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Inherited Sex-Reversal Mutations in *SRY* Define a Functional Threshold of Gonadogenesis: Biochemical and Evolutionary Implications of a Rare Monogenic Syndrome

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Abstract

Sex determination in therian mammals is regulated by heteromorphic chromosomes X and Y. Male development (the heterogametic sex) is initiated by the stage- and tissue-specific expression of a Y-specific gene adjacent to the pseudoautosomal region (SRY; sex determining region of the Y; interval 1A1). Expressed in supporting cells of the bipotential genital ridge just prior to its morphologic differentiation (38-40 days post-conception in late human embryogenesis; 12.5-14 days *post coitum* in mouse), SRY functions as an architectural transcription factor to trigger Sertoli-cell differentiation; these cells then orchestrate testis development leading to formation of Leydig cells. The principal downstream target of SRY is related autosomal gene *SOX9*, whose expression activates, in turn, a ramified network of male-specific gene expression. Although uncommon, mutations in human SRY are associated with disorders (or differences) of sex development (DSD): a spectrum of phenotypes leading to female somatic development with complete or partial gonadal dysgenesis (including ovotestis; 46, XY pseudohermaphroditism). Such mutations ordinarily occur *de novo* as meiotic errors in spermatogenesis. This review focuses on the rare subset of *inherited* mutations, i.e., variant *SRY* alleles shared between a fertile father and a sterile XY daughter. The known inherited mutations in SRY provide experiments of nature that unmask multi-faceted molecular functions of this specific DNA-bending protein and architectural transcription factor. Biochemical studies of such variants suggest that wild-type SRY functions just above a critical transcriptional threshold required for testis formation. Such rare pedigrees thus have broad implications for a gene-regulatory network functioning at the edge of ambiguity.

Abbreviations: ARD: Alanine-Rich Domain; CRM1: Chromosomal Maintenance 1; CTD: C-Terminal Domain; DSD: Disorders of Sex Development; ESD: Environmental Sex Determination; GRD: Glutamine-Rich Domain; GRN: Gene-Regulatory Network; GSD: Genetic Sex Determination; HMG: High Mobility Group; NTD: N-Terminal Domain; PDZ: Post synaptic Density Protein, *Drosophila* disc large tumor suppressor, and Zonula occludens-1 protein; PKA: Protein Kinase A; Sox: Sry-related HMG box; SRY: Sex-determining Region of the Y chromosome; TDF: Testis-Determining Factor; TES: Testis-Specific Enhancer; TESCO: Testis-Specific Enhancer of *Sox9* Core; TF: Transcription Factor; Amino acids are designated by standard one- and three-letter code.

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Introduction

Vertebrate sex determination may be based on genetic or environmental signals (**Figure. 1**). Whereas some classes exhibit both genetic- and environmental sex determination (GSD and ESD), the former predominates among birds and mammals [1]. Sex chromosomes can ordinarily be identified as heteromorphic pairs bearing sex-determining gene(s) (**Figure. 1**) [2] Male development of therian mammals, including humans, ordinarily directed by the Y chromosome, is initiated by the stage- and lineage-specific expression of *SRY* (Sex-determining region of the Y), a gene encoding the long-sought testis-determining factor (TDF) [3]. Assignment of SRY as the TDF was demonstrated by elegant studies of engineered XX mice in which an *SRY* transgene directed male development [4]. Over the past 25 years an extensive genetic database has emerged linking mutations in human *SRY* (or its translocation) to disorders of sex development (DSD; for review, see Ref [5,6]). Altered or impaired function of SRY leads to gonadal dysgenesis and, in turn, to a female somatic phenotype (Swyer's Syndrome; [7-12]). Although mutations in *SRY* most often represent meiotic errors in spermatogenesis [6,8], a small subset of inherited variants has also been described in which a fertile father and sterile XY daughter (or brother and sterile XY sister) share the same mutation [6,13-15].

This review focuses on rare cases of "inherited sex reversal" due to mutations in the DNA binding domain of SRY, designated the *high-mobility-group (HMG) box*. We first summarize molecular features underlying this syndrome and then suggest potential implications for understanding the evolution and operation of a sex-specific gene-regulatory network (GRN). We next discuss the general—and unexpected—relevance of such rare human pedigrees to classical anomalies in the natural history of rodents, the most speciose class of mammals [16] and a taxon unique among therian mammals for its exploration of non-canonical male sex-determining mechanisms [17]. Our conclusions seek to place these relationships within the broader context of mutation-driven evolution [18,19], a framework that highlights the importance of genomic contingency [20,21] in the emergence of biological novelty [22].

Anatomy of an HMG box

The HMG box of SRY is a conserved motif of DNA binding and DNA bending [22]. Characteristic of a metazoan superfamily of DNA-binding proteins, the HMG box is an L-shaped α-helical structure whose angular surface provides a template for sharp DNA bending [24,25]. The SRY HMG box (which recognizes specific DNA sequences [9, 26-28] as well as binding sharp preformed DNA bends [29-32] exhibits distinctive sequence- and structural features as the prototype of an extensive family of architectural transcription factors, designated Sox (Sry-related HMG box; [33]). In a landmark 2008 study Sekido and Lovell-Badge demonstrated in mouse models that the principal (and perhaps only) target of Sry is an autosomal *Sox* gene also implicated in the genetic pathway of male sex determination (*Sox9*) [34]. SRY-dependent transcriptional activation of *SOX9* in the pre-Sertoli cells of the bipotential gonadal ridge is envisaged as the central step in male gonadogenesis (**Figure. 2**). Proposed to function as an architectural transcription factor [35,36], Sry recognizes specific DNA sites in the upstream testis-specific enhancer (TES) of *Sox9* to effect its transcriptional activation [34].

The core DNA element of TES (designated TESCO) contains multiple consensus *Sry/Sox* target sites (5'-(A/T)TTGT(T/G)-3' and complement; (**Figure. 3**) as defined *in vitro* [9,37]. Binding of murine Sry (and similarly human SRY) to TESCO has been demonstrated by chromatin immunoprecipitation (ChIP) [34,38,39]. In mammalian cell lines (including an embryonic pre-Sertoli-like line derived from the male rat gonadal ridge; [40]) clinical mutations in SRY have been assessed for effects on TESCO occupancy [38,41] and transcriptional activation of the endogenous *Sox9* gene [15,38,41]. *De novo* DSD-associated mutations in the HMG box of human SRY generally impair in concert TESCO binding and *Sox9* transcriptional activation as exemplified by focused studies of a profoundly destabilizing mutation in the core of the major wing [41].

Although eukaryotic transcription factors are ordinarily modular proteins containing two or more conserved sequence motifs (such as DNA-binding domains, transcriptional activation motifs, transcriptional repression domains, and protein-protein interaction modules; [42,43]), SRY is anomalous: the HMG box is its only conserved domain (**Figure. 4*A***) [23,44]. Structural studies have shown that this domain (like canonical HMG boxes; [45]) contains an N-terminal β-strand followed by three α-helices (designated α_1, α_2 and α_3; **Figure. 4*B***) [10,11]. The resulting three-dimensional structure contains two wings. The major wing consists of α_1, α_2 and the N-terminal segment of α_3 whereas the minor wing is formed by packing of the N-terminal β-strand against a C-terminal portion of α_3. Both wings and a distinctive C-terminal basic tail (of sequence restricted to members of the SOX family; 33) contribute to an angular DNA-binding surface [12,46,47]. Sequence-specific DNA bending is effected in the minor groove of the DNA double helix [10,28]. The mechanism of DNA recognition and sharp DNA bending is remarkable for the partial intercalation of a "cantilever" side chain [48], which projects from the crux of the two wings; in human SRY this is an Ile (consensus position 13 in the human HMG box; residue 68 in full-length human SRY). The cantilever side chain inserts between consecutive AT base pairs in the DNA target site (consensus sequence 5'-A**TT**GTT-3' and complement; insertion site in bold) to disrupt base stacking but not base pairing [28,49]. The cantilever (otherwise conserved among Sry/Sox domains as Met, Leu, or Phe) is part of a "hydrophobic wedge" of side chains that dock within a widened DNA minor groove [11]. Such wedge insertion within the minor DNA groove—a mechanism of indirect read-out—stands in contrast to the sequence-specific recognition of functional groups in the major DNA groove by such α-helical DNA-binding motifs as homeodomains [50,51] and zinc fingers [52]. Sharp DNA bending by SRY/SOX HMG boxes also occurs at the 3' end of the DNA site (5'-ATTG*TT* and complement; italics) as mediated by non-intercalative contacts by the minor wing and basic tail [11,12]. Such contacts include hydrogen bonds to DNA bases and salt bridges to its phosphodiester backbone.

Clinical Mutations in SRY

Clinical mutations in *SRY* (the predominant cause of Swyer's

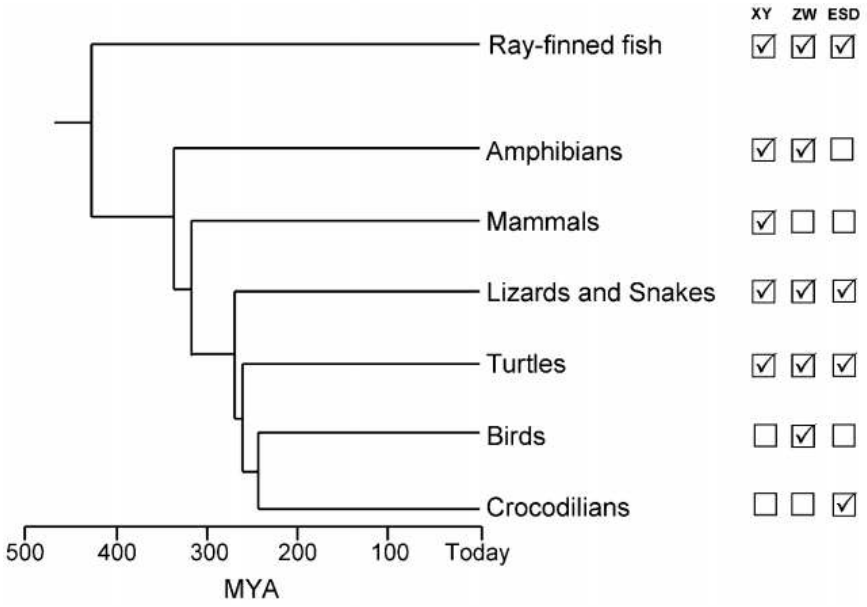

Figure 1 Sex determination mechanisms among selected euteleostomi. Checked boxes indicate known sex-determining mechanisms for each clade. Multi-box lineages possess multiple species with different sex determination mechanisms. Birds and mammals possess greater stability of genetic sex determination mechanisms whereas species in other lineages have diverse mechanisms.

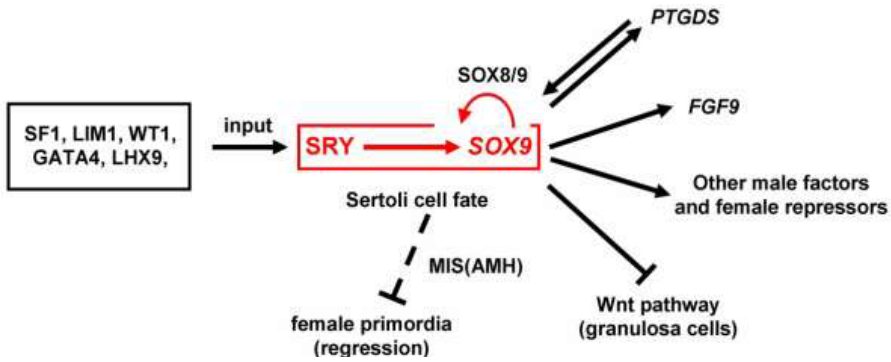

Figure 2 SRY-*SOX9* regulatory axis and related chronological flowchart. SRY→*SOX9* regulatory axis (*red* box) with genetic inputs (box at *left*) and outputs to a male-specific GRN (→) leading to inhibition of granulosa-cell fate (solid ⊥), and Müllerian regression (dashed ⊥). Red curved arrow indicates SOX8/9-mediating feedback maintaining *SOX9* expression. *SOX9* expression continues to be expressed postnatally supported by several positive-feedback loops. *SOX9* induces differentiation of Sertoli cells.
Abbreviations: FGF9: Fibroblast Growth Factor 9; GATA4: GATA binding protein 4; LHX: LIM homeobox 9; LIM1: homeobox protein Lhx 1; MIS: (AMH), Müllerian Inhibiting Substance (Anti-Müllerian Hormone); PTGDS: Prostaglandin D2 Synthase; SF1: Steroidogenic Factor 1; WT1: Wilm's Tumor 1; Wnt: Wingless-type.

syndrome in the presence of pure gonadal dysgenesis; between 1 in 30,000-50,000 live births; 53) account for only 15-20% of patients with DSD exhibiting some degree of XY intersexual phenotypes [54]. Almost all such mutations cluster in the SRY HMG box as distinct from N- or C-terminal flanking segments [15]. Patients with Swyer's syndrome typically exhibit complete bilateral gonadal dysgenesis [55] with female external genitalia, intact uterus and well-developed fallopian tubes. Partial gonadal dysgenesis is less commonly observed but may result in formation of an ovotestis [56]. Either histopathological form of Swyer's syndrome confers an enhanced risk of gonadoblastoma [5]. As in other forms of DSD (such as the androgen insensitivity syndrome; [57]), the same mutation in *SRY* may be associated with different phenotypic outcomes, even within a family [58]. Although genetic variation at autosomal loci presumably contribute to such genetic background effects, putative modifier genes have not yet been identified [59]. It is also possible that stochastic aspects of gene regulation in the differentiating gonadal ridge (a general feature of transcriptional control in the presence of small and variable numbers of regulatory molecules per cell; [60]) may contribute to the wide spectrum of DSD phenotypes, resulting in regional differences in cell fate and tissue organization as is illustrated with particular drama by the mixed states of differentiation in an ovotestis [56].

Despite such limitations to the present state of knowledge,

studies of SRY variants containing Swyer mutations *in vitro* and in cellular models have consistently demonstrated partial or complete impairment of SRY-dependent transcriptional activation of *Sox9*. This correlation has been shown in studies of the intact gene in its normal chromosomal context [15,38,41] as well as in studies of plasmid-encoded reporter genes in constructs driven by tandem TESCO-related control sites [6]. Most such mutations are associated with marked impairment of specific DNA binding [5]; the role of altered DNA bending is controversial [8,61].[1] Less common are mutations that perturb functions unrelated to specific DNA binding or DNA bending, such as efficiency of nuclear import or export [38,62 -64]. Yet other mutations display multiple biochemical or cell-biological defects that together impair transcriptional activation of *Sox9* in cellular models and presumably in the human gonadal ridge (**Table 1**). Least common are mutations in *SRY* lying outside the HMG box. Three such cases have been reported. One, N-terminal to the HMG box (R30I), has been proposed to impair phosphorylation of a flanking serine (or serines) in human SRY [65]. To our knowledge, the molecular consequences of the other two non-box mutations (S18N and L163X) have not been characterized.

Molecular Mechanisms and Implications of Inherited Human SRY Mutations

Mutations in human SRY associated with "inherited sex reversal" pose a seeming paradox: a single genetic variant shared between a fertile XY father and a sterile XY daughter. What molecular mechanisms might lead to such an ambiguity in developmental fate? To date, five such mutations have been identified by genetic testing of the proband (daughter) and her father (**Figure. 5*A***).[2] The mutation are broadly distributed within the HMG box (**Figure. 5*B***): V60L is located in the N-terminal β-strand, R76S and I90M lie in α2, F109S is part of the aromatic-rich core in the major wing, and Y127F adjoins the interface between the minor wing and a bent DNA surface. With the exception of R76 (whose guanidinium group participates in an electrostatic interaction with phosphodiester DNA backbone [11]), the wild-type side chains at these sites do not directly contact the DNA site [11].

Initial molecular studies of Swyer mutations were confounded by the range of DNA-related *in vitro* assays and by then-uncharacterized non-DNA-related cellular processes, including mechanisms of nucleocytoplasmic trafficking [38,62,64,66,67], identity of SRY target genes and delineation of SRY-responsive enhancer elements [34,64]. In recent years these gaps have been addressed by interdisciplinary approaches, combining *in vitro* biochemical analysis with an appropriate *in vivo* mammalian cell-culture assay. Such second-generation studies have insight into the diverse biological mechanisms of action for this intriguing subset of mutations [15,38].

A shared property of inherited SRY variants is maintenance of native-like specific DNA binding and sharp DNA bending—the primary biochemical functions of the HMG box. Such mutations nonetheless confer subtle (and in some cases multiple) perturbations to the biochemical and cellular activities of the protein (**Table 1**). Although the defect(s) in one variant may be unrelated to the defect(s) in another, a second common theme appears to be a twofold reduction in the extent of SRY-dependent transcriptional activation of *Sox9* as observed under conditions of physiological expression (100-10,000 protein molecules per cell; [60]). An illustrative example is provided by the seemingly conservative mutation V60L at position 5 of the HMG box; each of these residues contains a branched chain aliphatic side chain compatible with the environment of consensus residue 5 in a β-strand. Although biophysical studies have shown subtle "frustration" of minor-wing packing within the bent DNA-protein complex (manifest as an accelerated rate of disassembly; [15]), the key biological perturbation is partial impairment of nuclear localization. The wild-type V60 adjoins a bipartite nuclear localization signal and contributes to its binding to exportin 4 (here functioning in nuclear import) [68]. In cell culture rescue of nuclear localization by fusion of the variant SRY to an exogenous nuclear localization signal (the NLS of simian virus 40; SV40) was shown to restore native TESCO occupancy and *Sox9* activation [38]. We note in passing that V60L was first described as impeding specific DNA binding, a finding at odds with the phenotype of the proband's father and likely to represent a kinetic artifact of the DNA-binding assay employed in this early study (the electrophoretic mobility-shift assay; EMSA or "gel shift assay") [69].[3]

A complementary example is provided by the inherited mutation I90M, which is also permissive of native-like specific DNA binding and DNA bending [38]. This mutation disrupts nuclear export of SRY (via CRM1; [38]) and thus impairs nucleocytoplasmic shuttling. Whereas its initial studies were conducted under conditions of marked over-expression (thereby circumventing the subtle requirements of nucleocytoplasmic trafficking), I90M effectively prevents the cytoplasmic phosphorylation of the variant SRY [38], a post-translational modification that

[1]The *de novo* mutation M64I (position 9 of the HMG-box consensus; [10]) was originally characterized by Bianchi and colleagues as causing a selective defect in DNA bend angle [8]. Reassessment of this finding revealed that the decrement in DNA bending was due to inadvertent truncation of the C-terminal tail of the SRY HMG box [61]. In intact SRY M64I impairs nuclear entry in a pre-Sertoli cell line but does not perturb extent of DNA bending *in vitro*.

[2]It is important to note that several SRY mutations in the database could not be categorized as *de novo* or inherited due to the unavailability of the father for genetic testing. In addition, a given inherited mutation may be observed as a *de novo* mutation in another pedigree as the latter proband would be sterile (and so without opportunity for successive inheritance). For this reason the collection of *de novo* mutations may span a broad range of functional deficits on the biochemical level.

[3] The EMSA technique provides a rapid method for the detection of specific protein-DNA complexes based on retardation of migration of the complex relative to the free DNA site. False negatives may be obtained for complexes for which rapid disassembly leads to disappearance of the protein-bound band during the course of electrophoresis. Phillips and colleagues have shown that the inherited mutation V60L markedly accelerates the disassembly of an otherwise native-like SRY-DNA complex; near-native affinity is maintained by a compensating increase in the rate of protein-DNA association [15]. The phenotype of the proband appears to reflect partial impairment of nuclear entry associated with decreased N-terminal phosphorylation [38].

Table 1 Summary of Inherited Mutations in human SRY and State of Characterization[a].

Mut.	Clinical Assess.						Protein Pur.				Protein Charact.				Protein Partner				Cellular Studies								References
	CGD/PGD[b]	Ovotestis	Gonadal blastoma	Not Mosaic father	Father-daughter	Sister-brother	Intact	HMG box	Fusion	Extract	DNA affinity	Rate constants	Bending angle	Protein stability	Imp-α/β family	Exp-4	CaM	CRM1	TESCO reporter[c]	Endogenous *Sox9*	ChIP	Nuclear Import	Nuclear Export	Cellular degradation	Phosphorylation	Wnt/β-catenin inhibition study	
S18N	■			■	■	■	■				■								■[d]								(102,6)
R30I	■			■	■	■	■			■	■								■[d]						■		(65,6)
V60L	■			■	■		■	■		■	■[e]	■	■	■		■	■	■	■	■	■	■	■	■	■	■	(69,6,38)
I90M	■			■	■	■	■	■			■[f]	■	■[g]	■		■	■	■	■[h]	■	■	■	■	■	■	■	(6,103,8,58)
R76S	■				■																						(104)
F109S	■			■	■	■				■	■																(105)
Y127F	■			■	■					■	■																(106)
L163X	■				■														■								(107)

[a]For each inherited clinical mutation, results of prior studies are indicated by ■ or, in the case of possible technical error, by ■. Open spaces signify the absence of published data. Thus, aside from clinical genetics (gray zone at left), scant data are available.
[b]CGD/PGD: complete gonadal dysgenesis (■)/partial gonadal dysgenesis (■)
[c]TESCO reporter: *luciferase* or analogous reporter gene assays using co-transfection of SRY variants and human/mouse *TESCO*-dependent reporter plasmid.
[d]Studies employing an artificial TESCO-regulated reporter gene employed described in [65] (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).
[e]The results of [6] indicate the V60L *abolishes detectable specific DNA binding* as probed by a gel mobility-shift assay, 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.
[f,g]The results of [6] indicate that I90M markedly attenuates specific DNA binding, which is inconsistent with subsequent studies by this and other laboratories [65]. 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.
[h]The TESCO-dependent reporter gene assay employed in [65] indicated that I90M SRY exhibits enhanced transcriptional activity. 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 ChIP, analysis of nucleocytoplasmic shuffting, phosphorylation, proteosomal degradation, and and Wnt/β-catenin signaling

enhances TESCO occupancy and transcriptional activity [38]. Under expression of SRY is calibrated to match physiological levels [60] (achieved in transient transfection studies by diluting an expression plasmid by its empty parent plasmid), attenuated phosphorylation leads to a twofold reduction in *Sox9* transcriptional activation despite the enhanced nuclear accumulation of the variant SRY [38]. Together, the opposite effects of V60L (impaired nuclear entry) and I90M (impaired nuclear exit) suggest that dynamic nucleocytoplasmic trafficking contributes to the robustness of the SRY's function as a TDF in human embryogenesis. A requirement for such trafficking may be a general feature of the Sox family of architectural transcription factors [70].

Studies of the remaining inherited mutations, which to our knowledge await future studies, promise to provide analogous insight into which biochemical, structural or cell-biological properties of the protein contribute to the threshold of biological activity. We speculate, for example, that studies of the inherited mutation Y127F (like I60V, a seemingly conservative substitution when considered from the viewpoint of peptide chemistry) will reveal a subtle but critical role for the solvent-exposed *para*-hydroxyl group of Y127. This role is not apparent even in the high-resolution NMR structure of the bent SRY-DNA structure [11]. Whereas the present review focuses on mutations within the SRY HMG box, it is important to note that rare inherited mutations have also been identified outside of this domain. N-terminal to the box, for example, are two inherited mutations (S18N and R30I); to our knowledge, neither has been well characterized. It is possible that R30I affects phosphorylation of SRY at an adjoining SSS element (residues 31-33), proposed to function as a regulatory target of protein kinase A [71]. A nonsense mutation has also been reported 3' to the box (L163X). Such truncation may lead to accelerated degradation; the missing the C-terminal 41 residues includes a putative PDZ-binding motif proposed to mediate binding to an SRY-interacting protein [72]. These examples illustrate the opportunities that await further study of rare human mutations associated with remarkable phenotypes.

From Rare Human Pedigrees to Anomalies of Natural History

SRY acts as a developmental switch in therian mammals to initiate a male-specific GRN in the bipotential gonadal ridge [5]. We speculate that inherited mutations in human SRY define a *functional threshold* for the robust activation of this switch. Although studies are to date incomplete, each of the

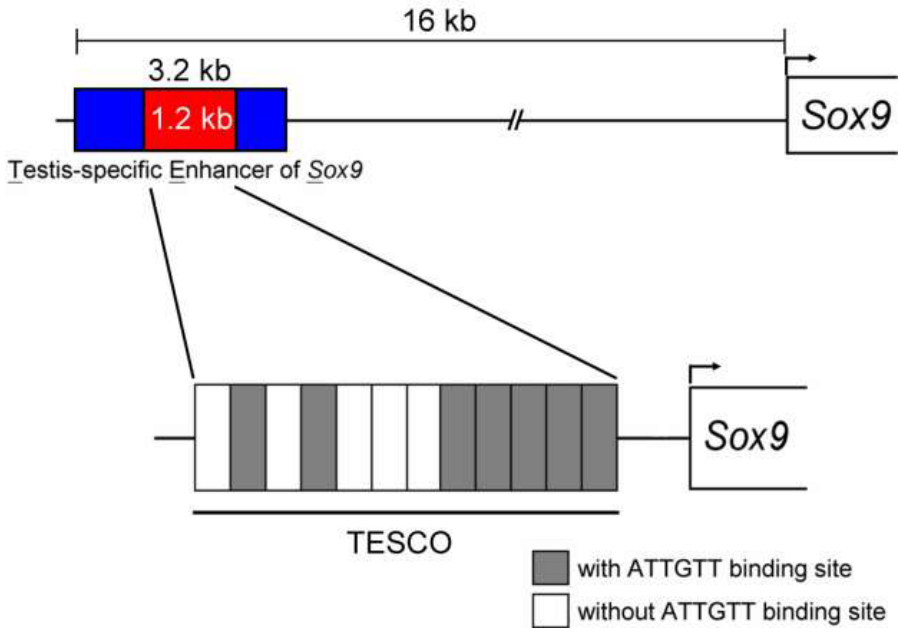

Figure 3 Schematic of the *Sox9* testis-specific enhancer (TES; 34) and its core region. TES (*blue*) is located 16kb upstream to the open reading frame of *Sox9*, the core of this sequence (TESCO, *red*) contains multiple identified Sry binding sites. Enlarged boxes indicate element fragments containing identifiable Sry binding sequences (*grey* boxes, indicating the presence of an ATTGTT sequence, *white* boxes represent fragments without identifiable Sry binding sequences).

well-characterized inherited mutations—independent of molecular mechanism—results in a twofold decrease in *Sox9* activation [15,38].[4] Remarkably, such a subtle reduction is in accordance with the low threshold of Sry function in intersexual mice wherein the phenomenon of Y chromosome-autosome incompatibility has long been the subject of study [73-75]. So striking and counterintuitive was the marginal threshold of murine Sry that Lovell-Badge and colleagues suggested, in a wry title, that this landmark example of a mammalian master switch directs gonadogenesis by a fleeting "wink and a nudge" [76]. Although a seeming violation of Waddington's classical principle of canalization [77], the subtle threshold of SRY/Sry may justify its scant biochemical description as "just a box" [78]. The twofold threshold of human SRY and mouse Sry renders syndromes of ambiguous gonadal specification analogous to a syndrome of transcription-factor haploinsufficiency.

In 2003 Eicher and colleagues first proposed—with prescient insight—a mechanistic analogy between the inherited form of Swyer's syndrome and the observation of murine XY females and XY hermaphrodites (ovotestes) due to Y-autosome strain incompatibility [73]. Despite present evidence supporting such an analogy, this proposal appeared in apparent conflict with the "just a box" hypothesis on molecular grounds: mice (unlike primates and other classes of mammals) exhibit an exceptional set of expanded *Sry* genes, which are generally found within the Muroidea superfamily of rodents (the largest subgroup within order Rodentia, which includes the ubiquitous genera *mus* and *rattus*; [79]). The origins of this expansion lie in an insertion of a CAG microsatellite (or, in some lineages, its contracted remnant) within the coding region of *Sry* downstream of the HMG box. Depending on reading frame, this introgressed DNA segment encodes a Gln- or Ala-rich domain (GRD or ARD, respectively) of variable length (depending on reading frame; [17,80]). Such variability presumably reflects microsatellite instability in the course of DNA replication. Expansion and contraction of such DNA repeats in vertebrate genomes typically occur at rates more than 100-fold greater than single base-pair transitions or transversions.

Microsatellite instability within muroid *Sry* is likely to have functional and evolutionary consequences. The CAG repeat motif encodes a key polypeptide component of the protein's gene-regulatory function: in general accordance with the Eicher hypothesis [73], deletion of this microsatellite motif in cell-based studies of truncated Sry constructs has been shown (as in inherited Swyer variants) to effect a twofold decrease in the extent of downstream *Sox9* activation [39]. Further, studies of the isolated mouse HMG box have revealed that its thermodynamic stability, DNA-binding, and DNA-bending properties are each perturbed relative to those of the human HMG box [39,81]. We have therefore proposed [39] that the microsatellite-encoded domain of rodent Sry functions as an intramolecular "genetic capacitor," a concept introduced by Rutherford and Lindquist in influential studies of heat-shock proteins in *Drosophila melanogaster* [82]. Although the molecular embodiment of the Sry capacitor (i.e., a GRD or ARD acting as an accessory transcriptional activation domain in a modular transcription factor) differs from heat-shock proteins, in each case, the system may function as a molecular mechanism to enable the accumulation and discharge of cryptic genetic variation [19,83]. The notion of a genetic capacitor has attracted broad attention in relation to the tempo

[4] Chen, Y-S., Racca, J., Phillips, N.B., & Weiss, M.A. (manuscript in preparation)

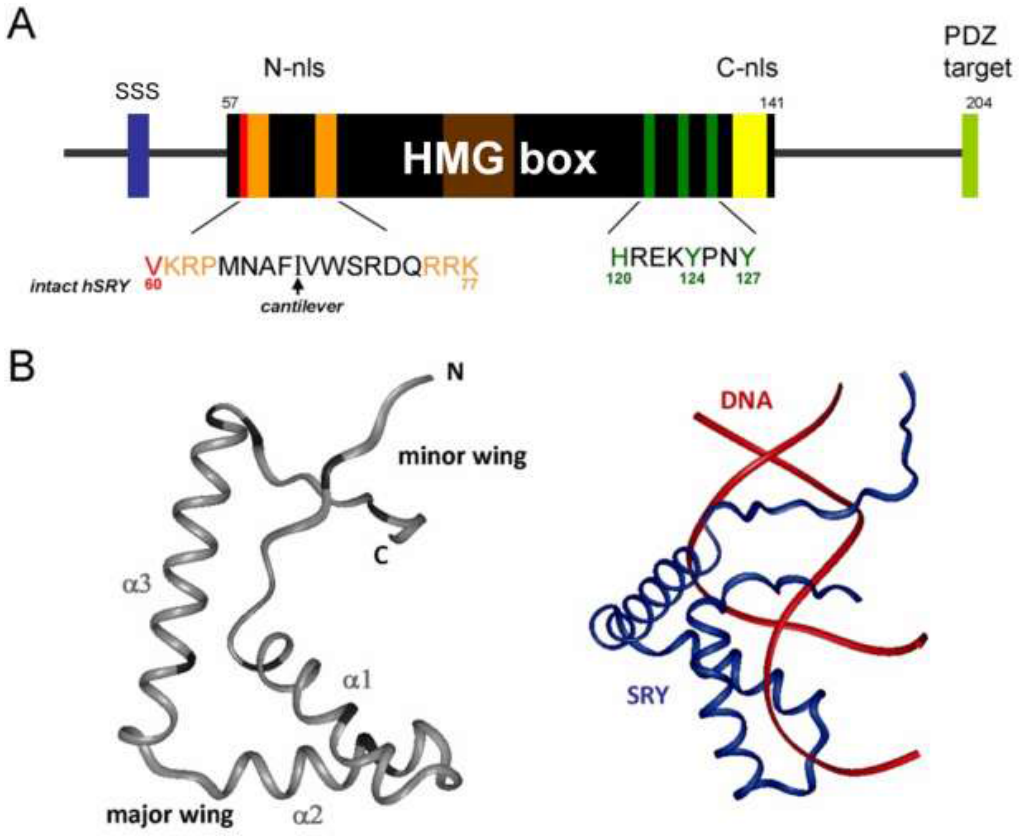

Figure 4 Schematic of full length human SRY and three dimensional structure of SRY HMG box. (A) Functional domains and motifs in human SRY. A serine repeat (residues 31-33 indicated by *purple*) are sites of phosphorylation [71]. The central HMG box (residues 55-141) is labeled. The N-terminal nuclear localization signal (N-nls) (*orange*) includes two sets of basic residues (sequence is listed below the orange box, key residues are colored orange), a site of an inherited mutation is located within this motif (V60L, position colored in *red*). The residue which intercalates into the base pairs required for DNA bending [32] is shown by an arrow, Ile in human SRY. A centralized motif for nuclear export (NES, *brown*) is required for nucleocytoplasmic shuttling of human SRY [38]. DNA-dependent reorganization of the protein "locks" the N- and C-termini involving a set of aromatic residues (*green* bars, sequence in the same color) and V60 at the N-terminus. A cluster of basic amino acids (*yellow*) in the C-terminal tail act as an electrostatic clamp [32] and also contain a classical nuclear localization signal (C-nls). At the extreme C-terminus of human SRY a PDZ-binding motif (*lime*) has been described [72], proposed to modulate protein-protein interactions. (B) Three dimensional structure of the human SRY HMG box. The domain contains a major wing (consisting of α-helix 1, α-helix 2, the first two turns of α-helix 3, and connecting loops) and a minor wing (the N-terminal β-strand, the remainder of α-helix 3, and C-terminal tail). Left Diagram of the HMG domain highlights the characteristic L-shaped of this DNA-binding and bending motif. Right Bent DNA-protein complex (DNA is in *red*) is shown oriented to highlight minor groove binding and DNA bending. Molecular coordinates were obtained from the Protein Databank (entry 1J46; [11]).

of morphologic change in the fossil record, notable for periods of stasis interrupted by sudden discontinuities on the geological time scale ("saltatory" evolution; [20]).

The plausibility of the genetic-capacitor model of rodent *Sry* evolution has been supported by model studies in cell culture in which deleterious effects of inherited Swyer mutations can be compensated by C-terminal fusion of the C-terminal GRD of mouse Sry in chimeric constructions [39]. Such intragenic complementation (i.e., rescue of gene-regulatory function) would provide a parsimonious explanation for (a) the marked (and anomalous) divergence of muroid Sry HMG-box sequences relative to the box sequences of other taxa of mammals [84] and (b) the biochemical "degeneration" of the mouse HMG box domain as evidenced by imprecision of DNA bending and loss of structural stability [39,81]. Microsatellite instability within the muroid *Sry* gene would then be expected to provide biochemical compensation for— or, on contraction below a functional threshold, unmask—such variation. Contraction of the microsatellite would be predicted to attenuate the encoded domain's biochemical compensation, which would in turn exert a powerful selective pressure under which other genetic mechanisms must emerge to maintain male development and fertility. We therefore envisage that the muroid *Sry* microsatellite, operating as a genetic capacitor in a GRN that itself is poised at the edge of ambiguity, provides a novel mechanism for the rapid evolution of biological novelty [39].

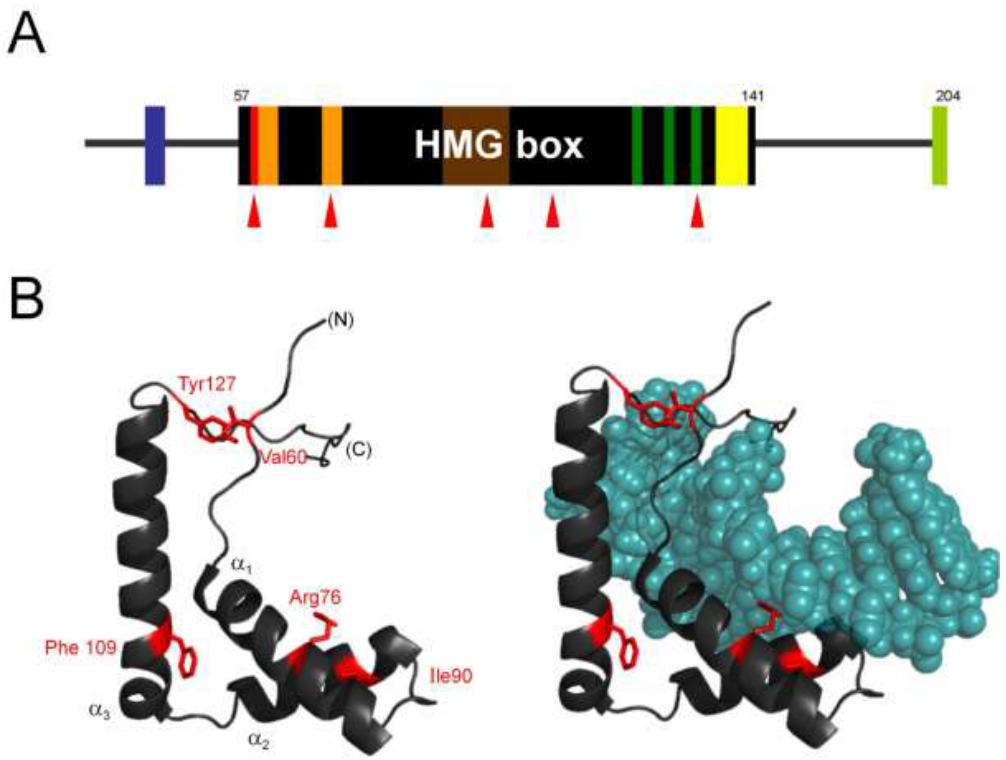

Figure 5 Location of inherited mutations in the HMG box of human SRY. (*A*). Domain schematic of full length human SRY (color scheme same as Fig 4*A)*, locations of the clinically identified inherited mutations are represented as *red* triangles. (*B*) Structural representation of the human SRY HMG domain, side chains of the inherited mutations are shown as *red* sticks. The termini and helices are labeled in the L-shaped domain. *Right,* is the same with the DNA shown (space filling model, *teal*). Coordinates from PDB 1J46 (11)

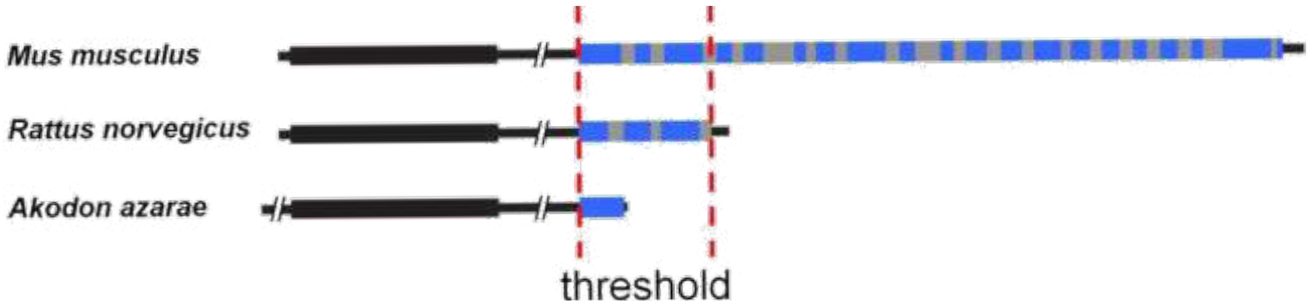

Figure 6 Diagram of various rodent Sry proteins. Schematic image highlighting a variety of Gln-rich tracts within the rodent family (GRD tracts are in *blue*, spacer sequences in *grey*); represented is mouse with 20 tracts, rat with 3 tracts, and *Akodon* containing a single Gln-rich tract. Previous literature suggests that a three-tract minimum is required for native *Sox9* activation defining a threshold (represented by the *red* dotted lines). *Akodon* Sry is predicted not to contain the minimum number of Gln rich-tracts to confer full transcriptional activity.

The microsatellite-capacitor model poses a challenge to the Modern Darwinian Synthesis [20,85] as discussed in detail elsewhere [19]. Such a challenge should not be lightly accepted. Unfortunately, experiments in natural history are intrinsically limited by our inability to "replay" the evolutionary tape, or (despite technical advances in retrieval of ancient DNA) obtain a systematic account of changing gene sequences and genomic structures as contemporaneous correlates of the discontinuous fossil record [20]. A strength of the *Sry* genetic-capacitor model is nonetheless provided by its successful retrodiction of otherwise anomalous features of the natural history of rodents [19]. Long enigmatic, these anomalies include the evolution of non-Sry-dependent mechanisms of male sex determination among Ryukyu spiny rats (genus *Tokudaia*; [86,87]) and the frank loss of the Y chromosome among voles (genus *Ellobius*; [88]). Of particular interest in relation to Swyer's syndrome, the muroid genus *Akodon* (a diverse group of South American grass mice) is

remarkable for the independent emergence of stable XY female subpopulations among several species [89,91]. Unlike Swyer patients, such XY *Akodon* females are fertile, presumably due to compensating variation on the X or elsewhere on the Y.

It would be of future interest to investigate representative *Akodon Sry* genes and the corresponding Sry proteins in relation to their human counterparts (as a canonical mammalian system) with emphasis on the database of Swyer mutations. Might Eicher's analogy between this human syndrome and the laboratory phenomenon of murine strain incompatibility [59,73-75] generalize to the *Akodon* radiation? This question is of compelling interest as the *Akodon Sry* genes of known sequence each exhibit intriguing features: (i) foreshortened N-terminal non-box domain with loss of putative phosphorylation sites (analogous to inherited mutation R30I?), (ii) a divergent HMG box with non-consensus NES (similar to human inherited clinical mutation I90M?), and (iii) a *single-block Gln-rich motif* [91], shown to be insufficient in mouse Sry to sustain GRD function and Sry-dependent *Sox9* activation (**Figure. 6**).[5] We predict that the single CAG repeat of the known *Akodon Sry* genes lies at the border of inactivity and so renders gonadogenesis ambiguous. This hypothesis in turn predicts that the biochemical and functional properties of Akodon Sry (in those species with stable XY female subpopulations) would resemble the properties of inherited mutations in human SRY in general accordance with the Eicher analogy.

Concluding Remarks: The Importance of a Rare Genetic Syndrome

The goal of this review is to highlight the broad implications of a rare human genetic syndrome. Such considerations have taken us far afield from medical genetics, and the scientific stakes could not be higher. From an evolutionary perspective the microsatellite-capacitor hypothesis may enable the rapid emergence of reproductive isolation among sympatric populations, long a subject of controversy in the Modern Synthesis [20,85,92]. Further, through the known phenomenon of environmental stress-induced microsatellite instability, our model might provide a "Lamarckian" mechanism of speciation, whereby environmental conditions could both precipitate and necessitate saltatory genetic change [19]. In this view, the contingent invasion of a compensatory trinucleotide repeat has set the stage for the accumulation of cryptic genetic variation in the specific DNA-binding domain of a master switch in a sex-specific GRN. Release of this variation—through "capacitive discharge" of the microsatellite (i.e., its contraction through slippage in DNA replication)—would cause developmental chaos in the bipotential gonadal ridge, leading to either the end of a lineage or to rapid speciation. The latter would create an opportunity for large scale genomic and/or genetic changes in the Sry-initiated GRN, including possible recruitment of another factor as the TDF [19]. This scenario is speculative but consistent with the fluidity of sex-determining mechanisms in other classes of vertebrates. It is possible that in the near future *Akodon* and other genera in Muroidea with non-canonical sex-determining mechanisms may provide natural systems amenable to both molecular genetics and ecological studies. Such studies would provide a counterpoint to the preservation of the Sry-TDF system (with conservation of its HMG box) in mammalian orders (and other rodent taxa) lacking the muroid microsatellite.

The medical genetics of a rare human genetic syndrome thus highlights a general feature of mammalian sex determination: the tenuous character of SRY as a developmental switch [38,39,93]. Such tenuousness is itself of theoretical interest. Whereas a considerable literature pertains to the origins of robustness [77] as general features of GRNs [94], we believe that the existence and advantages of tenuousness have not been sufficiently recognized. In particular, that the transcriptional threshold of wild-type SRY/Sry lies close to the border of ambiguity would magnify the evolutionary opportunities (and dangers) of subtle variation in the biochemical properties of this transcription factor. Indeed, we envisage that a developmental switch may evolve to the "edge of ambiguity" [95,96] as a mechanism to generate favorable phenotypic diversity [39]. This viewpoint echoes Farmer and Kauffman's celebrated metaphor (based on models of phase transitions in the mathematics of universal computation) of life as a network of interacting elements evolving at the edge of chaos [95].

Male diversity is itself of special interest in relation to the genetics of behavioral traits. In the nascent testis, quantitative variation in fetal Leydig-cell function (the source of fetal testosterone secretion) may be associated with a broad spectrum of hormone-dependent male brain patterning [97] and ultimately to variation in innate postnatal behaviors [98].

A tenuous developmental switch may have contributed, as a mechanism of phenotypic variation, to the evolution of social mammals, providing a molecular foundation for multilevel selection within the framework of sociobiology [99].

We envision that in the coming decades a next-generation "Modern Synthesis" [20] will integrate principles of evolutionary biology with a GRN view of development, including sexual dimorphism of the brain [100]. Looking beyond Swyer's syndrome, studies of other rare subgroups of DSD—interpreted as *differences* (rather than disorders; [101]) of sexual differentiation—promise to provide insight into the biological origins of gender identity in all of its richness and diversity.

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[5]Cell-based studies of mouse *Sry* have shown that its transcriptional potency depends on microsatellite length [39]. Whereas mouse Sry containing 20 repeat tracts, rat *Sry* contains 3 and the known *Akodon Sry* genes only one (**Figure. 6**).

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