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GATA4 binding to the *Sox9* enhancer *mXYSRa/Enh13* is critical for testis differentiation in mouse

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In mammals, *SOX9*/*Sox9* expression in embryonic gonads is essential for male gonadal sex determination. Multiple enhancers of *Sox9* have been identified, of which the *mXYSRa/Enh13* enhancer plays a crucial role in mice. SOX9 and SRY binding sites within the enhancer have been identified as functional. Simultaneous deletion of both sites in mice resulted in male-to-female sex reversal. However, the existence of other critical functional sequences remains unclear. This study identified an additional functional sequence by generating mice with partial deletions in *mXYSRa/Enh13*. Two nucleotide substitutions within the sequence were sufficient for male-to-female sex reversal. In vivo binding assay by CUT&RUN revealed that GATA4 binds to the sequence. In vitro luciferase assay showed that GATA4 promotes the enhancer activity and the substitution of the sequence reduces the effect. Taken together, the functional sequence in *mXYSRa/Enh13* is essential for testis differentiation and requires GATA4 binding.

In mammalian sex determination, male sex is determined by the expression of Y-linked *Sry* during the critical time window in the embryonic gonads. SRY is assumed to upregulate the autosomal gene, *Sox9*. In mice, when *Sry* is expressed in the embryonic gonads on embryonic day 10.5 (E10.5), *Sox9* expression is upregulated[1]. In addition, overexpression of *Sox9* in the gonads of XX embryos leads to testis differentiation and subsequent development of male genitalia[2]. Thus, SOX9 is thought to be a master regulator of testicular differentiation. Once the expression of *Sox9* in male embryonic gonads is elevated by SRY, *Sox9* expression is maintained by a positive feedback loop including fibroblast growth factor 9 (FGF9) and SOX9 auto-regulatory system, although *Sry* expression is temporal. High-level expression of *Sox9* in supporting cells triggers the differentiation of supporting cells into Sertoli cells, which induces testis differentiation and the subsequent development of male genitalia.

In humans, heterozygous mutations in *SOX9* cause campomelic dysplasia (CMPD, OMIM 114290), which is characterized by severe skeletal features, such as distinctive facies, Pierre Robin sequence with cleft palate, and shortening and bowing of long bones. Seventy percent of patients with CMPD with the XY karyotype display differences/disorders of sex development (DSD)[3–6]. Most patients with CMPD have pathogenic mutations in the *SOX9* coding sequence. However, some patients with CMPD have chromosomal rearrangements with breakpoints upstream or downstream of SOX9 without a mutation in the *SOX9* coding region[7], suggesting that there are regulatory sequences for *SOX9* expression in these regions.

In humans and mice, *SOX9*/*Sox9* is located in a "gene desert" spanning >2 Mb, and several regulatory regions have been found. The testis-specific enhancer (TES) was the first regulatory sequence associated with *Sox9* expression in murine embryonic gonads[8]. A mouse model harboring a deletion of TES was generated and analyzed[9]. Although the expression level of *Sox9* in embryonic gonads decreased to 45–60% compared to that in the wild-type, the mice developed testes. This suggests that the function of TES is redundant, and there should be additional regulatory sequences that

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upregulate *Sox9* expression above the threshold for testis development in embryonic gonads.

In 2015, another regulatory region for *SOX9*, XYSR, was identified 640 kb upstream of *SOX9* according to a genetic analysis of 46,XY patients with DSD[10]. As these patients did not develop skeletal phenotypes, this region was considered a testis-specific enhancer. Eventually, *Enh13* was identified as another *Sox9* enhancer 565 kb upstream of *Sox9*[11]. ATAC-seq, DNase I-seq, and chromatin immunoprecipitation (ChIP)-seq for histone mark H3K27Ac were used in mouse gonads to identify *Sox9* enhancers. In the same year, we identified the *mXYSRa* enhancer by generating a series of mice with deletions in the genomic region corresponding to human XYSR[12]. *mXYSRa* overlaps *Enh13*. XY mice with a homozygous deletion of *mXYSRa/Enh13* show male-to-female sex reversal, suggesting that *mXYSRa/Enh13* is the enhancer that dominantly upregulates *Sox9* expression in mouse embryonic gonads[11,12].

Gonen et al.[11] studied the molecular mechanisms underlying *Sox9* upregulation via *mXYSRa/Enh13*. They showed that SOX9 and SRY bind to *mXYSRa/Enh13* using ChIP-qPCR of embryonic gonads, and these binding sites (BSs) were designated as the SOX9-BS and SRY-BS, respectively. In 2023, we confirmed that SOX9-BS and SRY-BS were functional sequences of *mXYSRa/Enh13* by generating mutant mice, in which SOX9-BS and SRY-BS were substituted with G stretch sequences[13]. XY mice with homozygous mutations in either SOX9-BS or SRY-BS developed a male reproductive system, whereas XY mice with homozygous mutations in both SOX9-BS and SRY-BS developed small testes. This suggests that SOX9-BS and SRY-BS are functionally redundant and these two BSs are insufficient for upregulating *Sox9* to the level required for testis differentiation. In 2024, Ridnik et al.[14] also validated the functionality of these two BSs in vivo by deletion. The authors revealed that NR5A1-BS is one of a functional sequence in *mXYSRa/Enh13* that contributes to *Sox9* expression. In addition, mutant XY mice with either SOX9-BS or SRY-BS deletion carried a male reproductive system, whereas those with a double deletion displayed male-to-female sex reversal, suggesting that SOX9-BS and SRY-BS are functionally redundant, which is compatible with our results. However, the phenotype observed in our mice with the G stretch substitution was milder than that observed in mice with the deletion. The authors concluded that this inconsistency resulted from the binding of WT1, another transcription factor associated with gonadal formation, to the G stretch.

According to the results from our mouse model with substitutions at SOX9-BS and SRY-BS, we considered that there should be remaining functional sequences within *mXYSRa/Enh13* that are essential for *Sox9* upregulation. Therefore, we aimed to identify additional functional sequences at a single nucleotide level. To achieve this, we generated a series of mutant mice with either deletions or substitutions of the candidate sequences. Eventually, we revealed an additional functional sequence, the GATA4 BS, which is critical for testis differentiation, in addition to SOX9-BS and SRY-BS.

Results

Search for an additional functional sequence within the *mXYSRa/Enh13* region in vivo

To screen for additional functional sequences within the *mXYSRa/Enh13* region, we generated a series of mice with partial deletions in *mXYSRa*. To identify candidates efficiently, sperm were collected from male mice deficient for the entire *mXYSRa/Enh13* (4.7 kb), in the mixed genetic background of C57BL/6 and DBA/2[12], allowing us to analyze the phenotype at the F_0 generation (Fig. 1A, B). First, the fertilized eggs were divided into three groups, and gRNA mixes with hCas9 were microinjected into each group (Group 1: gRNA A, and H; Group 2: gRNA H and I; and Group 3: gRNA I and J) (Fig. 1A). All XY mice in Groups 2 and 3 displayed male-type genitalia, whereas XY mice in Group 1 developed female genitalia. The male-to-female sex-reversed mice in Group 1 had 384 bp deletion (Δ384 bp). Although the region included SOX9-BS, which was previously identified (Fig. 1B), an additional functional sequence is supposed to reside in this region because dysfunction of SOX9-BS alone does not cause male-to-female sex reversal[13,14].

Second, the same experiment was carried out using three other gRNA mixtures (Group 1-1:gRNA A and B, Group1-2: gRNA B and D, and Group1-3: gRNA D and H). Male-to-female sex-reversed mice were identified only in Group 1-3 (gRNA D and H, Δ106 bp). All XY mice in Group 1-1 had male-type genitalia. For Group 1-2, we failed to obtain any deleted mice. These results suggest that an additional functional sequence exists between gRNA D and H, where no BSs have been identified (Fig. 1B).

As a third step, the same experiment was performed using two gRNA mixes (Group 1-3-1: gRNA C and G and Group 1-3-2: gRNA E and H) to narrow down functional sequence, including the region from C to D. Male-to-female sex-reversed mice were identified in both groups (gRNA C-G:Δ99 bp; gRNA E-H:Δ61 bp), suggesting that the functional sequence is in the 49-bp overlapping region from E to G (Fig. 1B).

An additional generation of mutant mice with microinjection of gRNA F and hCas9 revealed that a nine-base-pair deletion resulted in a male-to-female sex reversal (Fig. 1B, Supplementary Fig. S1A, B, Supplementary Table S1).

Among these nine nucleotides, we identified a "GATA" sequence in the minus strand that could be a binding motif for GATA transcription factors (GATA-BS). Many critical sequences are evolutionarily conserved, therefore, we aligned mouse *mXYSRa* with its corresponding human sequence, eSR-A. As shown in Supplementary Fig. S2, "GATA" sequences are completely conserved in both species (Fig. 1B, Supplementary Fig. S2).

GATA4 binds to the putative GATA-BS in the *mXYSRa/Enh13* in embryonic gonads in vivo

We assumed that GATA4 is the transcription factor that binds to the putative GATA-BS among the GATA family and drives *mXYSRa/Enh13* activity for the following reasons. First, only GATA4 and GATA6 are expressed in embryonic gonads[15]. Secondly, in XY mice with conditionally deleted *Gata6* in the embryonic gonads and adrenal glands, gonadal histology appears normal[16]. Third, GATA4 is supposed to associate with *Sox9* upregulation in *cis* according to the previous study[17]. In the XX embryos of *Ods* mice, a transgene insertional mutation caused forced expression of *Sox9* in the embryonic gonads and led to testis differentiation. However, a homozygous mutation in the FOG2 interactive domain of *Gata4* suppressed *Sox9* expression in *Ods* mice. To validate GATA4 binding to *mXYSRa/Enh13* in gonads at E11.5, when *Sry* was expressed, CUT&RUN was used because it is suitable for the analysis of in vivo binding of a protein to genomic DNA from a small number of cells[18,19]. A total of 3,489 GATA4 binding regions were identified in the genome. The GATA4 motif was significantly enriched in GATA4 consensus sequence (Fig. 2A), indicating that CUT&RUN in this experiment detected GATA4-specific signals. GATA4 binding regions were found around the *Sox9* locus, one of which overlapped with *mXYSRa/Enh13* (Fig. 2B). Upon closer examination, the center of the GATA4 signal in *mXYSRa/Enh13* perfectly matched that of the putative GATA-BS (Fig. 2C). These results demonstrate that GATA4 binds to the region around the putative GATA-BS within *mXYSRa/Enh13* in the gonads at E11.5.

GATA4 is associated with enhancer activity of *mXYSRa* through the putative GATA-BS

To examine whether GATA4 is associated with the enhancer activity of *mXYSRa* through the putative GATA-BS, we performed an in vitro reporter assay. *Gata4* expression resulted in the upregulation of reporter activity irrespective of substitution at the GATA-BS, whereas the upregulation of reporter activity was higher in the wild-type reporter plasmid than in the reporter plasmid with the substitution (Fig. 3). According to the results of the CUT&RUN and reporter assays, we confirmed that the nine nucleotide-functional sequences elucidated from the serial analysis of mutant mice were GATA4 BS (GATA4-BS).

Identification of a functional sequence at a single nucleotide level in vivo

To verify whether the GATA4-BS functions in male gonadal sex determination in vivo, mice with one- and two-nucleotide substitutions within their

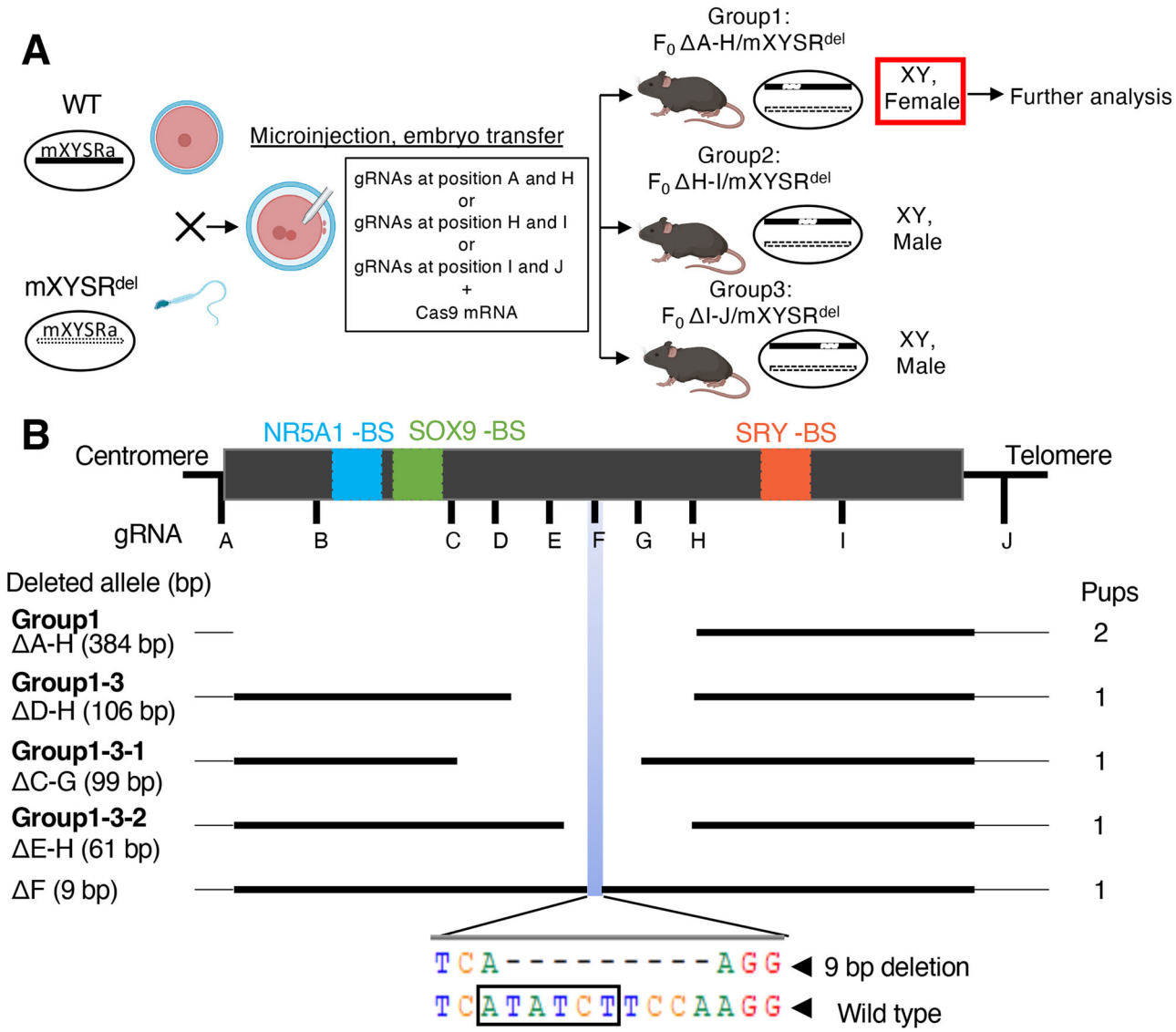

Fig. 1 | Phenotypic analysis of mice with partial deletions of the *mXYSRa/Enh13* region for identification of an additional functional sequence. A Overview of the method used to generate mutant mice. Left half shows in vitro fertilization and genome editing. Pink circle indicates an oocyte. Sperm is shown by the shape. Each genotype is indicated at left. Black and dotted rectangle indicate *mXYSRa* and deleted *mXYSRa*, respectively. Fertilized oocytes and mixtures (as a representative case, mixtures for initial screening are shown) for microinjection are depicted at middle. F_0 generation mice are shown at right. Deleted sequences are drawn with dotted rectangles. **B** Schematic diagram of *mXYSRa/Enh13* region and the position of responsible sequence. All the XY individuals of the mouse lines listed in this figure had a female-type reproductive system. Genome sequence is shown at top. Bold black box, blue box, green box and red box indicate *mXYSRa*, NR5A1-BS, SOX9-BS, and SRY-BS, respectively. Positions of gRNAs are shown below the genome sequence with the name. Genotypes of mice are shown below the genome with lines. Black bar: genomic sequence outside of *mXYSRa*; bold line: *mXYSRa*; no line: deleted sequence. gRNAs used, deleted sizes are indicated at left with Δ and parenthesis, respectively. The numbers of XY mice obtained are shown at right. Deleted sequence of mice microinjected gRNA F is shown at bottom with wild-type sequence. Dots indicates deleted bases. Rectangle indicates predicted GATA4 binding sequence.

GATA4-BSs were created. The microinjection protocol described previously was performed using gRNA F, hCas9 protein, and two ssODNs. ssODN1 was used for single nucleotide substitutions (ATATC > ATACC, the substituted nucleotide is underlined) and ssODN2 was used for two nucleotide substitutions (ATATC > ATGCC, the substituted nucleotides are underlined). As a result, two XY mice were identified as mutants containing mXYSR^{del} and one nucleotide substitution (GATA4-BS^{1sub}/mXYSR^{del}), whereas one mutant mouse with mXYSR^{del} and two nucleotide substitutions (GATA4-BS^{2sub}/mXYSR^{del}) was identified (Fig. 4A, B). The external genitalia were female, and the internal genitalia of all three mice were the uterus, oviducts, and ovaries (Fig. 4C).

GATA4-BS^{1sub}/mXYSR^{del} and GATA4-BS^{2sub}/mXYSR^{del} mice contain one copy of *mXYSRa*, thus it is unclear whether GATA4-BS mutations can cause male-to-female sex reversal. To examine this possibility, homozygous mutants of GATA4-BS$^{1sub/1sub}$ and GATA4-BS$^{2sub/2sub}$ were generated (designated as GATA4-BS$^{1sub/1sub}$;B6-DBA2 and GATA4-BS$^{2sub/2sub}$;B6-DBA2). The numbers of wild-type, GATA4-BS$^{1sub/+}$;B6-DBA2, and GATA4-BS$^{1sub/1sub}$;B6-DBA2 mice (XX and XY in total) were 11, 19, and 10, respectively, and wild-type, GATA4-BS$^{2sub/+}$;B6-DBA2, and GATA4-BS$^{2sub/2sub}$;B6-DBA2 mice were 5, 10, and 3, respectively. The scrotums were identified as XY GATA4-BS$^{1sub/1sub}$;B6-DBA2 and GATA4-BS$^{2sub/2sub}$;B6-DBA2. The AGD/BW ratio, a quantitative marker of androgen secretion, of some XY GATA4-BS$^{2sub/2sub}$;B6-DBA2 mice was significantly lower than in wild-type XY individuals ($p = 0.01069$) (Supplementary Fig. S3,S4, Supplementary Table S2).

Next, we generated GATA4-BS$^{1sub/1sub}$ and GATA4-BS$^{2sub/2sub}$ mice in a C57BL6/J background (designated as GATA4-BS$^{1sub/1sub}$;B6 and GATA4-BS$^{2sub/2sub}$;B6) because it is known that phenotypes of the reproductive

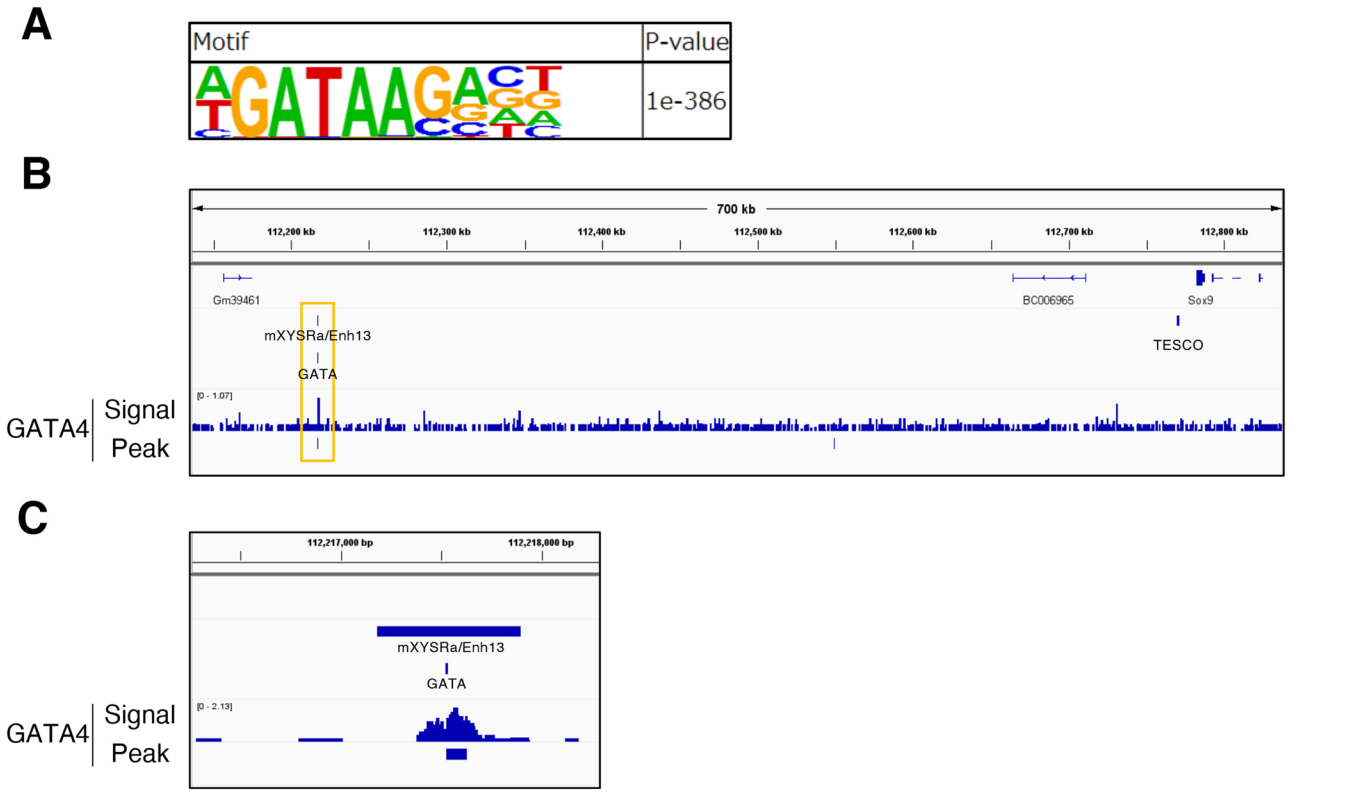

Fig. 2 | Binding of GATA4 to putative GATA-BS site within *mXYSRa/Enh13* in fetal gonads. A A nucleotide sequence extracted as the significantly enriched motif from the 3289 GATA4 binding regions in male gonads at E11.5. **B**, **C** Genome browser views of GATA4 distribution around the *Sox9* locus in male gonads at E11.5. One of GATA4 peaks overlapped with *mXYSRa/Enh13* (**B**). Regions highlighted by yellow rectangle was magnified (**C**).

system caused by mutations in genes associated with gonadal sex determination vary among genetic backgrounds due to the variation in signal transduction during gonadal sex differentiation and C57BL6/J mice are prone to cause male-to-female sex reversal[20]. We generated GATA4-BS^{1sub};B6 and GATA4-BS^{2sub};B6 using the strategy described above (Fig. 5A, B). GATA4-BS^{1sub};B6 and GATA4-BS^{2sub};B6 mice were obtained and phenotypes were analyzed at the F_2 generation or later. The numbers of wild-type, GATA4-BS$^{1sub/+}$;B6, and GATA4-BS$^{1sub/1sub}$;B6 mice (F_2 and F_3 generations) were 28, 67, and 27, respectively. Of the 27 GATA4-BS$^{1sub/1sub}$;B6 mice, the number of XY individuals was 10, all of which had male-type external genitalia at 8–12 weeks old. Of the ten, four had testes, and the remaining six had testes on one side and ovotestis on the other. Histological analyses showed that the former had normal testicular structures with disorganized seminiferous tubules at the pole, whereas the latter had small testes with disorganized structures on one side and ovotestes on the other, including small follicles and tubule-like structures (Supplementary Fig. S5E–J). The AGD/BW of GATA4-BS$^{1sub/1sub}$;B6 mice harboring the ovotestis was lower than that of the wild-type mice (Supplementary Table S2).

The total number of wild-type, GATA4-BS$^{2sub/+}$;B6, and GATA4-BS$^{2sub/2sub}$;B6 mice (F_2-F_5 generation) was 115, 199, and 102, respectively. Of the 102 GATA4-BS$^{2sub/2sub}$;B6 mice, 55 were XY mice. Forty-four GATA4-BS$^{2sub/2sub}$;B6 XY mice were analyzed, and they had female-type external and internal genitalia, except for one mouse with a testis on one side and an ovotestis on the other side (Fig. 5C). Comparisons of the AGD/BW of XY mice showed a significant decrease in the homozygous mutant group compared to the wild-type XY group ($p < 1 \times 10^{-7}$). No significant differences were observed when comparing wild-type XX and XY GATA4-BS$^{2sub/2sub}$;B6 mice (Fig. 5D, Supplementary Table S2). Regarding the histology of the gonads, the ovaries of XY GATA4-BS$^{2sub/2sub}$;B6 were smaller than those of the wild type, and follicles were sparsely observed (Fig. 5C).

Sox9 expression levels at E13.5 embryonic gonads in embryos with mutations in GATA4-BS

To examine the effect of GATA4-BS mutations on *Sox9* expression in embryonic gonads at the early sexual differentiation stage (E13.5), RT-qPCR and IF were performed. The expression level of *Sox9* in XY GATA4-BS$^{2sub/2sub}$;B6 mice was 90% lower than that in wild-type XY mice ($p = 0.0329$), and there was no significant difference in the expression levels between XY GATA4 BS$^{2sub/2sub}$;B6 and wild-type XX mice (Fig. 6A).

In XY GATA4-BS$^{1sub/1sub}$;B6, the SOX9 signal was present only at the center of the gonads, whereas the FOXL2 signal was present at both poles. In XY GATA4-BS$^{2sub/2sub}$;B6, the SOX9 signal was completely absent, and the FOXL2 signal was observed (Fig. 6B, Supplementary Fig. S6).

Discussion

In mammals, elucidation of the molecular mechanisms underlying *Sox9* expression is crucial for understanding gonadal sex determination and sexual differentiation. Thus, the identification of molecules associated with *mXYSRa/Enh13* is essential. In humans, the corresponding enhancer (eSR-A) of *mXYSRa/Enh13* has been identified in XYSR, with 80% sequence homology to *mXYSRa/Enh13*. Identification of molecules that interact with *mXYSRa/Enh13* in mice would facilitate the understanding of the corresponding mechanisms in humans and help to understand the etiology of human DSDs.

Mice with a single nucleotide substitution in the hemizygous state of *mXYSRa/Enh13* in a mixed genetic background of C57BL/6 J and DBA/2 J and two homozygous nucleotide substitutions in a C57BL/6 J background showed male-to-female sex reversal, suggesting that the targeted sequence should be a functional sequence of *mXYSRa/Enh13*. The CUT&RUN experiment identified GATA4 binding to the wild-type sequence of the substituted locus, and the luciferase assay showed that GATA4 upregulated the enhancer activity of *mXYSRa*, which was abolished when there were

mutations in GATA4-BS, suggesting that GATA4 binding promotes the enhancer activity of *mXYSRa/Enh13* against *Sox9*. The GATA4 direct upregulation of *Sox9* has long been proposed[17], and direct binding to *Sox9* enhancer is eventually validated through our study.

GATA4 is a critical factor in *Sry* expression. XY mice with a substitution in the coding region of the FOG2 binding sequence (*Gata4*$^{ki/ki}$ mice) were female because of reduced *Sry* expression. It is unlikely that the substitution of GATA4-BS affected the expression of *Sry* because the deletion of *Enh13*, including GATA4-BS, did not decrease *Sry* expression in the XY embryonic gonads at E11.5[11]. We assumed that the decrease in *Sox9* expression is the cause of the male-to-female sex reversal observed in GATA4-BS mutants.

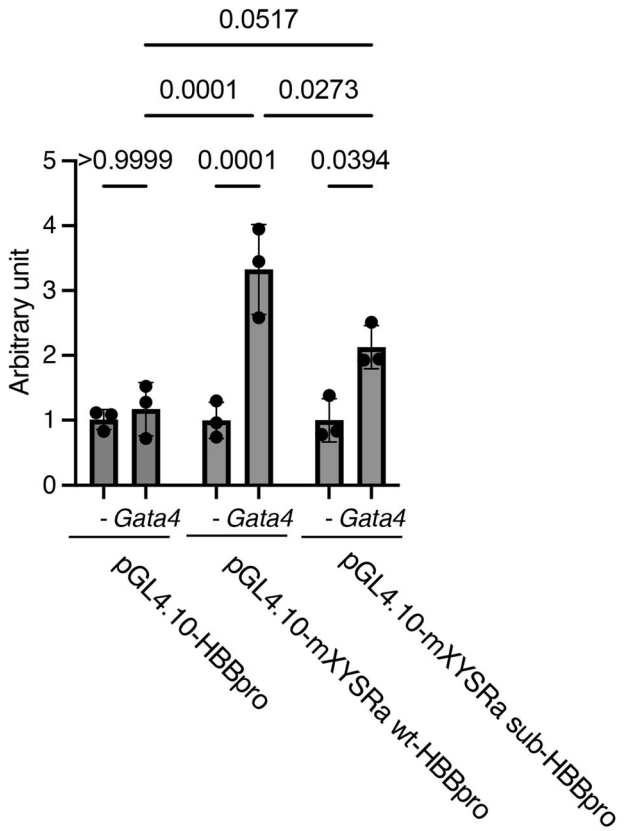

Fig. 3 | Luciferase assay. The value of firefly to Renilla (FF/RN) obtained by transfection without pcDNA3.1-Gata4 is set as 1. Mean ± SD is shown. Reporter plasmid used is shown at bottom. Gata4 and - indicate with and without pcDNA3.1-Gata4 being used for co-transfection, respectively. *P*-values are shown at the top.

Manuylov et al.[17] reported that GATA4 is required for *Sox9* expression in *Ods* mice. *Ods* mice overexpress *Sox9* owing to the insertion of a transgene and a 150-kb deletion ~1 Mb upstream of *Sox9*. The insertional mutation leads to *Sox9* expression in the embryonic gonads at a level sufficient for testis differentiation in XX individuals. In *Ods*: *Gata4*$^{ki/ki}$ embryos, *Sox9* expression was normalized to that of embryonic XX gonads, suggesting that *Sox9* overexpression in *Ods* is driven by GATA4. However, the precise mechanism remains to be elucidated. We first demonstrate the direct action of GATA4 on *Sox9* expression at high resolution. The deleted region in *Ods* mice is situated 1 Mb upstream of *Sox9*, while *mXYSRa* is located 566 kb upstream. Therefore, our findings suggest that GATA4 binding to *mXYSRa* does not directly explain the *Sox9* overexpression in *Ods* mice.

Our serial studies using *mXYSRa/Enh13* hemizygous mice and mice with homozygous mutations at the BSs revealed variations in potency among the BSs. Hemizygous GATA4-BS^{2sub} mice with a mixed genetic background developed ovaries, whereas some hemizygous SOX9-BS^{sub} mice and all hemizygous SRY-BS^{sub} mice developed testes. The GATA4-BS is thought to enhance *Sox9* expression predominantly, compared to SOX9-BS and SRY-BS. The expression patterns of SOX9 and FOXL2 in GATA4-BS$^{2sub/2sub}$;B6-DBA2 embryos observed by IF were similar to the pattern observed in the mice with deletion of both SRY-BS and SOX9-BS rather than that of G-stretch mutants, and phenotypes observed at the adult stage were quite similar between GATA4-BS$^{2sub/2sub}$;B6 and deletion mice of SRY-BS/SOX9-BS, suggesting that the contribution of GATA4-BS is similar to that of SRY-BS and SOX9-BS in the context of the *mXYSRa/Enh13* enhancer function.

To date, four functional elements in *mXYSRa/Enh13* have been identified, and the proteins bound to these elements are SRY, SOX9, NR5A1, and GATA4. The precise molecular mechanisms underlying the interactions between transcription factors and elements or between proteins need to be elucidated in the future. One limitation of this study is that not all functional sequence elements were comprehensively identified. Additionally, functionally redundant elements, such as SRY-BS and SOX9-BS were not identified. We aimed to identify a functional sequence element with strong activity so that the generation of mutant mice with gRNA B and D

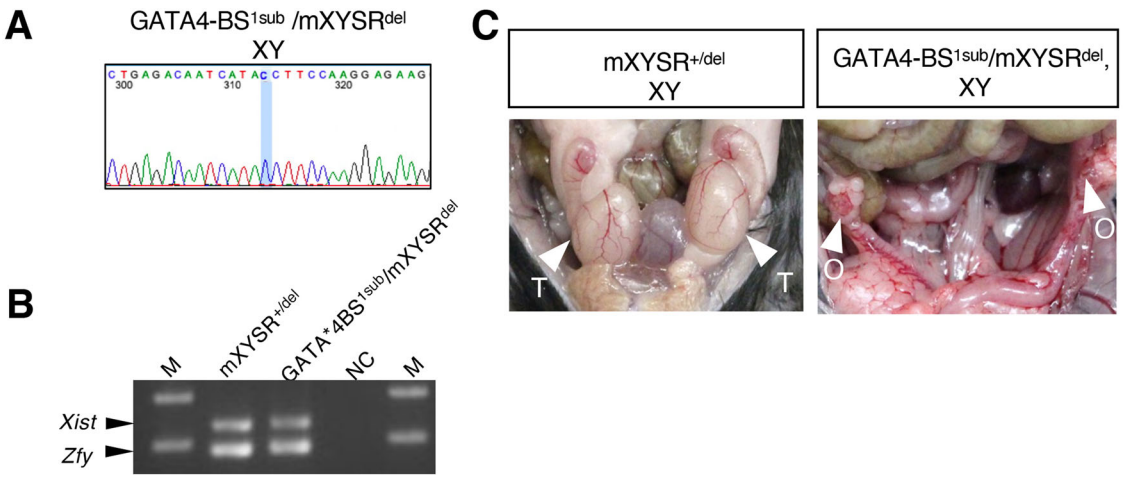

Fig. 4 | Phenotypic analysis of mice with GATA4-BS^{1sub} hemizygous of the *mXYSRa/Enh13* region. A A result of PCR direct sequence analysis of hemizygous GATA4-BS^{1sub}. Electropherogram is shown. Substituted base is labeled with blue. **B** A result of PCR sexing of XY GATA4-BS^{1sub}/mXYSR^{del}. An image of agarose gel electrophoresis is shown. Genes (X-linked *Xist* and Y-linked *Zfy*) detected are pointed at left. Genotypes are indicated at top. N: no template control; M: 100 bp DNA ladder marker. The full gel image is shown in Supplementary Fig. S7. **C** Phenotypes of GATA4-BS^{1sub} hemizygous F_0 individuals. Genotypes and sex chromosome compositions are indicated at top. T: testis; O: ovary.

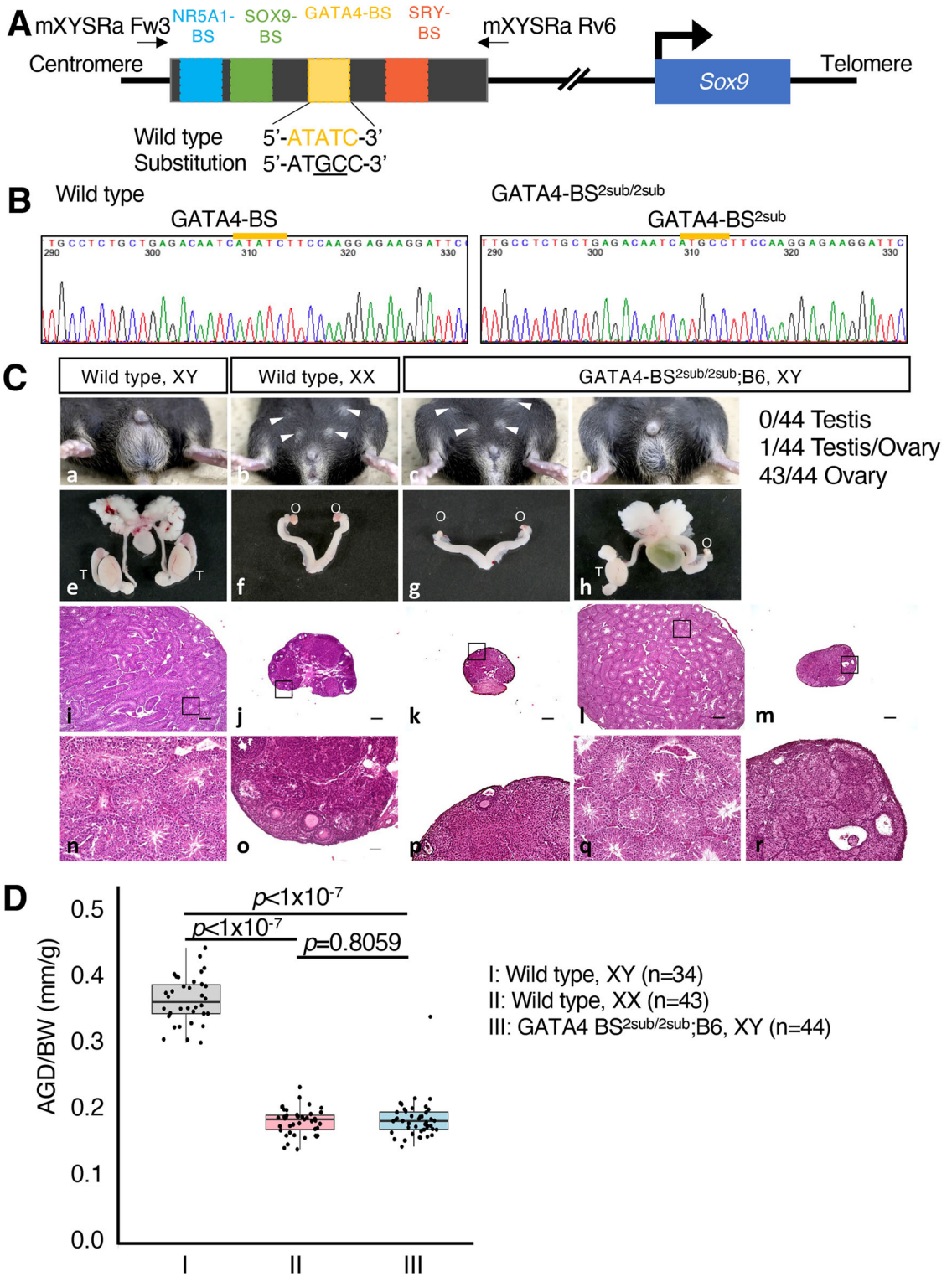

Fig. 5 | Phenotypic analysis of GATA4-BS$^{2sub/2sub}$ in C57BL/6 J genetic background at adult stage. A Schematic representation of genomic structure of *mXYSRa* and *Sox9* loci. Primers for genotyping are indicated at top with arrows. Wild-type sequence and substituted are shown at bottom. Substituted sequences are underlined. Black box: *mXYSRa*; light blue box: NR5A1-BS; green box: SOX9-BS; yellow box: GATA4-BS; red box: SRY-BS; blue box: *Sox9*; Bold arrow: orientation of transcription. **B** A result of PCR direct sequence analysis. Electropherogram are shown. Genotypes are shown at top. Wild-type and substituted GATA4-BS sequences are marked with yellow lines. **C** Phenotypic analysis of XY, GATA4-BS$^{2sub/2sub}$ mice. Genotypes and sex chromosome compositions are indicated at top. **a**–**d** External genitals. Arrow heads indicate nipples. **e**–**h** Internal genitals. T: testis; O: ovary. The numbers of XY, GATA4-BS$^{2sub/2sub}$ individuals with configuration of gonads among 44 mice are shown at right. **i**–**m** HE staining of gonads. Bar: 200 μm. **n**–**r** Close up images of region indicated with rectangles in (**i**–**m**), respectively. **D** Box-and-whisker plot of anogenital distance/body weight (AGD/BW) in adult mice. Genotypes, sex chromosome compositions and numbers used for analysis are shown at right. Dots indicates values of each mouse. *P*-values are shown at the top.

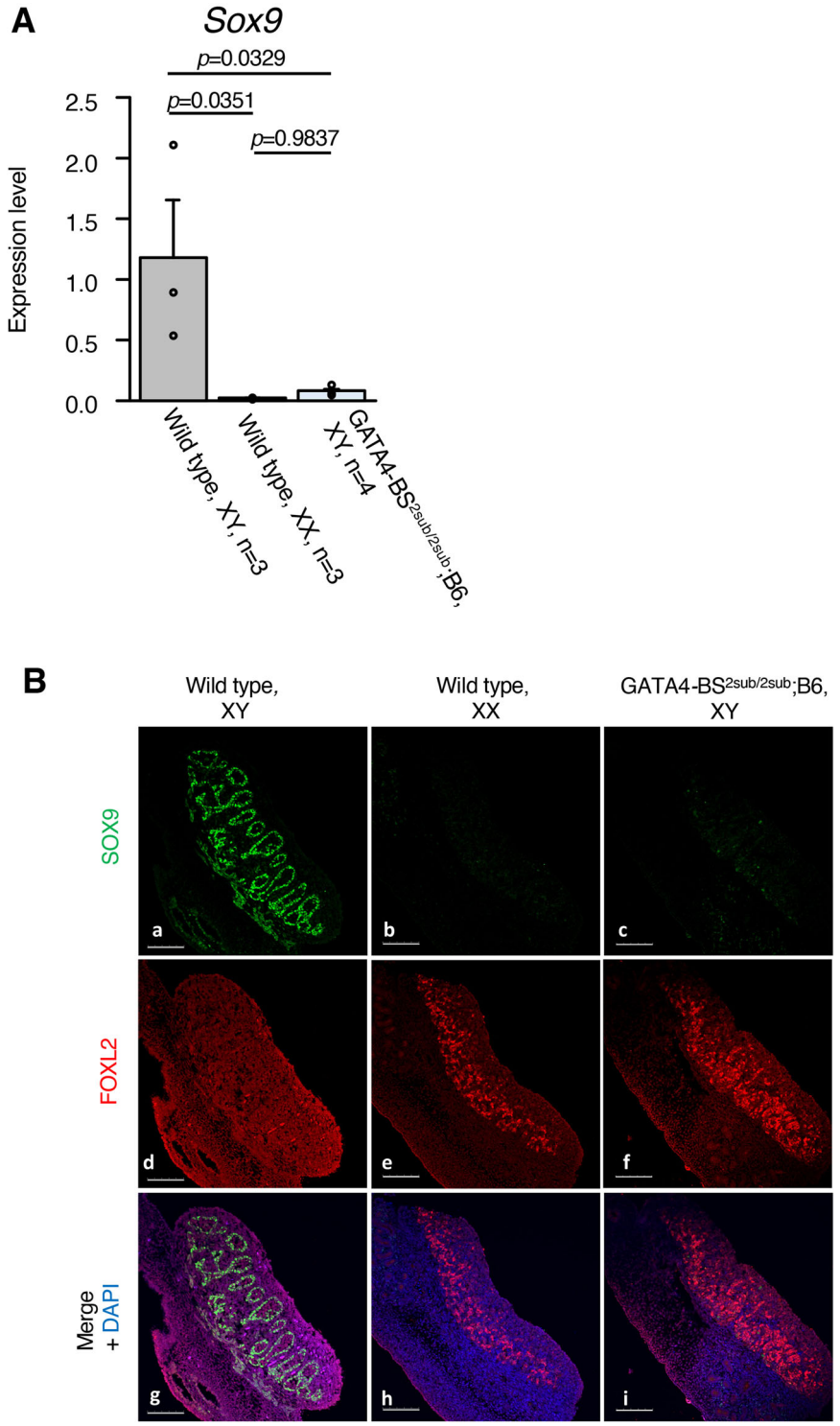

Fig. 6 | Expression analysis of GATA4-BS$^{2sub/2sub}$ in C57BL/6 J genetic background at E13.5. A RT-qPCR analysis. Genotypes, sex chromosome compositions and numbers used for analysis are shown at bottom. An expression level of XY, wild type is set as 1. Data are means ± SD. *P*-values are shown at the top. **B** IF analysis. Genotypes and sex chromosome compositions are indicated at top. **a**–**c** SOX9 expression. **d**–**f** FOXL2 expression. **g**–**i** Merged images of DAPI, SOX9 and FOXL2 signals. Bar: 100 μm.

was not repeated, considering that male-to-female reversal was caused by the deletion of the sequence from gRNA D to H. The possibility of the presence of other functional elements was not determined in our study.

Mice with GATA4-BS$^{2sub/2sub}$;B6 were XY females, suggesting that the two nucleotide substitutions were sufficient to abolish GATA4 binding to GATA4-BS. Single-nucleotide variants in regulatory regions can alter enhancer function[21], and we also elucidated the dysfunction of an enhancer due to one or two nucleotide variants.

The mice with GATA4-BS$^{2sub/2sub}$;B6-DBA2 were XY males, while GATA4-BS$^{2sub/2sub}$;B6 mice were XY females, suggesting that the function of *mXYSRa*/*Enh13* is influenced by genetic background. A similar situation has been reported in knockout mice for sexual differentiation-associated genes, such as *Dax1*[22], *Gata4*/*Fog2*[23], and *Jmjd1a*[24]. In all cases, male-to-female sex reversal was observed in C57BL/6 J mice. It is possible that the gonadal phenotypic difference between genetic backgrounds is derived from differences in the Y chromosome, as our mouse models with mixed genetic backgrounds succeeded the Y chromosome from DBA/2 J.

We confirmed the direct binding of GATA4 to the *Sox9* enhancer *mXYSRa*/*Enh13*. GATA4 upregulates *Sox9* expression through GATA4-BS, and the effect of GATA4-BS is similar to that of SRY-BS and SOX9-BS. Further studies are required to clarify whether SRY-BS, SOX9-BS, and GATA4-BS function in *Sox9* expression independently and/or cooperatively.

Methods

Animal experiment

C57BL/6 J and C57BL/6 J × DBA2/J F_1 (B6D2F1/J) mice were purchased from Sankyo Laboratory Services (Tokyo, Japan). Jcl:ICR female mice were purchased from CLEA Japan (Tokyo, Japan). Mice lacking the corresponding human XYSR region (mXYSR$^{+/del}$ mice) have been described in a previous study[12]. Embryonic day 0.5 (E0.5) was designated at noon of the day of vaginal plug observation. All animal protocols were approved by the Animal Protection and Use Committee of the National Center for Child Health and Development (Tokyo, Japan). We have complied with all relevant ethical regulations for animal use. All experiments were maintained under a 12 h light/dark cycle, and food and water were provided ad libitum.

Preparation of gRNAs for creation of *mXYSRa/Enh13* mutant mice

All single-guide RNA (sgRNA) sequences were designed using CRISPRdirect (http://crispr.dbcls.jp)[25]. All sgRNAs except gRNA F were synthesized as previously described[26]. Briefly, the protospacer sequences of gRNAs were cloned after inverse PCR and sgRNA was synthesized using the mMESSAGE mMACHINE T7 Transcription Kit (Thermo Fisher Scientific, Waltham, MA USA) with DNA templates amplified by PCR with protospacer sequences of gRNAs containing plasmids. sgRNAs were purified using the MEGAclear Transcription Clean-Up Kit (Thermo Fisher Scientific). The primers used in this study are listed in Table 1. gRNA F was prepared using a CUGA7 gRNA Synthesis Kit (Nippon Gene Co., Ltd., Toyama, Japan) according to the manufacturer's instructions. Human codon-optimized Cas9 (hCas9) and sgRNA cloning vectors were a gift from George Church (Addgene Plasmids #41815 and #41824, respectively). The resulting sgRNA sequences are shown in Table 2. hCas9 mRNA was prepared as described previously[26]. Briefly, the mMESSAGE mMACHINE T7 Transcription Kit was used with PCR products generated using the hCas9 plasmid and the primers Cas9 Rv and T7 Cas9 Fw. The hCas9 mRNA was purified using the MEGAclear Transcription Clean-Up Kit.

Creation of mutant mice

All mutant mice were created using the CRISPR/Cas9 system, and fertilized eggs were generated from zygotes collected from superovulated female and male mice. Nucleic acid mixtures were microinjected into fertilized oocytes at the pronuclear stage. The mixtures included hCas9/sgRNA, hCas9/two sgRNAs, and hCas9/sgRNA/single-stranded oligodeoxynucleotides (ssODNs). The first two and the last mix were microinjected into the cytoplasm and nucleus of the fertilized oocytes. Microinjected oocytes were cultured in KSOM medium (Arc Resources, Kumamoto, Japan) at 37 °C, and the next day, they were transferred to oviducts of pseudopregnant Jcl:ICR females. When a series of mutant mice with various deletions in *mXYSRa/Enh13* were generated using the CRISPR/Cas9 system, sperm were collected from male mice deficient in the entire *mXYSR* region (26.6 kb), including *mXYSRa/Enh13* in the mixed genetic background of C57BL/6 and DBA/2, and oocytes from wild-type B6D2F1 mice were used[12]. Mice in the F_0 generation were analyzed. The GATA4-BS^{1sub};B6-DBA2, and GATA4-BS^{2sub};B6-DBA2 alleles were generated from the same fertilized eggs used to generate mutants with various deletions in *mXYSR/Enh13*. Heterozygous GATA4-BS^{1sub} or GATA4-BS^{2sub} mutants in F_0 generation are once backcrossed to C57BL/6 mice. F_1 heterozygous females and males were mated and homozygous GATA4-BS^{1sub};B6-DBA2 or GATA4-BS^{2sub};B6-DBA2 mice in the F_2 generation were obtained.

Genotyping and sexing

Genomic DNA was extracted from the amniotic membrane, clipped tail, or ear. The primers used for genotyping were XYSR 1F3, XYSR 1 R, XYSR 6 F, mXYSRa Fw3, mXYSRa Rv6, and XYSR 6 R. The PCR products were sequenced directly using the ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific) or after cloning into plasmids. Electropherograms of the sequencing results were visualized using a Finch TV (1.3.0, Geospiza Inc.).

PCR sexing was performed using the primers previously reported by Obata et al.[27].

Alignment of genome sequences and estimation of binding motifs of transcription factors

Draft genome sequences were obtained from the University of California Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/). Human and mouse genome assemblies were February 2009 (GRCh37/hg19) and December 2011 (GRC38/mm10), respectively. Sequence homology searches and alignments were performed using CLUSTAL 2.1 Multiple Sequence Alignments (https://www.genome.jp/tools-bin/clustalw). The binding motifs of the transcription factors were searched using the GenetyxMac ver. 14.0.0.

Cleavage under targets and release using nuclease (CUT&RUN)

CUT&RUN was performed using permeabilized cells prepared from E11.5 male gonads of Jcl:ICR mice, as described by Takahashi et al.[28], with minor modifications. Briefly, male gonads were dispersed with collagenase type II (0.2 mg/mL, C6885, Sigma Aldrich, St Louis, MO, USA) and DNase I (0.2 mg/mL, #11284932001, Roche) at 37 °C for 45 min. Male gonadal cells (120,000) were bound to BioMag Plus Concanavalin A magnetic beads (#86057-3, Polysciences, Warrington, PA, USA) and then incubated with anti-Gata4 mouse antibody (1:100, sc-25310, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. After washing away unbound first antibody, anti-mouse IgG rabbit antibody (8 μg/mL, Cappel #55456) was added and incubated for 1 h at 4 °C. Then, pAG/MNase (700 ng/mL) was added to the sample and digestion was performed for 30 min on wet ice. Cleaved DNA fragments were collected and isolated using phenol-chloroform extraction and ethanol precipitation.

CUT&RUN fragments were processed for library construction using a NEBNext Ultra™ II DNA Library Prep Kit for Illumina (E7645, New England Biolabs, Ipswich, MA, USA) and NEBNext Multiplex Oligos for Illumina (E6440, New England Biolabs), following the manufacturer's instructions[29]. The libraries were sequenced using a NovaSeq 6000 (151-bp single-end; Illumina Inc., San Diego, CA, USA). Paired-end reads were quality controlled using FastQC and TrimGalore software. The reads were mapped using Bowtie2 version 2.2.5[30] to the mouse reference genome (UCSC GRC38/mm10), and genome annotation was downloaded from the UCSC Genome Browser. Bam and bigwig files were generated using SAMtools version 0.3.3[31] and bamCoverage of Deeptools version 3.4.3[32], respectively, and visualized using the IGV genome browser[33]. Peak calling and motif extraction were performed using HOMER version 4.11 (http://homer.ucsd.edu/homer/). Peaks overlapping the blacklisted regions[34] were eliminated.

Plasmid construction

The pGL4.10 (Promega, Madison, WI, USA) with the minimal HBB promoter (52 bp, chr11:5,227,060-5,227,111, hg38) was kindly provided by Professor Andrew Sinclair[35]. The plasmid was referred as 'pGL4.10-HBBpro'. pGL4.10-HBBpro was connected to *mXYSRa* (pGL4.10-mXYSRa wt-HBBpro), as previously described[13]. The sequences of *mXYSRa* with two nucleotide substitutions at the GATA4-BS were amplified by PCR using the previously reported primers SfiI_SalI_mXYSRa Fw2 and SfiI_mXYSRa Rv2[13] and cloned into pGEM-T Easy (Promega). The SalI recognition sequence was added to the plasmid via inverse PCR using the primers mXYSRa Sal, inverse PCR Fw1, and mXYSRa Sal, as listed in Table 1. The insert of the plasmid was subcloned in pGL4.10-HBBpro at XhoI site located 5′ end of the HBB minimal promoter, and the resulting plasmid is referred as pGL4.10-mXYSRa sub-HBBpro.

Open reading frames of *Gata4* cDNA were amplified using the primer pair Gata4F/Gata4R2, and the cDNA prepared from E13.5 male gonads of C57BL/6 J. The amplicon was digested with KpnI and BamHI and cloned

Table 1 | Primers used in this study

Primer name	Sequence
mXYSRa/Enh13_gRNA A_Fw	AGACTTGAAATTATTAAGAGTTTTAGAGCTAGAAATAGCAAG
mXYSRa/Enh13_gRNA A_Rv	AACTCTTAATAATTTCAAGTCTCGGTGTTTCGTCCTTTCCAC
T7_mXYSRa/Enh13_gRNA A_Fw	TTAATACGACTCACTATAGGAGACTTGAAATTATTAAGA
mXYSRa/Enh13_gRNA B_Fw	TCCTGTTTTGAAGCCCACCGTTTTAGAGCTAGAAATAGCAAG
mXYSRa/Enh13_gRNA B_Rv	AACGGTGGGCTTCAAAACAGGACGGTGTTTCGTCCTTTCCAC
T7_mXYSRa/Enh13_gRNA B_Fw	TTAATACGACTCACTATAGGTCCTGTTTTGAAGCCCACC
mXYSRa/Enh13_gRNA C_Fw	AGTGGAAAGGTTTTCTCAGGTTTTAGAGCTAGAAATAGCAAG
mXYSRa/Enh13_gRNA C_Rv	AACCTGAGAAAACCTTTCCACTCGGTGTTTCGTCCTTTCCAC
T7_mXYSRa/Enh13_gRNA C_Fw	TTAATACGACTCACTATAGGAGTGGAAAGGTTTTCTCAG
mXYSRa/Enh13_gRNA D_Fw	CAGAGCACAGGGTTTGAAGGTTTTAGAGCTAGAAATAGCAAG
mXYSRa/Enh13_gRNA D_Rv	AACCTTCAAACCCTGTGCTCTGCGGTGTTTCGTCCTTTCCAC
T7_mXYSRa/Enh13_gRNA D_Fw	TTAATACGACTCACTATAGGCAGAGCACAGGGTTTGAAG
mXYSRa/Enh13_gRNA E_Fw	TTGTCTCAGCAGAGGCAATGTTTTAGAGCTAGAAATAGCAAG
mXYSRa/Enh13_gRNA E_Rv	AACATTGCCTCTGCTGAGACAACGGTGTTTCGTCCTTTCCAC
T7_mXYSRa/Enh13_gRNA E_Fw	TTAATACGACTCACTATAGGTTGTCTCAGCAGAGGCAAT
mXYSRa/Enh13_gRNA F	CTAATACGACTCACTATAGTGAGACAATCATATCTTCCAGTTTTAGAGCTAGAAATAGCA
mXYSRa/Enh13_gRNA G_Fw	CTCCTGGAATCCTTCTCCTGTTTTAGAGCTAGAAATAGCAAG
mXYSRa/Enh13_gRNA G_Rv	AACAGGAGAAGGATTCCAGGAGCGGTGTTTCGTCCTTTCCAC
T7_mXYSRa/Enh13_gRNA G_Fw	TTAATACGACTCACTATAGGCTCCTGGAATCCTTCTCCT
mXYSRa/Enh13_gRNA H_Fw	GCTGTGGGAGAGTCGCAGAGTTTTAGAGCTAGAAATAGCAAG
mXYSRa/Enh13_gRNA H_Rv	AACTCTGCGACTCTCCCACAGCCGGTGTTTCGTCCTTTCCAC
T7_mXYSRa/Enh13_gRNA H_Fw	TTAATACGACTCACTATAGGGCTGTGGGAGAGTCGCAGA
mXYSRa/Enh13_gRNA I_Fw	GTGTGTTCTAATCAGCCAGGTTTTAGAGCTAGAAATAGCAAG
mXYSRa/Enh13_gRNA I_Rv	AACCTGGCTGATTAGAACACACCGGTGTTTCGTCCTTTCCAC
T7_mXYSRa/Enh13_gRNA I_Fw	TTAATACGACTCACTATAGGGTGTGTTCTAATCAGCCAG
mXYSRa/Enh13_gRNA J_Fw	ATAACAAACATTTACTGATGTTTTAGAGCTAGAAATAGCAAG
mXYSRa/Enh13_gRNA J_Rv	AACATCAGTAAATGTTTGTTATCGGTGTTTCGTCCTTTCCAC
T7_mXYSRa/Enh13_gRNA J_Fw	TTAATACGACTCACTATAGGATAACAAACATTTACTGAT
gRNA Rv	AAAAGCACCGACTCGGTGCC
GATA4_BS_sub_SSODN1	GCTCTGAAATCGTGCCCATTGCCTCTGCTGAGACAATCATrcCTTCCAAGGAGAAGGATTCCAGGAGCTGTGGGAGAGT
GATA4_BS_sub_SSODN2	ACTCTCCCACAGCTCCTGGAATCCTTCTCCTTGGAAGgyATGATTGTCTCAGCAGAGGCAATGGGCACGATTTCAGAGC
XYSR 1F3	CCCATGTACAGTTCACGCTTC
XYSR 1 R	CAGCCCCATAATAAGCAAGG
XYSR 6 F	CCTTTGCTACCCAAACCTCA
XYSR 6 R	TTTGTGCGCAGACTATCAGG
mXYSRa Fw1	AATCACAAAAGGCACTGAGG
mXYSRa Fw2	TACATCTGCCCCAAGGAAGC
mXYSRa Fw3	AAGAAGGTAGACCCAGAGAC
mXYSRa Rv6	TGCTAGAATAAAGCTAAGCC
mXYSRaTg Fw1 for cloning	ATAGCTGAGGAATTAGAAGG
mXYSRaTg Rv1 for cloning	TGGCTTCCAATTCTCTGCGG
Gata4F	GCGGTACCGTGACAGTTCCGCACACCCG
Gata4R2	GCGGATCCGGCTTTTCCAGTGCTCCACC
Sfil_SalI_mXYSRa Fw2	GCGGCCAAACAGGCCGTCGACAGAAGGTAGACCCAGAGACA
Sfil_mXYSRa Rv2	GCGGCCTGTTTGGCCTAAGCCAAATCTTGAAACAT
mXYSRa SalI inverse PCR Fw1	GCTTAGTCGACGGCCAAACAGGCCGCATCGAATT
mXYSRa SalI inverse PCR Rv1	GGCCGTCGACTAAGCCAAATCTTGAAACATAAAG

Underline: Spacer sequence of gRNA; small case letters: substitution sequence.

Table 2 | Sequences of sgRNA

gRNA	Sequence
gRNA A	GAGACUUGAAAUUAUUAAGAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC
gRNA B	GUCCUGUUUUGAAGCCCACCGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC
gRNA C	GAGUGGAAAGGUUUUCUCAGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC
gRNA D	GCAGAGCACAGGGUUUGAAGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC
gRNA E	GUUGUCUCAGCAGAGGCAAUGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC
gRNA F	UGAGACAAUCAUAUCUUCCAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC
gRNA G	GCUCCUGGAAUCCUUCUCCUGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC
gRNA H	GGCUGUGGGAGAGUCGCAGAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC
gRNA I	GGUGUGUUCUAAUCAGCCAGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC
gRNA J	GAUAACAAACAUUUACUGAUGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC

Underline: Spacer sequence of gRNA.

into pcDNA3.1(+) (Thermo Fisher Scientific). The resulting plasmid was referred to as pcDNA3.1-Gata4.

In vitro reporter assay

HEK293T cells were seeded at a density of 1.8×10^5 cells/well in a 24-well plate. The next day, co-transfection was carried out using pGL4.10-mXYSRa wt-HBBpro or pGL4.10-mXYSRa sub-HBBpro (100 ng), pRL-SV40 Renilla luciferase reporter plasmid (12.5 ng, Promega), and expression plasmids (100 ng of pcDNA3.1-Gata4). The amount of transfected DNA was standardized using pcDNA3.1(+). Transfection was carried out using the Lipofectamine 2000 reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Firefly and Renilla luciferase activities were measured utilizing with the Dual-Glo Luciferase Assay System (Promega) 2 days after transfection. The ratio of firefly to Renilla (FF/RN) was calculated, and each FF/RN was standardized by the FF/RN without GATA4.

Anogenital distance analysis

Phenotypic sex was examined by macroscopic observation of the external and internal gonads at 8-week-old or older. Anogenital distance (AGD) and body weight (BW) were measured twice and once, respectively. AGD/BW was calculated as the average AGD divided by the BW. Testis weights were measured and divided by the body weight for comparison. Statistical analysis and data visualization were performed using the R package ggplot2 (version 3.0.0).

Hematoxylin and eosin (H&E) staining

Testes or ovaries collected from 8 week-old or older mice were fixed with Bouin's fluid and embedded in paraffin. Sections were cut at 7 µm. Hematoxylin and eosin staining was performed as follows: Tissue sections on slides were deparaffinized with three sequential xylene treatments and rehydrated using a graded series of ethanol solutions of decreasing concentrations, ranging from 100%–70%. Sections were stained with hematoxylin and rinsed under running water to remove excess dye. These sections were then counterstained with eosin. After staining, the sections were dehydrated using a graded series of ethanol solutions of increasing concentrations, and cleared with three changes of xylene. Finally, tissue sections were mounted using Mount Quick (Daido Sangyo, Saitama, Japan). Images were captured using a BZ-X710 microscope (KEYENCE CORPORATION, Osaka, Japan).

RT-qPCR

RNA was prepared from E13.5 gonads using ISOGEN (Nippon Gene Co., Ltd.), and complementary DNA was synthesized with oligo dT and SuperScript II reverse transcriptase (Thermo Fischer Scientific). SYBR Green PCR Master Mix (Invitrogen) was used for quantitative PCR using the primers described by Ogawa et al.[13]. Relative gene expression levels of *Sox9* were calculated by ΔΔCt method using Ct values of β-actin for normalization. Statistical analyses were performed using two-tailed Student's *t*-test.

Immunofluorescence (IF)

IF of the embryonic gonads was performed as previously described[13]. Briefly, all samples were fixed in 4% paraformaldehyde in phosphate buffer saline and embedded in paraffin. Tissue sections were sliced at 5 µm thickness from the embedded paraffin, and the slides were treated with 1 M citric acid buffer as antigen retrieval, then, primary antibodies and second antibodies were applied, and counterstained with 4,6-diamidino-2-phenylindole (DAPI) solution (1:1000,19178-91; Nacalai Tesque, Inc., Kyoto, Japan). The primary antibodies used were rabbit anti-SOX9 (1:1000, ab5535; Merck Millipore, Burlington, MA, USA), goat anti-FOXL2 (1:500, ab5096; Abcam, Cambridge, UK), donkey anti-rabbit IgG (H + L) conjugated with Alexa Fluor 488 (1:500, A21206; Life Technologies, Carlsbad, CA, USA), and donkey anti-goat IgG (H + L) conjugated with Alexa Fluor 594 (1:500, A11058; LifeTechnologies) Fluorescent images were obtained using a BZ-X710 microscope (Keyence, Osaka, Japan) or Olympus IX83-FV3000 confocal microscope (Olympus, Tokyo, Japan). The images were analyzed using Adobe Photoshop 2024 (25.5.0).

Statistics and reproducibility

The experiments were not performed randomly, and no statistical methods were used to determine the sample size. All biological experiments were repeated at least thrice. Statistical analyses were performed using two-way analysis of variance (ANOVA), Tukey's multiple comparison test, or Dunnett's multiple comparison test. $p < 0.05$ was considered statistically significant. Figures were generated using GraphPad Prism version 10.0.2. for Macintosh, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com. Statistical significance was set at $p < 0.05$.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author. The CUT&RUN raw data were deposited in DDBJ database with the accession number DRR568671. The plasmids of pGL4.10-mXYSRa sub-HBBpro (RDB20788), pcDNA3.1-Gata4 (RDB20787), and plasmids coding gRNAs (RDB20778-RDB20786) are available on RIKEN BioResource Research Center (RIKEN BRC). Source data used for generating graphs in Fig. 3, Fig. 5D and Fig. 6A are found in Supplementary Data 1.

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Author contributions

Y.O. performed mouse experiments. M.T. performed microinjections. Y.O., I.T., A. T.-H. and S.T. maintained the mice for genotyping. S.Y. and T.B., K.-I.M, T.S., and J.S. performed the CUT&RUN analyses. Y.O., I.T., A.T.-H. S.T. performed luciferase assays. Y.O., S.Y., A.T.-H. and S.T. wrote article. All the authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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