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Sex reversal following deletion of a single distal enhancer of *Sox9*

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Cell fate decisions require appropriate regulation of key genes. *Sox9*, a direct target of SRY, is pivotal in mammalian sex determination. In vivo high-throughput chromatin accessibility techniques, transgenic assays, and genome editing revealed several novel gonadal regulatory elements in the 2-megabase gene desert upstream of *Sox9*. Although others are redundant, Enh13, a 557–base pair element located 565 kilobases 5′, is essential to initiate mouse testis development; its deletion giving XY females with *Sox9* transcript levels equivalent to XX gonads. Our data are consistent with the time-sensitive activity of SRY and indicate a strict order of enhancer usage. Enh13 is conserved and embedded within a 32.5-kilobase region whose deletion in patients is associated with XY sex reversal, suggesting it is also critical in humans.

The regulation of genes with important roles in embryonic development can be complex, involving multiple, often redundant enhancers, repressors, and insulators (*1*, *2*). The genes may have a poised epigenetic state prior to their expression, and their activation or repression may involve positive or negative feed-forward loops. This complexity is likely to be amplified when the gene has functions in more than one tissue, given that the regulatory elements required for each are often interspersed and necessitate dynamic alterations in chromatin conformation (*1*, *2*). The developing gonads comprise an interesting system in which to explore questions of gene regulation during development (*3*). Most of the cell lineages are bipotential, with the ability to give rise to cell types typical of either ovaries or testes, and many genes that become associated with male or female fate begin by being expressed at equivalent, although usually low, levels in supporting cell precursors of both XX and XY gonads (*4*–*6*).

In mammals, the *Sry* gene encodes a protein that is transiently expressed and initiates testis and subsequent male development by triggering cells of the supporting cell lineage to differentiate into Sertoli rather than granulosa cells typical of ovaries (*7*). *Sox9*, the main target of SRY, is critical for the differentiation of Sertoli cells and then functions along with other transcription factors, notably *Sox8* and then *Dmrt1*, for their maintenance (*4*–*6*). Both gain- and loss-of-function studies in mouse and human demonstrate that *Sox9* plays a key role in testis determination (*8*–*13*). Notably, humans heterozygous for null mutations develop campomelic dysplasia (CD) [Online Mendelian Inheritance in Man (OMIM) entry 114290] (*11*), a severe syndrome where 70% of XY patients show female development (*12*, *13*).

Sox9 functions in many embryonic and adult cell types (*14*), and genetic and molecular evidence suggests that its regulatory region is spread over at least a 2 Mb gene desert 5′ to the coding sequence (*15*). The only enhancer known to be relevant for expression in Sertoli cells was TES, a 3.2-kb element mapping 13 kb 5′, and its 1.4-kb core, TESCO (*16*). Targeted deletion of TES or TESCO reduced *Sox9* expression levels in the early and postnatal mouse testis to about 45% of normal but did not result in XY female development (*17*). We therefore used several unbiased approaches to systematically screen for additional gonad enhancers upstream of mouse *Sox9*. We first made use of DNaseI-seq data obtained with embryonic day 13.5 (E13.5) and E15.5 sorted Sertoli cells (*18*). From 33 putative enhancers, we chose only those positive at both stages for in vivo validation by transgenic assays (14 enhancers) (Fig. 1A and fig. S1). In parallel, we carried out ATAC-seq on XY and XX gonads, which permitted the use of fewer sorted cells at E10.5, an early bipotential stage, and E13.5, when gonadal sex is already determined (figs. S1 and S2 and methods). Most putative enhancers discovered by DNaseI-seq were evident in the E13.5 XY ATAC-seq data;

however, we used this to include two more in the in vivo screen: enhancer 1 (Enh1) and Enh14 (Fig. 1A and fig. S1). Chromatin immunoprecipitation-seq (ChIP-seq) was also performed for H3K27ac, a histone modification that marks active enhancers (fig. S1).

All 16 putative enhancers were cloned upstream of an *hsp68* minimal promoter and the reporter gene *LacZ* and used to generate transgenic mice (*2*, *19*) (table S1). For initial screens, we performed transient analyses at E13.5. Twelve failed to give any gonadal β-galactosidase (β-Gal) activity, although many showed staining in other tissues in which *Sox9* is normally expressed, such as chondrocytes, brain, and spinal cord (fig. S3). The remaining four showed gonad expression, and these constructs were reinjected to generate stable lines in order to better study their activity in both males and females during development. Enh8 [672 base pairs (bp) long, 838 kb 5′] conferred robust β-Gal activity in the ovary, whereas it was barely present in the testis at E13.5 (Fig. 1A and fig. S4B). This may be due to Enh8 being taken out of its original genomic context; notably, ATAC-seq reveals a much stronger peak in granulosa cells compared to Sertoli cells (fig. S4A).

In contrast, Enh14 (1287 bp long, 437 kb 5′) showed robust testis-specific β-Gal activity (Fig. 1A and fig. S5B). DNaseI-seq, ATAC-seq, and H3K27ac ChIP-seq all suggest that this enhancer is active and open only in Sertoli cells (figs. S1 and S5A). To test this candidate, we used genome editing to delete Enh14. However, Enh14 deletion did not alter expression of *Sox9*, its target gene *Amh*, or *Foxl2*, a marker of granulosa cells, in E13.5 XY gonads (fig. S5D), indicating that Enh14 has a redundant role, at least in the embryo. Enh32 (970 bp long, 10 kb 5′) is also testis-specific, but very weak and restricted to a domain close to the mesonephros (Fig. 1A and fig. S6, B and C). ATAC-seq, DNaseI-seq, and H3K27ac ChIP-seq data suggest that this is a Sertoli cell enhancer, although weak peaks were seen in the granulosa cell samples (figs. S1 and S6A).

The remaining enhancer, Enh13 (557 bp long, 565 kb 5′), is highly conserved among mammals and located toward the distal 5′ end of a 25.7-kb region in mice showing conserved synteny with a 32.5-kb region upstream of human *SOX9* termed XY SR, whose deletion is associated with sex reversal (*20*) (Fig. 1, A and B, and fig. S1). Enh13 shows the strongest Sertoli cell–specific peak within this region, in both the DNaseI-seq and ATAC-seq data. H3K27ac ChIP-seq data mark Enh13 as active in both Sertoli and granulosa cells, which may support the observation that some transgenic lines also exhibit β-Gal activity in the ovary (Fig. 1C and figs. S1 and S7A). β-Gal expression is clearly within Sertoli and granulosa cells (fig. S7C). ATAC-seq data from E10.5 genital ridges show that Enh13 is not open at this stage, irrespective of chromosomal sex (Fig. 1B and fig. S1), suggesting that it opens coincident with specification of the supporting cell lineage from SF1-positive cells of the coelomic epithelium (*5*).

Genome editing was used to derive mice homozygous for deletions of Enh13. Irrespective of whether this was carried out on a TES mutant background or by itself it always led to XY female development (Fig. 2 and figs. S8 to S10). The latter result was surprising, because if TES accounts for 55% of *Sox9* expression in early Sertoli cells, any additional enhancer(s) should not account for more than 45%, and, when deleted, levels of *Sox9* would remain higher than the threshold of about 25% below which sex reversal might be expected (*17*). Nevertheless, at embryonic stages, whereas XY Enh13$^{+/-}$ embryos still undergo normal testis development, XY Enh13$^{-/-}$ embryos produce ovaries indistinguishable from those of XX wild-type embryos, with no signs of testis cords or a coelomic vessel (Fig. 2, B and C, and fig. S10). Immunofluorescence analysis for SOX9 and FOXL2 of E13.5 and 6-week-old XY Enh13$^{+/-}$ and Enh13$^{-/-}$ gonads showed that the former are still testes whereas the latter are fully sex-reversed ovaries (Fig. 2C and fig. S10D). Similar analysis of XX Enh13-deleted gonads did not show any obvious phenotype (fig. S11).

The Enh13 deletion was generated on a C57BL/6J genetic background, which is sensitized toward XY female sex reversal (*21*). To test the strength of the deleted allele, we therefore backcrossed it onto a mixed C57BL/6J × CBA background. As before, XY heterozygotes presented as normal fertile males whereas homozygotes showed full male-to-female sex reversal (fig. S12).

We could detect no difference in gonadal phenotypes between Enh13$^{-/-}$:TES$^{+/+}$ and Enh13$^{-/-}$:TES$^{-/-}$ embryos or mice, suggesting that homozygosity for the Enh13 deletion alone reduced *Sox9* levels well below the critical threshold required for testis development, which had been determined at E13.5 (*17*) (figs. S8 and S10). However, examining levels of gene expression at this stage when there is sex reversal will be uninformative because factors such as WNT4 and FOXL2 repress *Sox9* once ovary development begins (*5*). We therefore analyzed *Sox9*, *Sry*, *Sf1*, and *Foxl2* mRNA at E11.5, during the brief period when gonadal sex is being determined. qRT-PCR revealed that XY Enh13$^{+/-}$ and Enh13$^{-/-}$ genital ridges expressed 58 and 21% of the wild-type levels of *Sox9* mRNA, whereas XY TES$^{+/-}$ and TES$^{-/-}$ genital ridges showed 55 and 50%, respectively (Fig. 3A). In control genital ridges, XX contained 18% of the *Sox9* mRNA levels found in XY (Fig. 3A). Therefore, E11.5 XY Enh13$^{-/-}$ gonads express *Sox9* at levels close to those of XX gonads at the same stage, explaining the observed complete sex reversal. Deleting one or two copies of TES had relatively little effect at E11.5, especially compared to at E13.5, and is in contrast to the results with Enh13 deletions at E11.5 (Fig. 3A) (*17*). This again supports the conclusion that Enh13 plays a more significant role than TES during

early gonadal development.

Sry expression is normally down-regulated as SOX9 levels increase, but it can persist if testis differentiation fails (*22*, *23*). This is consistent with a direct or indirect repressive effect of SOX9 on *Sry*. At E11.5, *Sry* mRNA levels were higher compared to wild-type in both Enh13$^{+/-}$ and Enh13$^{-/-}$ XY gonads (168 and 152%, respectively) (Fig. 3B). In contrast, the TES deletion did not significantly alter *Sry* expression (Fig. 3B), which is expected because Sertoli cell differentiation is proceeding in these mutants. SF1 is known to interact first with SRY and later with SOX9 to regulate *Sox9* expression levels and also many of their downstream target genes (*16*). We found no significant changes in levels of *Sf1* mRNA in any of the enhancer deletions at E11.5 (Fig. 3C). Using *Foxl2* as an early marker of granulosa cell differentiation (*24*), mRNA levels were 3.6-fold higher in XX than XY wild-type gonads at E11.5 (Fig. 3D). Compared to the latter, Enh13$^{+/-}$ and Enh13$^{-/-}$ XY gonads showed a 2-fold and 3-fold increase, respectively, with the homozygotes having very close to XX control levels. Therefore, Enh13$^{-/-}$ XY gonads reveal an early commitment to the ovarian pathway.

There was a 30 to 50% decrease in *Sox9* mRNA levels in E11.5 XX Enh13$^{-/-}$ gonads compared to wild type, reflected by reduced immunofluorescence for SOX9 protein (fig. S11). These data indicate that Enh13 also plays a role in the very early expression of *Sox9* in the XX gonad, consistent with both the small peak seen with ATAC-seq and with occasional reporter activity in the transgenic mouse assays (Fig. 1, B and C, and fig. S7).

The sequence of Enh13 is highly conserved among mammals (Fig. 1B) and contains consensus binding sites for transcription factors known to regulate early gonad development and sex determination (fig. S13) (*6*). Mouse Enh13 contains a single consensus SRY binding site as well as a SOX9 site to which SRY can also bind (Fig. 4A and fig. S13). ChIP-qPCR on E11.5 gonads dissected from *Sry-Myc* transgenic embryos was performed using a specific antibody against the MYC tag (*22*). There was an 11-fold enrichment in SRY-MYC–positive versus negative gonads with primers spanning the SOX9 consensus site and a sixfold enrichment with primers spanning the SRY site, whereas primers against the strongest SRY binding site in TESCO (*22*) showed fivefold enrichment (Fig. 4, A and B). This reveals the strong binding of SRY to Enh13 at E11.5, with a preference for the SOX9 consensus site, possibly due to the adjacent SF1 binding site. Preferential binding of SRY to Enh13 over TESCO at E11.5 supports the hypothesis that the former is more critical because it initiates up-regulation of *Sox9* while the latter is secondary.

ChIP assays revealed SOX9 to be bound at similar levels to both Enh13 and TESCO in cells from E13.5 testes (Fig. 4C). In addition, SOX9 ChIP-seq data using bovine embryonic testis (*25*) revealed a strong peak localizing to the conserved syntenic region of Enh13 (537 bp long, 570 kb 5′) (Fig. 4D). This suggests that, like TES, Enh13 is used by SOX9 to autoregulate its expression, and that this interaction is conserved in mammals. Unlike several other gonadal enhancers, Enh13 appears well conserved, has a clear role in mice to initiate up-regulation of *Sox9* expression, in response to SRY activity, and it may contribute to its maintenance. This makes it very likely to play a similar role in humans given its location within the XY SR region (*20*, *26*). If so, heterozygosity for Enh13 deletions in humans should mimic heterozygosity for null mutations in SOX9, with XY female sex reversal occurring in about 70% of cases but perhaps without other CD phenotypes.

It is clear from our data and that of others that the upstream regulatory region of *Sox9* is very complex. Our screens using gonadal cells revealed 33 potential enhancers distributed over 1.5 Mb. Transgenic assays used to test the most promising 16, revealed four that gave expression in the gonads, whereas the majority did not. This "hit rate" of 25% agrees with some other studies (*19*) and merits caution in interpreting data based solely on accessibility of chromatin and histone marks. However, some are bona fide enhancers in other locations; for example Enh29, mapping about 70 kb 5′, is equivalent to "SOM", an enhancer active in many tissues except the gonads (*27*). Others may have distinct roles; indeed Enh11 contains a putative CTCF binding site. In addition, several putative enhancers, notably Enh4, 5, 8, and 9, appear open in both granulosa and Sertoli cells, with Enh8 even more so in the former. These could contribute to the low level of *Sox9* seen in supporting cell precursors, but they may also represent sequences required to repress *Sox9*, which might not be detected by transgenic reporter assays.

The notion of redundancy or "shadow enhancers" within a regulatory region is well established (*28*, *29*), and recent data suggest that deletion of single, even "ultraconserved" enhancers from developmentally important genes can have at most subtle if not undetectable effects (*30*, *31*). It is therefore remarkable to see that deleting Enh13 alone phenocopies the loss of *Sox9* itself within the supporting cell lineage (*9*, *32*). Substantial evidence points to the time-dependent action of SRY on *Sox9*. If *Sox9* fails to reach a critical threshold within a few hours, then ovary-determining/anti-testis factors, such as Wnt signaling, accumulate to a sufficient level to repress *Sox9* and make it refractory to male-promoting factors, including SRY, even though expression of the latter persists in XY gonads when Sertoli cells fail to differentiate (*33*). We suggest that Enh13 is an early-acting enhancer, such that without it *Sox9* transcription fails to increase to a level where the other enhancers can act before the gene is silenced. It is only later that TES, and perhaps other enhancers such as Enh14, and Enh32, begin to act in a more redundant fashion, alt-

hough it is conceivable that each has a major role to play during distinct phases of Sertoli cell development from the fetal to the adult testis. It will be of interest to determine how Enh13 activity cascades into the recruitment of the other enhancers.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/cgi/content/full/science.aas9408/DC1
Materials and Methods
Figs. S1 to S13
Tables S1 to S4
References (*34*–*44*)

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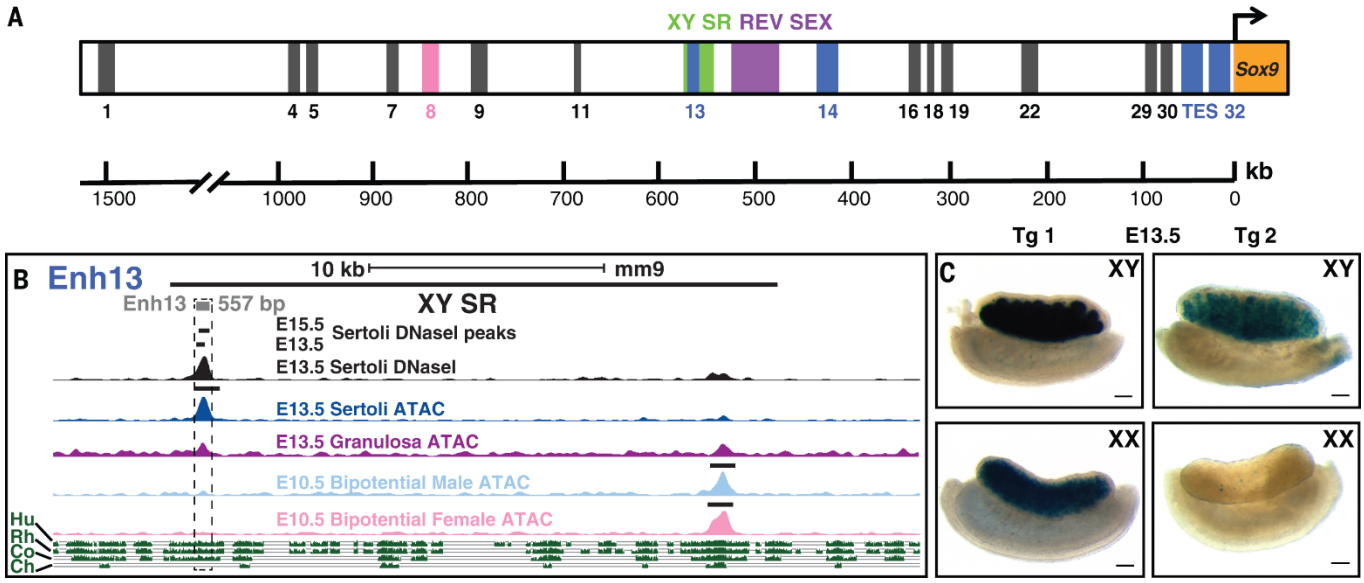

Fig. 1. Enh13 is a testis-positive enhancer of *Sox9* located within the XY SR region. (**A**) A schematic representation of the gene desert upstream of the *mSox9* gene and the locations of the putative enhancers identified by ATAC-seq and DNaseI-seq that were screened in vivo using transgenic reporter mice. Enhancers that did not drive gonad expression of LacZ are shown in gray. Enhancers that drove testis-specific and ovary-specific LacZ expression are shown in blue and pink, respectively. The mouse regions that show conserved synteny with the human XY SR and REV SEX are depicted in green and purple boxes, respectively. (**B**) Enh13 (gray box) is located at the 5′ side of the 25.7-kb mouse equivalent XY SR locus (black rectangular box). DNaseI-seq (black) on E15.5 and E13.5 XY sorted Sertoli cells and ATAC-seq on E13.5 sorted Sertoli cells (blue) and granulosa cells (purple), as well as E10.5 sorted somatic cells, at Enh13 genomic region are presented. Peaks correspond to nucleosome depleted regions, and are marked by black box if they are significantly enriched compared to flanking regions as determined by MACS, and present in at least two biological replicates. The gray box overlaying each peak indicates the cloned fragment. Green lines represent sequence conservation between mouse, human, rhesus, cow, and chicken (sequence conservation tracks obtained from UCSC). (**C**) β-Gal staining (blue) of E13.5 testes and ovaries from two representative independent stable Enh13 transgenic lines. Scale bars, 100 μm.

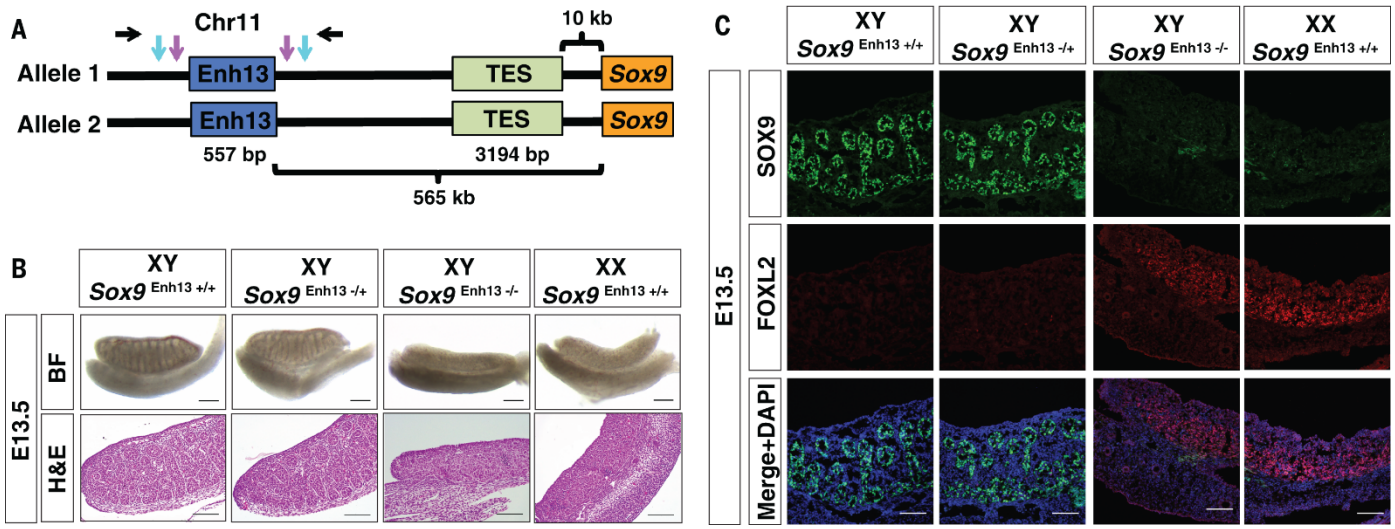

Fig. 2. Deletion of Enh13 leads to complete XY male-to-female sex reversal. (**A**) A schematic representation of the locations of Enh13 and TES upstream of *Sox9*. Turquoise and purple arrows represent the external and internal sgRNAs used to delete Enh13. Black arrows represent the PCR primers used to genotype Enh13 deleted embryos and mice. (**B**) Bright-field pictures and hematoxylin and eosin (H&E)–stained sections of E13.5 XY Enh13$^{+/+}$, Enh13$^{+/-}$, and Enh13$^{-/-}$ and XX Enh13$^{+/+}$ gonads. (**C**) Immunostaining of E13.5 gonads from XY wild type, Enh13$^{+/-}$, and Enh13$^{-/-}$ and XX wild type. Gonads were stained for Sertoli marker SOX9 (green), granulosa marker FOXL2 (red), and 4′,6-diamidino-2-phenylindole (DAPI) (blue). Sex-reversed gonads are indistinguishable from wild-type XX gonads, while the heterozygous deletion does not appear to alter testis morphogenesis. Scale bars, 100 μm.

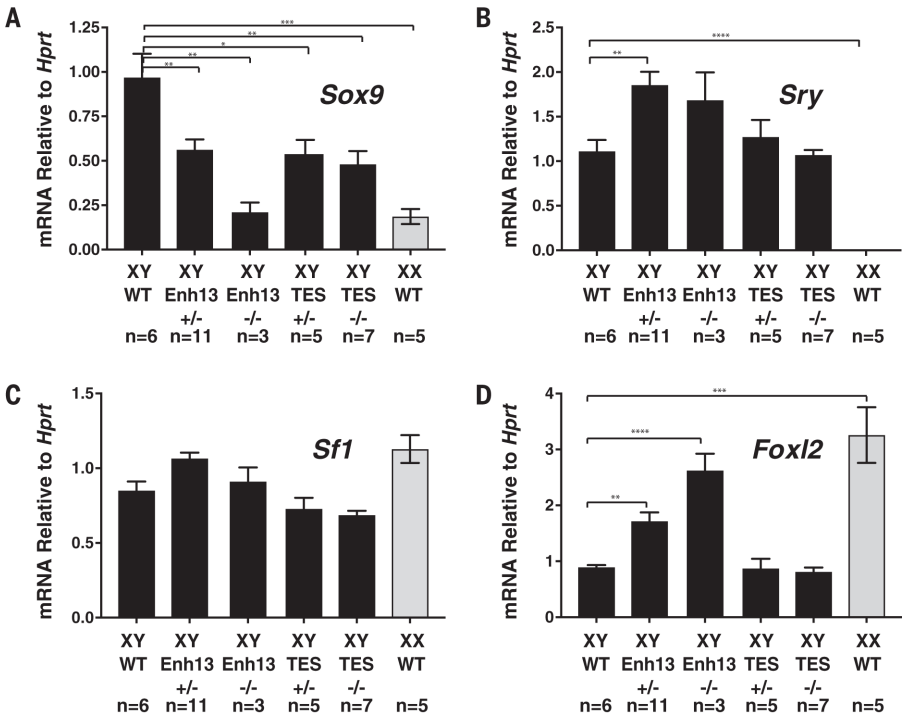

Fig. 3. Enh13 regulates the expression of the *Sox9* gene in vivo. (**A** to **D**) Real-time quantitative PCR of genes involved in male (*Sox9*, *Sry*, and *Sf1*) and female (*Foxl2* and *Sf1*) sex determination in XY Enh13 deleted and TES deleted gonads at E11.5 (18 tail somites). Data are presented as mean $2^{-\Delta\Delta Ct}$ values, normalized to the housekeeping gene *Hprt*. Sample size represents the number of individuals and is indicated below each genotype. Error bars show SEM of $2^{-\Delta\Delta Ct}$ values. *P* value is presented above the relevant bars (unpaired, two-tailed *t* test on $2^{-\Delta\Delta Ct}$ values; $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$). Dark gray bars: XY; light gray bars: XX.

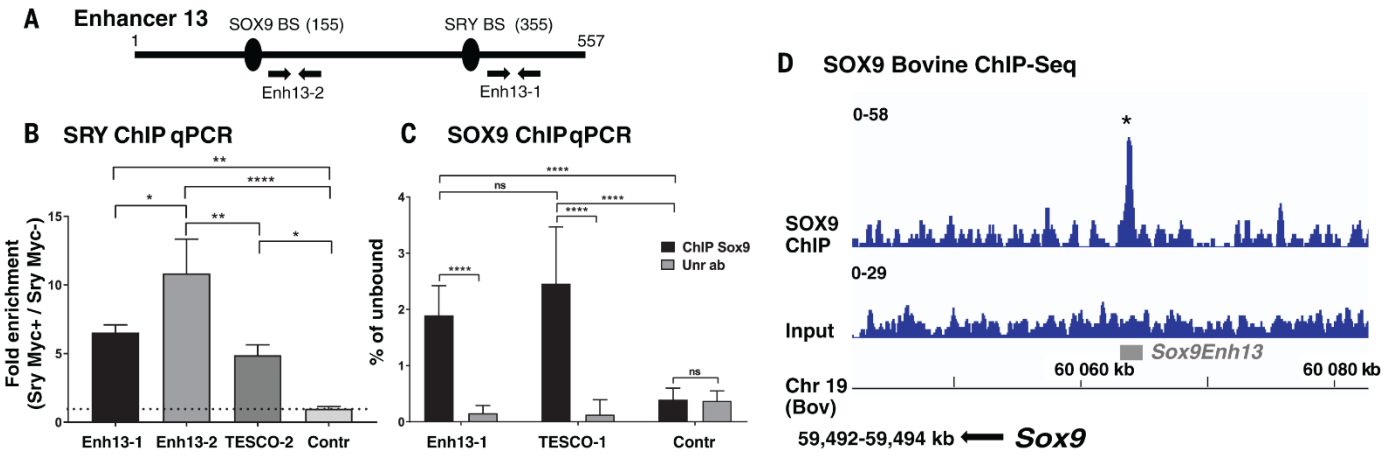

Fig. 4. Enh13 is bound by SRY and SOX9 in vivo. (**A**) A schematic representation of the location of the primers used for ChIP experiments in Enh13. (**B**) ChIP-qPCR assay of E11.5 mouse genital ridge following immunoprecipitation with anti-cMYC antibody. Data are presented as fold enrichment of SRY-MYC–positive relative to SRY-MYC–negative genital ridges, meaning that values greater than 1 represent specific enrichment. The primers used span the putative SRY binding site in Enh13, TESCO [around SRY R6 site; see (*16*)] and a negative control region on Chr11. Data are the mean ± SD ($n = 2$); ****$P < 0.0005$ (Student t test). (**C**) ChIP-qPCR assay of E13.5 mouse testes following immunoprecipitation with anti-SOX9 antibody. The primers used span the putative SOX9 binding site in Enh13, TESCO [around SOX9 R1 site; see (*16*)] and a negative control region on Chr11. Data are the mean ± SD ($n = 3$); ****$P < 0.0005$ (Student t test). (**D**) ChIP-seq with anti-SOX9 antibody using E90 fetal bovine testis. The bovine Enh13 is indicated by the gray box (at Cow Bostau8 chr19: 60,063,628-60,064-165). The star represents the peaks with FDR <0.05 in the two bovine datasets. *y*-axis numbers represent counts. The input tracks represent sequencing reads of chromatin input. The *bSox9* gene is indicated by the arrow and is 570 kb downstream (to the left) of Enh13.

Sex reversal following deletion of a single distal enhancer of *Sox9*

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