Inherited human sex reversal due to impaired nucleocytoplasmic trafficking of SRY defines a male transcriptional threshold

Yen-Shan Chen*, Joseph D. Racca*, Nelson B. Phillips*, and Michael A. Weissab,c,1

Departments of *Biochemistry, #Biomedical Engineering, and ^Medicine, Case Western Reserve University, Cleveland, OH 44106

Edited by Patricia K. Donahoe, Massachusetts General Hospital, Boston, MA, and approved August 14, 2013 (received for review January 16, 2013)

Human testis determination is initiated by SRY (sex determining region on Y chromosome). Mutations in SRY cause gonadal dysgenesis with female somatic phenotype. Two subtle variants (V60L and I90M in the high-mobility group box) define inherited alleles shared by an XY sterile daughter and fertile father. Whereas specific DNA binding and bending are unaffected in a rat embryonic pre-Sertoli cell line, the variants exhibited selective defects in nucleocytoplasmic shuttling due to impaired nuclear import (V60L mediated by Exportin-4) or export (I90M mediated by chromosome region maintenance 1). Decreased shuttling limits nuclear accumulation of phosphorylated (activated) SRY, in turn reducing occupancy of DNA sites regulating Sertoli-cell differentiation [the testis-specific SRY-box 9 (Sox9) enhancer]. Despite distinct patterns of biochemical and cell-biological perturbations, V60L and I90M each attenuated Sox9 expression in transient transfection assays by twofold. Such attenuation was also observed in studies of V60A, a clinical variant associated with ovotestes and hence ambiguity between divergent cell fates. This shared twofold threshold is reminiscent of autosomal syndromes of transcription-factor haploinsufficiency, including XY sex reversal associated with mutations in Sox9. Our results demonstrate that nucleocytoplasmic shuttling of SRY is necessary for robust initiation of testicular development. Although also characteristic of ungulate orthologs, such shuttling is not conserved among rodents wherein impaired nuclear export of the high-mobility group box and import-dependent phosphorylation are compensated by a microsatellite-associated transcriptional activation domain. Human sex reversal due to subtle defects in the nucleocytoplasmic shuttling of SRY suggests that its transcriptional activity lies near the edge of developmental ambiguity.

Significance

Mutations in human SRY (sex determining region on Y chromosome) associated with somatic sex reversal provide a model for the perturbation of a genetic switch in organogenesis. Inherited alleles, associated with either testicular or ovarian differentiation, provide unique probes of threshold biochemical properties, defining mechanistic borders between functional and nonfunctional transcription factors. This study exploited two such alleles to demonstrate that bidirectional nucleocytoplasmic trafficking (import–export shuttling) enables robust operation of this switch via phosphorylation at a site external to the DNA-binding motif of the transcription factor. In accordance with studies of intersexual mice, our results suggest that human SRY functions at the edge of ambiguity.

*Seven inherited Swyer alleles of SRY have been reported (1), a Y-chromosomal gene encoding an architectural transcription factor (TF) (2). SRY contains a central high-mobility group (HMG) box, a conserved motif of specific DNA binding and bending (Fig. L4) (3, 4). Assignment of Sry as the testis-determining factor is supported by transgenic mouse models (5) and human mutations leading to gonadal dysgenesis with female somatic phenotype (Swyer syndrome) (6). Expressed in the pre-Sertoli cells of the differentiating gonadal ridge, Sry activates Sox9, an autosomal gene broadly conserved among vertebrate sex-determining pathways (7). Direct binding of Sry to specific DNA sites within the testis-specific core enhancer of Sox9 (TESCO) activates a male-specific gene-regulatory network in the fetal gonadal ridge (7). Sustained Sox9 expression orchestrates programs of cell–cell communication, migration, and differentiation leading to gonadogenesis (8), regression of the female primordia (following Sertoli-cell secretion of anti-Müllerian Hormone/Müllerian Inhibiting Substance; AMH/MIS) (9), and somatic virilization (through fetal Leydig-cell secretion of testosterone) (10). Mutations in this pathway are associated with disorders of sexual development (11). Structure–function relationships in human SRY (hSRY) have been investigated through comparative biochemical and biophysical studies of Swyer variants (6). Most often arising de novo in spermatogenesis and clustering in the HMG box (Fig. L4), such mutations typically impair specific DNA binding by direct or indirect perturbation of an angular protein–DNA interface (6, 12). The present study focuses on inherited mutations (V60L and I90M; asterisks in Fig. L4) that by contrast allow near-native DNA binding and bending (13, 14). Such mutations are compatible with alternative developmental outcomes: testicular differentiation leading to virilization (fertile 46,XY father) or nascent ovarian differentiation leading to gonadal dysgenesis (sterile 46,XY daughter) (13). In the structure of the wild-type (WT) SRY–DNA complex (12), V60 and I90 pack within the minor and major wings (Fig. 1B) of the HMG box (consensus positions 5 and 35, respectively; Fig. 1C). The clinical substitutions each represent a subtle interchange of nonpolar side chains. Known inherited mutations in SRY are summarized in SI Appendix, Table S1.*

Inherited mutations in hSRY provide experiments of nature probing the threshold molecular properties of a developmental switch beyond DNA binding and bending. To this end, our studies exploited a model of the central SRY–Sox9 regulatory axis (Fig. 1D) in a rodent fetal pre-Sertoli cell line (14–16). Our findings demonstrate that hSRY undergoes nucleocytoplasmic

www.pnas.org/cgi/doi/10.1073/pnas.1300828110

PNAS | Published online September 3, 2013 | E3567–E3576
shuttling (NCS) and that such NCS is coupled to an activating N-terminal serine phosphorylation (17, 18). Respective V60L and I90M variants exhibit selective impairment of nuclear import (as mediated by Exportin-4) or nuclear export (mediated by CRM1) but otherwise retain substantial gene-regulatory activity (as mediated by Exportin-4) or nuclear export (mediated by CRM1) (23). Although use of a cytomegalovirus (CMV) promoter measured following transient transfection of WT or mutant SRY constructs. Although use of a cytomegalovirus (CMV) promoter under standard transfection conditions yielded \(10^5\) to \(10^6\) molecules per transfected cell, such overexpression could be attenuated by dilution of the plasmid with an empty vector to achieve an appropriate TF concentration (\(10^5\) to \(10^6\) molecules per cell). A subset of key findings was replicated in a human male cell line.

Inherited mutations of sexual development in hSRY (V60L and I90M; first and second red asterisks in Fig. 1A, respectively) were chosen based on their compatibility with native-like structure and DNA binding (Table 1) (14). Structural environments are shown in Fig. 1 (12). Conserved as Val among mammalian SRY/Sry domains, V60 (consensus position 5 in the minor wing) adjoins a bipartite nuclear localization signal (NLS). A second mutation at this site (V60A; father uncharacterized) was associated with ovotestes (33). V60L and V60A do not affect box stability and permit DNA-dependent folding of the minor wing with near-native DNA bending (14). I90 projects from the second \(\alpha\)-helix (\(\alpha_2\)) into the major-wing core as part of an aliphatic motif (19, L94, M100, and L101; consensus positions 35, 39, 45, and 46) proposed to function as a Sox nuclear export signal (NES) (14, 20). Although I90M destabilizes the free box (\(\Delta_{\text{TM}} 4^\circ\)C and \(\Delta\Delta G_{\text{M}} 0.5\) kcal/mole) (Table 1 and SI Appendix, Fig. S1), near-native specific DNA affinity (\(K_d\)) and DNA bending are nonetheless maintained (Table 1 and SI Appendix, Figs. S2 and S3). Like V60L and V60A box–DNA complexes (14), the I90M
complex exhibits a reduced kinetic lifetime with compensating changes in on-rate constants similar to wild type.

Transcriptional Regulation in a Pre-Sertoli Cell Model. CH34 cells were used to probe hSRY-dependent activation of Sox9; transcription efficiency was 32.6 (+ 1.2)% as inferred from control cotransfection of a plasmid encoding green fluorescent protein (GFP). Transient transfection of WT hSRY under standard conditions (1 μg per well) activated expression of Sox9 by eightfold relative to an empty vector (black bar in Fig. 2 A). Extent of transcriptional activation decreased to fivefold on successive dilution of the hSRY plasmid by the empty vector (maintaining total transfected DNA constant) to a final dilution of 0.02 μg of hSRY plasmid and 0.98 μg of empty vector per well (50-fold dilution; white bar in Fig. 2 A). Such dilution provided a control for overexpression artifacts.

Although Sox9 is the principal target of Sry (7), additional qPCR assays were undertaken to characterize a downstream gene-regulatory network in relation to in situ transcriptional profiling of the murine XY gonadal ridge (28). Whereas transient expression of hSRY did not affect mRNA accumulation of non–sex-related Sox genes and housekeeping genes (boxes at Lower in Fig. 1 D), specific up-regulation of Sox9, fibroblast growth factor 9 (Fgf9), and prostaglandin D2 synthetase (Ptgd2) were observed in accord with their known roles in testicular differentiation (34). No such changes in mRNA accumulation were observed on transient transfection of an empty plasmid or a control plasmid expressing a stable hSRY variant devoid of specific DNA-binding activity (I68A) (35). Further, following 24-h incubation in serum-rich medium, no significant changes were observed in the expression of rat Sry itself.

Activities and Stabilities of hSRY Variants. Comparative studies of variant hSRY constructs (V60L, V60A, and I90M) demonstrated that (i) the two minor-wing substitutions exhibited decreased transcriptional activity at each dilution tested whereas (ii) major-wing substitution I90M enhanced Sox9 expression when overexpressed but exhibited progressive loss of function with serial dilution (at right in Fig. 2 A). Remarkably, at highest dilution (50-fold; white bars in Fig. 2 A), the three substitutions were each associated with twofold loss of Sox9 activation relative to wild type at the same dilution. These trends were also observed in the downstream gene-regulatory network modulated by the SRY-Sox9 axis (SI Appendix, Fig. S4). The anomalous dilution-related properties of I90M hSRY motivated assessment of proteolytic stability and cellular NCS as factors that could lead to overexpression artifacts.

Cellular turnover of hemagglutinin (HA)-tagged hSRY constructs (transfected without dilution) was evaluated following cycloheximide inhibition of translation (Fig. 2 B). Comparison of anti-HA Western-blot intensities demonstrated that V60L and V60A variants are more susceptible to degradation than are WT or I90M hSRY (graph in Fig. 2 B). Such differential degradation could be circumvented through addition of proteosome-inhibitor MG132 (SI Appendix, Fig. S5). Subcellular localization was investigated using immunofluorescence microscopy (Fig. 2 C); control GFP cotransfection studies demonstrated that WT and variant constructs achieved similar transfection efficiencies; 900 cells were counted in each case (triplicate by blinded coworkers). Representative images are shown in Fig. 2 C. Lower in relation to corresponding nuclear staining of the same cells with 4',6-diamidino-2-phenylindole (DAPI) (Fig. 2 C, Upper). The variant proteins exhibited contrasting perturbations relative to wild type (Fig. 2 C, Lower). Whereas V60L and V60A exhibited pan-cellular distributions with significant reduction in exclusive nuclear staining (gray bars in Fig. 2 D), I90M hSRY exhibited a reduced pancellular fraction (white bars in Fig. 2 D) with increase in exclusive nuclear staining. Partial restoration of nuclear localization of V60L and V60A hSRY was achieved by N-terminal fusion of an exogenous nuclear localization signal (NLS) derived from SV40 large T Antigen (36) (“+NLS” at right in Fig. 2 D). No significant changes in nuclear accumulation of WT hSRY were observed in control studies of NLS-hSRY (SI Appendix, Fig. S6). I90M NLS-hSRY immunofluorescence studies were repeated on addition of MG132 after 24-h posttransfection (Fig. 2 E). As expected, proteosomial inhibition did not affect nuclear localization of WT or I90M hSRY (Fig. 2 D and E). In the absence of the fused NLS, MG132 treatment led to small decreases in extent of residual GFP-positive cells lacking detectable hSRY expression (100-sum of gray and white bars in Fig. 2 D and E); this trend did not achieve statistical significance. Strikingly, the combination of MG132 treatment and SV40 NLS fusion led to near-complete rescue of nuclear localization of V60L and V60A hSRY (right-hand bars in Fig. 2 E); representative cellular images see Fig. 2 C. The efficiency of NLS/MG132 “double rescue” motivated reinvestigation of the functional properties of V60L and V60A hSRY under conditions wherein WT and variant proteins were expressed at similar levels and with similar patterns of subcellular localization (Fig. 2 F). Whereas NLS fusion and/or addition of MG132 had no significant effects on transcriptional activation of Sox9 by WT or I90M hSRY at any dilution, defective activation of Sox9 by V60L or V60A hSRY was partially overcome by either maneuver, and double rescue led to native-like Sox9 expression (Fig. 2 F, Right).

Chromatin immunoprecipitation (CHIP) studies were undertaken of SRY/Sry binding sites in the TESCO element of Sox9 (Fig. 3 A, with primer sets defined in Fig. 3 B; ref. 7). Occupancy (as probed by CHIP band intensities relative to input controls following transfection without plasmid dilution) of I90M hSRY 
was indistinguishable from wild type (lanes 5 and 6 in Fig. 3C; see also Lower histogram). Control studies of inactive I68A hSRY (35) demonstrated an absence of enhancer binding: further control studies used de novo clinical mutants R62G and R75N in the N-terminal bipartite NLS of the HMG box (37). R62G and R75N (which impair both NLS1 specific DNA binding) demonstrated weak ChIP band intensities (Fig. 3C, lanes 1 and 2 in gel panel and the Lower histogram). Whereas TESCO-specific ChIP band intensities for V60L and V60A hSRY in the absence of NLS/MG132 were reduced to ca. half of the WT level (Fig. 3C), double rescue restored near-native enhancer occupancy (Fig. 3D). As in the Sox9 qPCR assay, double-rescue of V60A hSRY was more complete than that of V60L hSRY (set c in Fig. 3D). These findings imply that the minor-wing variants, once bound to the Sox9 enhancer, retain native-like gene-regulatory properties in a cellular milieu in accordance with their native-like biophysical properties (14).

### Analysis of hSRY Protein–Protein Interactions

The above findings motivated investigation of how the mutations affect binding of hSRY to the NCS machinery. V60L and V60A adjoin the N-terminal bipartite basic NLS of the HMG box (21); this motif (residues 61–77 of hSRY; KRPMAAFIVWSRDOHRKK; basic residues in bold) binds to Exportin-4 (Exp4) to mediate nuclear import (37) rather than importin-α or -β (38). Coimmunoprecipitation (co-IP) studies were thus undertaken based on cotransfection of HA-tagged hSRY and FLAG-tagged Exp4 (Fig. 4A). The studies revealed that, whereas I90M mutation does not affect binding to Exp4 (lanes 1 and 6 in Fig. 4B), a graded series of perturbations were observed among the other variants (lanes 2–5). Measurement of relative band intensities (in triplicate) (SI Appendix, Fig. S7) defined the order V60L [mildest impairment; 60(±13)% relative to WT hSRY] and V60A [41(±13)%]

relative to control mutations R75N [23(±6)%] and R62G [most severe; 17(±7)%] as previously characterized (37).

Nuclear export of SOX proteins is mediated by CRM1 via a conserved NES in the HMG box (20). Binding of hSRY (NES consensus IxxxLxxxxxR; residues 35–46 in the consensus HMG box) to endogenous CRM1 was demonstrated in CH34 cells by co-IP (lane 7 in Fig. 4C). Analogous CRM1-dependent NCS was observed in studies of goat Sry and deer Sry, whose HMG boxes bear active NES variant IxxxLxxxxxR (SI Appendix, Fig. S8). Binding of hSRY to CRM1 was unaffected by V60L or V60A (lanes 9 and 10 in Fig. 4C) but markedly impaired by I90M (lane 8) in accordance with its enhanced nuclear accumulation. Binding of hSRY to CRM1 and resulting NCS were also impaired by mSry-related substitution IxxxLxxxxxR (AxxxxAxxxxxAL) (SI Appendix, Fig. S9).

Mutation-specific perturbations of hSRY Exp4 or hSRY–CRM1 interactions stand in contrast to the absence of perturbations in assays of hSRY–calmodulin (CaM) binding. Although such binding (mediated by the N-terminal segment of the hSRY HMG box) has been proposed to direct nuclear entry (38), co-IP studies of endogenous CaM (outlined in Fig. 5A) revealed similar levels of binding to diverse mutations in the HMG box, including V60L and V60A (Fig. 5 B and C). Biochemical and biophysical studies of V60L and V60A hSRY–CaM complexes likewise demonstrated similar affinities and structural features (SI Appendix, Fig. S10).

Protein–protein interactions involving SRY have also been implicated in male-specific inhibition of Wnt/β-catenin signaling (Fig. 5D) (39). Such signaling is operative in ovarian development, and its inappropriate activation in the XY gonadal ridge (via stabilization of β-catenin) can be associated with human sex reversal (40). Although in pre-Sertoli cells this pathway is not well characterized, a functional SRY assay has been described in nongonadal
Fig. 5. expressing S37A MG132 to equalize protein levels. Comparison of the CH34 cells plasmids to minimize overexpression and in the presence of transfections were performed with 50-fold dilution of SRY fold increase in luciferase activity (lanes 1 and 2 in Fig. 5). 

Chen et al. PNAS outline of CRM1 and Exp4 co-IP assays. (A) Schematic

Fig. 4. Molecular mechanisms of nucleocytoplasmic shuttling. (A) Schematic outline of CRM1 and Exp4 co-IP assays. (B) Biochemical studies of the binding of epitope-tagged hSRY variants to nuclear import (Exp4) or nuclear export machinery (CRM1). Internal loading controls are provided by α-tubulin (Bottom). (C) Exp4 co-IP assay. Whereas the de novo mutations in the N-terminal NLS exhibit marked impairment, V60L exhibits only mild impairment, and V60A intermediate; no defect was observed for I90M. (C) CH34 CRM1 co-IP assay. Mutations at position 60 did not impair binding to CRM1; I90M causes almost complete lack of binding.

Demonstrated by maintenance of luciferase activity on transient transfection of SRY chimeras bearing inactive control mutations I68A or G95R (lanes 7 and 8 in Fig. 5F). Inherited mutations V60L and I90M as well as ovotestis-associated mutation V60A gave rise to intermediate levels of luciferase activity (lanes 4–6 in Fig. 5F). Whereas NLS fusion did not affect the inhibitory activity of the I90M construct (lanes 6 and 11 in Fig. 5F), partial rescue of nuclear localization enabled the V60L and V60A constructs to achieve near-native inhibition of luciferase activity (lanes 9 and 10).

Nuclear Trafficking Affects hSRY Phosphorylation. SRY in primates contains potential phosphorylation sites N-terminal to the HMG box (hSRY residues 29–36; RRSSSSFLC) recognized by protein kinase A (PKA); phosphorylation augments specific DNA binding (17). To investigate whether altered NCS affects phosphorylation of hSRY (HA-tagged) and thus enhancer binding, we evaluated by co-IP the extent of phosphorylation in cytosolic and nuclear fractions; an anti-phosphoserine antiserum was used to pull down phosphoproteins for Western blot by anti-HA antiserum (Fig. 5A). Molecular markers for fidelity of fractionation were provided by glyceraldehyde-3-phosphate dehydrogenase (GAPDH; cytosol) and β-catenin with the parent cell line revealed a 40-fold increase in luciferase activity (lanes 1 and 2 in Fig. 5F). This activity was inhibited by 3.5-fold on transient transfection of the hSRY plasmid and in the presence of MG132 to equalize protein levels. Comparison of the CH34 cells expressing S37A β-catenin with the parent cell line revealed a 40-fold increase in luciferase activity (lanes 1 and 2 in Fig. 5F). Inhibition required specific DNA binding as demonstrated by maintenance of luciferase activity on transient transfection of SRY chimeras bearing inactive control mutations I68A or G95R (lanes 7 and 8 in Fig. 5F). Inherited mutations V60L and I90M as well as ovotestis-associated mutation V60A gave rise to intermediate levels of luciferase activity (lanes 4–6 in Fig. 5F). Whereas NLS fusion did not affect the inhibitory activity of the I90M construct (lanes 6 and 11 in Fig. 5F), partial rescue of nuclear localization enabled the V60L and V60A constructs to achieve near-native inhibition of luciferase activity (lanes 9 and 10).
however, the total phosphorylation and relative phosphorylation of I90M hSRY were decreased in the nucleus despite its enhanced accumulation in that fraction. The twofold reduction in Sox9 transcriptional activation observed under these conditions (50-fold plasmid dilution) presumably reflects a defect in phosphorylation, which more than offsets any increase in transcriptional-regulatory activity that would otherwise be associated with its enhanced nuclear accumulation. V60L and V60A hSRY were predominantly detected in the cytosolic fraction in accordance with impairment of nuclear entry (Fig. 6B, comparison of lanes 3 and 4 with lanes 7 and 8, respectively). Whereas the relative extent of phosphorylation in the cytosolic fraction is similar to that of WT hSRY, a striking reduction was observed in each case in the absolute amount of phosphorylated protein in the nuclear fraction (lanes 7 and 8 in Fig. 6B, Top).

To investigate the relationship between N-terminal phosphorylation of hSRY and transcriptional activation of Sox9, phospho-dead and phospho-mimic variants (Fig. 6C) were used...
in qPCR assays (Fig. 6D). The assays were conducted as above with 50-fold plasmid dilution and in the presence of MG132. The nonbox Ala or Asp substitutions did not affect nuclear localization (SI Appendix, Fig. S11) in accordance with past studies (17). WT and variant PKA sites were introduced into three epitope-tagged hSRY constructs, bearing either a WT HMG box, the I90M HMG box, or a variant HMG box in which three putative NES residues (consensus positions 35, 39, and 45) were each substituted by Ala. The latter construct (designated NESØ in Fig. 6D) thus yielded an hSRY variant containing six Ala substitutions, three in the nonbox PKA site (S31A, S32A, and S33A) and three in the major wing (I90A, L94A, and M100A). In the presence of the native PKA motif, I90M caused the expected twofold reduction in Sox9 mRNA accumulation (relative to WT hSRY) as did the NESØ construct (left-hand set of bars in Fig. 6D). Strikingly, mimicry of PKA phosphorylation site by tandem Asp substitutions in each case led to enhanced and equal Sox9 expression (right-hand set of bars in Fig. 6D). This finding provides evidence that (i) serine phosphorylation enhances the activity of hSRY in an appropriate cellular milieu and (ii) defective phosphorylation of I90M hSRY underlies its partial loss of activity. Conversely, in the phospho-dead context, WT hSRY exhibited the same twofold loss of function as was conferred by I90M or the NES Ala substitutions (middle group of bars in Fig. 6D). These findings suggest that unphosphorylated hSRY retains partial activity whether the defect in phosphorylation is due to mutation of the PKA site or to mutations in the HMG box that impair nuclear exit.

Extension to Human Cell Lines. In an effort to confirm a subset of key findings in a human cellular milieu, transient-transfection studies were conducted in male prostatic cell line PC-3 (41). This line expresses SFI but not SRY. On transient transfection of the undiluted WT SRY plasmid, transcription of the endogenous SOX9 gene was activated by fourfold (SI Appendix, Fig. S12). Although this fold-activity is lower than that in CH34 cells, limiting the extent of feasible plasmid dilution, transient transfection of WT or variant hSRY plasmids without dilution yielded a pattern of relative activities [a twofold decreased for V606A and 35% (±4)% increased for I90M] that mirrored results of CH34 assays (SI Appendix, Fig. S12). The enhanced activity of I90M SRY on overexpression was mitigated on 20-fold dilution, consistent with our finding in CH34 cells that such enhancement is a consequence of overexpression. Analogous studies in NT2-D1 cells [derived from a human embryonal carcinoma cell line (42) and used in cotransfection studies of SRY by Harley and coworkers (13)] were not feasible due to insufficient transcriptional activation of its endogenous SOX9 gene by transfected SRY despite its comparable overexpression. Such differences in fold-SOX9/Sox9 transcriptional activation presumably reflects lineage-specific patterns of global gene expression (including other TFs and noncoding RNAs) and local chromatin structure (such as histone modifications within TES (43)).

Discussion

Metazoan development is ordinarily robust. Developmental stability in the presence of cryptic genetic variation or environmental fluctuations, known as Waddington canalization (30), has stimulated interest in biochemical mechanisms of genetic capacitors (as exemplified by heat shock proteins) (44) and topological properties of gene-regulatory networks (45). A paradigm for developmental stability is provided by the Hox cluster, invariant even among unrelated body plans (46). Sex is different. Metazoans exhibit an extraordinary diversity of sex-determining genes and signals, including temperature and social cues (47, 48). Interssexual phenotypes, readily obtained in model organisms (49), abound in the wild, especially in the presence of environmental endocrine disruptors (50). Further, the ubiquity of bisexual and homosexual behaviors among social mammals (including in diverse human cultures across historical epochs) poses a Darwinian paradox in relation to reproductive fitness (51).

In this study, we exploited clinical mutations in SRY associated with variable phenotypes (14, 52, 53) to measure the transcriptional threshold of testis determination. Our studies thus focused on subtle variants inherited by sterile XY daughters from fertile fathers. Potential artifacts of TF overexpression were mitigated through dilution of the hSRY expression plasmid (driven by the strong CMV promoter) by the parent empty vector to obtain a level of hSRY expression (104 to 105 molecules per cell) typical of factors that regulate cell-fate decisions (24). Molecular analysis of the clinical variants in a mammalian embryonic gonadal-ridge cell line uncovered perturbed NCS due to impaired binding of hSRY to Exp4 (V606A or CRM1 (190M). Impaired nuclear import or export is in each case associated with twofold reduction in male-pattern Sox9 transcription and twofold enhancement of female-pattern Wnt/β-catenin signaling. These SRY variants are thus poised at a critical boundary of activity (54), highlighting the narrow margin of “decision making” in the differentiating gonadal ridge. Such twofold perturbations are analogous to syndromes of autosomal TF haploinsufficiency (55). We anticipate that this margin will be a general characteristic of inherited Swyer mutations (SI Appendix, Table S1) irrespective of biochemical mechanisms of perturbation.

V606L and V606A SRY retain near-native biochemical properties (Table 1). Structural accommodation of the variant side chains reflects the flexibility of the minor wing of the HMG box. To probe gene-regulatory functions, we investigated the intrinsic gene-regulatory activity of these variants following “correction” of their altered trafficking (by NLS fusion) and accelerated degradation (by proteosome inhibition). Under these conditions, occupancy of the testis-specific Sox9 enhancer by WT or variant proteins was similar and led to near-complete double rescue of Sox9 mRNA accumulation; native-like inhibition of Wnt/β-catenin signaling was likewise imposed. I90M impairs binding of SRY to CRM1, a mediator of nuclear export (56), leading to enhanced nuclear accumulation. Although transcriptional activation of Sox9 is increased under conditions of overexpression (in accordance with ref. 13), successive plasmid dilution unmasked a twofold defect in Sox9 activation.

Nucleocytoplasmic Shuttling of SRY. On plasmid dilution, impaired nuclear export of I90M hSRY attenuates its gene-regulatory activity, suggesting that impaired nuclear export is a consequence of posttranslational modification. Insight may be obtained from consideration of evolutionary patterns of conservation and divergence (SI Appendix, Fig. S13). Among primates, SRY alleles are highly conserved and share potential N-terminal phosphorylation sites. Their HMG boxes in each case contain consensus NES sequences (IxxxLxxxxxML). The divergent boxes of rat and mouse SRY by contrast contain an unfavorable NES consensus NES sequences (IxxxLxxxxxML). The divergent boxes of rat and mouse SRY by contrast contain an unfavorable NES (driven by the strong CMV promoter) by the parent empty vector to obtain a level of hSRY expression (104 to 105 molecules per cell) typical of factors that regulate cell-fate decisions (24). Molecular analysis of the clinical variants in a mammalian embryonic gonadal-ridge cell line uncovered perturbed NCS due to impaired binding of hSRY to Exp4 (V606A or CRM1 (190M). Impaired nuclear import or export is in each case associated with twofold reduction in male-pattern Sox9 transcription and twofold enhancement of female-pattern Wnt/β-catenin signaling. These SRY variants are thus poised at a critical boundary of activity (54), highlighting the narrow margin of “decision making” in the differentiating gonadal ridge. Such twofold perturbations are analogous to syndromes of autosomal TF haploinsufficiency (55). We anticipate that this margin will be a general characteristic of inherited Swyer mutations (SI Appendix, Table S1) irrespective of biochemical mechanisms of perturbation.

V606L and V606A SRY retain near-native biochemical properties (Table 1). Structural accommodation of the variant side chains reflects the flexibility of the minor wing of the HMG box. To probe gene-regulatory functions, we investigated the intrinsic gene-regulatory activity of these variants following “correction” of their altered trafficking (by NLS fusion) and accelerated degradation (by proteosome inhibition). Under these conditions, occupancy of the testis-specific Sox9 enhancer by WT or variant proteins was similar and led to near-complete double rescue of Sox9 mRNA accumulation; native-like inhibition of Wnt/β-catenin signaling was likewise imposed. I90M impairs binding of SRY to CRM1, a mediator of nuclear export (56), leading to enhanced nuclear accumulation. Although transcriptional activation of Sox9 is increased under conditions of overexpression (in accordance with ref. 13), successive plasmid dilution unmasked a twofold defect in Sox9 activation.
dead” (RRAAFL) and “phospho-mimic” (RRDDDFL) constructs verified that negative charges enhance transcriptional potency. In either context, the extent of WT or 90M hSRY-dependent Sox9 expression is indistinguishable at reduced (phospho-dead) or elevated (phospho-mimic) levels. Because divergent rodent Sry proteins lack this N-terminal PKA site, we speculate that its C-terminal glutamine-rich expansion provides an alternative mechanism to enhance Sox9 transcription [see also ref. 22].

Coupling between NCS and posttranslational modification occurs in diverse systems (19, 20). Such coupling may be a hallmark of mammalian Sox proteins (19, 57) as exemplified by Sox9, which contains two PKA sites, one N-terminal to the HMG box and the other adjoining NLS2 in its C-terminal tail. The latter phosphorylation enhances NLS2 binding to importin-β and thereby to a threshold of nuclear import (20). Autocrine regulation of PKA activity by prostaglandin D2 in the differentiating Sertoli cell may provide a mechanism to amplify and maintain Sox9 expression as shown in engineered mice (34). Although phosphorylation-regulated nuclear localization is not a feature of hSRY, its acetylation (K136 in NLS2) provides an alternative mechanism to enhance binding to importin-β (58).

Mouse Sry Exhibits an Analogous Threshold. The inherited Swyer syndrome resembles Y-chromosome incompatibility among mouse strains wherein Y chromosomes derived from diverse mouse strains can cause abnormal gonadal development in B6 strain C57BL/6J (27, 28). Aberrant interaction between such alleles and autosomal genes leads to strain-dependent intersexual phenotypes (59). Such phenotypes could not be correlated with changes in Sry sequence (28) but instead depend on the extent and timing of Sry expression (27, 28). As found here, changes of twofold or less in Sry expression in the differentiating gonadal ridge were associated with developmental abnormalities. Such genetic-background dependence strongly suggests that Sry-Sox9-directed transcriptional program in mice lies just a threshold of function.

The tenuous biochemical thresholds of SRY function in mice and humans—mammals with otherwise divergent SRY/Sry genes (28)—demonstrate that lack of robustness in nascent Sertoli-cell specification has been independently maintained in lineages separated by 80 million y. Such conservation seems to violate Waddington’s Principle: that fundamental developmental pathways are canalized, at least in their upstream steps, and so robust (30, 31). It is possible that the apparent fragility of hSRY is illusory. The transcriptional set point of hSRY, for example, may be more tightly controlled by upstream factors (such as GATA4 and WTI) (60) and transcriptional coregulators (such as SF1 in sex-specific transcriptional preinitiation complexes) (7) that twofold reductions in hSRY-directed Sox9 activation would be unlikely. Similarly, it is possible that variation in hSRY activity is buffered by feedback and feed-forward regulatory circuits in the Sox9-directed gene-regulatory network (34).

Multilevel Selection in Mammalian Evolution? The similar transcriptional thresholds of murine and human SRY (despite their biochemical divergence; see a recent study (22) that suggests that its thin thread of function (29) is the product of selection. The shared tenuousness of the switch poses an intriguing problem given the general robustness of developmental processes (61). It is possible that an hSRY of higher transcriptional potency could impair individual fitness, such as through induction of gonadal neoplasias (13). Alternatively, higher potency could lead to intragenomic conflict with female-specific genes and so impair daughter fitness (62). Genes that contribute to variation in male-specific traits, including hormone-dependent behaviors and social competencies (63), may also be subject to intragenus selection (such as in male dominance hierarchies) or intersexual selection (female mate choice) (64).

We speculate that genetic variation in fetal testosterone production influenced the evolution of eutherian mammals, especially species (like humans and mice) that evolved within social groups. Given epidemiological linkages between human fetal testosterone exposure (as measured in midtrimester amniotic fluid) and behavioral styles in childhood (65), it is possible that genetic or stochastic variation in fetal Leydig-cell function could ultimately affect male choice, reproductive success, or social integration within the framework of multilevel selection (66). Indeed, nonrobustness is a hallmark of human genetics at successive stages of male differentiation (67). Heterozygous nonsense and missense mutations in SF1 associated with gonadal dysgenesis likewise suggest (in the absence of adrenal abnormalities) a syndrome of haploinsufficiency (68, 69). Mutations in SOX9, moreover, result in a syndrome of TF haploinsufficiency (campomelic dysplasia), wherein abnormalities of bone coincide (in XY patients) with male, intersex, or female somatic phenotypes (70). Such phenotypic variability suggests that the twofold transcriptional threshold characteristic of hSRY extends to its immediate downstream target. Similarly, hemizygosity of chromosome 9p24.3 (which contains three DM genes related to the Doublesex gene of Dipterans) (71) is associated with 46,XY gonadal dysgenesis in the presence of WT SRY and SOX9 alleles (72). This trend extends to the androgen receptor itself. Studies of the X-linked androgen insensitivity syndrome have demonstrated that the same receptor mutation can be associated with complete feminization (“testicular feminization”), partial insensitivity, or minimal perturbations in virilization or fertility (73). Together, these clinical entities highlight the anomalous nonrobustness of the male program.

Concluding Remarks. Our studies address an overarching issue in human development: biochemical properties of TFs that distinguish critical boundaries between organized and disorganized states of cellular differentiation or downstream pathways of pattern formation (54). Inherited alleles of SRY provide probes of this boundary in gonadogenesis. Our results, demonstrating that NCS and NCS-coupled phosphorylation of hSRY contribute at the margin to its genetic function, highlight the tenuous transcriptional threshold of human Sertoli-cell specification. A similar threshold pertains to testicular differentiation in mice (11) despite the marked biochemical divergence of murine Sry (22).

Given general trends toward the evolution of developmental stability (30), why have human and murine SRY evolved to the edge of ambiguity? We speculate that sex determination differs from canonical embryonic patterning through its coupling to variation in extent of testosterone secretion by fetal Leydig cells, in turn enabling male neurodevelopmental diversity. This perspective highlights the complementary and potentially conflicting roles of within-group and between-group selection as a feature of multilevel selection in social mammals (66). Implicit in this view are connections between genotype, development, differentiation of the central nervous system, and complex behaviors, including empathy and other social competencies as defined in longitudinal studies of human fetal testosterone exposure (65). A connection between multilevel selection and social competencies was anticipated by Darwin’s surmise that “although a high standard of morality gives but a slight or no advantage to each individual man and his children over the other men of the same tribe,… an increase in the number of well-endowed men and advancement in the standard of morality will certainly give an

---

Positioning of XX and XY fetuses in litter-bearing mammals is a feature of a principle: that fundamental developmental path-...
immense advantage to one tribe over another” (66, 74). The anomalous nonrobustness of male sex determination and sexual differentiation in social mammals, as evidenced by inherited alleles of human SRY at the edge of ambiguity, may relate Darwin’s insight to the tenuous biochemistry of a genetic switch.

Materials and Methods

Plasmids. Plasmids expressing hSRY or variants were constructed by PCR (14). Following the initiator methionine, the cloning site encoded an HA tag in triplicate. In selected constructs, an element encoding a nuclear localization signal (NLS) sequence PRKKRV as derived from SV40 large T antigen was inserted after HA-related codons.

Cell Culture. CH34 cells were cultured in Dulbecco’s modified Eagle medium as described (14). For protease-inhibitor studies, transfected cells were maintained for 24 h in serum-free conditions and then treated with MG132 for 6 h followed by 18 h incubation in 5% (vol/vol) serum-containing medium. PC-3 cells were cultured in the F-12K medium (ATCC) with 10% (vol/vol) FBS in 5% CO2 atmosphere. NT2-D1 cells were grown in DMEM in an atmosphere of 5% CO2; the complete growth medium contained FBS to a final concentration of 10%, SOX9- and TBP-specific PCR primers were in accordance with human genomic sequences.

Transient Transfection. Transfections were performed as described (14). Transfection efficiencies (30–35% in the case of CH34 cells) were determined by ratio of GFP-positive cells to untransfected cells following cotransfection with 1 μg of GFP in equal amounts. Subcellular localization was visualized by immunostaining 24 h posttransfection following treatment with 0.01% trypsin (Invitrogen) and plating on 12-mm coverslips. SRF expression was monitored in triplicate by Western blot in relation to α-tubulin (SI Appendix, Fig. S14).

Cycloheximide-Chase Assay and Western Blot. Twenty-four hours posttransfection, cells were evenly into control plates and treated with cycloheximide to a final concentration of 20 μg/mL in regular medium for indicated times; cells were then lysed by radio immunoprecipitation assay (RIPA) buffer (Hoffmann LaRoche). After protein normalization, cell lysates were subjected to 12% SDS/PAGE and Western blot using anti-HA antibody (Sigma-Aldrich) at a dilution ratio of 1:5,000 with α-tubulin as a loading control. Experiments were performed in triplicate.

Sox9 Activation Assay. SRY-mediated transcriptional activation of Sox9 and other endogenous CH34 genes was measured in triplicate by qPCR as described (14). Cellular RNA was extracted using RNeasy (Qiagen).

Immunocytochemistry. Transfected cells were plated evenly on 12-mm coverslips, fixed with 3% para-formaldehyde in PBS on ice for 30 min, treated with cold-permeability buffer solution (PBS containing 10% goat serum and 1% Tween 20) for 10 min, blocked with 10% goat serum for indicated times; cells were then lysed by radio immunoprecipitation assay (RIPA) buffer (Hoffmann LaRoche). After protein normalization, cell lysates were subjected to 12% SDS/PAGE and Western blot using anti-HA antibody (Sigma-Aldrich) at a dilution ratio of 1:5,000 with α-tubulin as a loading control. Experiments were performed in triplicate.

Chromatin Immunoprecipitation. Cells were transfected with SRY variants, exposed to MG132, and subjected to ChIP. In brief, recovered cells were cross-linked in wells by formaldehyde, collected, and lysed after quenching the cross-linking reaction. Chromatin lysates were sonicated to generate 300- to 400-bp fragments and immunoprecipitated with anti-HA antibody (Sigma-Aldrich) coupled with Protein A slurry (Santa Cruz) after preclariﬁng; a non speciﬁc antibody (Santa Cruz) served as control. After reversal of cross-linking at 65 °C overnight, fragments were treated with proteinase K and RNase (Hoffmann LaRoche), followed by extraction with 1:1 phenol–CIAA solution. A high-fidelity PCR protocol was provided by Hoffmann LaRoche. Experiments were performed in triplicate.

Phosphorylation Assay. HA-tagged SRY variants in cytosolic or nuclear fractions (below) were immunoprecipitated with rabbit polyclonal anti-phosphoserine antisem (Abcam). Western blot following 12% SDS/PAGE used HRP-conjugated anti-HA antibody (Hoffmann LaRoche). Loading controls were provided by cytosolic enzyme GAPDH (Sigma-Aldrich) and nuclear proteins histone H1 and YY1 (Santa Cruz).

Wnt/β-Catenin Luciferase Assay. CH34 cells were engineered to stably express S37A β-catenin as described (39). Assays, adapted from previous studies (39), used a chimeric SRF containing residues 1–155 of hSRY followed by residues 128–396 of mouse SRF. Experiments were performed in triplicate using the Dual-Luciferase Reporter Assay System (Promega); lysates were simultaneously analyzed for ﬁreﬂy luciferase activity encoded by TOPFlash (Mili pore) and farnella luciferase activity (Promega). A negative control for β-catenin-directed luciferase expression was provided by a TOPFlash variant containing an inactive β-catenin-responsive promoter (FOPFlash; Millipore). Experiments were performed in triplicate.

Communoprecipitation Assays. CH34 cells expressing HA-tagged SRY variants were treated with MG132 and lysed using complete Lysis-M buffer containing a protease inhibitor mixture (Hoffmann LaRoche). In SRY–CaM studies, lysates were precipitated with monomodal anti-HA agarose beads (Sigma Aldrich). Following 12% SDS/PAGE, Western blots used an anti-CaM antisem (Abcam). In SRY–CRM1 studies, lysates were analyzed by immunoprecipitation using anti-CM1 antibody with agonase-conjugated protein G (Santa Cruz). Pellets were subjected to 10% SDS/PAGE, and HRP-conjugated anti-HA antisem (Hoffmann LaRoche) was used to investigate the CRM1-bound HA-SRY variants. In a reverse protocol, lysates were treated with monocodal agarose-conjugated anti-HA antisem (Sigma-Aldrich), subjected to 10% SDS/PAGE electrophoresis, and analyzed by anti-CRM1 antibod (Santa Cruz) to detect SRF-bound CRM1. In SRY–Exp4 studies, transfected cells were cotransfected with pcMX–FLAG-human Exp4, MG132 treated; cell lysates were immunoprecipitated by monocodal anti-FLAG agarose (Sigma-Aldrich). After analysis by 10% SDS/PAGE and electro blotting, hybridization solutions containing HRP-conjugated anti-HA anti sem (Hoffmann LaRoche) were used to investigate Exp4-bound SRF. Anti FLAG antisem was used to monitor Exp4 expression; respective antisera against HA tag and α-tubulin (Sigma Aldrich) provided SRF input and general loading controls.

Cellular Fractionation. Cells were pelleted and suspended in 10 mM Hepes (pH 7.9), 20 mM KCl, 3 mM MgCl₂, 0.5% Nonidet P-40, 5% glycerol, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). Lysates were kept on ice, sheared by five passages through a 25-gauge needle, and centrifuged at 2,500 × g for 15 min at 4 °C; supernatants provided cytosolic extract. Pellets were suspended in nuclear lysis buffer (20 mM Hepes (pH 7.9), 0.225 M NaCl, 1 mM EDTA, 3 mM MgCl₂, 0.5% Nonidet P-40, 10% glycerol, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride), sheared by needle passage, kept on ice for 15 min, and subjected to 13,000 × g centrifugation for 15 min at 4 °C.

ACKNOWLEDGMENTS. We thank Prof. P. K. Donahoe for cell line CH34 and encouragement; P. Janki and S. Jeong for assistance. M.A.W. thanks B. Baker, R. Singh for participation in early stages of this work; and P. DeHaseth, H.-Y. Kao, D. Samols, and P. Sequeira for advice. M.A.W. thanks B. Baker, F. A. Jenkins, Jr., and P. Koopman for discussion. This work was supported in part by National Institutes of Health Grant GM080505 (to M.A.W.). This article is dedicated to the memory of the late Prof. Farish A. Jenkins, Jr. (Harvard University and the Harvard-MIT Program in Health Sciences and Technology) for his encouragement, humanity, and scientiﬁc example.


Supporting Information (Chen, Y. S. et al.)

Fig. S1. I90M (residue 35 in HMG box) does not perturb folding or stability. (A) Far-UV CD spectra of the native- (●) and mutant domain (I90M, □) are similar at 4 °C. (B) Guanidine-induced unfolding of native (●) and I90M domains (□) as monitored by CD at 222 nm. Estimates of (ΔGf) were extracted by a two-state model. (C) Thermal unfolding of wild-type (●) and I90M (□) SRY domains (CD 222 nm). Midpoint temperatures were obtained from the derivative of the curve.

Fig. S2. FRET-based equilibrium binding and kinetics. (A) Stopped-flow experiment coupled to a fluorimeter, enabling measurement of FRET-based dissociation rate. One syringe contained a preformed protein-DNA complex with the 15-bp DNA probe containing 5'-donor (fluorescein 6-FAM; green circle) on one strand and 5'-acceptor (TAMRA; red circle) on the other; the other syringe contained a 20-fold excess of unmodified DNA site. (B) FRET-based stopped-flow measurement of dissociation rates (k_{off}). Time-dependent increase in donor fluorescence was due to dissociation of FRET-labeled complex. Data and fitted solid lines at 15 °C are shown for wild type- (blue) and I90M-complexes (red). Dissociation reactions were monitored for 90 sec. Dissociation rate constants, k_{off}, were determined by fitting traces to a single exponential equation and averaging the results of three replicates. Values of k_{off} are 0.14(±0.001) sec^{-1} for I90M and 0.041(±0.001) sec^{-1} for wild-type. (C) FRET-based equilibrium-binding studies of wild-type and I90M domains enabled determination of K_d. The K_d of the variant domain (15.0(±1.4) nM) is indistinguishable from wild-type (14.2(±2.6) nM).
**Fig. S3.** Emission spectra of free and SRY-bound DNA after excitation at 490 nm. (A) DNA singly labeled with donor 6-FAM in complex with wild type SRY (green) and in complex with I90M (light blue); DNA doubly labeled with 6-FAM and TAMRA (acceptor), free DNA (black), in complex with wild type SRY (dark blue) and in complex with I90M (orange). Emission spectra of DNA singly labeled with TAMRA (free, complexed with wild-type domain and complexed with the I90M domain) are shown in yellow (beneath overlapping spectra). (B) Emission of normalized rhodamine (TAMRA) spectra in double labeled DNA due to FRET indicating similar degree of bending by wild type (dark blue), and I90M (orange). The free DNA spectrum is shown in black.

**Fig. S4.** SRY-regulated testicular gene-regulatory network and transcriptional activation of Sox9. Transcriptional assays of selected genes activated by SRY variants in rat embryonic gonadal cell line. RT-Q-rt-PCR was employed to probe mRNA abundances of (A) Sox family members, (B) candidate male gene-regulatory network-related genes, and (C) housekeeping genes. qPCR was analyzed following transfection of SRY variant expression plasmids, empty vector, or control plasmid expression a stable but inactive SRY variant (I68A). (A) Fold mRNA accumulation of Sox family, including endogenous Sry and transfected SRY-activated Sox9. (B) Fold mRNA accumulation of sex-related factors in the program of SRY-mediated gene-regulatory network. Results show that CH34 cells express low endogenous levels of Fgf9, PtgdS, Sox8, and Wnt5 gene expression. (C) Fold mRNA accumulation of sex-unrelated housekeeping genes; these gene were not affected by expression of transfected SRY; statistical analyses: *Wilcoxon test, p<0.05.
**Fig. S5.** MG132 rescues expression of V60L and V60A to achieve levels similar to wild-type SRY. *Top,* Western blots probing extent of total-cellular expression of wild-type or variant SRY constructs following 24 hours in serum-rich medium in the absence of proteosome inhibitor MG132. In the absence of the SV40 NLS, V60L and V60A bands are ca. twofold less intense than that of wild-type; addition of the SV40 NLS enhances total-cellular expression; loading controls are as described in Figure 3. *Bottom,* addition of MG132 leads to similar band intensities in each lane. Note that these data do not probe nucleus-specific accumulation of SRY. NLS-tagged constructs are 0.7 kDa larger than non-NLS-tagged constructs. It is not known why V60A constructs exhibit slightly faster mobilities. This figure was taken from the left-hand (lanes 1-3) and right-hand (lanes 4 and 5) portions of a single gel and blot; the middle lanes (not pertinent to this study) were omitted.

**Fig. S6.** Subcellular localization of wild-type SRY as analyzed by immunostaining. (A) Nuclear staining by DAPI (blue) and anti-HA immunofluorescence, which specifically detects the epitope-tagged transfected SRY (green). Wild-type SRY containing in-frame N-terminal SV40 NLS exhibits native-like extent of nuclear localization without further increase in the nuclear fraction. Images were obtained following MG132 treatment to equalize levels of total SRY protein expression. (B) Histogram showing quantitative summary of extent of nuclear localization (gray) or pancellular distribution (white) in the without/with MG132. The dose of the SRY-expression plasmid was undiluted (1 μg/well). Horizontal brackets designate statistical comparisons as in Figure 3 (main text).

**Fig. S7.** SRY-Exportin-4 co-IP assays. Histogram provides a quantitative summary of Western blots repeated in triplicate. Mutations in the SRY HMG box lead to a range of perturbations to the interaction between SRY and Exportin-4, which is mediated by the N-terminal bipartite NLS of the HMG box (see Fig. 3F in the main text). Error bars represent 1 standard deviation: *, p-values less than 0.05, and “ns,” not significant.
Fig. S8. Subcellular localization and CRM1 binding of mammalian SRYs. (A) Histogram indicating fractions of transfected CH34 cells exhibiting exclusive nuclear localization of SRY (filled bars) versus pancellular distribution (open bars); the transfected plasmid dose was in each case 1µg. Whereas the localization patterns of human, goat, and deer SRY share indistinguishable (gray bars), mouse and rat Sry, exhibit near-exclusive nuclear localization (red bars; see companion paper in this issue; Chen Y.-S., et al. *Proc Natl Acad Sci USA* (2013)). At bottom is summarized CRM1-binding activity as observed in panel B (for experimental design, see Fig. 4E). Horizontal brackets indicate statistical comparisons (asterisk, p<0.05; ns, p>0.05 and in this case, the ns p-values were all > 0.15). (B) CRM1 co-IP assay in transfected CH34 cells. Whereas human SRY (hSRY), goat Sry (gSry; *Capra hircus*) and deer Sry (dSry; *Hydropotes inermis*) exhibit similar CRM1-co-IP signals (lanes 1, 2, and 3, respectively), mouse Sry (mSry; *Mus musculus*) and rat Sry (rSry; *Rattus norvegicus*) lack such binding in association with a variant NES motif (IxxxLxxxxxSL). Top boxes, background bands (X) and SRY-CRM1 co-IP signal (arrow); middle boxes, species-specific Sry/SRY input bands; and bottom boxes, α-tubulin control for protein loading. Images were taken from a single gel.

Fig. S9. Subcellular localization and CRM1 binding of hSRY variants with NES modifications. (A) Histogram showing fractions of transfected CH34 cells exhibiting exclusive nuclear localization of SRY (filled bars) versus pancellular distribution (open bars). Wild-type and variant NES motifs are as labeled; the transfected plasmid dose was in each case 1µg. Variant NES motifs (IxxxLxxxxxSL and AxxxAxxxxxAL) in hSRY confer near-exclusive nuclear localization as is characteristic of mouse Sry (mSry; see companion paper in this issue). At bottom is summarized observed (+) or impaired (-) binding to CRM1. Horizontal brackets indicate statistical comparisons (asterisk, p<0.05; ns, p>0.05 and in this case, the ns p-values were all > 0.15). (B) Binding of SRY variants to CRM1 as probed by co-IP assay (for experimental design, see Fig. 4E). Top boxes, background band (X) and SRY-CRM1 co-IP signal (arrow); middle boxes, mSry input band (upper band in lane 4) and hSRY input band (lower bands in lanes 1-3); and bottom boxes, α-tubulin control for protein loading. Lanes 1-4 provide: (1) wild-type human SRY (wild-type NES motif designated at top –ML); (2) variant hSRY bearing mouse-specific NES substitution IxxxLxxxxxSL (designated at top –SL); (3) variant hSRY bearing Ala-substituted NES substitution AxxxAxxxxxAL (designated –AL); and (4) control studies of mouse Sry (mSry) bearing impaired NES sequence IxxxLxxxxxSL (designated at top –SL). Images were taken from a single gel.
Fig. S10. V60L and V60A do not perturb binding of calmodulin (CaM). (A) Model of the N-terminal segment of SRY (red) bound to CaM (gray) (Harley VR, et al. FEBS Lett 391:24-8 (1996)). Calcium ions in CaM are shown as silver balls. Arrow indicates V60 (position 5 in HMG-box consensus). (B) CD spectra of wild-type, V60L, V60A SRY domains and their CaM complexes. Top group, spectra of free domains at 20 °C: wild-type (●), V60L (□), and V60A (◇). Middle group, spectrum of CaM alone (●). Bottom group, spectra of SRY-CaM complexes: wild-type (+), V60L (△), or V60A SRY (●); the latter were obtained at 20 °C under the same conditions as above after incubating at 4 °C for 30 min. SRY-CaM complexes exhibit indistinguishable patterns. (C) Trp fluorescence spectra of SRY HMG box and variants either free or as CaM-bound complexes. Bottom group, free SRY domains: wild-type (▲), V60L (●), and V60A (◇). Top group, SRY-CaM complexes: wild-type (▲), V60L (◇), and V60A (○). In presence of CaM, similar enhancement and shift in emission wavelength maxima (representing reversal of the quenching and blue shift characteristic of the folded free domain) are observed. (D) Titration of SRY domains with increasing [CaM] as monitored by Trp fluorescence. $F_0$ is the tryptophan fluorescence at 350 nm of SRY HMG box alone (2 μM); $F$ is the observed fluorescence upon CaM addition. CaM titration curves with the variants are similar, indicating similar CaM-binding affinities: wild-type (■), V60L (◇), and V60A (○). (E and F) DNA gel-mobility shift assays (GMSA) depicting competition between CaM and 32P-labeled DNA (36 bp) for wild-type (E) and V60A SRY (F) domain. Similar extent of competition was observed. Domains were either pre-complexed with the specific DNA site prior to the addition of [CaM] (lanes 1-9 in panels E and F; 0, 0.08, 0.3, 0.6, 1.3, 2.5, 5.0, and 20 μM) or pre-complexed (lanes 2'-9' in panels E and F) prior to the addition of DNA. Bands representing free DNA and shifted 1:1 complex C1 are indicated by arrows at right; higher-order complexes C2 and C3 are also indicated.
**Fig. S11.** Evidence that nucleocytoplasmic trafficking of hSRY does not depend on phosphorylation. (A) Schematic depiction of hSRY analogs containing triplicate substitutions within its N-terminal PKA phosphorylation site (PALRRSSFLCTE; putative phosphorylation sites underlined): Top, wild-type motif; Middle, Ser • Ala substitutions (AAA) to render motif non-phosphorylatable ( "phospho-dead" ); Bottom, Ser • Asp substitutions (DDD) to mimic the negative charges of phospho-serine ("phospho-mimic"). Domains are defined as in Fig. 7C in main text. (B) Non-box AAA or DDD substitutions do not affect nuclear localization. Histogram provides fractions of transfected CH34 cells exhibiting exclusive nuclear localization of SRY (gray filled bars) versus pancellular distribution (open bars). The transfected plasmid dose was in each case without dilution ("1X"). The hSRY AAA or DDD variants thus exhibit indistinguishable subcellular localization. Horizontal brackets indicate statistical comparisons as in Figure 3 (ns, p>0.05 and in this case, the ns p-values were all > 0.2.
Fig. S12. Transient transfection of hSRY in PC-3 cells activates endogenous human SOX9. (A) Histogram showing results of wild-type and variant rt-Q-PCR assays (plasmid dose 1 μg) with an empty vector and inactive hSRY variant I68A as negative controls. V60L and V60A attenuate SOX9 activation by ca. twofold in accordance with their relative activities in CH34 cells (see main text).

At this high plasmid dose I90M has 30% higher activity than wild-type hSRY; the p-value is 0.035. Statistical comparisons: p-value (*) < 0.05; “ns” indicates p-value > 0.05. (B) Western blots probing extent of total cellular expression of hSRY and variants. Top: similar SRY band intensities are shown in each lane. Bottom: α-tubulin loading controls. We note that PC-3 cells, derived from a human prostate-cancer cell line, has a Wnt/β-catenin-responsive SOX9 gene (Wang H, et al. (2007) Cancer Res 67:528-36) but lacks endogenous SRY (Dasari V, et al. (2002) J Urology 167:335-8). (C) Effect of intermediate plasmid dilution on SRY-dependent transcriptional activation of SOX9: undiluted expression plasmid as in panel A (“1X”; azure) versus 20-fold dilution by empty plasmid (“20X”; sky blue). Whereas such dilution does not affect the ca. twofold attenuation of the activities of V60L and V60A SRY relative to wild-type SRY, the apparent increase in the activity of I90M SRY relative to wild-type SRY (as observed in panel A on transfection of undiluted plasmids) is mitigated in accordance with intermediate-dilution levels in CH34 cells. This shared trend suggests that the apparent increase in the activity of I90M SRY (in undiluted transfections) represents in both cell lines an artifact of TF over-expression as indicated in the main text.

Technical note: Because the magnitude of wild-type SRY-dependent activation of the endogenous human SOX9 in PC-3 cells is smaller than the magnitude of corresponding SRY-dependent activation of the endogenous rat Sox9 gene in CH34 cells (presumably due to baseline differences between the two cell lines in stage- and tissue-specific patterns of gene expression and SOX9/Sox9 chromatin structure), it was not technically feasible to test the effects of further dilutions in PC-3 cells (i.e., > 20-fold). The increased efficiency of SRY-dependent Sox9 activation in CH34 cells by contrast enabled studies of 50-fold dilution (see main text). This technical difference highlights the utility of using a mammalian cell line that derives from the appropriate embryonic stage and site of hSRY/mSry expression as emphasized by Haqq, C.M. and Donahoe, P.K. (1998) Physiol Rev. 78:1-33).
Fig. S13. Potential N-terminal phosphorylation sites and NES of primate SRY alleles. (A) Phylogenetic tree of representative primates; Mya: million years ago (panel is modified from Ferguson-Smith MA, et al. (2007) Nat Rev Genet 8:950-62. (B) Known or predicted phosphorylation sites (PKA); serines in magenta (residues 31-33 in hSRY) depict observed phosphorylation sites (Desclozeaux M, et al. (1998) J Biol Chem 273:7988-95). Serines in bold (non-human primates) represent potential phosphorylation sites as predicted by NetPhosK 1.0 (website http://www.cbs.dtu.dk/services/NetPhosK). R30, a site of clinical mutation in hSRY adjoining the PKA site, is underlined. (C) Alignment of representative primate SRY HMG box sequences in relation to rodent Sry proteins (rat and mouse). Boxed are NLS-1, NES, and NLS-2. Critical NES residues are in bold. Sites of mutations impairing NCS of hSRY (as investigated in previous studies) are underlined (from Left with full-length residue numbers: R62(G), R75(N), and R133(W/G)). V60 and I90 (focus of the present study) are highlighted in yellow boxes. Highlighted in red at bottom is a non-conservative substitution in the HMG box of rat and mouse Sry (M→S) proposed to disable its NES.
**Figure S14.** Effect of Plasmid Dilution on HA-tagged SRY Expression in CH34 Cells. CH34 cells transfected without dilution (“1X”). Protein expression is reduced by 320-fold following 50-fold plasmid dilution (maximum feasible dilution). Top, representative gel images of SRY and α-tubulin (housekeeping loading control); the panel was taken from a single gel and blot. Because of the greater abundance of tubulin, total cellular protein extracts were diluted by 100-fold (“100X”) to obtain bands of similar intensity. Bottom, summary table providing SRY signal strength relative to tubulin as a function of plasmid dilution.

**Technical note.** Plasmid dilution in CH34 cells enables an appropriate intracellular concentration of SRY to be achieved as follows. A typical mammalian cell contains 300 pg of total protein (Lodish, H., et al. (2000) *Molecular Cell Biology*. 4th Edition. New York: W. H. Freeman & Co.), and has a tubulin content of 2.5-3.3% of total protein (Hiller, G. and Weber, K., (1978) *Cell* 14, 795-804). Relative Western blot intensities enabled estimation of the SRY concentration following transient transfection (either without dilution (1X) or following 50-fold dilution by the empty parent vector (50X; maximum dilution)) by a four-step calculation. This procedure assumes that the affinities of the anti-HA and anti-tubulin antisera are similar as indicated by the vendor; our qualitative conclusions are robust to fivefold errors.

**Step 1.** We assumed that a typical mammalian cell contains by weight the following amount of tubulin: 300 x 10^{-12} grams x 3% = 9 x 10^{-12} grams;

**Step 2.** We next estimated the number of tubulin molecules (molecular weight 110 kDa) as [9 x 10^{-12} grams/110 x 10^3 grams per mole] x 6.02 x 10^{23} molecules per mole = 5 x 10^7 molecules;

**Step 3.** We in turn estimated the number of transfected SRY molecules per cell in the 1X transfection using its Western-blot signal strength relative to α-tubulin: 5 x 10^7/100 (the tubulin-blotting dilution ratio)/3 (relative band intensity)/32.6% (transfection efficiency), which yields an estimate of 5 x 10^5 molecules (which are predominantly in the nucleus);

**Step 4.** Finally, we estimated the number of transfected SRY molecules per cell following 50-fold plasmid dilution using relative signal strength between 1X and 50X HA-SRY Western blots: 5 x 10^5 molecules/320, which yields an estimate of between 1000 and 2000 molecules per cell. This degree of expression is within the middle of the range of cellular abundances expected of a lineage- and stage-specific transcription factor (10^2-10^4 molecules per nucleus).
and green boxes with a 68-bp fragment. (B) Transfection of NT2-D1 cells with hSRY or variants (100 ng plasmid) stimulates expression of firefly luciferase. NT2-D1 cells, derived from a human testis carcinoma, express FGF9, SF1, and SOX9 and so may in part resemble gonadal cell types (Knower KC, et al. (2007) Sex Dev 1:114-26). As in CH34 cells, mutations V60L and V60A impaired luciferase activity by ca. twofold. NES mutation I90M enhanced activity by 60% relative to wild type hSRY. Statistical comparisons: p-value (*) < 0.05, (**) < 0.01; “ns” indicates p-value > 0.05.

Technical note: The apparent enhancement of the transcriptional activity of I90M SRY in this reporter assay is in accordance with its enhanced activity on over-expression in CH34 cells (i.e., in the absence of plasmid dilution; see main text) and in PC-3 cells (also in the absence of plasmid dilution; see Supplemental Fig. S12). These reporter results, which in part replicate the findings of Harley and colleagues (Knower KC, et al. (2011) PLoS One 6(3):e17751), are likely to reflect the non-physiological nature of the assay with respect to TF expression level, non-native organization of the SRY/SF1 transcriptional pre-initiation complex, and an overall cellular milieu unrepresentative of the embryonic bipotential XY gonadal ridge. The co-transcription system of Knower et al. (2011) employed a 3.2-kb human TES fragment. See Figure S16 for estimate of extent of epitope-tagged SRY overexpression (ca. 1.5 million molecules/cell).
Figure S16. Expression of HA-tagged SRY following transient transfection in human embryonal carcinoma cell line NT2-D1. Cells were transfected without dilution (100 ng plasmid per well; defined as “1X” condition) or following dilution with parent empty plasmid as indicated. Protein expression was reduced by 20-fold following 50-fold plasmid dilution (maximum feasible dilution). Top, representative gel images of SRY and α-tubulin (housekeeping loading control); the panel was taken from a single gel and blot. Because of the greater abundance of tubulin, total cellular protein extracts were diluted by 50-fold (“50X”) to obtain bands intensities differing by ca. twofold. Bottom, summary table providing SRY signal strength relative to tubulin as a function of plasmid dilution.

Technical note. A calculation formally analogous to that outline in the caption to Figure S14 indicates that under 1X transfection conditions in NT2-D1 cells (as employed in Knower KC, et al. (2011) PLoS One 6:e17751); reference 13 in the main text) the abundance of HA-tagged SRY is ca. 1.5 million molecules per cell. Stage- and tissue-specific TF expression is typically in the range $10^2$-$10^4$ molecules per nucleus (Goentoro L, Shoval O, Kirschner MW, & Alon U (2009) Mol Cell 36:894-9).
Table S1. Summary of inherited mutations in human SRY and state of characterizations

<table>
<thead>
<tr>
<th>Mut.</th>
<th>Clinical Assess.</th>
<th>Protein Pur.</th>
<th>Protein Charact.</th>
<th>Protein Partner</th>
<th>Cellular Studies</th>
<th>Transgenic</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA-binding</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Offset</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CSIP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*For each inherited clinical mutation, results of prior studies are indicated by ■, or, in the case of possible technical error, by □. The results of the present study (and its antecedent in the J. Biol. Chem.) are indicated by ■. Artifactual conclusions in prior studies corrected in the present study are indicated by □. Open spaces signify the absence of published data. Thus, aside from clinical genetics (gray zone at left), scant data are available.

PGD: pure gonadal dysgenesis

*Model reportor: luciferase or analogous reporter gene assays using co-transfection of SRY variants and 5'-ATTGTT-dependent reporter plasmid.

*Model reporter: luciferase or analogous reporter gene assays using co-transfection of SRY variants and human/mouse TESCO-dependent reporter plasmid.

Chimeric SRY/Sry constructions: see Supplemental Figure S6 in companion paper (Chen, Y.-S., Racca, J.D., Phillips, N.B., & Weiss, M.A. Proc. Natl. Acad. Sci. USA (2013))

*Studies employing an artificial TESCO-regulated reporter gene employed described in ref. 2 (below) suggest that inherited mutation R30I exhibits native transcriptional activity, which poses a paradox in light of the proband’s phenotype and data presented in ref. 1 (below).

*The results of ref. 3 indicate the V60L abolishes detectable specific DNA binding as probed by a gel mobility-shift assay (see Appendix), which suggests a transcriptional threshold of >50 in contradiction to the factor of two obtained here in accord with studies of mouse models. The present in vivo and in vitro data demonstrate that the specific DNA-binding affinity of V60L is similar to that of wild-type SRY.

*The results of ref. 3 indicate that I90M markedly attenuates specific DNA binding, which is inconsistent with subsequent studies by this and other laboratories (ref 2). Our present in vivo and in vitro data demonstrate that the specific DNA-binding affinity of I90M is similar to that of wild-type SRY.

*The TESCO-dependent reporter gene assay employed in ref. 2 indicated that I90M SRY exhibits enhanced transcriptional activity (see Appendix). The present studies suggest that this result is likely to be an artifact of TF over-expression. Whereas enhanced activity makes sex reversal difficult to understand, the present study resolves this paradox through plasmid dilution and multifactorial characterization of cellular biochemistry, including endogenous Sox9 activation, endogenous TESCO CSIP, analysis of nucleocytoplasmic shuttling, phosphorylation, proteosomal degradation, and and Wnt/β-catenin signaling (multiple green boxes above).

References


Table S2. Nucleocytoplasmic trafficking of SOX factors

<table>
<thead>
<tr>
<th>SOX family</th>
<th>studies of nuclear import</th>
<th>studies of nuclear export</th>
<th>trafficking-mediated activity</th>
<th>post-translational modification</th>
<th>clinical/animal model mutations</th>
<th>identified transport protein</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOX1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>exportin4, importin β, CRM1</td>
<td>(1-3)</td>
</tr>
<tr>
<td>SOX2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>(4-6)</td>
</tr>
<tr>
<td>SOX3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(1,7,8)</td>
</tr>
<tr>
<td>SOX4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(1,2)</td>
</tr>
<tr>
<td>SOX5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>(1,2)</td>
</tr>
<tr>
<td>SOX6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>(1,2)</td>
</tr>
<tr>
<td>SOX7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>(2,9)</td>
</tr>
<tr>
<td>SOX8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>(2,10,11)</td>
</tr>
<tr>
<td>SOX9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>calmodulin, importin β, CRM1</td>
<td>(4,11,12)</td>
</tr>
<tr>
<td>SOX10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>calmodulin, CRM1</td>
<td>(4,13)</td>
</tr>
<tr>
<td>SOX11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>(2,14)</td>
</tr>
<tr>
<td>SOX12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>(2,15)</td>
</tr>
<tr>
<td>SOX13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>(2,16)</td>
</tr>
<tr>
<td>SOX14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>(2,17)</td>
</tr>
<tr>
<td>SOX15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>(2,18)</td>
</tr>
<tr>
<td>SOX17</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>(1,2,19)</td>
</tr>
<tr>
<td>SOX18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>(1,2,20,21)</td>
</tr>
<tr>
<td>SOX21</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(1,22,23)</td>
</tr>
<tr>
<td>SOX30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>(1,2)</td>
</tr>
</tbody>
</table>

*For each member of the Sox family, the availability of prior studies of trafficking is indicated by + (yes) or – (no). Columns indicate known components of the nuclear import or export machinery implicated in trafficking of that factor.

References


