

Gonadal Determination and Adrenal Development Are Regulated by the Orphan Nuclear Receptor Steroidogenic Factor-1, in a Dose-Dependent Manner

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The orphan nuclear receptor steroidogenic factor-1 (SF-1, NR5A1) regulates the transcription of multiple genes involved in steroidogenesis, reproduction, and male sexual differentiation. A heterozygous loss-of-function SF-1 mutation (G35E) has been described in a patient with adrenal failure and complete 46XY sex-reversal, indicating that haploinsufficiency of this factor is sufficient to cause a severe clinical phenotype. This mutation in the P-box region of the DNA-binding domain markedly impairs SF-1 binding to most response elements. In an infant with a similar clinical phenotype, we identified an SF-1 mutation (R92Q) in a highly conserved residue of the A-box, a region that functions as a secondary DNA-binding domain. Strikingly, the affected infant was homozygous for

the R92Q mutation, but three relatives (parents, sister) were phenotypically normal despite being heterozygous for the mutation. In functional assays, the R92Q mutant exhibited partial loss of DNA binding and transcriptional activity when compared with the G35E P-box change, consistent with its phenotypic expression only when transmitted as a homozygous trait. Taken together, these two naturally-occurring SF-1 mutations reveal the relative functional importance of the P-box and A-box regions for monomeric binding by nuclear receptors. In addition, these patients reveal the exquisite sensitivity of SF-1-dependent developmental pathways to gene dosage and function in humans. (*J Clin Endocrinol Metab* 87: 1829–1833, 2002)

STEROIDOGENIC FACTOR-1 (SF-1) (*FTZf1*; Ad4BP, NR5A1) is an orphan nuclear receptor that regulates the transcription of multiple target genes involved in gonadal and adrenal development, male sexual differentiation, steroidogenesis, and reproduction (1–3). In mice, homozygous targeted disruption of the gene encoding Sf-1 (*Ftzf1*) ($-/-$) causes a complex phenotype of gonadal and adrenal agenesis, 46XY sex-reversal, persistent müllerian structures in males, obesity, and abnormalities of the ventromedial hypothalamus and pituitary gonadotropes (4–6). Heterozygous animals ($+/-$) have a milder phenotype (7). Thus, SF-1 plays a pivotal role, to orchestrate the development of the reproductive axis and the adrenal gland at multiple levels.

Unlike most nuclear receptors, which bind to DNA as homo- or heterodimers, SF-1 binds to target gene promoters as a monomer and recognizes variations of an extended half-site motif (PyCA AGGTCA) (1, 8, 9). Previously, we described a heterozygous *de novo* mutation in SF-1 in a patient with primary adrenal failure, testicular dysgenesis, and 46XY sex-reversal, including the presence of a uterus (10). This mutation (G35E) affects an amino acid in the proximal (or P-box) of the first zinc finger of SF-1. The P-box motif binds to the half-site core sequence (AGGTCA) and is critical for determining DNA-binding specificity by nuclear receptors (11, 12). Consistent with this, mutant G35E SF-1 showed impaired binding to, and transactivation of, several known target genes (10, 13). Because no strong dominant negative

effect was seen when mutant and wild-type (WT) SF-1 were cotransfected in transient gene expression assays (13), haploinsufficiency of SF-1 seems to cause a clinically severe phenotype. This concept underscores the importance of gene dosage effects of transcription factors in human development and function and is supported by the recent description of a second *de novo* heterozygous SF-1 mutation (R255L) in a phenotypically normal female with adrenal insufficiency and apparently normal ovarian differentiation (14).

Identifying mutations in other regions of SF-1 could help to determine important structural domains of this nuclear receptor. Here, we report a homozygous mutation in the A-box of SF-1 in a baby born to consanguineous parents. This mutation affects a region of SF-1 that modulates DNA binding by monomers (8, 13) and produces a phenotype with an autosomal recessive mode of inheritance. This A-box change produces a partial loss of function, compared with the P-box mutation. Thus, these studies highlight the importance of quantitative effects of transcription factors that control development.

Materials and Methods

Mutational analysis

After obtaining written consent, genomic DNA was extracted from blood leukocytes of family members. All six coding exons of *FTZf1* were amplified by PCR using specific oligonucleotide primer pairs (15). Direct DNA sequencing of PCR products was performed using a dRhodamine dye terminator sequencing kit and ABI377 automated sequencer (PE Applied Biosystems, Foster City, CA).

Restriction enzyme analysis was performed by *MspI* (Promega Corp., Madison, WI) digestion of a PCR fragment amplified by the following

Abbreviations: ERR, Estrogen-related receptor; NGFI-B, nerve growth factor-induced-B; SF-1, steroidogenic factor-1; WT, wild type.

primer pair: forward, 5'-GGAGCCATGAAAGGGTGTG-3'; reverse, 5'-CTGTCTCCAGCTTGAAGCCAT-3'. Digests were separated on a 3% NuSieve GTG agarose gel (BMA Products, Rockland, ME).

Functional analysis of mutant SF-1

An expression vector (pCMX) containing the SF-1 A-box mutation was created using an overlapping PCR strategy with human *FTZF1* cDNA as a template. The construction of the WT SF-1 and P-box mutant (G35E) SF-1 expression vectors has been reported previously (10).

Transient gene expression studies were performed by cotransfecting human embryonic kidney ts201 cells with empty (-), WT or mutant (G35E; R92Q) SF-1 expression vector (20 ng), and luciferase reporter constructs (500 ng) containing mouse *Cyp11a* (P450scc) (-81 to +42) (SF-1 sites: 5' GGGAGGTC, 3' TCAAGGCTA), mouse *Ahch* (Dax-1) (-134 to +26) (composite SF-1 sites: TCGAGGTCATGGCCA), or rat *Cyp19* (aromatase) (-294 to +20) (SF-1 site: CCAAGGTC) promoters, as described previously (10, 13). Luciferase assays were performed 48 h later. The results of triplicate transfections are expressed as mean \pm SEM.

Electrophoretic mobility shift assays were performed by incubating 3 μ L *in vitro* translated SF-1 proteins (TNT reticulocyte lysate system, Promega Corp.) with a 32 P-radiolabeled probe (20 fmol) corresponding to the 3' murine *Cyp11a* (P450scc) SF-1 binding site (TCAAGGCTA), as described previously (10, 13).

Modeling of SF-1-DNA interactions

The interaction between the P- and A-boxes of SF-1 and a typical DNA response element was modeled on the crystal structure of nerve growth factor-induced-B (NGFI-B) bound to DNA as a monomer [protein database reference Id: 1CIT; (9)] (WebLab ViewerLite software, Molecular Simulations Inc., San Diego, CA).

Results

Case report

The proband (Fig. 1A) is a phenotypically female baby (birth weight, 4.5 kg, full term) who presented 1 d after birth with a hypoglycemic convulsion. Examination revealed marked hyperpigmentation. Progressive hypotonia, jaundice, weight loss, and failure to thrive developed during the neonatal period, and hyponatremia (sodium, 129 mM) and

hyperkalemia (potassium, 6.5 mM) were detected. Detailed investigations, at 15 d of age, revealed evidence of primary adrenal failure (Table 1). Although the fetal adrenal steroid dehydroepiandrosterone sulfate was detectable [30 μ g/dl; normal range, 5–111 (0.9 μ M; normal range, 0.15–3.3)], 17-hydroxyprogesterone was low [2 ng/dl; <300 (0.06 nM; <10)] and abdominal CT scan demonstrated left adrenal hypoplasia and right adrenal agenesis. The patient's karyotype was found to be 46XY, and a uterus was seen on pelvic ultrasound and confirmed by magnetic resonance imaging. The parents are first cousins (Fig. 1A). Their first child died from unknown causes at 4 d of age.

Mutational analysis

Direct DNA sequencing in the index case revealed a CCG-to-CAG nucleotide transversion in exon 4 of *FTZF1*, which results in a homozygous R92Q mutation in the A-box of SF-1 (Fig. 1B, bottom).

Analysis of the family showed that both parents and one sister are heterozygous for this mutation (Fig. 1B, middle), whereas the oldest sister is normal (Fig. 1B, top). The autosomal recessive mode of inheritance for this mutation was confirmed by restriction enzyme analysis (Fig. 1A). The parents seem to have normal fertility and reproductive function. No impairment of adrenal function was detected in the heterozygous individuals investigated (Table 1), and the mother had an adequate cortisol response to corticotropin stimulation (basal cortisol, 23.5 μ g/dl; peak cortisol, 50 μ g/dl).

Functional analyses

The R92Q mutation affects a codon within the A-box of SF-1 (Fig. 1B). This A-box region is thought to be involved in stabilizing monomeric binding by SF-1 to the response elements of target genes (Fig. 2A; Refs. 8 and 13), and the

FIG. 1. Homozygous R92Q mutation in SF-1 in a baby with primary adrenal failure, 46XY sex-reversal, and a uterus. A, Details of the kindred. The parents are first cousins. Their first child died, at 4 d of age, from unknown causes. Two girls are apparently normal. The index case, shown by the arrow, had a hypoglycemic convulsion, shortly after birth, and evidence of progressive primary adrenal failure in the neonatal period. B, Direct sequencing revealed a homozygous R92Q (CGG to CAG) mutation in the A-box of SF-1 in the index case. Both parents and one sister are heterozygous for this change. This nucleotide transversion removes an *MspI* site. Restriction enzyme digestion produces a 187-bp fragment from the mutant allele and a 154-bp fragment from the normal allele. This approach was used to confirm the autosomal recessive inheritance pattern in the family. The zinc-finger region of SF-1 is shown in pink. This region contains the P-box motif and G35E mutation reported previously (10).

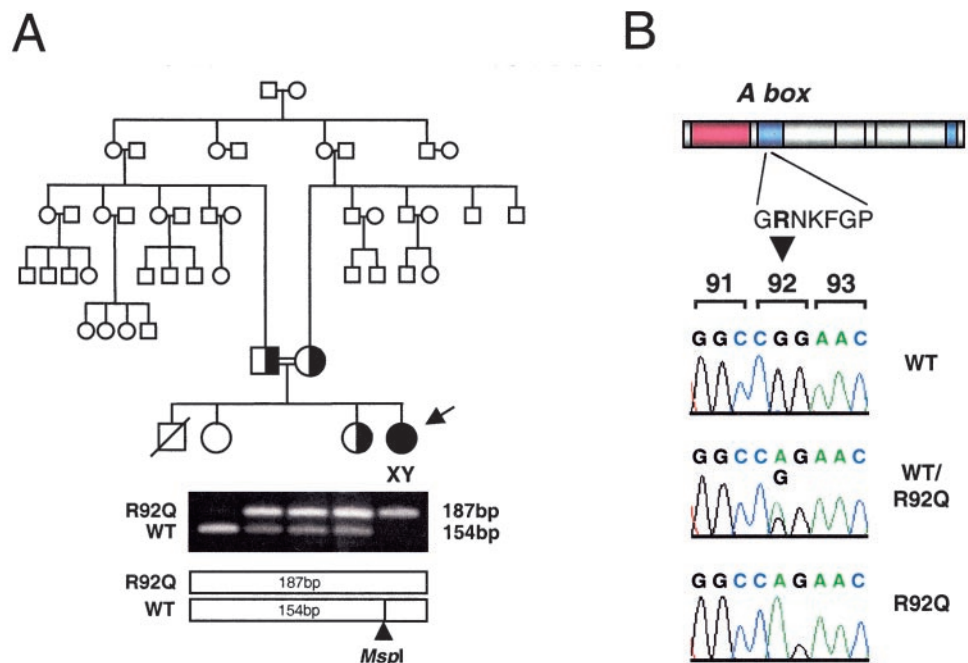


TABLE 1. Investigations of adrenal and gonadal function

	Genotype	Age	Cortisol ^a (μg/dl)	ACTH ^a (pg/ml)	Aldosterone ^a (ng/dl)	LH (U/liter)	FSH (U/liter)	T (ng/dl)	E2 (pg/ml)
Proband (XY)	R92Q	15 d	1.1	9312	1.8				
		36 d				0.01	1.4	10	
Mother	R92Q/WT	30 yr	23.5 ^b	74	6.7	32	8.4		92
Sister	R92Q/WT	5 yr	27	53	19.0	0.01	1.7		
Normal ranges:		Infant	3–23	10–69	5–90				
		1–10 yr	3–23	10–69	5–80				
		Adult	9–23	10–69	3–30				

^a Cortisol, ACTH and aldosterone values obtained at 0800 h.

^b Basal cortisol value shown. The peak cortisol response following stimulation with corticotropin (Synacthen, 0.25 mg intramuscularly) was 50 μg/dl. Father unavailable for further investigation.

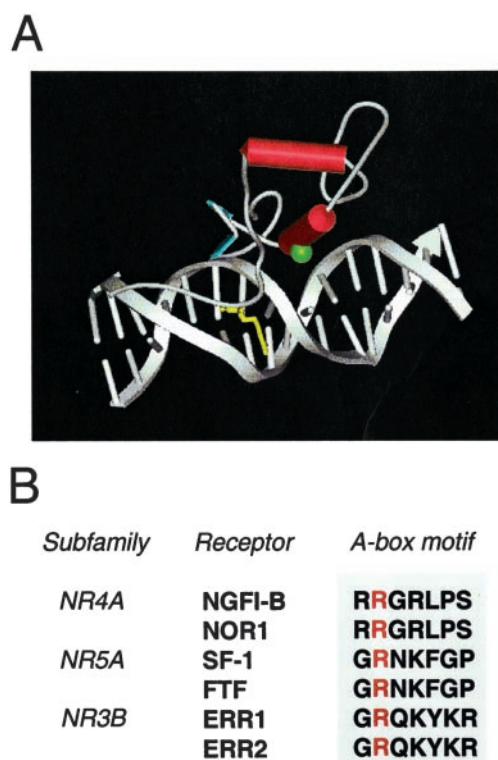


FIG. 2. A, Model of SF-1 binding based on the crystal structure of NGFI-B bound to DNA as a monomer (9). The position of amino acid 35 within the P-box is shown in green, and the amino acid 92 within the A-box is shown in yellow (9). The P-box amino acids bind to the half-site sequence (variations on AGGTCA) within the major groove of DNA, whereas the A-box is believed to bind to the 5'-flanking sequence (T/CCA) within the minor groove of DNA. B, The A-box arginine affected is highly conserved in orphan nuclear receptors that bind DNA as monomers. NOR1, Neuron-derived orphan receptor 1; FTF, fetoprotein transcription factor.

arginine at this position is highly conserved among members of this subgroup of nuclear receptors (Fig. 2B; Ref. 9). Consistent with this, the R92Q mutant SF-1 exhibits reduced transactivation of the *Cyp11a* (P450_{scc}) promoter (Fig. 3B) and partially impairs binding to a probe corresponding to the 3' SF-1 binding site of this promoter [SCC1 (10)] (Fig. 3C). However, this loss of function is not as severe as that seen with the G35E P-box mutant reported previously (10). This quantitative difference between the P- and A-box mutants was confirmed by studying the *Ahch* (Dax-1) and *Cyp19* (aromatase) promoters, where the G35E P-box mutant shows partial activation (Fig. 4, A and B).

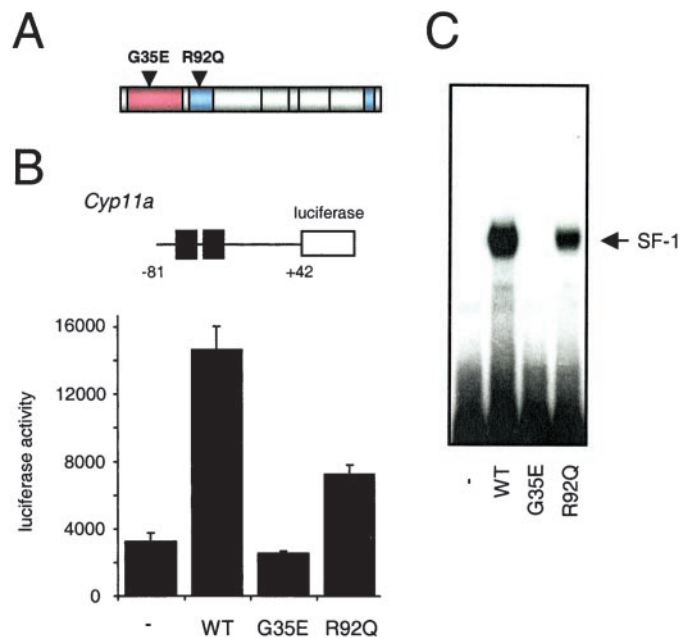


FIG. 3. The R92Q mutation reduces the functional activity of SF-1. A, Expression vectors containing WT, G35E P-box mutant SF-1 (G35E), and R92Q A-box mutant SF-1 (R92Q) were created for use in functional studies. Empty vector was used as a negative control (-). B, The R92Q A-box mutant shows impaired activation of a critical SF-1 target gene, *Cyp11a* (P450_{scc}). C, Reduced binding to a probe corresponding to the SF-1 binding site (TCA AGGCTA) of this promoter. However, this loss of function was not as severe as that seen with the G35E P-box mutant reported previously (10).

Discussion

SF-1 is a key regulator of endocrine development and function, because it influences the transcription of multiple (>20) genes involved in gonadal and adrenal development, steroidogenesis, and reproduction (*e.g.* *DAX1*, *MIS*, *MIS receptor*, *STAR*, *CYP11A*, *CYP19*, *LHβ*). Consequently, abnormalities in SF-1 have profound effects on the development of the male (46XY) fetus. The phenotype of the sex-reversed patient reported here is remarkably similar to a previous patient (10): namely, early-onset primary adrenal failure caused by glucocorticoid and mineralocorticoid insufficiency; 46XY sex-reversal caused by gonadal dysgenesis, leading to insufficient testosterone for virilization and the development of a uterus caused by reduced production and action of a müllerian-inhibiting substance (antimüllerian hormone); and potentially impaired gonadotropin secretion. Strikingly,

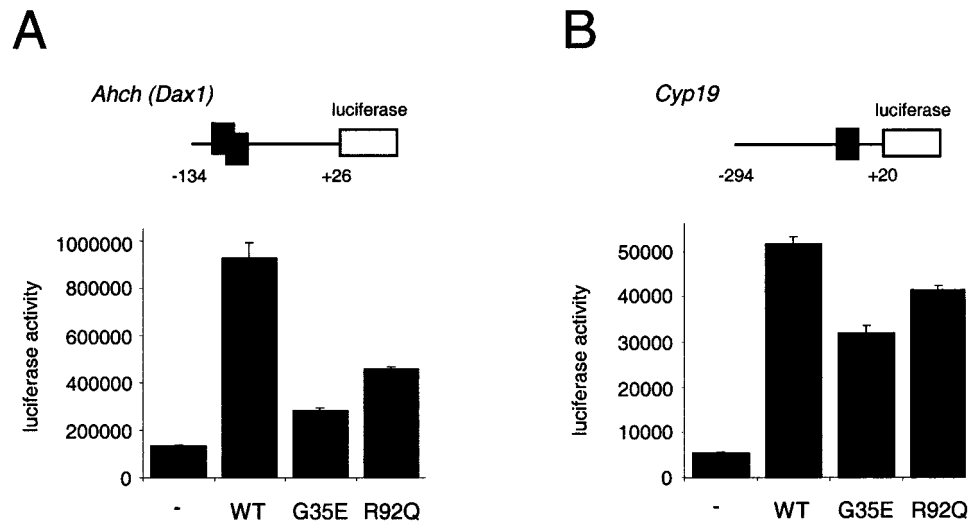


FIG. 4. Similar quantitative differences between the P-box (G35E) and A-box (R92Q) mutants were obtained when the (A) *Ahch* (Dax-1) and (B) *Cyp19* (aromatase) promoters were studied. Loss of transactivation is less severe with the R92Q mutant.

however, the genetic basis for the phenotype is autosomal dominant in one case (10) but recessive in the family reported here, even though both mutations affect the DNA-binding properties of SF-1. Individuals who are heterozygous for the R92Q A-box mutation do not have a significant clinical phenotype, whereas the homozygous proband has profound adrenal insufficiency and sex-reversal with retained müllerian structures. Therefore, the R92Q mutation has an autosomal recessive mode of inheritance. These findings underscore the importance of gene dosage and residual function of SF-1 as a determinant of clinical phenotype.

SF-1 binds to the promoters of target genes as a monomer and recognizes variations of the DNA sequence, PyCA AGGTCA (1, 8). However, the variability in this sequence is great, and many SF-1-responsive genes have variant response elements, a feature that seems to modulate SF-1 transcriptional activity (16). Although all of the naturally occurring SF-1 mutations described to date alter DNA binding (10, 14), each occurs within a different domain of the transcription factor. The interaction between P-box codon 35 (Fig. 2A, green) and the major groove of the DNA helix dictates binding to the AGGTCA motif (8, 9, 12, 13). As a result, mutations in the P-box sequence (G35E) interfere with SF-1 binding, especially to promoters containing variant half-site sequences (e.g. *Cyp11a* 3', TCA AGGCTA) (Fig. 3; Ref. 13). The A-box is a conserved feature of several nuclear receptors that bind to DNA as monomers [e.g. NGFI-B, neuron-derived orphan receptor 1, and human estrogen-related receptor (ERR)1] (8, 9). This A-box region interacts primarily with the PyCA flanking sequence and the first part of the half-site, which is located within the minor groove of the DNA helix (8, 9, 13). This interaction is probably weakened by the loss of charge resulting from the R92Q mutation. However, because this protein-DNA interface is less important than that between the P-box and major groove, only partial loss of function and binding by the R92Q mutant is seen, compared with the G35E mutant (Figs. 3 and 4).

It is becoming evident that mutations in nuclear receptors and other transcription factors play an important role in human genetic disease (17). The case described here highlights how different mutations in DNA-interacting regions of

SF-1 can produce distinct functional effects and variable phenotypic penetrance. A similar spectrum of inheritance patterns and phenotypes is seen with mutations in Pit-1, a transcription factor that controls the development of pituitary cell lineages (18–20), as well as with mutations in several membrane receptors [such as mutations in the melanocortin 4 receptor in patients with monogenic obesity (21), and the calcium-sensing receptor in patients with hypocalcemic hyperparathyroidism/neonatal severe hyperparathyroidism (22)]. We suggest that gene dosage and residual function, as well as alterations in the specificity for target genes, can profoundly influence the phenotypic consequences of mutations in many developmental transcription factors. Alternative or milder phenotypes are likely to result from mutations in other regions of SF-1.

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References

- Parker KL, Schimmer BP 1997 Steroidogenic factor 1: a key determinant of endocrine development and function. *Endocr Rev* 18:361–377
- Lala DS, Rice DA, Parker KL 1992 Steroidogenic factor 1, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor 1. *Mol Endocrinol* 6:1249–1258
- Honda S, Morohashi K, Nomura M, Takeya H, Kitajima M, Omura T 1993 Ad4BP regulating steroidogenic P-450 gene is a member of steroid hormone receptor superfamily. *J Biol Chem* 268:7494–7502
- Luo X, Ikeda Y, Parker KL 1994 A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell* 77:481–490
- Shinoda K, Lei H, Yoshii H, Nomura M, Nagano M, Shiba H, Sasaki H, Osawa Y, Ninomiya Y, Niwa O, Morohashi K-I, Li E 1995 Developmental defects of the ventromedial hypothalamic nucleus and pituitary gonadotroph in the *Ftz-F1* disrupted mice. *Dev Dyn* 204:22–29

6. Sadovsky Y, Crawford PA, Woodson KG, Polish JA, Clements MA, Tourtellotte LM, Simburger K, Milbrandt J 1995 Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. *Proc Natl Acad Sci USA* 92:10939–10943
7. Bland ML, Jamieson CA, Akana SF, Bornstein SR, Eisenhofer G, Dallman MF, Ingraham HA 2000 Haploinsufficiency of steroidogenic factor 1 in mice disrupts adrenal development leading to an impaired stress response. *Proc Natl Acad Sci USA* 97:14488–14893
8. Wilson TE, Fahrner TJ, Milbrandt J 1993 The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. *Mol Cell Biol* 13:5794–5804
9. Meinke G, Sigler PB 1999 DNA-binding mechanism of the monomeric orphan nuclear receptor NGFI-B. *Nat Struct Biol* 6:471–477
10. Achermann JC, Ito M, Ito M, Hindmarsh PC, Jameson JL 1999 A mutation in the gene encoding steroidogenic factor-1 causes XY sex-reversal and adrenal failure in humans. *Nat Genet* 22:125–126
11. Mader S, Kumar V, de Verneuil H, Chambon P 1989 Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. *Nature* 338:271–274
12. Umesono K, Evans RM 1989 Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell* 57:1139–1146
13. Ito M, Achermann JC, Jameson JL 2000 A naturally-occurring steroidogenic factor-1 (SF-1) mutation exhibits differential binding and activation of target genes. *J Biol Chem* 275:31708–31714
14. Biason-Lauber A, Schoenle EJ 2000 Apparently normal ovarian differentiation in a prepubertal girl with transcriptionally inactive steroidogenic factor 1 (NR5A1/SF-1) and adrenocortical insufficiency. *Am J Hum Genet* 67:1563–1568
15. Wong M, Ramayya MS, Chrousos GP, Driggers PH, Parker KL 1996 Cloning and sequence analysis of the human gene encoding steroidogenic factor 1. *J Mol Endocrinol* 17:139–147
16. Ito M, Park Y, Weck J, Mayo KE, Jameson JL 2000 Synergistic activation of the inhibin alpha promoter by steroidogenic factor-1 and cAMP. *Mol Endocrinol* 14:66–81
17. Johnson W, Jameson JL 1998 Transcriptional control of gene expression. In: Jameson JL, ed. *Principles of molecular medicine*. Totowa, NJ: Humana Press; 25–41
18. Pfäffle RW, DiMattia GE, Parks JS, Brown MR, Wit JM, Jansen M, Van der Nat H, Van den Brande JL, Rosenfeld MG, Ingraham HA 1992 Mutation of the POU-specific domain of Pit-1 and hypopituitarism without pituitary hypoplasia. *Science* 257:1118–1121
19. Radovick S, Nations M, Du Y, Berg LA, Weintraub BD, Wondisford FE 1992 A mutation in the POU-homeodomain of Pit-1 responsible for combined pituitary hormone deficiency. *Science* 257:1115–1118
20. Scully KM, Jacobson EM, Jepsen K, Lunyak V, Viadiu H, Carriere C, Rose DW, Hooshmand F, Aggarwal AK, Rosenfeld MG 2000 Allosteric effects of pit-1 DNA sites on long-term repression in cell type specification. *Science* 290:1127–1131
21. Farooqi IS, Yeo GS, Keogh JM, Aminian S, Jebb SA, Butler G, Cheetham T, O'Rahilly S 2000 Dominant and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency. *J Clin Invest* 106:271–279
22. Pollak MR, Chou YH, Marx SJ, Steinmann B, Cole DE, Brandi ML, Papapoulos SE, Menko FH, Hendy GN, Brown EM, Seidman CE, Seidman JG 1994 Familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. Effects of mutant gene dosage on phenotype. *J Clin Invest* 93:1108–1112

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