as number of colonies per cm^2.
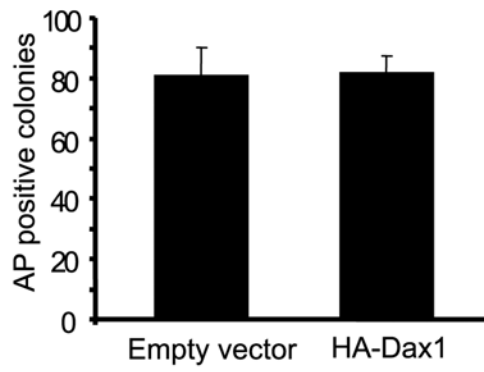
(C) mES cells in 24 wells were harvested in duplicate and Dax1 expression levels determined by QPCR. One representative experiment is shown; expression in multiple experiments ranged from 75- to 500-fold over empty vector control.
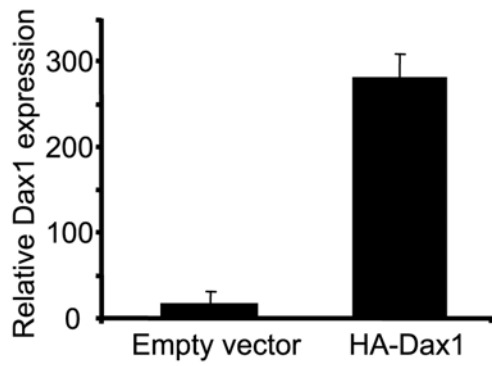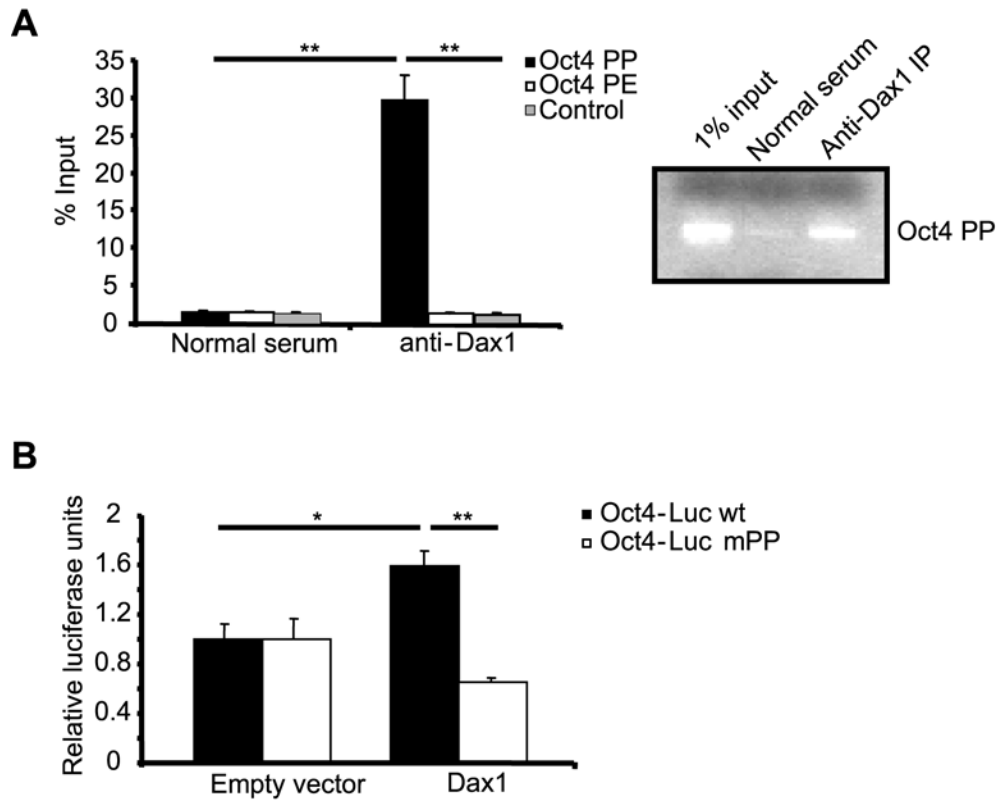
Figure 3.2
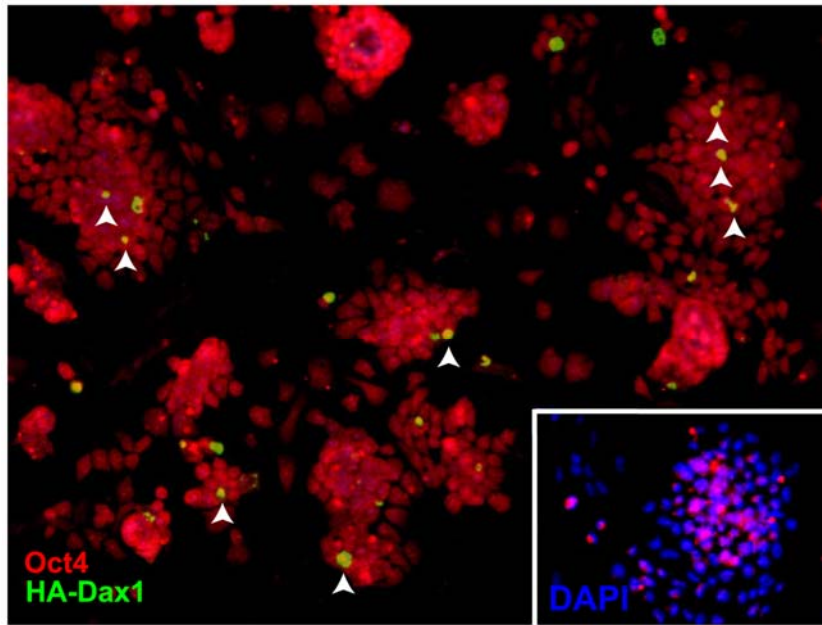
Oct4
HA-Dax1
DAPI

Figure 3.3. **Dax1 binds to and upregulates Oct4 transcription through the LRH-1 proximal promoter site.**

(A) Chromatin immunoprecipitation (ChIP) was performed in mES cells as described in methods. IP was performed with normal serum or anti-Dax1 antibody and QPCR carried out with primer sets flanking the Oct4 proximal promoter LRH-1 site (PP), proximal enhancer site (PE), or control site. Results are shown from independent experiments quantitated by either QPCR or PCR and agarose gel analysis. *p<0.02, **p<0.006

(B) mES cells were transfected with either 200 ng wildtype Oct4-Luc reporter or reporter in which the LRH-1 PP site is mutated. Cells were co-transfected with 200 ng empty vector or Dax1 and 48 hr later harvested and luciferase assay carried out.

Figure 3.3

**Figure 3.4.** **Overexpression or knockdown of Dax1 results in alteration of endogenous Oct4.**

(A) For overexpression, mES cells were transiently transfected in a 6 well plate with 3 µg empty vector or pcDNA3 Dax1 and 48 hours later cells were harvested and RNA isolated. For knockdown, mES cells were transfected in a 10 cm plate with 10 µg of a vector containing shRNA against Dax1 or scrambled control, and a GFP expression cassette. 24 hours after transfection, cells were harvested and GFP positive cells sorted and then RNA isolated. For both, cDNA synthesis and QPCR were carried out as described in methods. Oct4 values were normalized to GAPDH and data presented as fold over empty vector control. $*p<0.005$

(B) mES cells were transfected in 10 cm dishes with 10 ug empty vector or pcDNA3 Dax1 and 48 hours later cells were harvested and protein isolated. Western blot analysis was performed as described in methods with anti-Dax1, anti-Oct4 and anti-β-actin antibodies.

(C) mES cells were transfected in a 10 cm plate with 10 µg of a vector containing shRNA against Dax1 or scrambled control, and a GFP expression cassette. 24 hours after transfection, cells were harvested and GFP positive cells sorted and then cells were harvested and protein isolated. Western blot analysis was performed as described in methods with anti-Dax1, anti-Oct4 and anti-β-actin antibodies.
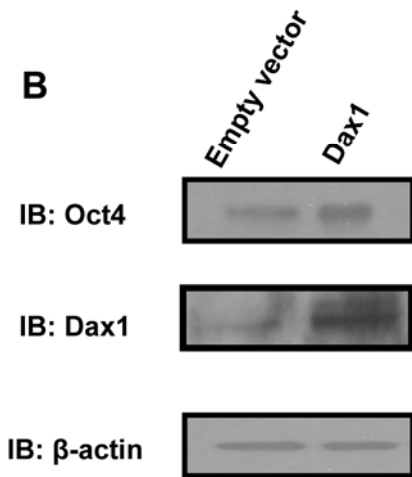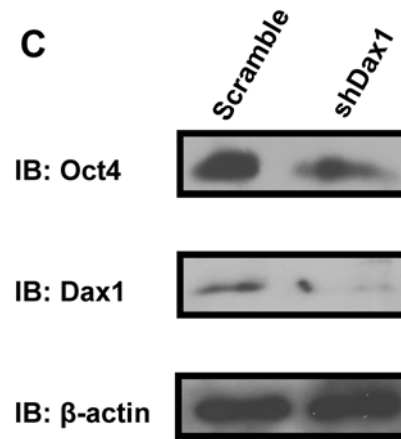
Figure 3.4

A
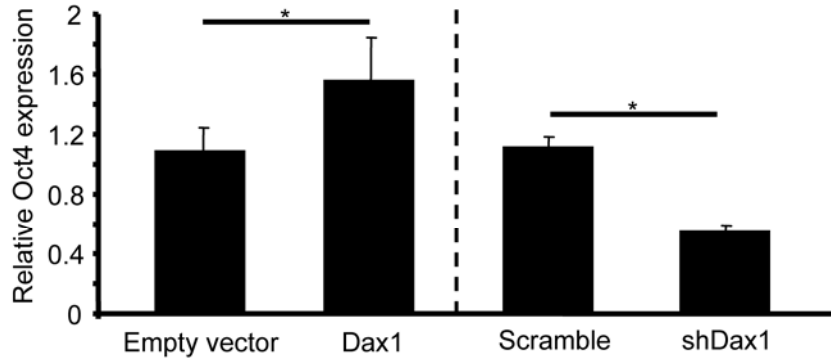
B

C

Figure 3.5. Dax1 regulation of Oct4 is mediated by SRA.

(A) mES cells were transfected in three 10 cm dishes with 10 µg pDax-1-Myc or pCMV-3tag-4A vector per dish, and RNA-immunoprecipitation performed as described in methods. The Final RNA immunoprecipitated was used to synthesize cDNA and QPCR performed for SRA and GAPDH (as a non-specific normalization control). $*p<0.02$

 (B) mES cells stably expressing shRNA against SRA or scrambled were generated as described in methods. RNA was harvested, cDNA synthesized and QPCR carried out to analyze the amount of SRA knocked down. Results are presented as relative to scramble control.

(C) Scramble or SRA KD mES cells were transfected with 200 ng Oct4-Luc reporter and co-transfected with empty vector or 300 ng pcDNA3 Dax1. 48 hr after transfection cells were harvested and lysates analyzed for luciferase activity. Reporter alone for each cell line was set as 1 luciferase unit. $**p<0.006$

(D) Scramble or SRA KD mES cells were transfected in 6 well plates with 3 µg empty vector or Dax1. 48 hr after transfection cells were harvested for RNA, cDNA synthesized, and QPCR carried out. Change in Oct4 was normalized and results are presented as fold change over empty vector control.

Figure 3.5
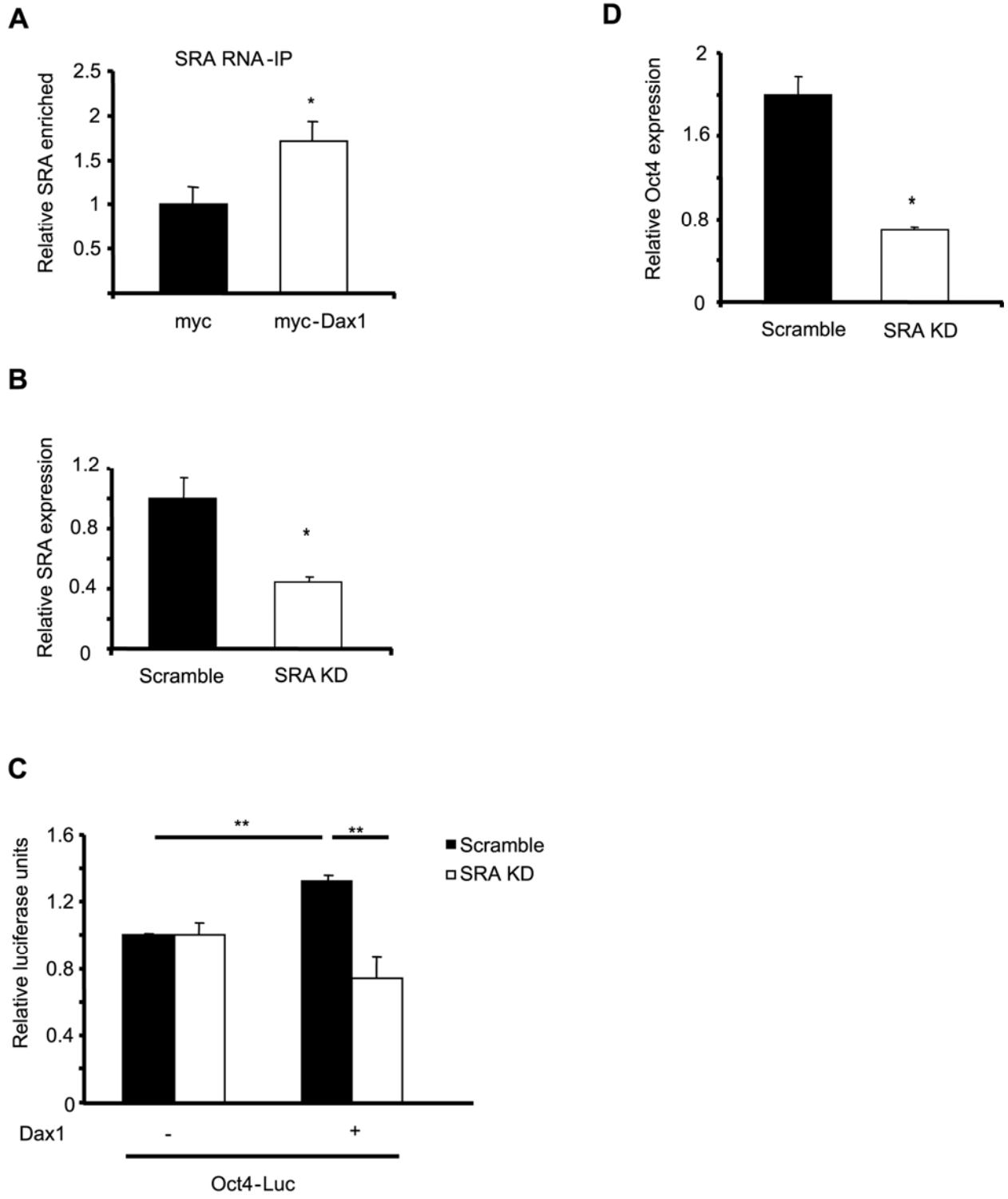
Table 3.1 QPCR primers

	Forward	Reverse
Dax1	accgtgctctttaacccaga	ccggatgtgctcagtaagg
Oct4	tgaggctacaggacacctttc	gtgccaaagtccggacct
SRA	ggctggagggaagttgtcaatac	ccactggtgatctaaaagttcttg
GAPDH	aatgtgtccgtcgtggatct	cccagctctccccatacata

Table 3.2   ChIP primers

	Forward	Reverse
Oct4 promoter LRH PP (1)	cctccgtctggaagacacaggcagatagcg	cgaagtctgaagccaggtgtccagccatgg
Oct4 promoter LRH PE {Gu, 2005 #13}	gctggggaagtcttgtgtga	gcttccagcctagttcctgg
Control	agagggtcaaggatggaatgatt	cagtgtgctccctcccacc

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CHAPTER 4

CONCLUSION

Embryonic stem (ES) cells hold enormous potential for use in therapeutic medicine. With the potential to give rise to any cell type, these cells may one day be used to grow tissues and organs for medical use. There are two main areas of research that are necessary to allow us to understand ES cells before these uses can be realized. First, we must elucidate the mechanisms that both keep these cells undifferentiated and allow their maintenance indefinitely. Understanding these mechanisms requires knowing the factors important for these mechanisms. Second, we need to determine the mechanisms by which these cells differentiate into the vast array of cell types that are present in the adult organism. This thesis has begun to expand our knowledge of the regulation and role in mouse embryonic stem (mES) cells of Dax1, a factor that is required for the maintenance of pluripotency.

Chapter 2 describes two mechanisms by which Dax1 expression is regulated specifically in mES cells. First, we found that the nuclear receptor binding site located at -128 in the Dax1 promoter, which has been shown to be bound and regulated by Sf1 in

steroidogenic cells, is required for Dax1 transcription in mES cells (1-3). Interestingly, though Sf1 is not expressed in these cells, the closest nuclear receptor family member of Sf1, LRH-1, is expressed robustly (4). We show that LRH-1 binds and activates the Dax1 promoter *in vitro* and LRH-1 overexpression and knockdown alter endogenous Dax1 levels. Additionally, based on prior work that suggested that Nanog binds to the Dax1 intron, we performed chromatin immunoprecipitation walking studies within the Dax1 intron and defined a novel Nanog binding site (5). Thus, Nanog binds to the Dax1 intron, and overexpression or knockdown of Nanog alters endogenous Dax1 levels, showing that Nanog regulates Dax1 transcription through the intron. Combined studies of the proximal promoter and the Nanog binding region from the intron show that Nanog and LRH-1 cooperate to activate Dax1 transcription.

These studies add to the known mechanisms of regulation of Dax1 expression in mES cells. All the critical ES factors that have been defined have multiple regulators of their expression. This serves to create networks of redundancy that back up each other in order to withstand transient changes in gene expression while still maintaining pluripotency. All studies on Dax1 transcription so far have focused on the proximal promoter and intron. However, long range enhancers in the genome may also regulate Dax1 expression but have yet to be found. In addition, while this thesis focuses on mES cells, it is interesting to consider the data generated using these cells and how it may contribute to the understanding of the roles of Dax1 in steroidogenic cells. While the intron has been shown to be important for regulation of Dax1 in mES cells, no studies have determined whether it is involved in Dax1 transcription in steroidogenic cells (6). In both systems, Dax1 has several regulators of its expression. Perhaps this suggests that

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Dax1 mRNA or protein have short half-lives, requiring multiple methods to keep Dax1 expressed. In steroidogenic cells, Dax1 is negatively regulated by AR (7). So far, no negative regulators of Dax1 expression have been identified in mES cells. However, negative regulation to keep Dax1 expression at a certain level would imply that Dax1 dosage is important, and high expression would result in differentiation. This phenomenon has been shown in one study, but its significance in mES cell biology is unclear. Finally, whether negative regulators are required to prevent Dax1 expression during and after differentiation of mES cells is unclear. However, decreased expression of factors that activate its expression, which are also specifically expressed in the undifferentiated cell, may be the mechanism by which Dax1 expression is lost.

Chapter 3 defines a specific role for Dax1 in transcriptional regulation in mES cells. In steroidogenic cells Sf1 upregulates Dax1 transcription, and then Sf1 and Dax1 interact in a protein complex whereby Dax1 alters Sf1 target gene transactivation (8-11). Based on this paradigm, we determined that Dax1 and LRH-1 proteins interact in mES cells. LRH-1 regulates Oct4 expression in mES cells, and we hypothesized that Dax1 would inhibit LRH-1-mediated Oct4 activation as a mechanism by which Oct4 levels are kept within the pluripotency window (4,12). While typically Dax1 has been shown to be a repressor, recent work shows that Dax1 can also act as an activator (13). Indeed, in our system, Dax1 appears to co-activate LRH-1-mediated Oct4 transcription. For its action as a co-activator, Dax1 interacts with the functional RNA SRA (13). SRA acts as a scaffold to recruit co-activators to nuclear receptors to activate transcription (14). We found that Dax1 and SRA interact in mES cells, and knockdown of SRA results in loss of

Dax1's ability to co-activate Oct4 expression. Thus, Dax1 may help preserve

pluripotency by contributing to the precise maintenance of Oct4 expression levels.

Our studies, however, do not show explicitly that Dax1 activation of Oct4

expression is a mechanism by which Dax1 maintains pluripotency. As Dax1 loss results

in multi-lineage differentiation and not to trophectoderm (as would be expected with loss

of Oct4), it is likely that other Dax1 mechanisms also contribute to its maintenance of

pluripotency (12,15). However, whether maintenance of Oct4 levels by some other

mechanism with Dax1 loss would reduce differentiation remains to be determined. In

addition, Dax1 has been shown to interact with Nanog, another regulator of Oct4

expression (16). While we were unable to demonstrate Dax1 localization to the Nanog

binding site in the Oct4 promoter (data not shown), it remains possible that Dax1 also

participates in Oct4 regulation in complex with Nanog.

As Dax1 can act as both an activator and repressor, it likely functions as both in

mES cells. One study showed that Dax1 fused to the Gal4 DNA binding domain for the

most part repressed transcription from the thymidine kinase or β-globin promoters in

mES cells, suggesting Dax1 acts as a repressor in mES cells (15). In contrast, recent

ChIP-microarray data which mapped the whole genome binding targets of several critical

mES cell factors found that Dax1 binds to a similar pattern of sites as Nanog and Oct4

(17). Additionally, they show that, in general, the sites with at least three of these factors

bound tend to be within genes which are expressed in mES cells, suggesting that Dax1

activates at these sites. Determination of Dax1 regulation of specific genes is likely the

best method to understand the roles Dax1 plays in mES cells.

It is likely that the binding partners of Dax1 will alter its activity. This thesis presents the first data on the role of SRA in mES cells. Whether Dax1 interacts with SRA on other genomic sites remains to be determined. In addition, SRA may interact with the other nuclear receptors which are expressed in mES cells, LRH-1 and ERRβ. As SRA interacts with Sf1, it is likely that SRA interacts with LRH-1, and these experiments are in progress. Which co-repressors and co-activators Dax1 interacts with in mES cells also remains unknown.

The mechanisms that determine the binding partners of Dax1 also warrant study. Recent studies indicate that high concentrations of Dax1 allow Dax1 to be a transcriptional activator, but it is unclear why this is the case (13). Perhaps Dax1 protein is first recruited into complexes with corepressors due to a higher binding affinity with these proteins, and once these complexes are saturated with Dax1 it can then bind to coactivators or SRA. Dax1 is one of the top twenty enriched mRNAs in mES cells, so its abundant expression may facilitate its ability to act as a coactivator (18).

Though much study is needed to even scratch the surface of the role of Dax1 in mES cells, certain directions warrant study more than others. The data in this thesis and elsewhere define many mechanisms by which Dax1 transcription is regulated in mES cells. However, only this thesis and one other study have shown specific mechanisms by which Dax1 regulates transcription in mES cells. Therefore, study of Dax1's target genes and interacting partners is critical. Studies utilizing mass spectrometry defined a small interactome of proteins in mES cells, demonstrating that Dax1 interacts with Nanog and six other factors: Rif1, Tif1β, Pelo, Zfp609, REST, and ERR2 (16). Understanding the role Dax1 plays in complex with these factors could be a starting point for expanding

known Dax1 target genes. In addition, Dax1 likely interacts with many other factors, and more mass spectrometry identification of these proteins would open more areas of inquiry.

This thesis reports that Dax1 regulates expression of a key pluripotency factor, Oct4. In addition, a study that demonstrated that Dax1 interacts with Oct4 showed that Dax1 regulates other key pluripotency factors, specifically Nanog and Rex1 (19). However, if Dax1 acts as a repressor it may also be important in the repression of differentiation factors. Given that ChIP-chip and Dax1 knockdown microarray data are available, searching for these possible targets in the available data would be a starting point for understanding other mechanisms by which Dax1 maintains pluripotency.

Finally, a very interesting area to research would be the study of the role of Dax1 in adult stem cells of the adrenal gland. Though not described in this thesis, studies from the Hammer lab suggest that Dax1 may play a role in the maintenance of a population of adrenal stem cells which repopulate the gland throughout adult life (unpublished observations). While most critical ES cell factors are not expressed in the adrenal, it is a provocative idea that some targets of Dax1 transcription might be shared in embryonic and adrenal stem cells. Specifically, Dax1 appears to be expressed in the transiently amplifying or progenitor cells of the adrenal gland (unpublished observation). Work in our lab has shown that in the aged Dax1 null mouse, the adrenal cortex begins to fail and the secretion of hormones is reduced (Josh Scheys, manuscript in preparation). The mechanism by which Dax1 helps maintain the pool or stem cells is unknown, and few target genes of Dax1 have been elucidated in the adrenal, but it is interesting to speculate on the types of genes Dax1 may regulate. In these cells, Dax1 may repress the levels of

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the steroidogenic enzyme genes in order to maintain the differentiation ability of the progenitor cells. In contrast, Dax1 may activate expression of genes involved in the maintenance of these cells, such as genes involved in proliferation. The populations of adrenal stem and progenitor cells have not been isolated or characterized. However, it is not necessary to isolate these cells to identify Dax1 targets specifically in progenitor cells; using whole adult male adrenal glands for ChIP-chip exeriments (in which Dax1 is specifically expressed only in subcapsular cells) would specifically identify genes that Dax1 regulates in progenitor cells. These types of experiments are critical for advancing our understanding of Dax1 in the adrenal gland and adrenal stem cell biology.

In conclusion, the studies described herein have expanded our knowledge of transcriptional regulation of Dax1 in mES cells and begun to characterize a specific role for Dax1 in mES cells. Clearly, Dax1 is intricately involved in the network of transcription factors that is critical for mES cell self renewal and pluripotency. While much work remains to fully understand of function of Dax1 in these cells, studies such as these are necessary for our understanding of not only the actions of Dax1 but the maintenance of pluripotency.

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