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LETTER TO THE EDITOR

SDX on the X chromosome is required for male sex determination

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Dear Editor,

Sex determination is one of the most fundamental development processes, as gender is the first and most important identity of human. In most mammals, biological sex is determined by differences in sex chromosomes,[1] while the autosomes are identical in both male and female. During the past 30 years, two genetic pathways of sex determination have been revealed in mammals: the testis-determining (SRY-SOX9-FGF9) and ovary-promoting (RSPO1-WNT/β-catenin-FOXL2) pathways.[1] Previous study have shown that *SRY* is the only gene in the Y chromosome necessary for sex determination.[2] Among sex reversed patients, about 15% of 46 XY females can be attributed to mutations of *SRY*.[3] However, it remains elusive whether there is another sex chromosome gene necessary for sex determination. Interestingly, the African pygmy mouse *Mus minutoides* showed XY-female sex reversal due to disruption of unknown genes on the X chromosome.[1] To identify the potential sex determinants from the X chromosome, we employed an innovative proteomics approach and identified a novel sex-determining gene *SDX* on the X chromosome. Our study demonstrates that knockout of *SDX* results in development of ovotestis in fetal $SDX^{-/Y}$ gonads and complete male-to-female sex reversal in adult $SDX^{-/Y}$ mice. These findings suggest that *SDX* on the X chromosome is required to cooperate with SRY to assure male sex determination.

We previously developed a method to purify the chromatin-associated meiotic proteins. Many of the identified proteins have been proved to be required for meiosis, including MEIOB and YTHDC1/2.[4] Our studies on MEIOB have been emphasized in a textbook (Spermatogenesis, ISBN-13:978-1498764117), and widely applied in clinical diagnosis and treatment of human infertility. Surprisingly, YTHDC1/2 (YT521-B in *Drosophila*) can not only regulate meiosis in mice but is also necessary for sex determination in *Drosophila*.[5] This hints that our method for meiotic protein identification may be applicable to identifying sex determinants, as many key sex-determining proteins function as transcription factors which are bona fide chromatin-associated proteins, and many of those proteins regulate both sex determination and meiosis, such as FGF9, RSPO1, DMRT1. Here we have optimized the method which can identify sex-determining proteins from the embryonic gonads (Supplementary information, Fig. S1a). To assess the purity of the chromatin-associated proteins, we used histone H3 as the positive control, and cytoplasmic proteins VDAC1 and GAPDH as the negative controls (Supplementary information, Figs. S1b, S15). The identification of SOX9 showed that our method is competent for screening sex determinants. Combined with our previous proteomics data on chromatin-associated meiotic proteins, we focused on a candidate: 9430086K21Rik (also known as PWWP3B and MUM1L1), which showed high abundance in both embryonic gonads and testis at postnatal day 18. According to the sex-determining function described below, we renamed this gene as *SDX* (*Sex-Determining gene on the X chromosome*). Using our homemade antibody, we verified that SDX associated with chromatin, which further proves the success of our proteomic method (Supplementary information, Figs. S1b, S15).

We further analyzed the expression pattern of SDX during sex determination. Real time quantitative PCR (RT-qPCR) results showed that *SDX* expressed as early as in the initiation of sex determination (Supplementary information, Fig. S1c). SOX9 is a direct downstream target of SRY and a Sertoli cell marker. Co-immunostaining results demonstrated that SDX localized predominantly to the nuclei of somatic cells in E11.5 and E13.5 male gonads (Fig. 1a, b). Interestingly, SDX colocalized with SOX9 in Sertoli cells at both embryonic and postnatal testis (Fig. 1a, b; Supplementary information, Fig S4d). In contrast, SDX protein was not detected in fetal or postnatal ovaries (Supplementary information, Fig. S2). These data indicate that SDX may play a critical role in sex determination and maintaining functions of Sertoli cells.

To reveal the biological function of SDX in sex determination, we used a regular background mouse model, C57BL/6 (B6), to generate the conditional knockout mice, in which the only coding exon (exon 7) is flanked by loxP sites (Supplementary information, Fig. S3). We then obtained the complete *SDX* knockout mice ($SDX^{-/Y}$) using a germ cell-specific *cre* transgene (Supplementary information, Fig. S4a). Phenotypic analysis showed that approximately 25% (5 out of 20) (Fig. 1e) of $SDX^{-/Y}$ adult mice were completely sex-reversed with development of both female internal and external genitalia (Fig. 1c; Supplementary information, Fig. S6). The $SDX^{-/Y}$ females carried the Y chromosome, which was confirmed with three Y-chromosome specific genes (*ZFY, DDX3Y, RBMY*) (Fig. 1d). Unreversed $SDX^{-/Y}$ males showed severe spermatogenic defects, and their testes were much smaller than the wild type littermates (Supplementary information, Figs. S5, S6). Interestingly, some of the reversed $SDX^{-/Y}$ female and unreversed $SDX^{-/Y}$ males were fertile, but all the $SDX^{-/Y}$ males became infertile at around 8 months old. $SDX^{-/-}$ female ovaries developed normally, which is consistent with the absence of the SDX protein in the ovaries (Supplementary information, Fig. S2).

To study the etiology of male-to-female sex reversal, we examined the testicular Sertoli cell marker SOX9 and the ovarian somatic cell marker FOXL2 in embryonic gonads at E13.5 and E15.5. At E13.5, all the $SDX^{-/Y}$ gonads contained both SOX9- and FOXL2-positive signals, which was distinguished from the $SDX^{+/Y}$ male gonads showing SOX9 signal only and $SDX^{+/+}$ female gonads showing FOXL2 signal only (Fig. 1f, h). When developing into E15.5, 27% (3 out of 11) of the $SDX^{-/Y}$ gonads contained FOXL2-positive cells exclusively (Fig. 1g, i), which recapitulated the

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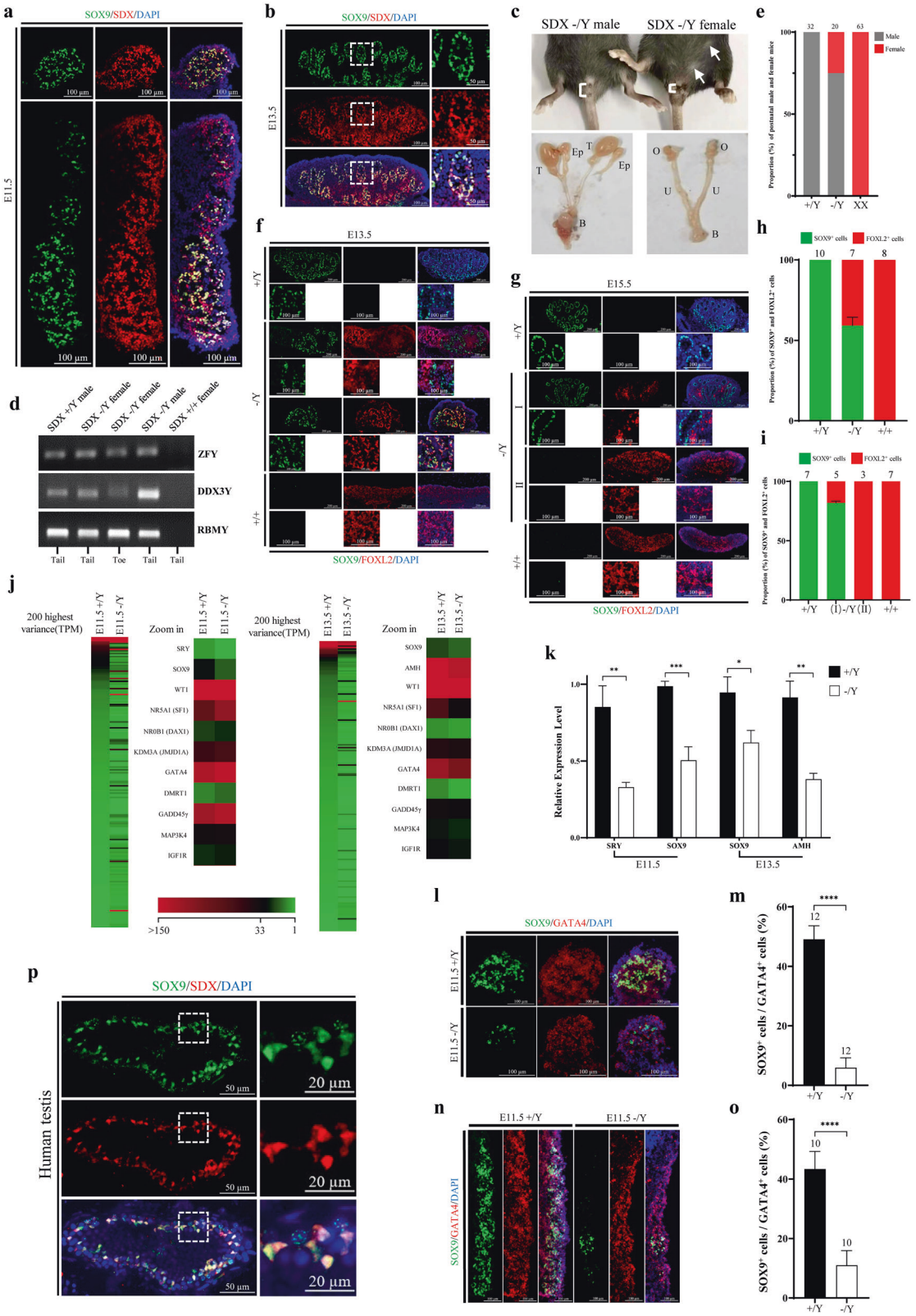

expression pattern of wildtype female gonads, indicating a complete male-to-female sex reversal. Nevertheless, 73% (8 out of 11) of *SDX*$^{-/Y}$ gonads at E15.5 still preserved both SOX9- and FOXL2-positive cells (Fig. 1g, i). At E13.5, some somatic cells in *SDX*$^{-/Y}$ gonads expressed both SOX9 and FOXL2 proteins (Fig. 1f), indicating a bidirectional possibility (Sertoli cell or granulosa cell) of those cells. However, at E15.5, SOX9 and FOXL2 localized in different cells (Fig. 1g), suggesting completion of cell fate determination. To further confirm the early failure of the testis-determining pathway in the *SDX*$^{-/Y}$ gonads, we co-immunostained

Fig. 1 Characterization of the role of SDX in male sex determination. a, **b** Coimmunostaining profiles of SOX9 and SDX in E11.5 XY gonads (**a**) and in E13.5 ones (**b**). Scale bar, 100 μm, and the one in enlarged box, 50 μm. **c** External and internal genitalia of normally sex differentiated (left) (#B266) and completely sex-reversed (right) (#B264) $SDX^{-/Y}$ mice. T, testis; Ep, epididymis; O, ovary; U, uterus; B, bladder. The distance between anus and penis or vagina is indicated. White arrows represent mammary glands. **d** Identification of Y chromosome in SDX-deficient mice, indicated by the existence of genes *ZFY*, *DDX3Y* and *RBMY* detected through PCR. **e** Proportion analysis of males and females in $SDX^{+/Y}$, $SDX^{-/Y}$, and $SDX^{+/+}$ mice. **f**, **g** Immunofluorescence analysis of SOX9 and FOXL2 in E13.5 gonads (**f**) and E15.5 gonads (**g**). Scale bar, 200 μm, and 100μm in enlarged box. **h**, **i** Quantification of SOX9- and FOXL2-positive cells in E13.5 gonads (**h**) and in E15.5 gonads (**i**). **j** Genome-wide expression profiling of $SDX^{+/Y}$ and $SDX^{-/Y}$ gonads at E11.5 and E13.5. **k** RT-qPCR analyses of SRY and SOX9 in E11.5 XY gonads, as well as SOX9 and AMH in E13.5 ones. Repeated data were acquired from three different batches of samples, each of which included 4~6 XY gonads. Expression levels in $SDX^{+/Y}$ were defined as 1. **l**, **n** Coimmunostaining profiles of SOX9 with GATA4 in $SDX^{+/Y}$ and $SDX^{-/Y}$ gonads at E11.5, which were cut both vertically (**l**) and parallelly (**n**) referring to the long axis. Scale bar, 100 μm. **m**, **o** The ratios of SOX9-positive cells to the cells positive for GATA4 in E11.5 XY gonads in both vertical sections (**m**) and parallel sections (**o**). **p** Co-immunostaining analysis of SOX9 and SDX in adult human testes. Scale bar, 50 μm, and the one in enlarged box, 20 μm. Numbers of examined animals or embryos are shown above the bars. Expression levels are normalized to GAPDH. All data are presented as means ± SE. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ (two-tailed unpaired Student's *t*-test).

FOXL2 and AMH (a specific marker of Sertoli cells). As expected, both AMH- and FOXL2-positive cells resided in the $SDX^{-/Y}$ gonads at both E13.5 and E15.5, and FOXL2-positive only $SDX^{-/Y}$ gonads could also be detected at E15.5 (Supplementary information, Fig. S7). These data demonstrate that SDX is necessary for male sex determination.

Next, we performed transcriptome analysis for the $SDX^{+/Y}$ and $SDX^{-/Y}$ gonads during male sex differentiation. At E11.5, many genes were dramatically altered in the $SDX^{-/Y}$ gonads (Fig. 1j). Expression levels of SRY and its downstream target gene *SOX9* were significantly reduced in the $SDX^{-/Y}$ gonads (Fig. 1j, k). SRY was reduced to approximately 30% of the level of the wildtype control during the period of sex determination (Fig. 1k). Consistent with a recent report,[6] we confirmed that *SRY* encodes two transcripts (*SRY-T*, *SRY-S*), and both of them were significantly decreased in the $SDX^{-/Y}$ gonads (Supplementary information, Fig. S10). Considering that the RNA-Seq data and RT-qPCR data were acquired from mixed samples including completely and incompletely reversed gonads, the complete sex reversed gonads would have even much lower expression levels of SRY and SOX9. In some of the $SDX^{-/Y}$ gonads, the proportion of SOX9-positive cells was obviously lower than the threshold (25%),[7] which was reported to be necessary for male development (Fig. 1l–o). In the $SDX^{-/Y}$ gonads at E13.5, expression levels of Sox9 and AMH were also significantly lower than those in the $SDX^{+/Y}$ male gonads (Fig. 1k), indicating a catastrophe in the male development pathway.

To determine whether SDX regulates the male pathway of SRY-SOX9-FGF9 in a direct manner rather than by affecting the upstream modulators of SRY, we investigated the expression profiles of these proteins upstream of SRY in sex determination pathway. Both the RNA-Seq and RT-qPCR data demonstrated that these known upstream factors, such as WT1, SF1, JMJD1A, showed no significant difference between gonads in knockout and wildtype mice (Fig. 1j; Supplementary information, Figs. S8, S9). We also analyzed the transcription levels of ovary-related genes, which showed no significant difference (Supplementary information, Fig. S11). Only transcriptions of SRY and its downstream factors, such as SOX9 and AMH, were significantly reduced (Fig. 1k). A further co-immunofluorescence analysis revealed that the number of SOX9-positive cells was dramatically declined in the $SDX^{-/Y}$ gonads at E11.5 (Fig. 1l–o), which was consistent with the RNA expression data.

Since SDX is necessary for male sex determination in mice, we reasoned that it may function similarly in human sex determination. Through the Bayesian phylogeny analysis, we found that SDX is highly conserved in eutherian mammals, especially between mouse and human (Supplementary information, Fig. S12). In most mammalian species, *SDX* is on the X chromosome (Supplementary information, Fig. S12). The SDX protein showed 84% identity for its PWWP domain between human and mice, which is the only domain characterized so far (Supplementary information, Fig. S13). We further immunostained SOX9 and SDX in human testis, and found that SDX completely co-localized with SOX9 in human Sertoli cells, which is the same as that in mice (Fig. 1p; Supplementary information, Fig. S4d). These data strongly imply that SDX plays an important role in human sex determination.

It is commonly recognized that the Y chromosome determines male sex. Individuals develop as females by default without the Y chromosome. In mammals, the XX, XY or XO individuals can all survive, but the YY or YO individuals cannot. Thus, it is technical challenging to study whether X chromosome alone is necessary for sex determination in vivo. Although two genes (*DAX1* and *SOX3*) on the X chromosome are implicated in sex determination, neither is indispensable for male sex determination. Deletion of SOX3 had no effect on sex development.[8] Deficiency of DAX1 could not lead to sex reversal in human and regular background mice, although it resulted in XY female sex reversal of XY^{pos} mice,[9] which are susceptible to XY female sex reversal due to a much lower SRY expression level as compared with regular mouse models, such as C57BL/6. Moreover, DAX1 showed conserved anti-testis function when overexpressed in human and mice.[10] Here, we have identified a novel male sex-determining gene *SDX* on the X chromosome, revealing that the X chromosome plays an essential role in male sex determination.

Intriguingly, the adult $SDX^{-/Y}$ mice exhibited as either male or female. We have not found any intermediates with ovotestis, or partial male and partial female. Previous studies showed that masculinizing signals initiated by SRY expression spread from the center of the bipotential gonad to the polar regions.[11] Interference of the masculinizing signal spreading resulted in ovotestis development, with polar ovarian tissue at either side of a central testicular region.[12] Indeed, all the $SDX^{-/Y}$ gonads exhibited as ovotestis at E13.5; some of those gonads contained testis-specific tissues only in the center, but had ovarian tissues in the polar regions (Fig. 1f). At E15.5, some of the $SDX^{-/Y}$ gonads solely contained FOXL2-positive cells, and those mice would develop as completely sex-reversed females. For the other $SDX^{-/Y}$ mice, the ones with E15.5 ovotestis containing more SOX9-positive cells than threshold would resolve into testis, and they would develop as males. This is consistent with previous reports that fetal ovotestis often resolve into testis in mice.[13]

Based on our study and previous reports of the sex-determining factors on the sex chromosomes, we proposed a model of how sex chromosome genes determine sex differentiation in mammals (Supplementary information, Fig. S14). As a commander of the mammalian sex determination, the X and Y chromosomes together set the direction and coordinate with the autosomes in male or female development. The decision is mainly made by *SRY* on the Y chromosome and *SDX* on the X chromosome. SDX gives a strong signal for male sex determination by promoting SRY

expression. DAX1 gives a weak signal for male development through SOX9, as DAX1 overexpression showed no XX male sex reversal.[10]

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AUTHOR CONTRIBUTIONS

J.Z. carried out most of the experiments. P.C. made antibodies and analyzed phenotypes. Z.Y. and W.Q. performed the bioinformatics analysis. M.L. and J.Z. designed the project and wrote the manuscript. All members participated in the manuscript discussion.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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