Structure–function analysis of mouse Sry reveals dual essential roles of the C-terminal polyglutamine tract in sex determination

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The mammalian sex-determining factor SRY comprises a conserved high-mobility group (HMG) box DNA-binding domain and poorly conserved regions outside the HMG box. Mouse Sry is unusual in that it includes a C-terminal polyglutamine (polyQ) tract that is absent in nonrodent SRY proteins, and yet, paradoxically, is essential for male sex determination. To dissect the molecular functions of this domain, we generated a series of Sry mutants, and studied their biochemical properties in cell lines and transgenic mouse embryos. Sry protein lacking the polyQ domain was unstable, due to proteasomal degradation. Replacing this domain with irrelevant sequences stabilized the protein but failed to restore Sry's ability to up-regulate its key target gene SRY-box 9 (*Sox9*) and its sex-determining function in vivo. These functions were restored only when a VP16 transactivation domain was substituted. We conclude that the polyQ domain has important roles in protein stabilization and transcriptional activation, both of which are essential for male sex determination in mice. Our data disprove the hypothesis that the conserved HMG box domain is the only functional domain of Sry, and highlight an evolutionary paradox whereby mouse Sry has evolved a novel bifunctional module to activate *Sox9* directly, whereas SRY proteins in other taxa, including humans, seem to lack this ability, presumably making them dependent on partner proteins(s) to provide this function.

sex development | Y chromosome | testis | TESCO

SRY is the male sex-determining factor in most mammals, including mice and humans (1, 2). It functions by binding to and activating the testis-specific enhancer core sequence (TESCO) of SRY-box 9 (*Sox9*) (3). Sox9 protein in turn induces somatic precursor cells to develop into Sertoli cells (4), which orchestrate the development of the gonads as testes (4). Without *Sox9* activation, the fetal gonads develop as ovaries. Despite the pivotal role of this step in male sex determination, little is known about the actual molecular mechanisms by which SRY activates *SOX9* transcription (5).

Unlike most known transcriptional activators, SRY proteins from most species lack an obvious transactivation domain (TAD). For example, human SRY consists of a conserved high-mobility group (HMG) box DNA-binding domain flanked by poorly conserved N- and C- terminal domains (NTD and CTD, respectively; Fig. S1). The NTD and CTD bear no homology to known TADs. Moreover, neither domain showed intrinsic transactivation activity when the full-length human SRY protein was tethered to a GAL4 DNA-binding domain and tested in vitro (6). Thus, it has been postulated that human SRY may have to recruit a partner protein containing a TAD to activate *SOX9* transcription (6).

Mouse Sry is exceptional, lacking an NTD and containing an unusual C terminus comprising a bridge domain and a polyglutamine (polyQ) tract encoded by a CAG-repeat microsatellite (Fig. S1). In mice, this polyQ tract consists of 8 (*Mus domesticus*) to 21 blocks (*Mus musculus*) of 2 to 13 glutamine residues interspersed by a short histidine-rich spacer sequence (7, 8). The longer *musculus*-type polyQ tract can function as a TAD when fused to a GAL4 DNA-binding domain and tested in vitro (6). Recently, it has also been shown that a threshold length of the polyQ tract (at least three glutamine blocks) is required for Sry to transactivate *Sox9* in a rat pre-Sertoli cell line (9). These results suggest that mouse Sry, unlike human SRY, may use its polyQ domain to activate *Sox9* transcription, but in vivo support for this concept is lacking.

Transgenic expression of human (10) or goat SRY (11), neither of which bears a sequence related to any known TAD or the mouse polyQ tract, has been reported to cause male sex reversal of XX mouse embryos (Fig. S1). These results have been interpreted as implying that a TAD may not be required for mouse Sry to activate *Sox9* and that the polyQ domain may not be necessary for testis determination in mice (10, 12, 13). Arguing against this view, we have previously shown that two mutant mouse *Sry* transgenes, in which a stop codon was introduced either just before the sequence encoding the polyQ tract or just after the HMG box (Fig. S1), failed to give XX male sex reversal in transgenic mouse embryos, indicating that the polyQ domain is indeed required for mouse testis determination (14). Two possibilities may account for these findings: Either the polyQ domain is both necessary and sufficient for Sry's ability to activate *Sox9* and effect male sex determination, or the truncated Sry mutant proteins were not expressed or degraded, possibly due to conformational change. It was not possible to exclude the latter possibility at the time, due to the lack of suitable antibodies.

Significance

The sex-determining factor SRY is thought to function by up-regulating expression of its key target gene SRY-box 9 (*SOX9*) in pre-Sertoli cells of the developing gonads, but evidence for a transactivation domain is lacking for human SRY and is limited to in vitro evidence for mouse Sry. The latter is unusual in possessing a polyglutamine tract at its C terminus. We demonstrate, using a combination of biochemical, cell-based, and transgenic mouse assays, that this domain plays essential roles in both protein stabilization and transactivation of *Sox9*, and is required for male sex determination in mice. Our data indicate that mouse Sry has evolved a novel bifunctional module, revealing an unexpected level of plasticity of sex-determining mechanisms even among mammals.

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In the current study, we reinvestigated this issue using contemporary tools. We analyzed the expression and transactivation ability of a series of GFP-tagged Sry mutants in cultured cells and in transgenic mouse models. Our data show that the polyQ domain not only prevents mouse Sry from proteasomal degradation, but also acts as a TAD, enabling Sry to induce *Sox9* transcription directly. This TAD is critical for male-determining function in vivo, suggesting that mouse Sry has acquired a functional module not represented in other mammalian genera, and revealing an unexpected level of plasticity of sex-determining mechanisms even among mammals.

Results

The polyQ Domain Protects Mouse Sry Protein from Proteasomal Degradation. To analyze the functions of the polyQ domain, we first generated constructs encoding either the wild-type Sry or an Sry mutant lacking this domain. To facilitate detection of the proteins, we placed an EGFP coding sequence in frame at the N terminus of the *Sry* ORF in these constructs (gSry and gSryΔQ, respectively; Fig. 1*A*).

When stably overexpressed in 15P-1, a mouse Sertoli-like cell line (15), gSry protein was readily detected in the cell nuclei by immunofluorescence (Fig. 1*B*) or Western blot (Fig. 1*C*). In contrast, the Q-domain deletion mutant (gSryΔQ) was not detected (Fig. 1 *B* and *C*), despite its mRNA transcript being about threefold more abundantly expressed than that of gSry (Fig. 1*D*). These results suggest that gSryΔQ protein is degraded in these cells, and that the polyQ domain stabilizes the Sry protein.

To clarify how gSryΔQ is degraded, we treated the 15P-1/gSryΔQ cells with either proteasomal or lysosomal inhibitors, and quantitated the GFP fluorescence using flow cytometry. Treatment with proteasomal inhibitors epoxomicin or MG-132 significantly increased both the percentage of GFP$^+$ cells (Fig. 1*E*) and the mean fluorescence intensity (MFI) of the GFP$^+$ cells (Fig. 1*F*) relative to vehicle-treated controls. In contrast, treatment with two lysosomal inhibitors, chloroquine or E64, did not result in consistent changes in the percentage of GFP$^+$ cells or fluorescence intensity (Fig. S2). Similar results were obtained using a different mouse Sertoli-like cell line, TM4 (16) (Fig. S3). These results indicate that Sry protein lacking the polyQ domain is degraded via the proteasomal pathway.

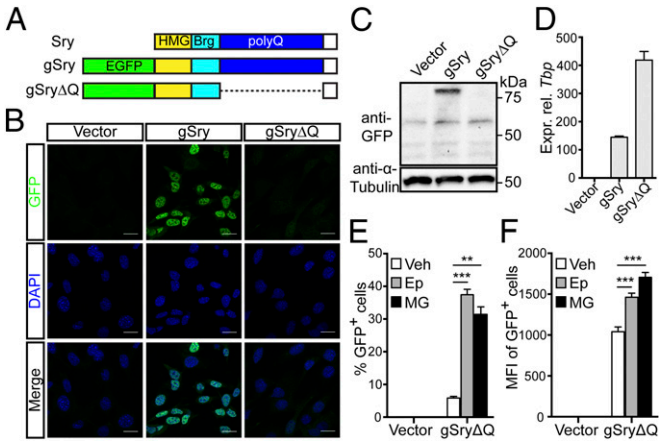

Fig. 1. The polyQ domain protects mouse Sry protein from proteasomal degradation. (*A*) Structure of wild-type Sry, gSry, and gSryΔQ proteins. Brg, bridge domain. (*B–D*) Expression analysis of gSry and gSryΔQ in 15P-1 stable cell lines. gSry but not gSryΔQ protein was detected by immunofluorescence (*B*) or Western blot (*C*). (Scale bar: 20 μm.) Predicted molecular weight: gSry, 77.1 kDa; gSryΔQ, 47.9 kDa. A blot using anti–α-tubulin served as loading control. qRT-PCR revealed that the expression of gSryΔQ mRNA was higher than that of gSry (*D*). $n = 1$. Error bars indicate SEM of technical replicates. (*E* and *F*) The 15P-1 cells stably expressing either an empty vector or gSryΔQ were treated with a vehicle control (Veh), epoxomicin (Ep) or MG-132 (MG), and analyzed for GFP fluorescence using flow cytometry. Treatment of 15P1/gSryΔQ cells with either Epox or MG-132 caused a significant increase in both the percentage (*E*) and MFIs of GFP$^+$ cells (*F*). Data are presented as mean ± SEM ($n = 3$). Statistical significance was determined using Student *t* test. $^{**}P < 0.01$ and $^{***}P < 0.001$.

Protein Stabilization by the polyQ Domain Is Sequence Independent but Size Dependent. To assess whether the polyQ domain's protein-stabilizing function is dependent on a specific sequence, we next generated a mutant construct in which the polyQ tract was removed and the coding sequence of the irrelevant marker protein mCherry inserted in its place (gSryΔQ+Ch; Fig. 2*A*). The presence of mCherry polypeptide restored Sry protein expression levels similar to those found by assaying gSryΔBQ (Fig. 2 *B* and *C*). The fact that the degradation of gSryΔQ can be rescued by the presence of either the polyQ domain (in gSry) or ballast polypeptide such as mCherry indicates that the protein-stabilizing function of the polyQ domain is sequence independent.

We also tested the stability of a construct in which the polyQ domain was replaced by the TAD of the transcriptional activator protein VP16 (Fig. 2*A*). This gSryΔQ+VP mutant protein was barely detected in stable 15P-1 cells (Fig. 2 *B* and *C*). Because the VP16 domain is much shorter than the polyQ domain and the mCherry polypeptide (Fig. 2*A*), both of which confer stability in 15P-1 cells (Figs. 1 and 2), we hypothesized that the efficiency of protection from degradation is dependent on the size of the C-terminal ballast. To test this hypothesis, we made two other mutant constructs by inserting either the PQA domain from mouse Sox9 (gSryΔQ+PVP) or mCherry (gSryΔQ+ChVP), to act as stuffer sequences between the bridge and VP16 domains of gSryΔQ+VP (Fig. 2*A*), thus extending the length of the Sry C terminus. Both extended proteins were more stable than gSryΔQ+VP (Fig. 2 *B* and *C*), despite their lower expression at the transcript level (Fig. 2*D*), supporting our hypothesis. gSryΔQ+Ch mutant was more stable than either gSryΔQ+PVP or gSryΔQ+ChVP (Fig. 2 *B* and *C*), suggesting that sequence-dependent differences in conformation of the C terminus also influence Sry protein stability.

The polyQ Domain Is Essential for Sry to Transactivate a TESCO Reporter in Vitro. To initiate the male program, Sry binds to the TESCO enhancer element upstream of *Sox9* and activates *Sox9* transcription in the presence of Sf1 (also known as Nr5a1) (3). We took advantage of an established in vitro TESCO–luciferase reporter assay system (3) and characterized the ability of the Sry mutants to activate this reporter.

We first confirmed that gSry synergised with Sf1, when cotransfected into HEK293 cells, to induce the TESCO reporter to a degree similar to that of wild-type Sry in this assay (Fig. S5). In contrast, the mutants in which the polyQ tract was removed or

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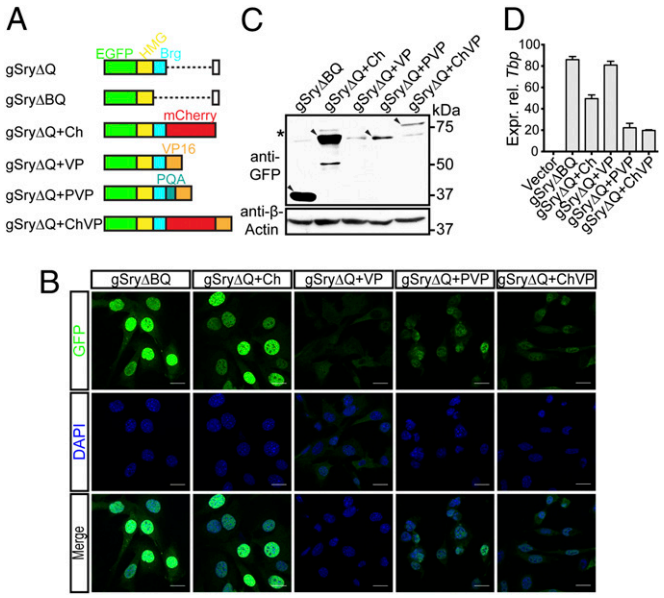

Fig. 2. The polyQ domain's protein-stabilizing function is sequence independent but size dependent. (*A*) Structure of the Sry mutants. (*B* and *C*) Expression analysis of the Sry mutant proteins in 15P-1 stable cell lines by immunofluorescence staining (*B*) or Western blot (*C*). (Scale bars: 20 μm.) (*C*) Arrowheads indicate the respective protein bands. The asterisk indicates a nonspecific band that overlaps with gSryΔQ+Ch and gSryΔQ+PVP. gSryΔQ+PVP showed a size similar to gSryΔQ+Ch and larger than its predicted molecular weight, likely due to posttranslational modifications. A blot using anti–β-actin served as loading control. Predicted molecular weight: gSryΔBQ, 37.4 kDa; gSryΔQ+Ch, 72.0 kDa; gSryΔQ+VP, 56.0 kDa; gSryΔQ+PVP, 59.7 kDa; gSryΔQ+ChVP, 80.1 kDa. (*D*) qRT-PCR revealed that the gSryΔQ+VP transcript was expressed at a higher level compared with gSryΔQ+PVP or +ChVP. $n = 1$. Error bars indicate SEM of technical replicates.

replaced, i.e., gSryΔQ, gSryΔBQ, and gSryΔQ+Ch failed to induce TESCO reporter activity (Fig. 3). The loss of ability to activate the TESCO reporter of gSryΔQ mutant was rescued by an ectopic VP16 TAD: Both gSryΔQ+VP and +ChVP showed fully restored ability to induce the TESCO reporter (Fig. 3). gSryΔQ+PVP appeared to have a higher transactivation activity compared with gSry, gSryΔQ+VP, or +ChVP (Fig. 3). This is consistent with a previous report (20) that the PQA domain, although not a TAD per se, contributes to maximizing the transactivation activity of Sox9's TAD. Taken together, these results indicate that the polyQ domain is required for mouse Sry to activate its endogenous target TESCO in an in vitro assay system, by functioning as a TAD.

A Truncated *domesticus*-Type polyQ Domain Stabilizes Sry Protein and Transactivates the TESCO Reporter. Sry protein from *M. domesticus* contains a truncated polyQ tract of only eight glutamine blocks, due to a premature stop codon in the CAG microsatellite (7). Previously, it has been shown that the shortened polyQ tract does not possess transactivation activity when tethered with the GAL4 DNA-binding domain and tested with an artificial reporter containing multiple GAL4-binding sites (21). This raises the questions of whether *domesticus*-type polyQ domain stabilizes Sry protein and functions as a TAD, similar to its longer *musculus*-type counterpart, and whether *domesticus* Sry requires a TAD to function in male sex determination at all.

To address these questions, we generated two constructs (Fig. 4*A*): gDomSry encoding EGFP-tagged *domesticus* Sry (Tirano) (7) and gSryΔQ+DomQ, where gSryΔQ (containing *musculus* HMG + bridge domains) is fused to a *domesticus* polyQ domain, and examined protein stability and ability to activate the TESCO reporter. Consistent with our hypothesis that a C-terminal ballast peptide sequence is required for stabilizing Sry protein, both mutants were readily detected when stably expressed in 15P-1 cells (Fig. 4*B*). Importantly, both gDomSry and gSryΔQ+DomQ were able to synergize with Sf1 to transactivate the TESCO reporter, albeit more weakly than gSry containing a longer *musculus*-type polyQ domain (Fig. 4*A*). The discrepancy between our results and the previous study (21) is likely due to the different reporter constructs used.

These results suggest that the *domesticus*-type polyQ domain functions as a TAD and, although its transactivation activity is weaker than its *musculus* counterpart, is sufficient to activate TESCO, induce *Sox9* expression and effect male sex determination in *M. domesticus*. This conclusion is supported by recent evidence that truncated polyQ domains containing three glutamine blocks are sufficient to enable Sry to induce *Sox9* expression in a rat pre-Sertoli cell line (9).

The polyQ Domain Is Essential for both Sry Protein Stabilization and Transactivation of Its Target Gene *Sox9* in Vivo. Having shown that the polyQ domain possesses a dual role in protein stabilization and transcriptional activation in vitro, we sought to confirm these findings in vivo, by assaying protein stability and sex-determining function in developing mouse fetal gonads. We cloned a set of *Sry* mutants into a novel hyperactive self-inactivating *piggyBac* transposon-mediated vector (22, 23) in which the expression of the transgene is controlled by a constitutively active human ubiquitin C (*UBC*) promoter (Fig. 5*A*). Each construct was microinjected into one-cell mouse zygotes to generate transgenic embryos, with wild-type *Sry* (in the same *piggyBac* vector) serving as a positive control (1, 14).

We recovered the embryos at 13.5 d *post coitum* (dpc) and determined whether XX transgenic embryos developed testes (judged by the presence of testis cords). We assayed transgene expression in the XX transgenic gonads using quantitative RT-PCR (qRT-PCR) at 13.5 dpc; because the constitutive *UBC* promoter controls the transgene expression in this system, we assumed that expression levels would not vary substantially from 11.5 dpc (the critical time point for activating *Sox9*) to 13.5 dpc. Protein expression of each of the *Sry* constructs was assayed by immunofluorescence staining using a GFP antibody. To confirm the sex reversal phenotype, we also analyzed expression of Sox9 and forkhead box L2 (Foxl2), markers for testis or ovary development (4) respectively, in XX transgenic gonads at both the transcript and protein levels.

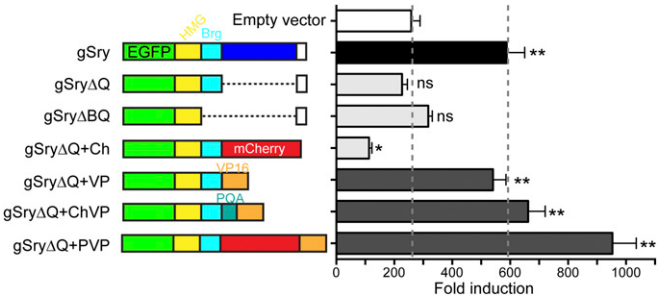

Fig. 3. Mouse Sry requires a functional TAD to activate the TESCO reporter. HEK293 cells were cotransfected with the TESCO-lucII reporter construct in conjunction with an expression construct encoding each of the Sry mutants and either an empty plasmid or an Sf1 plasmid. For simplicity, only the +Sf1 data are presented here as means ± SEM ($n = 3$ to 4). Dashed lines indicate the levels of baseline (empty vector + Sf1) or synergistic activation by gSry+Sf1. Statistical significance was determined using Student *t* test. Statistical significance (*$P < 0.05$ and **$P < 0.01$) compared with cells transfected with the empty vector and an Sf1 plasmid. Unlike gSry, none of the mutant gSryΔQ, gSryΔBQ, and gSryΔQ+Ch were able to synergize with Sf1 to activate the reporter. The transactivation ability was only restored when an ectopic VP16 TAD was appended (gSryΔQ+VP, +ChVP, or +PVP). ns, not significant.

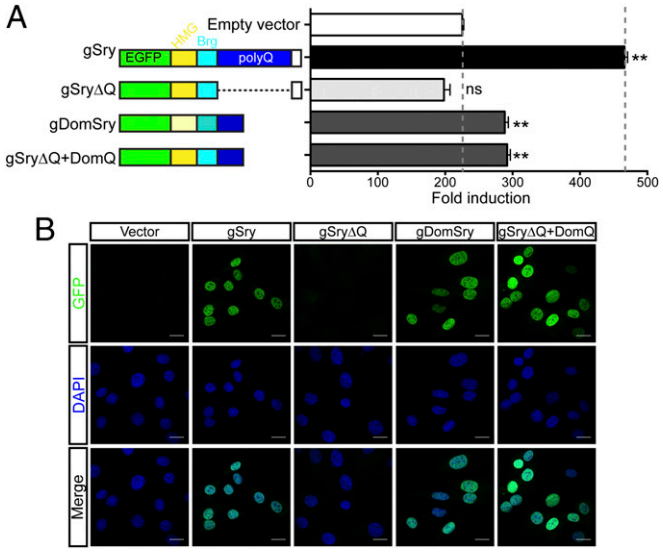

Fig. 4. A truncated polyQ tract from *M. domesticus* stabilizes Sry protein and transactivates the TESCO reporter. (*A*) Both gDomSry and gSryΔQ+DomQ synergized with Sf1 to activate the reporter, albeit more weakly than gSry. TESCO reporter assays were performed as in Fig. 3. Only the +Sf1 data are presented as means ± SEM ($n = 3$). Dashed lines indicate the levels of baseline (empty vector + Sf1) or synergistic activation by gSry+Sf1. $^{**}P < 0.01$, determined using Student *t* tests, compared with cells transfected with the empty vector and an Sf1 plasmid. ns, not significant. (*B*) Both gDomSry and gSryΔQ+DomQ proteins were readily detected by immunofluorescence staining in 15P-1 stable cell lines. (Scale bars: 20 μm.)

Transgenic constructs encoding wild-type Sry or gSry were able to induce *Sox9* expression (Fig. 5*C*) and testis cord formation in 1 of 8 and 1 of 11 cases of XX transgenic embryos, respectively (Table 1), demonstrating the validity of our transgenic system. In the sex-reversed XX gonads, *Sox9* was up-regulated to levels similar to that of wild-type XY testes (Fig. 5 *C* and *E*). Conversely, *Foxl2* was repressed in the sex reversed XX gonads to levels close to those seen in wild-type XX ovaries (Fig. 5*D*). qRT-PCR results revealed a clear threshold level of expression for these two transgenes to induce testis development (Fig. 5*B*), consistent with the notion proposed previously that Sry's ability to induce Sertoli cell differentiation is determined by whether a threshold level of expression is exceeded in the fetal bipotential genital ridges (24, 25).

In contrast, Sry mutant constructs encoding gSryΔQ, gSryΔBQ, and gSryΔQ+Ch, were not capable of inducing *Sox9* expression (Fig. 5*C*) and testis development (Table 1), or repressing *Foxl2* expression (Fig. 5*D*) in XX transgenic gonads, even at expression levels above the threshold (Fig. 5*B*). Consistent with our in vitro findings, gSryΔQ protein was barely detected in transgenic gonads (Fig. 5*E*) despite its transcript being expressed at levels comparable to or higher than gSry (Fig. 5*B*), confirming that the polyQ domain plays a role in stabilizing Sry protein in vivo. In the cases of gSryΔBQ and gSryΔQ+Ch, the mutant proteins were expressed at much higher levels than gSry in transgenic gonads (Fig. 5 *B* and *E*) but nonetheless failed to induce male sex determination (Table 1 and Fig. 5*E*), demonstrating the mere presence of a protein bearing the mouse Sry HMG box is not sufficient to bring about testis development.

Consistent with our in vitro analyses, gSryΔQ+PVP protein was detected in XX transgenic gonads and was able to induce Sox9 expression (Fig. 5 *C* and *E*) and effect testis development (Table 1 and Fig. 5*E*), demonstrating that the presence of a functional TAD is a prerequisite for mouse Sry to activate *Sox9* expression and direct male sex determination in vivo. gSryΔQ+PVP caused male sex reversal at an expression level lower than the threshold of wild-type Sry or gSry (Fig. 5*B*), possibly due to its higher transactivation activity as exhibited in TESCO reporter assays (Fig. 3).

Discussion

Although all SRY proteins contain regions other than the conserved HMG box, it has long been debated whether the non–HMG-box domains play any meaningful roles in sex determination, perhaps because these regions are diverse and poorly conserved, and little is known about the molecular functions they may possess (26). The non–HMG-box domains bear no apparent sequence homology to known TADs and, as exemplified by human SRY,

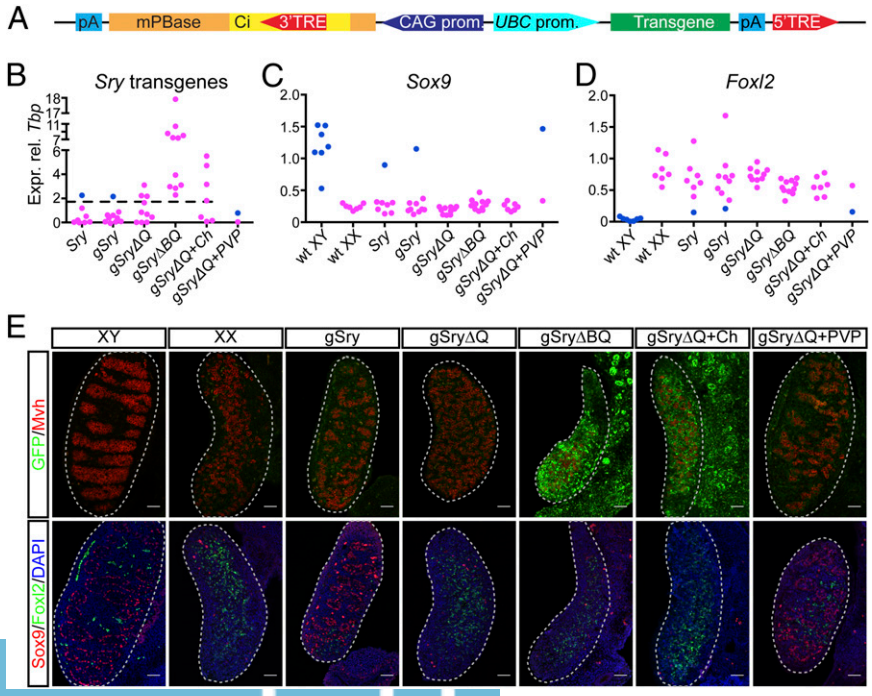

Fig. 5. Analyses of transgenic mouse gonads at 13.5 dpc. (*A*) Schematic diagram of pmhyGENIE3. Ci, chimeric intron; mPBase, mouse codon-optimized *piggyBac* transposase; pA, polyadenylation signal; 5′/3′-TRE, 5′/3′-terminal repeat element. (*B–E*) mRNA expression of the respective transgene (*B*), *Sox9* (*C*), and *Foxl2* (*D*) in XX transgenic embryos recovered at 13.5 dpc was analyzed using qRT-PCR. Wild-type XY and XX embryos were included as controls in *C* and *D*. Each data point represents an individual embryo, with blue dots representing phenotypic males and pink dots representing phenotypic females. The dashed line in *B* indicates the deduced expression threshold for wild-type Sry or gSry to give rise to testis development. This threshold does not apply to gSryΔQ+PVP, which was able to effect testis development at lower expression levels, likely due to its increased transactivation activity. (*E*) Sagittal sections of dissected gonads at 13.5 dpc from wild-type (XY and XX) and XX transgenic fetuses with expression exceeding the threshold level in *B* were analyzed by immunofluorescence. Mvh is a germ cell marker, whereas Sox9 and Foxl2 are markers for Sertoli and granulosa cells, respectively. Gonads are outlined by dashed lines. (Scale bars: 50 μm.)

Table 1. Sex reversal observed in XX transgenic embryos injected with different *Sry* constructs

Tg construct	Embryos recovered	Total Tg	Tg rate, %	XX Tg	High expressers*	Sex reversed	$P^{\dagger}$
Sry	30	18	60	8	1	1	N/A
gSry	44	22	50	11	1	1	N/A
gSryΔQ	40	30	75	10	3	0	0.105
gSryΔBQ	28	20	71.4	11	11	0	0.032
gSryΔQ+Ch	41	10	24.4	7	4	0	0.082
gSryΔQ+PVP	26	6	23.1	2	N/A	1	N/A

N/A, not applicable; Tg, transgenic.

*Number of XX transgenic embryos in which expression of the respective transgene exceeded the deduced threshold level of wild-type Sry and gSry (Fig. 5*B*).

†Barnard's exact test *P* value, compared with gSry.

evidently lack transactivation activity (6). Although the unique mouse Sry C-terminal polyQ domain can function as a transcriptional activation domain in vitro (6, 9), the relevance of this finding for in vivo function has been difficult to discern, given the absence of an identifiable TAD from SRY in nonrodent species. Our previous study showing that mouse Sry mutants lacking the polyQ tract failed to give male sex reversal in transgenic mice was unable to exclude the trivial explanation of protein instability (14). We have now exploited a novel *piggyBac* transgenesis system, the efficiency of which allowed a detailed structure–function analysis of mouse Sry in vivo. The results generated by the combination of in vitro and in vivo approaches (summarized in Fig. S6), in particular the reliance on biochemical and cell-based assays to provide mechanistic insight, and transgenic mouse assays to prove in vivo functional relevance, demonstrate that the polyQ domain not only stabilizes mouse Sry protein, but more importantly, functions as a TAD essential to activate *Sox9* transcription and effect male sex determination in vivo.

The presence of Sry at a suitable level within the pre-Sertoli cells is a prerequisite for male sex determination, and it is now clear that the polyQ domain plays an important role in achieving this situation by preventing Sry from being degraded. Our data indicate that the protein-stabilizing function is not an intrinsic property of the polyQ domain, but rather determined by the presence of potentially any ballast sequence of an appropriate size at the C terminus. The notion of such a size threshold of Sry's C terminus for stabilizing the protein accords with results from a recent study showing that a threshold length of the polyQ tract is required for mouse Sry to induce *Sox9* expression in Sertoli-like cell lines (9). Where this threshold lies may depend on the actual amino acid composition, such that a high percentage of glutamine residues may enable shorter polyQ tracts to stabilize Sry in *M. domesticus* and rats, whereas a longer VP16 TAD with only one glutamine failed to stabilize gSryΔQ+VP protein (Figs. 2 and 4*B*). The mechanisms by which the polyQ domain ameliorates protein instability are still unknown, but it is likely that it masks the sites within the bridge domain that otherwise target Sry to proteasomes for degradation. The results presented here not only formally disprove the possible interpretation of our previous study (14) that the inability of SryStop1 mutant (Fig. S1; similar to gSryΔBQ in the current study) to give male sex reversal might be solely due to protein instability, but also provide in vivo confirmation that the polyQ domain possesses additional essential roles in sex determination besides protein stabilization, namely transcriptional activation.

The roles of the non–HMG-box sequences have remained controversial. A popularly held view is that the HMG box is the only part of Sry needed for activating *Sox9* transcription, perhaps because it is the only conserved domain in Sry, and also is the only common part of several transgenes that give rise to XX male sex reversal in transgenic mouse models (10, 12, 13, 27–29). The assumption underlying this hypothesis is that all SRY proteins use the same or a similar set of conserved mechanisms to activate *SOX9*. In direct challenge to this view, we show that the ability of mouse Sry to effect male sex determination was only restored when a TAD was supplemented, either in the form of the polyQ domain (in wild-type Sry or gSry) or the VP16 TAD (in gSryΔQ+PVP). These results demonstrate that mouse Sry requires a TAD to function in sex determination, thereby excluding models suggesting that the HMG box in the only functional part of SRY.

Given the requirement for a TAD in mice, how is it that human and goat SRY can bring about testis development in XX transgenic mice embryos (10, 11) even though neither protein appears to possess a TAD? We consider it likely that both human and goat SRY can bind to mouse TESCO (the sequence of which is conserved among multiple mammalian species) via the conserved HMG box, and activate *Sox9* by recruiting a partner protein containing a TAD. We hypothesize that in a rodent ancestor, the acquisition of the polyQ domain by the insertion of a CAG microsatellite made the recruitment of a TAD-supplying partner become redundant and unnecessary, and subsequently Sry's ability to recruit such a partner was lost during evolution, possibly due to the rapid degeneration of Y-chromosomal genes. This hypothesis is supported by the recent observation that the polyQ tract allows accumulation of variation elsewhere in the Sry protein, including the disappearance of NTD and deleterious amino acid substitutions in the HMG box (9). Although no longer needed, this partner protein may still be expressed in the mouse fetal genital ridges at the time of sex determination, potentially enabling human/goat *SRY* to direct male sex development when expressed as a transgene in mice. Identification of such partner protein(s) of human SRY in transgenic mouse models may advance our understanding the basis of unexplained clinical mutations involved in human primary sex reversal (26).

The diversity and plasticity of the sex-determining mechanisms in the animal kingdom have long been appreciated (30, 31). In vertebrates, sex can be determined genetically by different systems of sex chromosomes (including XY and ZW) or through environmental cues such as temperature or social situation. In most mammals, sex is determined by SRY (4, 5, 13). Although the sequence, structure, and temporal expression profile of SRY varies considerably among different mammalian species, its function is conserved, namely to activate its conserved target gene, *SOX9*, in the developing testes. Perhaps because both the regulator (SRY) and the target (*SOX9*) are conserved, it has been assumed that the molecular mechanisms involved in this regulation are invariant. Our data support an alternative model in which mouse Sry employs a biochemical mechanism fundamentally different to that of nonrodent SRY. In this regard it is significant that in some rodent species no *Sry* gene or Y chromosome is present at all (32, 33). We speculate that the rapid degeneration associated with Y-chromosomal genes and/or the CAG microsatellite instability may have resulted in truncation of the polyQ tract as in *M. domesticus* species, or in more severe cases, complete loss of the polyQ domain in those species with no *Sry* gene or Y chromosome. In the latter cases, Sry is essentially rendered inactive. This loss of function would have

necessitated the emergence of new (Sry-independent) sex-determining mechanisms in these species.

Materials and Methods

Plasmids. EGFP coding sequence was inserted in frame at the N terminus to the mouse *Sry* coding region. All Sry mutant constructs were generated using QuikChange mutagenesis method (Agilent).

Establishment of Stable Cell Lines. The retrovirus was produced by cotransfecting 293T cells with a pMIH vector (34) each containing the Sry construct and a packaging vector pEQ_{ECO} (35). The 15P-1 cells (obtained from American Type Culture Collection) were infected with individual pMIH virus and subjected to hygromycin selection. The difference in mRNA expression levels of the respective gSry constructs in the stable cell lines is likely due to the variation in titres of the individual pMIH virus.

Immunofluorescence Staining. Immunofluorescence staining was performed on cultured cells or paraffin sections of dissected gonads as described (29, 36), using the antibodies listed in Table S1. Images were taken on a LSM710 confocal microscope (Zeiss).

Western Blot. Western blot analysis was conducted as described (36), using the antibodies listed in Table S1. Proteins were visualized using WestPico reagent (Pierce) on a ChemiDoc machine (Bio-Rad).

qRT-PCR. RNA was extracted from cells or a single gonad using RNeasy kit (Qiagen). cDNA was synthesized using a high-capacity cDNA kit (Life Tech). qPCR was conducted with SYBR Green mix (Life Tech) on a ViiA7 machine (Life Tech). Expression was normalized to *Tbp*. The primers are described in Table S2.

Flow Cytometry. The 15P-1/vector and 15P-1/gSryΔQ cells were treated with vehicle control (ethanol), 1 μM epoxomicin, or 5 μM MG-132 for 16 h, and analyzed on a FACSCanto II flow cytometer (Becton Dickinson).

Luciferase Reporter Assays. HEK293 cells were transfected with a pTESCO-δ51-LucII reporter construct (3), a Sry construct, and either an empty pcDNA3 or a pcDNA-Sf1 plasmid (37). The plasmid dilution studies described in ref. 9 provide assurance that the overexpression of Sry and Sf1 is unlikely to cause spurious results in this case. A cytomegalovirus-Renilla luciferase plasmid was included as a control for transfection efficiency. Cell lysates were harvested after 48 h and luciferase activities analyzed using a Dual Luciferase kit (Promega) on a POLARstar Omega luminometer (BMG Labtech).

Microinjection and Genotyping. Four nanograms per microliter of each pmhyGENIE3 plasmid were injected into one pronuleus of one-cell zygotes as described (14). Embryos were cultured overnight and transferred to pseudopregnant CD1 mice. Embryos were recovered at 13.5 dpc and genotyped by PCR for genetic sex (38) and presence of the transgene, using the primers listed in Table S2. All animal procedures were approved by the University of Queensland Animal Ethics Committee.

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