Nuclear receptors Sf1 and Dax1 function cooperatively to mediate somatic cell differentiation during testis development

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Summary

Mutations of orphan nuclear receptors *SF1* and *DAX1* each cause adrenal insufficiency and gonadal dysgenesis in humans, although the pathological features are distinct. Because Dax1 antagonizes Sf1-mediated transcription in vitro, we hypothesized that *Dax1* deficiency would compensate for allelic loss of *Sf1*. In studies of the developing testis, expression of the fetal Leydig cell markers *Cyp17* and *Cyp11a1* was reduced in heterozygous *Sf1*-deficient mice at E13.5, consistent with dose-dependent effects of *Sf1*. In *Sf1/Dax1* (*Sf1* heterozygous and *Dax1*-deleted) double mutant gonads, the expression of these genes was unexpectedly reduced further, indicating that loss of *Dax1* did not compensate for reduced *Sf1* activity. The Sertoli cell product *Dhh* was reduced in *Sf1* heterozygotes at E11.5, and it was undetectable in *Sf1/Dax1* double mutants, indicating that *Sf1* and *Dax1* function cooperatively to induce *Dhh* expression. Similarly, *Amh* expression was reduced in both *Sf1* and *Dax1* single mutants at E11.5, and it was not rescued by the *Sf1/Dax1* double mutant. By contrast, *Sox9* was expressed in single and in double mutants, suggesting that various Sertoli cell genes are differentially sensitive to *Sf1* and *Dax1* function. Reduced expression of *Dhh* and *Amh* was transient, and was largely restored by E12.5. Similarly, there was recovery of fetal Leydig cell markers by E14.5, indicating that loss of *Sf1/Dax1* delays but does not preclude fetal Leydig cell development. Thus, although Sf1 and Dax1 function as transcriptional antagonists for many target genes in vitro, they act independently or cooperatively in vivo during male gonadal development.

Key words: *Sf1*, *Dax1*, Gonad, Mouse embryo, Nuclear receptor

Introduction

Sf1 and Dax1 are nuclear receptors found predominantly in the endocrine tissues that control reproduction: the ventromedial hypothalamus, pituitary gonadotropes, adrenal gland and gonad (Ikeda et al., 1996). Sf1 is considered to be a master regulator of the reproductive system because it regulates the expression of a large array of genes required for gland development and hormone synthesis (Parker et al., 2002). Sf1 protein acts by binding to a cognate DNA-response element (Morohashi et al., 1992; Rice et al., 1991) in the promoter regions of these genes. Unlike most nuclear receptors, Sf1 binds DNA as a monomer (Wilson et al., 1993). Sf1 interacts with a number of transcriptional co-activators (Val et al., 2003), and phosphatidyl inositol lipids may serve as ligands to regulate transcriptional activity (Krylova et al., 2005).

Dax1 is atypical among nuclear receptors because it lacks the highly conserved zinc-finger DNA-binding domain (Zanaria et al., 1994). Instead, the amino terminus of Dax1 contains a repeated peptide sequence comprising variations of a hydrophobic LXXLL-like motif (Zhang et al., 2000) that mediates protein-protein interactions (Suzuki et al., 2003). Dax1 may bind to certain DNA hairpin structures and to RNA (Lalli et al., 2000), but its major transcriptional regulatory action involves direct interactions with other nuclear receptors, including Sf1 (Holter et al., 2002; Ito et al., 1997; Zhang et al., 2000). The carboxy terminus of Dax1 contains a transcriptional repressor domain (Ito et al., 1997; Zazopoulos et al., 1997) that interacts with several different co-repressors (Altincicek et al., 2000; Crawford et al., 1998). One model of Sf1 and Dax1 action proposes that the N terminus of Dax1 interacts with Sf1, and recruits repressors to the Sf1 transcription complex, thereby inhibiting the expression of Sf1-regulated genes such as *Star*, *Dax1*, *Lhb*, *3βHSD* (*Hsd3b* – Mouse Genome Informatics), *Cyp19*, *Cyp11a1* and *Amh* (Lalli et al., 1998; Nachtigal et al., 1998; Salvi et al., 2002; Suzuki et al., 2003; Tabarin et al., 2000; Wang et al., 2001; Zazopoulos et al., 1997).

Sf1 is expressed in the urogenital ridge at E9.5 (Ikeda et al., 1994; Ikeda et al., 2001). In the male gonad, Sf1-positive cells can be found in a population of coelomic epithelial cells that give rise to both Sertoli and interstitial cell precursors (Schmahl et al., 2000). In the Sertoli population, one target of Sf1 is anti-Müllerian hormone (*Amh*/*Mis*) (Hatano et al., 1994; Shen et al., 1994). Later in testis development, *Sf1* expression intensifies in the interstitial Leydig population where it regulates the expression of multiple steroidogenic enzyme genes necessary for testosterone production (Hatano et al., 1994; Ikeda et al., 1996; Morohashi et al., 1993). *Dax1*

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expression peaks in the male gonad at E11.5 and then remains relatively low until E17.5 when it increases significantly (Ikeda et al., 2001). At E11.5, Sf1 and Dax1 proteins overlap in the XY bipotential gonad, and by E12.5, they are co-localized mainly within the testis cords (Ikeda et al., 2001). The spatial and temporal overlap of *Sf1* and *Dax1* expression during the crucial time of gonadal differentiation raises the possibility of a functional interaction, but this has not been explored directly in vivo.

Loss-of-function mutations in *Sf1* and *Dax1* suggest important roles in gonadal development. Homozygous deletion of *Sf1* in mice prevents adrenal gland and gonadal development, reflecting increased programmed cell death in the cell populations that normally give rise to these tissues (Luo et al., 1994). In the male, the failure of testis differentiation results in absence of anti-Müllerian hormone production and, consequently, there is persistence of Müllerian structures at birth. Prior to regression of the gonad rudiment in the *Sf1* homozygous knockout, primordial germ cells are detectable in the gonadal ridge (Luo et al., 1994). Thus, the primary role of *Sf1* in gonad development involves the somatic cell lineages.

Dax1-null hemizygous male mice have testicular dysgenesis and delayed regression of the fetal X-zone in the adrenal gland (Yu et al., 1998b). Although *Dax1* had been suggested as a possible ovarian determining gene, ovaries develop in females with deletion of *Dax1* on both X chromosomes. Adult female mice lacking *Dax1* exhibit multi-oocyte follicles but they are fertile. The loss of *Dax1* function in the testis has been shown to impair testis cord formation. This is caused in part by reduced numbers of peritubular myoid (PTM) cells (Meeks et al., 2003a), which normally surround the testis cords and, together with Sertoli cells, form the basement membrane of the developing seminiferous tubules. In the adult, the efferent duct epithelium is hyperplastic and there is obstruction of seminiferous tubules (Jeffs et al., 2001). Sertoli cells appear to be incompletely differentiated and germ cells progressively degenerate. As a consequence, male *Dax1* null mice are infertile. Leydig cell development is also altered in the absence of *Dax1*. Fetal Leydig cells are restricted to the coelomic side of the interstitial compartment rather than extending across the full diameter of the gonad (Meeks et al., 2003a). In the adult, Leydig cells are hyperplastic and aromatase expression is elevated, leading to increased intratesticular estradiol production (Wang et al., 2001). On a genetic background with a weakened *Sry* allele (*Mus domesticus poschiavinus*), the phenotype associated with *Dax1* deletion changes from dysgenetic testes to complete sex reversal (Meeks et al., 2003b), indicating that *Dax1* functions in parallel with, or downstream of, *Sry* in the sex determination cascade to mediate normal testis development. Thus, *Dax1* plays a more crucial role in testis differentiation than it does in ovary development.

Dax1-deficient mice exhibit overactivity of some Sf1-regulated genes, consistent with the idea that Dax1 antagonizes Sf1 function. For example, *Cyp19*, an Sf1-regulated steroidogenic enzyme gene, is overexpressed in the testis of adult male *Dax1*-knockout mice (Wang et al., 2001). *Cyp21*, a key enzyme for mineralocorticoid and glucocorticoid synthesis, is overexpressed in the adrenal gland of *Dax1*-deficient mice (Babu et al., 2002). These findings are consistent with a model in which Dax1 represses Sf1-mediated transcription.

The phenotypes seen in *Sf1* and *Dax1* knockout mice are largely predictive of the clinical manifestations in humans with mutations in *SF1* or *DAX1* (Achermann et al., 2001b). *SF1* mutations cause adrenal insufficiency and XY gonadal dysgenesis. These features occur even with heterozygous mutations and vary across a wide phenotypic spectrum (Jameson, 2004). Thus, in humans, the function of *SF1* is strikingly dose dependent (Achermann et al., 2002). Human *DAX1* mutations cause X-linked adrenal insufficiency, hypogonadotropic hypogonadism and gonadal dysgenesis (Bardoni et al., 1994; Muscatelli et al., 1994; Zanaria et al., 1994). Some mutations with partial loss of *DAX1* activity are associated with delayed onset and milder clinical features (Ozisik et al., 2003b; Salvi et al., 2002; Tabarin et al., 2000), suggesting that *DAX1* action is also dose dependent (Achermann et al., 2001a; Reutens et al., 1999).

The phenotypic similarities associated with *Sf1* and *Dax1* loss-of-function mutations are somewhat at odds with their proposed antagonistic actions at the transcriptional level. To further explore their functional relationship in vivo, we examined testis development in the context of combined loss of function of *Sf1* and *Dax1*. We hypothesized that *Sf1* heterozygosity would reduce the expression of some Sf1 target genes, such as steroidogenic enzymes genes (Lala et al., 1992; Morohashi et al., 1992). Moreover, because Dax1 acts as a repressor of Sf1-mediated transcription (Ito et al., 1997; Lalli et al., 1997), we predicted that *Dax1* deficiency might partially or completely compensate for *Sf1* heterozygosity in the *Sf1/Dax1* double mutant. In contrast to a model in which *Dax1* acts as a universal antagonist of *Sf1* action, combined loss of *Sf1* and *Dax1* further impaired Sertoli cell differentiation and fetal Leydig cell development. In the embryonic gonad, we found that *Sf1* and *Dax1* act coordinately to enhance the expression of the Sertoli-derived factors *Dhh* and *Amh*. Dhh is a paracrine signaling factor that regulates fetal Leydig cell development (Yao et al., 2002). Amh is a key regulator of Müllerian regression and testis differentiation (Behringer et al., 1990; Behringer et al., 1994; Ross et al., 2003; Vigier et al., 1985). Thus early male gonadal development depends on the cooperative function of *Sf1* and *Dax1*.

Materials and methods

Animal husbandry and embryo dissection

All procedures were approved by the Northwestern University Animal Care and Use Committee, Master Protocol #2004-0494. $Dax1^{+/-}$ females were mated to $Sf1^{+/-}$ males to attain animals of all four genotypes. For analysis of embryos, females were placed with individually housed males at 17.00 h. Vaginal plugs were identified at 08.00 h the following morning. Twelve o'clock noon corresponded with embryonic day 0.5. Visceral yolk sac taken from each embryo was used to obtain genomic DNA. *Sf1*, *Dax1* and *Sry* genotyping was performed for all embryos as previously described (Babu et al., 2002; Yu et al., 1998b). Only males within each of the four genotype groups were studied.

In situ hybridization

In situ hybridization was performed by a standard protocol (Wilkinson, 1998). Plasmid constructs for synthesis of probes to detect *Amh, Sox9*; *Cyp11a1*, *Cyp17*; and *Dhh* were generously provided by B. Capel (Duke University), P. Koopman (University of Queensland), S. Tevosian (Dartmouth College) and A. McMahon (Harvard University), respectively. Whole-mount tissue was viewed

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with a Leica MZLFIII (Leica, Heerbrugg, Switzerland) dissecting microscope and images were taken with a Color MagnaFire (Optronics, Goleta, CA) digital camera. Littermates were used as wild-type controls.

TUNEL apoptosis detection

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) of fragmented DNA in apoptotic cells on tissue sections of embryonic gonads was performed using the Fluorescein-FragEL kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions. De-paraffinized sections were treated with Proteinase K. Incubation with terminal deoxynucleotidyl transferase catalyzed the addition of fluorescein-labeled deoxynucleotides to exposed 3′-OH ends of DNA fragments. Nuclei were counterstained with Propidium Iodide mounting medium (Vector Laboratories, Burlingame, CA). Fluorescence microscopy images were captured on a Zeiss Axioskop (Zeiss, Thornwood, NY) with a Color MagnaFire (Optronics, Goleta, CA) digital camera.

Immunohistochemistry

Paraffin wax-embedded tissue was sectioned at 5 μm using a Jung RM 2025 (Leica, Nussloch, Germany) microtome. Sections were deparaffinized by serial washes in xylenes and ethanol, followed by antigen retrieval in sodium citrate buffer (pH 6.0) at high temperature. 3β-hydroxysteroid dehydrogenase antibody (1:2500) was provided by Ian Mason (Edinburgh, Scotland). Secondary antibodies were applied for two hours at room temperature (Goat Cy3-anti-rabbit from Jackson Immunoresearch, West Grove, PA, DAB Vector, Burlingame, CA). Sections were washed and mounted with DAPI Hard Set (Vector, Burlingame, CA). Hematoxylin (Fisher Scientific, Fairlawn, NJ) and Eosin (Surgipath, Richmond, IL) (H&E) staining was performed following de-paraffinization of tissue and xylene-to-ethanol washes. Sections were viewed with a Zeiss Axioskop. Pictures were taken with a Color MagnaFire (Optronics, Goleta, CA) digital camera.

Results

The effect of *Sf1* haploinsufficiency is not rescued by loss of *Dax1*

In wild-type males, fetal Leydig cells can be identified by in situ hybridization for the steroidogenic enzymes genes *Cyp17* and *Cyp11a1* at E13.5 (Fig. 1A-H). The Leydig cell population was diminished in *Sf1*$^{+/-}$ mice, particularly in the periphery of the gonad. In the *Dax1*$^{-/Y}$ testis, Leydig cells were restricted to the coelomic surface on the dorsal side of the gonad. Both markers were expressed on the ventral side, indicating a spatial requirement for *Dax1* in the dorsolateral aspect of gonad near the mesonephros (S.Y.P., unpublished). In the *Sf1/Dax1* double mutants, the *Cyp17* and *Cyp11a1* markers were not detected. Thus, in contrast to the hypothesis that elimination of *Dax1* might compensate for loss of *Sf1* activity, these results indicate a combinatorial role for *Sf1* and *Dax1* in fetal Leydig cell development. These effects of *Sf1* haploinsufficiency are consistent with a gonad-specific knockout of *Sf1*, which showed failure to express the steroidogenic pathway genes *Cyp11a1* and *Star* expression at E14.5 and 16.5, respectively (Jeyasuria et al., 2004).

Desert hedgehog expression is coordinately induced by Sf1 and Dax1

Desert hedgehog (Dhh) is produced by the Sertoli cells (Bitgood et al., 1996). Its cognate receptor patched 1 (Ptc1) is located on the Leydig cell membrane and initiates an intracellular cascade to induce *Cyp11a1* expression in an *Sf1*-dependent manner (Yao et al., 2002). We hypothesized that loss of *Sf1* and *Dax1* might alter *Dhh* expression, providing a basis for impaired Leydig cell development. *Dhh* was measured by whole-mount in situ hybridization starting at E11.5. In wild-type mice, *Dhh* was expressed uniformly in the male gonad (Fig. 2A-D). In heterozygous *Sf1* mutants, *Dhh* was expressed in the central region of the gonad but it was diminished in the anterior and posterior poles. In the *Dax1* mutant, *Dhh* expression was lower than in wild type, but it was present throughout the entire length of the gonad. In *Sf1/Dax1* double mutants, *Dhh* expression was undetectable at E11.5. TUNEL staining was performed to assess whether the reduced expression of *Dhh* was associated with programmed cell death, as observed in *Sf1* homozygous knockouts (Luo et al., 1994). However, there was minimal apoptosis in wild type, single, or double mutants (Fig. 2I-L). *Sf1* homozygous tissue (see insert) served as positive control. By E12.5, *Dhh* was localized to the embryonic testis cords and its expression was comparable in

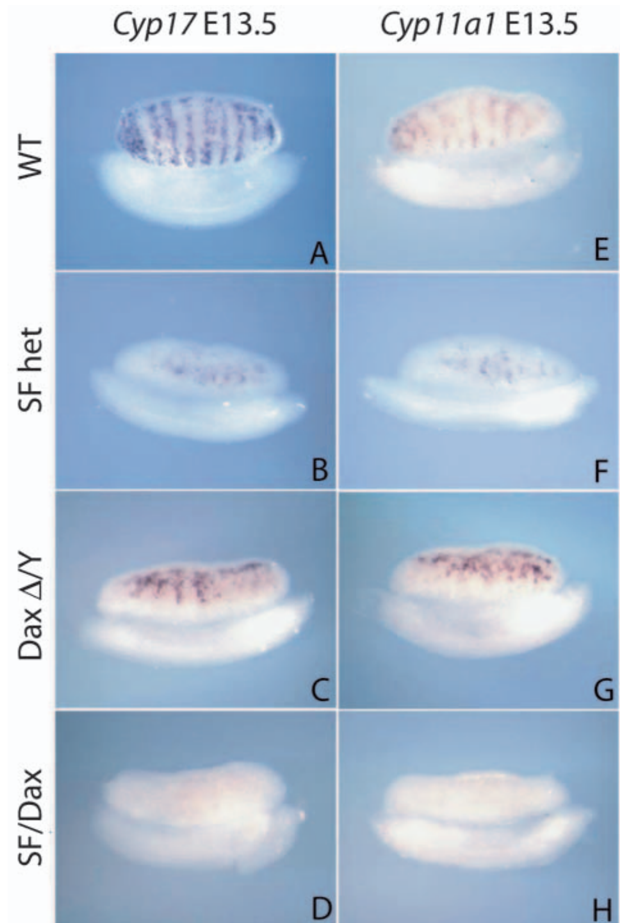

Fig. 1. Expression of fetal Leydig cell markers in embryonic testes of single and double *Sf1* and *Dax1* mutants. In situ hybridization for steroidogenic enzymes *Cyp17* (A-D) and *Cyp11a1* (E-H) identifies the Leydig cells of the embryonic testis at 13.5 dpc. Leydig cells are present in columns between testis cords in wild-type mice (A,E). Leydig cells are decreased in *Sf1*$^{+/-}$ gonads in agreement with dosage-sensitive regulation (B,F), and are restricted to the coelomic surface of the *Dax1*$^{-/Y}$ testis (C,G). *Sf1/Dax1* double mutant mice are devoid of Leydig cells markers (D,H).

all four genotypes (Fig. 2E-H). The reduced *Dhh* expression associated with loss of *Sf1* and *Dax1* is therefore transient, suggesting coordinated regulation by *Sf1* and *Dax1* without affecting cell survival.

Anti-Müllerian hormone expression requires Sf1 and Dax1 independently

Amh activation is directly regulated by Sf1 (Hatano et al., 1994; Shen et al., 1994). Other transcription factors that act with Sf1 on the *Amh* promoter include Wt1, Sox9, Sox8 and Gata4 (De Santa Barbara et al., 1998; Nachtigal et al., 1998; Schepers et al., 2003; Viger et al., 1998). Dax1, however, represses Sf1-mediated activation of *Amh* when tested in vitro (Nachtigal et al., 1998). *Amh* transcripts were first detected at E11.5 in the wild-type male gonad (Fig. 3A). The *Sf1* heterozygous gonad did not express *Amh* (Fig. 3B), whereas sparse staining was seen in the anterior region of the *Dax1* null gonad at E11.5 (Fig. 3C). The double mutant also showed minimal *Amh* expression (Fig. 3D). By E12.5, *Amh* levels returned to normal in the single *Sf1* mutant (Fig. 3E,F). However, *Amh* expression remained low in the *Dax1* mutant and in the *Sf1/Dax1* double mutant (Fig. 3G,H), compatible with distinct roles for *Sf1* and *Dax1* in *Amh* expression.

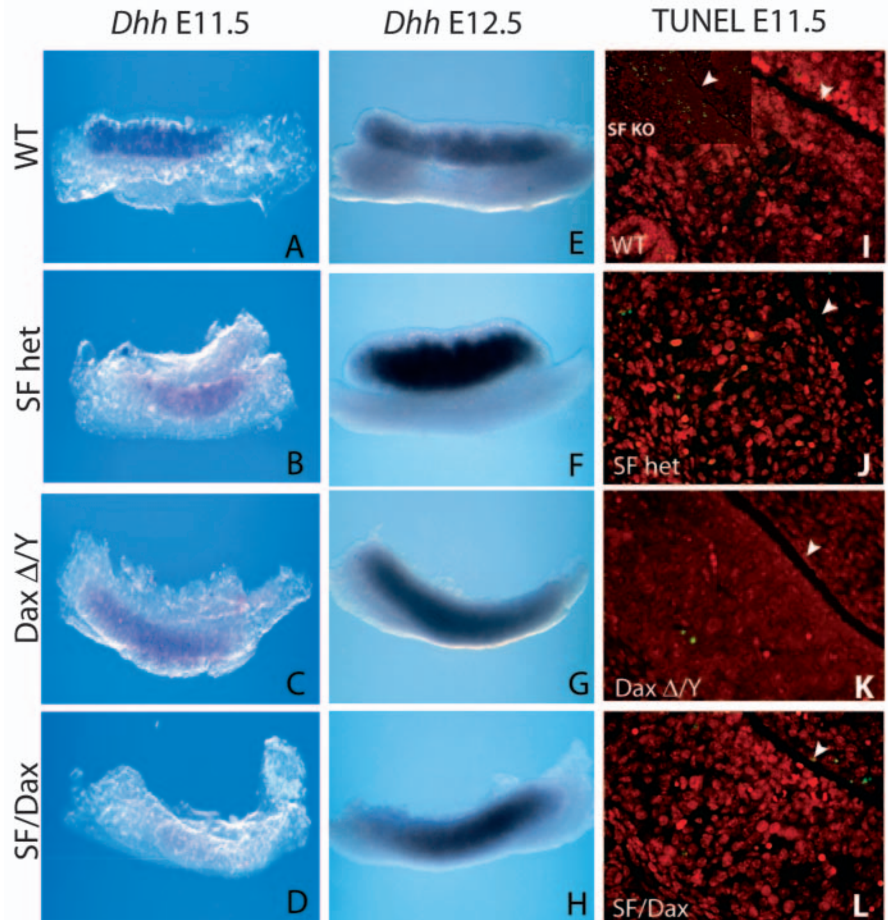

Fig. 2. Desert hedgehog expression in embryonic testes of single and double *Sf1* and *Dax1* mutants. In situ hybridization of the Sertoli marker *Dhh* is shown at E11.5 (A-D) and E12.5 (E-H) in genital ridges. The coelomic surface is at the top; anterior is to the left. At E11.5, both $Sf1^{+/-}$ and $Dax1^{-/Y}$ single mutants expressed reduced amounts of *Dhh* (B,C). Expression of *Dhh* is undetectable in the gonad of the double mutant (D). By E12.5, *Dhh* expression is comparable in wild-type and single mutant gonads (E-G), and is restored in the double mutant (H). (I-L) Lack of apoptotic death in cells of the E11.5 gonad by TUNEL staining (green) of lateral sections, compared with the *Sf1* knock out shown in the inset in I. Arrowhead indicates the coelomic epithelial surface.

Sox9 expression in the *Sf1/Dax1* double mutant

Sox9 expression was examined at E11.5 to assess whether reduced expression of *Dhh* and *Amh* is selective or whether it reflects a more general delay in Sertoli cell differentiation. In the wild type, *Sox9* was strongly expressed at E11.5 (Fig. 4A). There was some reduction of *Sox9* expression in the *Sf1* heterozygote (B) and in the *Sf1/Dax1* double mutant gonad (D), but less reduction in the *Dax1* null (C). At 12.5 dpc, *Sox9* expression was similar in Sertoli cells of all genotypes (Fig. 4E-H). Thus, *Sox9* expression is partially impaired in single or double mutants. However, the reduction of *Sox9* is not as pronounced as *Dhh* or *Amh*.

Testis cord morphogenesis is not affected in the *Sf1/Dax1* double mutant

Testis cord morphogenesis is a characteristic feature of male gonad development (Tilmann and Capel, 2002). In the wild-type male at E12.5, testis cord morphogenesis is apparent by cross-sectional histology. Sertoli cells surrounding primordial germ cells are enclosed by flattened, peritubular myoid cells within primitive tubules (Fig. 5A). The appearance of testis cords is not disrupted in the *Sf1* heterozygous gonad (Fig. 5B). However, in the *Dax1* null gonad, the number of testis cords is reduced and many are incompletely enclosed, reflecting impaired peritubular myoid differentiation (Fig. 5C) (Meeks et al., 2003a). A similar phenotype is observed in the *Sf1/Dax1* double mutant gonad (Fig. 5D), indicating that the *Dax1* mutant phenotype is not rectified by allelic loss of *Sf1*.

Fetal Leydig cell differentiation recovers after a period of delay

Although temporal regulation of Leydig cell differentiation is delayed in the double mutant, steroidogenic enzyme gene expression begins to recover as early as E14.5 (Fig. 6A-H). Thus, Leydig cell recovery allows the progression of steroidogenesis during embryonic development. This finding is consistent with the recovery of *Dhh* by E12.5 following a period of delay. Immunostaining for 3β-hydroxysteroid dehydrogenase (3βHSD) was performed two weeks after birth, prior to the proliferation of adult Leydig cells. Similar amounts of 3βHSD were found in Leydig cells of all four genotypes (Fig. 6I-L).

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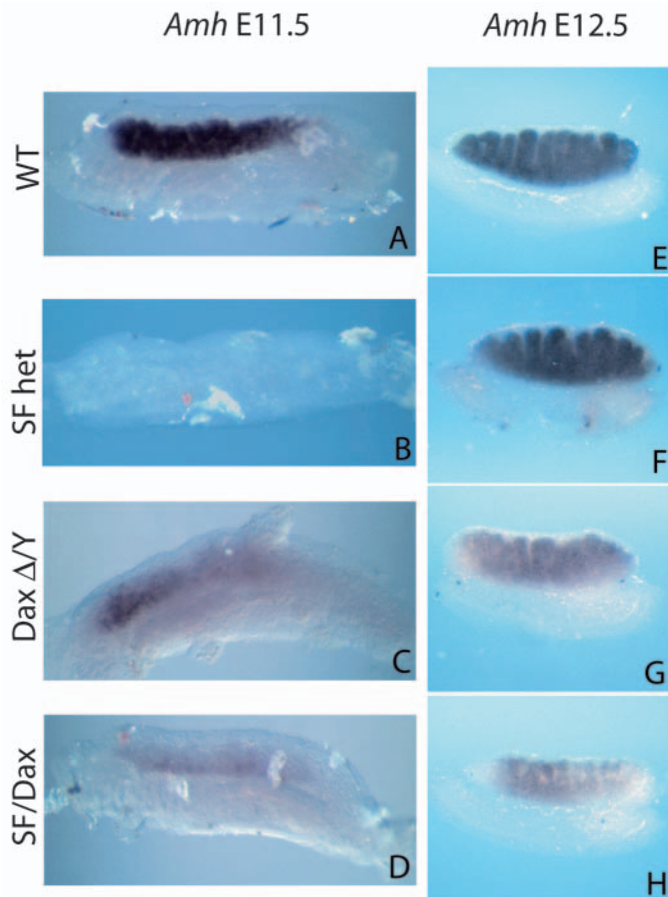

Fig. 3. *Amh* expression in embryonic testes of single and double *Sf1* and *Dax1* mutants. *Amh* expression is localized to the primitive gonadal ridge in the wild-type male at E11.5 (A). *Sf1* heterozygous gonads show early loss of *Amh* expression (B), but recover by E12.5 (F). *Dax1* null mutant gonads also have markedly reduced *Amh* expression (C), followed by immediate recovery at E12.5 (G). The recovery of *Amh* expression is further delayed in the double mutants when compared with the single mutants (D,H).

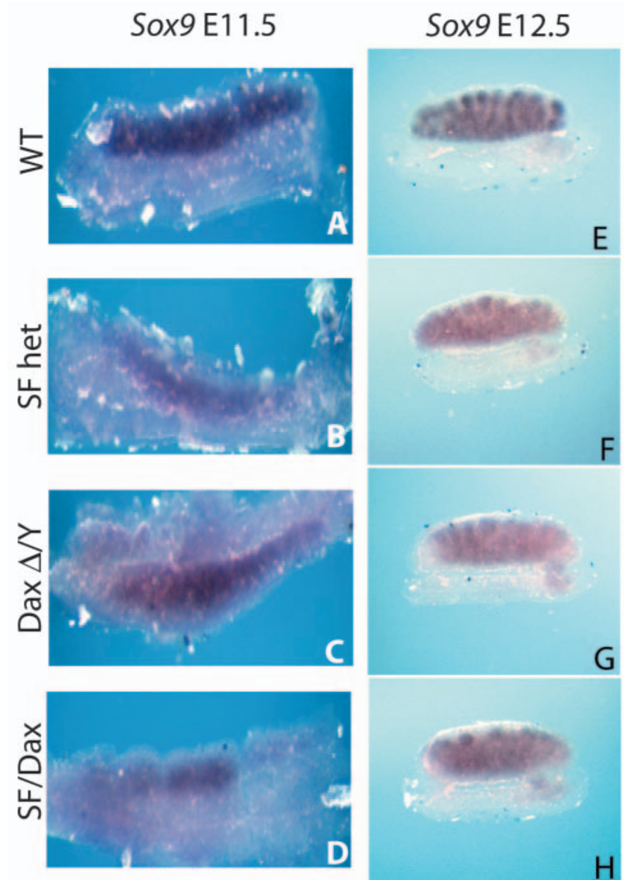

Fig. 4. Sox9 expression in embryonic testes of single and double *Sf1* and *Dax1* mutants. In situ hybridization for *Sox9* was performed at 11.5 dpc (A-D) and at 12.5 dpc (E-H). *Sox9* expression is detected in the wild-type male gonad during the bipotential stage at E11.5 (A). *Sox9* expression in Sertoli cells is diminished in the *Sf1* heterozygous mutant, the *Dax1* null mutant and the double mutant (B-D), when compared with wild type. At 12.5 dpc, normal testis cord organization and the presence of Sertoli cells is indicated by *Sox9* expression in both wild-type and $Sf1^{+/-}$ gonads (E,F). Although Sertoli cells are present, testis cords are not easily identifiable in either the $Dax1^{-/Y}$ gonad or the *Sf1/Dax1* double mutant gonad (G,H).

Discussion

In this report, we examined the consequences of combined loss of function of nuclear receptors *Sf1* and *Dax1* in the context of male gonad development. Previous studies indicate that *Sf1* acts in a dose-dependent manner (Babu et al., 2002; Bland et al., 2004; Bland et al., 2000; Ozisik et al., 2002), and that Dax1 binds to Sf1 to inhibit its transcriptional activity (Ito et al., 1997). We initially hypothesized that loss of *Dax1* might complement *Sf1* haploinsufficiency and partially rescue features associated with reduced *Sf1* action. Unexpectedly, however, combined loss of *Sf1* and *Dax1* generated a more severe phenotype than that seen with the individual mutations, suggesting that these factors function in partially independent and complementary pathways. The most striking feature of the combined *Sf1/Dax1* mutant is a delay in the development of fetal Leydig cells and their expression of steroidogenic enzyme genes. These features are heralded by delayed Sertoli cell expression of *Dhh* and *Amh*. Dhh is known to act in a paracrine manner to induce steroidogenic enzyme gene expression in fetal Leydig cells via its cognate receptor Ptc1 (Yao et al., 2002). In addition to its primary role in the regression of the Müllerian duct, Amh is thought to promote testis cord formation (Behringer et al., 1990), perhaps by inducing mesonephric cell migration (Ross et al., 2003), and to antagonize adult Leydig cell function (Behringer et al., 1994; Racine et al., 1998). Hence, the compound loss of *Sf1* and *Dax1* was associated with a transient delay in Sertoli cell function, as *Dhh* and *Amh* expression recover, followed by fetal Leydig cell differentiation.

In this study, the dose-dependent effects of *Sf1* seen previously in the adrenal gland (Babu et al., 2002; Bland et al., 2004; Bland et al., 2000) were also found in the developing testis. Heterozygous *Sf1* mice have delayed adrenal development and reduced adrenal size but adrenal function is ultimately normal, indicating that compensatory mechanisms activate Sf1 target genes and stimulate adrenal growth. In the developing testis, heterozygous *Sf1* mice have reduced

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expression of Leydig and Sertoli marker genes but these cell types recover, indicating a transient delay when *Sf1* expression is reduced. The effects of *Sf1* deficiency in the early stage of gonad development are more pronounced in the periphery of the gonad than in the central region. Immunohistochemical analyses have shown that *Sf1* is expressed throughout the developing bipotential gonad at 11.5 dpc (Ikeda et al., 2001). In the coelomic epithelial layer of the male gonad, a population of Sf1-positive cells are proliferative and contribute to the sexually dimorphic growth seen in the testis (Schmahl et al., 2000). In addition, Sf1 acts on numerous target genes (Val et al., 2003) that are expressed during gonadal development. Thus, the delay in Sertoli and Leydig cell differentiation observed in the *Sf1* heterozygous male gonad might reflect reduced numbers of progenitor cells, impaired differentiation, reduced transactivation of marker genes, or several of these mechanisms. A gonad-specific knockout of *Sf1* similarly failed to express *Cyp11a1* and *Star* at E14.5 and 16.5, which resulted in hypoplastic testes and cryptorchidism (Jeyasuria et al., 2004). Furthermore, the number of proliferating cells observed at E12.5 in the XY gonad-specific *Sf1* knockout was significantly lower than in wild type. It is notable that humans with heterozygous *SF1* mutations exhibit a spectrum of adrenal insufficiency and gonadal dysgenesis, including XY sex reversal (Jameson, 2004; Ozisik et al., 2003a). Thus, the dose-dependent effects of *SF1* are even more pronounced in humans than in murine models.

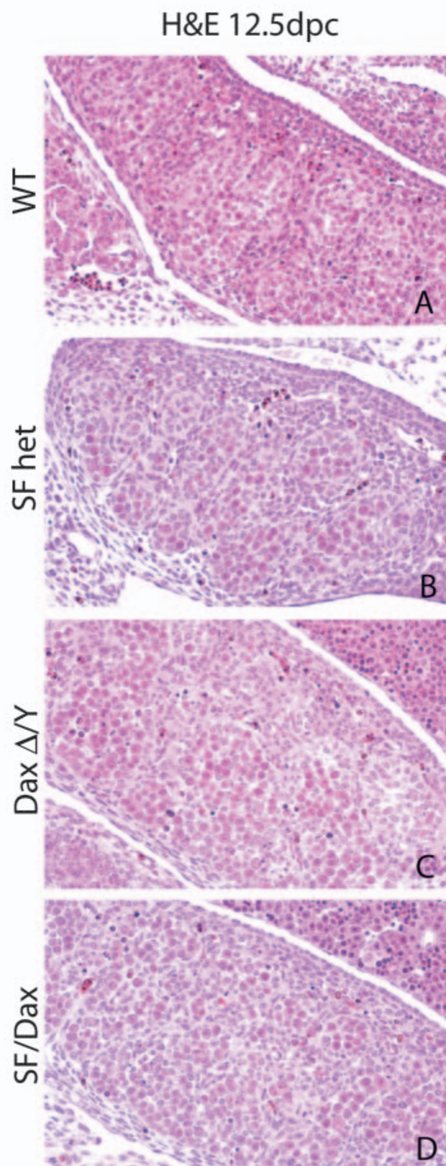

Fig. 5. Testis cord morphogenesis in *Sf1* and *Dax1* mutant male gonads. Testis differentiation is characterized by morphogenesis of primitive testis cords in tissue stained by Hematoxylin and Eosin. Cross-sectional histology of wild-type gonad tissue shows round cord structures containing Sertoli and germ cells enclosed by flattened peritublar myoid cells (A). *Sf1* heterozygous gonads retain cord morphology (B). *Dax1* null (C) and *Sf1/Dax1* double mutant (D) gonad tissue both show a more disorganized patterning, reflecting *Dax1* deficiency in both cases.

The delay in Sertoli and Leydig cell function associated with *Sf1* haploinsufficiency provides an opportunity to assess functional interactions with *Dax1*. The consequences of *Dax1* deficiency on testis development have been documented previously (Meeks et al., 2003a). Although the loss of either *Sf1* or *Dax1* function alters early testis development, their effects are distinct. Absence of *Dax1* predominantly alters the differentiation of peritubular myoid cells, and the patterning of fetal Leydig cells along the ventromedial to dorsolateral axis. *Sf1* regulates the temporal differentiation of Sertoli and Leydig cells, and there is a predominant spatial dependence exhibited in the anterior and posterior poles of the gonads. *Sry* expression begins in the central region of the gonad and extends anteriorly, followed by completion at the posterior end (Bullejos and Koopman, 2005). It is possible that factors downstream of *Sry* follow this spatiotemporal pattern.

Because the *Dax1* promoter contains Sf1-regulatory elements (Burris et al., 1995; Hoyle et al., 2002; Kawabe et al., 1999; Yu et al., 1998a), the defects observed in the *Sf1* heterozygote may be explained in part by reduced *Dax1* expression. Semi-quantitative RT-PCR analysis of urogenital ridge tissue confirmed there was a decreased number of *Dax1* transcripts in *Sf1* heterozygous gonads at E11.5 by about 50% (data not shown), consistent with previous reports (Hoyle, 2002). Nevertheless, the phenotypic features of the *Sf1* heterozygous gonad are more pronounced than those seen in *Dax1* null mutant, indicating distinct functions.

Although in vitro studies of Dax1 inhibition of Sf1 transactivation provide a relatively straightforward model for how these factors interact, the distinct effects of individual *Sf1* and *Dax1* mutations presage the consequences of the combined *Sf1/Dax1* mutation. Indeed, we found that testis development was affected to a greater degree in the combined *Sf1/Dax1* double mutant. In particular, the fetal Leydig cell markers *Cyp17* and *Cyp11a1* were absent at E13.5. The fetal Leydig cells ultimately recover in the *Sf1/Dax1* mutant and their population is normal by 2 weeks after birth, prior to the proliferation of adult Leydig cells. Sertoli cells appear to have selective roles for *Sf1* and *Dax1*. *Amh* expression was reduced in the *Sf1* heterozygote, consistent with the presence of Sf1-regulatory elements in this gene (Hatano et al., 1994; Shen et al., 1994). Unexpectedly, *Dax1* null gonads have reduced *Amh* production in vivo, although *Dax1* has been shown to mediate repression at the transcriptional level in vitro (Nachtigal et al., 1998). By E12.5, both of the single mutants recover *Amh*

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expression, but recovery is more delayed in the double mutant. Of note, adult Leydig cells are present in *Sf1/Dax1* double mutant mice and steroidogenesis is normal in the adult testis (data not shown). In spite of delayed steroidogenic enzyme gene expression, testis descent and secondary male reproductive features were unaffected in single and double mutants, and there was no persistence of Müllerian ducts (data not shown).

The phenotype of the combined *Sf1/Dax1* mutation is reminiscent of the *Dhh* knockout, which also exhibits a delay in fetal Leydig cell development and function (Yao et al., 2002). Another feature of the *Dhh* knockout male gonad is a defect in peritubular myoid cell development (Clark et al., 2000; Pierucci-Alves et al., 2001), a feature also seen in the *Dax1* null mutant (Meeks et al., 2003a). *Dhh* is expressed by Sertoli cells and acts via the Ptc1 receptor on fetal Leydig cells. At E11.5, *Dhh* expression was greatly reduced in *Sf1/Dax1* double mutant gonads but was partially recovered by E12.5. Hence, a temporal delay in *Dhh* expression in *Sf1/Dax1* mutants from E11.5 to E12.5 precedes the delay in fetal Leydig cell differentiation from E13.5 to E14.5. In addition to *Sf1* and *Dax1*, *Pdgfra* also acts upstream of *Dhh.* Homozygous knockouts of *Pdgfra* show delayed *Dhh* expression from 11.5 to 12.5, with a concomitant decrease in *Cyp11a1* (Brennan et al., 2003).

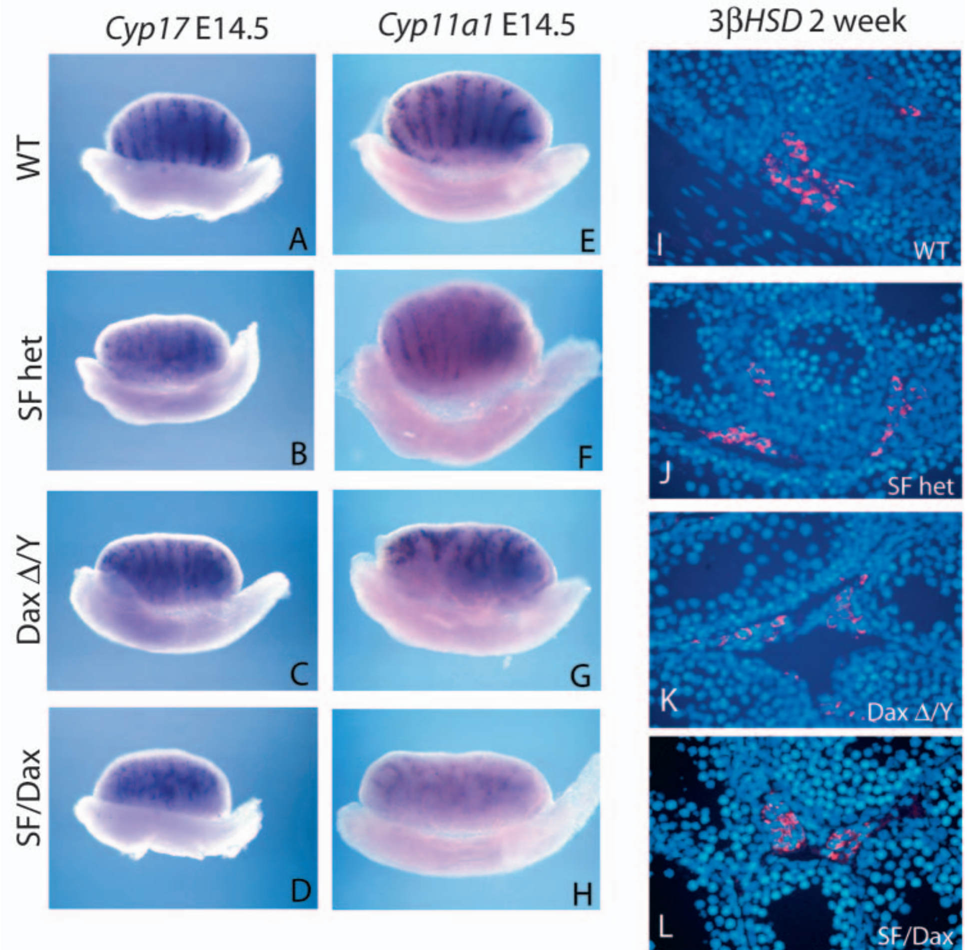

Fig. 6. Leydig cell development recovers by neonatal age. Consistent with *Dhh* recovery, low levels of *Cyp17* and *Cyp11a1* expression are detectable as early as E14.5. Expression of *Cyp17* (A-D) and *Cyp11a1* (E-H) recover by E14.5. Recovery of fetal Leydig cell differentiation was assessed by analysing 3βHSD protein levels at 2 weeks after birth, prior to the proliferation of adult Leydig cells. Two-week-old testis stained for *3βHSD* (I-L) confirms the presence of Leydig cells. Therefore, prior to the proliferation of adult Leydig cells, fetal Leydig cell differentiation is complete.

The delay in *Dhh* and *Amh* expression suggests that *Sf1* and *Dax1* converge on Sertoli cells to modulate key molecular pathways in male differentiation. However, *Sox9* expression is relatively preserved, suggesting differential effects on specific genes. The crucial genetic interaction of *Sf1* and *Dax1* appears to occur early in the bipotential stage at 11.5 dpc or before. The precise mechanism for this interaction is currently unknown. It remains plausible that Dax1 exerts a repressive function for a subset of Sf1-regulated genes that somehow regulate the timing of Sertoli cell differentiation, or *Dhh* and *Amh* expression. Given the multiple functions of Sf1 in male gonad development (coelomic epithelial proliferation, Amh production, and steroidogenesis), *Dax1* repression of *Sf1* target genes might be highly variable and depend on both the amount of *Sf1* protein present and the number of *Sf1* DNA-binding elements on the target gene promoter and (Hanley et al., 2001). At present, the regulatory elements of the *Dhh* promoter have not been characterized but may contain regulatory elements that allow cooperative rather than antagonistic actions of *Sf1* and *Dax1*. Alternatively, *Sf1* and *Dax1* may act indirectly to delay *Dhh* expression by altering cell lineage restriction prior to *Dhh* expression. This could include paracrine effects on Sertoli cells that express *Dhh*. The observation that single mutations of *Sf1* and *Dax1* influence the spatial expression of genes in the developing gonad is consistent with effects on positional cues or cell-cell interactions.

In a previous study, we analyzed the effect of allelic loss of *Sf1* on a *Dax1* null background with respect to adrenal development and function (Babu et al., 2002). Double mutation of *Sf1* and *Dax1* restored adrenal weight and corticosterone production. Allelic loss of *Sf1* corrected the overexpression of *Cyp21* and the *Acthr* in the *Dax1* null adrenal gland. These findings in the adrenal gland are reminiscent of the selective repression of *Cyp19* in adult Leydig cells of the testis (Wang et al., 2001). Taken together, these studies of *Sf1* and *Dax1* interactions in vivo suggest antagonistic interactions for some target genes, such as *Cyp21*, *Acthr* and *Cyp19*. However, target genes, such as *Dhh* and *Amh*, require cooperative functions of

Sf1 and *Dax1*. It is also likely that these nuclear factors function independently, as evidenced by the distinct features found in single gene mutant phenotypes of the adrenal gland and gonad. These discrete actions of *Sf1* and *Dax1* are also consistent with the clinical consequences of human *SF1* and *DAX1* mutations, each of which impair adrenal and testis development but exhibit distinct histological characteristics.

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