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Materials and Methods

Chemicals and Media

Pregnant mares' serum gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) were purchased from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise stated. Round spermatid and oocyte collection and subsequent manipulation, including microinjections were done in HEPES-buffered CZB medium (HEPES-CZB) (*19*). Culture of round spermatid-injected oocytes and embryos was done in CZB medium (*20*).

Animals

Six-to-twelve week-old B6D2F1 (C57BL/6J x DBA/2) females (NCI, Raleigh, NC) were used as oocyte donors for injections and CD-1 (Charles River, Wilmington, MA) or Swiss Webster (NCI, Raleigh, NC) mice were used as vasectomized males and surrogate/foster females for embryo transfer.

The mice of interest in this study were mice with a single X chromosome (XO) and limited Y gene complement:

1. **$X^{Eif2s3y}O$*Sox9*** (abbreviated as $X^{E}O$*Sox9*) are males carrying an autosomally-encoded *Sox9* transgene driven by *Wt1* (Wilms tumor 1) promoter (*3*) and the X chromosome-located transgene encoding spermatogonial proliferation factor *Eif2s3y* (*5*); the X chromosome carrying the *Eif2s3y* transgene is designated as X^{E}. We obtained cryopreserved sperm samples from XY*Sox9* transgenic males from Andreas Schedl (Inserm, France). These samples were used for intracytoplasmic sperm injection (ICSI) to generate live XY*Sox9* males. The $X^{E}O$*Sox9* males were then produced in 3 steps (Fig. S1A). In step 1, XY*Sox9* males were bred to sex reversed XY^{Tdym1} females, which carry an X chromosome and a Y chromosome with an 11-kb deletion removing the testis determinant *Sry* (*dl1Rlb*) (*21, 22*). This resulted in obtaining XY^{Tdym1}*Sox9* males, in which sex determination was driven by *Sox9* in the absence of *Sry* but with the remaining Y chromosome genes present. In step 2, the XY^{Tdym1}*Sox9* males were crossed to $X^{E}X^{E}$ females, in which both X chromosomes carried the *Eif2s3y* transgene, in order to produce $X^{E}Y^{Tdym1}$*Sox9* males. In step 3, the $X^{E}Y^{Tdym1}$*Sox9* males were bred to $X^{Paf}O$ (abbreviated as $X^{P}O$) (*23*) carrying the X-linked coat marker *Patchy-fur* (*24*).
2. **XO*Sry,Eif2s3x*** are males carrying an autosomally-encoded *Sry* transgene (*25*) and an autosomally encoded *Eif2s3x* transgene. We first generated mice transgenic for *Eif2s3x*. The bacterial artificial chromosome (BAC) clone containing *Eif2s3x* and 21 kb upstream of its transcription site was placed in pBeloBAC11 vector, resulting in a ~118 kb construct that was used for pronuclear microinjections. Six founders had germline transmission and were propagated. The males from lines with significant *Eif2s3x* expression in testes and other tissues were used to obtain XX*Eif2s3x* transgenic females. The XO*Sry*,*Eif2s3x* males were then produced in two steps (Fig. S1B). In step 1, the XX*Eif2s3x* females were bred to XY^{Tdym1}*Sry* males that have the X chromosome carrying an *Eif2s3y* transgene (*5*) and a Y chromosome with an 11-kb deletion removing the testis determinant *Sry* (*dl1Rlb*) (*21, 22*), complemented by an autosomally-located *Sry* transgene [Tg(Sry)2Ei] (*25*). In step 2, the

$XY^{Tdym1}Sry$,*Eif2s3x* males (*(*$XY^{Tdym1}Sry$ transgenic for autosomally encoded *Eif2s3x*) obtained in step 1 were bred with $X^{P}O$ females.

3. **XO*Sox9,Eif2s3x*** are males carrying an autosomally-encoded *Sox9* transgene driven by *Wt1* (Wills tumor 1) promoter (*3*) and an autosomally encoded *Eif2s3x* transgene. The XO*Sox9,Eif2s3x* males were produced by crossing XX*Eif2s3x* females with $XY^{Tdym1}Sox9$ males and then the resulting $XY^{Tdym1}Sox9$,*Eif2s3x* to $X^{P}O$ females (Fig. 1SC).

In addition to these three primary genotypes of interest, we also examined mice with two Y chromosome genes, $\mathbf{X^{Eif2s3y}O}$***Sry*** (abbreviated as $X^{E}O$*Sry*). These males carry an autosomally-encoded *Sry* (*25*) and X chromosome encoded *Eif2s3y* (*5*) transgenes, in the context of a single X chromosome. The $X^{E}O$*Sry* males were produced 'in house' by breeding $X^{Paf}O$ or $X^{Paf}Y^{*X}$ females (*23*) carrying the X-linked coat marker *Patchy-fur* (*24*) and $X^{Eif2s3y}Y^{Tdym1}Sry$ males that have the X chromosome carrying an *Eif2s3y* transgene (*5*) and a Y chromosome with an 11-kb deletion removing the testis determinant *Sry* (*dl1Rlb*) (*21, 22*), complemented by an autosomally-located *Sry* transgene [Tg(Sry)2Ei] (*25*).

The breeding crosses yielded a variety of progeny genotypes; the males of interest were identified among the progeny by genotyping for Y chromosome markers, scoring fur appearance, and evaluation of testes size. All mice with limited Y gene complement were on partial MF1 genetic background. The XY^{RIII} on MF1 background and XY B6D2F1 males were used as wild-type controls.

The mice were fed ad libitum with a standard diet and maintained in a temperature and light-controlled room (22ºC, 14h light/10h dark), in accordance with the guidelines of the Laboratory Animal Services at the University of Hawaii and guidelines presented in National Research Council's (NCR) "Guide for Care and Use of Laboratory Animals" published by Institute for Laboratory Animal Research (ILAR) of the National Academy of Science, Bethesda, MD, 2011. The protocol for animal handling and treatment procedures was reviewed and approved by the Animal Care and Use Committee at the University of Hawaii.

Testicular material collection and preparation

Testes were collected twice from each male following initial semi-castration. Each dissected testis was weighed, photographed if needed, and divided into 3 parts: half for Bouin fixation and subsequent sectioning, one-fourth for preparation of testicular cell suspension for injections, and one-fourth for storage at -80ºC and subsequent molecular or cytogenetic analyses.

Histology analysis

For histology analysis, part of the testes were fixed in Bouin overnight and then stored in 70% ethanol prior to embedding in paraffin wax, sectioning at 5 µm, and staining with hematoxylin-eosin (H&E) and Periodic acid Schiff and hematoxylin (PAS-H). The stages of seminiferous tubules were identified based on the composition of cells near the basal membrane according to the method described by Ahmed (*26*). This was necessary because of meiotic and post-meiotic arrests present in males with limited Y gene complement, which prevented staging based on the changes of acrosome and nuclear morphology of spermatids. For quantitative analysis of spermatogenesis

progression, for each male 10 tubules were examined per stage category and the numbers of spermatogonia, round spermatids, and Sertoli cells were counted. The data were expressed as germ cell/Sertoli cell ratios. For XO*Sry*, which could not be staged due to lack of spermatogenesis, 60 randomly chosen tubules were used for cell counts.

Round spermatid injection (ROSI)

All ROSI offspring were derived using spermatids from males on partial (transgenics) or full MF1 (XY) genetic background and oocytes from B6D2F1 females. The metaphase II (MII) oocytes for ROSI were collected from superovulated (5 iu eCG and 5 iu hCG given 48 hours apart) female mice and incubated at 37ºC, 5% CO_2 until injection. Testicular cell suspension was diluted with HEPES-CZB containing 1% (w/v) polyvinyl pyrrolidone (PVP) on the injection dish. Spermatids were injected individually into the oocytes. The total duration of ROSI was no longer than 1 hour. The oocytes were activated shortly after injection by incubation in Ca^{2+}-free CZB medium supplemented with 2.5 mM $SrCl_2$ at 37ºC, 5% CO_2 for 4 hrs, after which time they were transferred into standard CZB medium for subsequent culture. At ~6-8 hrs after injection the oocytes were assessed for polar body extrusion and pronuclei development. Normally fertilized oocytes exhibiting two pronuclei (PN) and extruded second polar body (PBII) as well as abnormally fertilized oocytes (with deviations from normal in regard of pronuclei and extruded polar body number) were differentiated. Twenty four hours after injection the oocytes in each group were scored for cleavage. Embryo transfer was performed with the 2-cell embryos derived from normally fertilized oocytes. Surrogate mothers were subjected to caesarian section on day 20 of pregnancy to allow for scoring of the numbers of fetuses and implantation sites.

ROSI progeny analyses

All offspring were raised by foster mothers until weaning, allowed to mature, and bred (if applicable). All offspring were genotyped by PCR to identify presence of *Eif2s3y*, *Eif2s3x*, *Sox9*, and *Sry* transgenes, and the sex chromosomes (primer sequences are shown in Table S4). Some of the offspring from XO*Sox9*,*Eif2s3x* males were also tested for a battery of other Y chromosome genes to show that none were present. The overall health and well-being of some of the ROSI progeny were monitored carefully during the first 8 months of age, which included monthly body weight measures. Some of the ROSI offspring were maintained beyond 8 months of age, with the oldest progeny being now at 20 months of age. None from the mice that were maintained died prematurely or developed tumors.

Transgene copy number

Genomic DNA for *Eif2s3x* transgene copy number assessment was isolated from mouse tails using phenol chloroform extraction and ethanol precipitation. 5 ng/µl of DNA was used to amplify single copy X chromosome genes *Eif2s3x*, *Amelx* and *Prdx4* by qPCR using *Power* SYBR Green PCR Master Mix on QuantStudio 12K Flex machine (Applied Biosystems). The following conditions were used: 95^{o}C for 10 min, followed by 37 cycles of 95^{o}C for 10 sec and 60^{o}C for 60 sec. *Eif2s3x* was amplified with *Eif2s3x-sp-CNAFP* and *Eif2s3x-sp-CNARP* primers (*Eif2s3x* (sp1), Table S4), which recognize both endogenous and transgenic *Eif2s3x* but do not recognize either of the *Eif2s3x* retroposons

or the Y chromosome gene *Eif2s3y*. XO samples were used as a reference control since they have a single X chromosome and are expected to show single copies of *Eif2s3x*, *Amelx* and *Prdx4*, and no copies of *Eif2s3y*. XX and XY samples were included to verify primer specificity. *Amelx* and *Prdx4* were run for each sample as quality and copy number controls. A minimum of three samples per genotype were tested in quadruplicate per assay. Copy number estimation for each gene was calculated with the ΔΔCt method using *Atr* as a housekeeping gene. ΔCt values were calculated as difference between tested gene and *Atr*. ΔΔCt values were calculated by subtracting ΔCt of tested gene from the reference samples. The copy numbers were calculated by raising 2 to the power of ΔΔCt ($2^{\Delta\Delta Ct}$). *Eif2s3x* transgene copy number was inferred from a difference in *Eif2s3x* amplification readout between XO and transgenic samples, and established using a typical mathematical rounding, i.e. readout <1.5 was scored as 1 and readout >/=1.5 as 2. Primer sequences are shown in Table S4.

Real-time RT-PCR

For real-time reverse transcriptase polymerase chain reaction (RT-PCR), total testis RNA was extracted using Trizol and DNAse I treatment (Ambion, Austin, TX,USA), and purified using an RNeasy kit (Qiagen, Valencia, CA, USA). Reverse transcription of polyadenylated RNA was performed with Superscript Reverse Transcriptase III, according to the manufacturer's guidelines (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using SYBR Green PCR Master mix on an ABI QuantStudio 12K Flex machine (Applied Biosystems, Carlsbad, CA, USA). PCR reactions were incubated at 95°C for 10 min followed by 40 PCR cycles (10 s at 95°C and 60 s at 60°C).

The analysis of ovarian markers (Fig. S5H-J) was performed using primers for *FoxL2*, *Rspo1*, *Wnt4* with *Actb* and *Sdha* as loading controls. For analysis of *Eif2s3* expression (Fig. 2A-C&F, Fig. S10&S11), four types of PCR reactions were performed: (1) '*Eif2s3x*' amplifying endogenous and transgenic *Eif2s3x* transcripts, and the *Eif2s3x* X-linked retroposon *Gm2223*; (2) '*Eif2s3x* (sp2)' amplifying specifically endogenous and transgenic *Eif2s3x* transcripts but not the *Gm2223* or the *Eif2s3x-ps1* retroposons; (3) '*Eif2s3y*' amplifying endogenous and transgenic *Eif2s3y* transcripts; (4) *'Eif2s3x/y'* amplifying transcripts from both *Eif2s3x* and *Eif2s3y*, both endogenous and transgenic, and *Gm2223*. At least three mice per genotype were analyzed, all reactions were carried out in at least triplicates per assay, and two ubiquitously expressed genes (*Actb* and/or *Sdha*) were used as loading controls. ΔCt value for each individual sample was calculated by subtracting the average Ct of loading control(s) from the average Ct of a tested gene. ΔΔCt value was calculated by subtracting the ΔCt of each tested male from the average ΔCt of control samples (XO*Sry* or XY males in *Eif2s3* expression analysis and XX in ovarian marker analysis), which served as references. The data were expressed as a fold value of expression level. For *Eif2s3x/y* specific and global expression analysis PCR primer efficiencies were taken into consideration. A 5x serial dilution of cDNA template was used to obtain the efficiency (E) for each primer pair. The average CT values were plotted against $\log_5$ cDNA concentrations, and the slope (a) was obtained to calculate (E) which equals to $5^{-1/a}$. Each plot yielded a trend line that had an R^2 value greater than 0.99. The fold expression was calculated using the formula $E^{\Delta Ct}$. Primer sequences are shown in Table S4.

Statistical analyses

Fisher's Exact Test was used to assess the differences between the genotypes for ROSI data and to analyze the progeny genotype frequencies. One-way or two-way ANOVA with post-hoc tests were used to analyze body weight data. Student's t-test was used for all other analyses.

Supplementary Text

Replacement of *Sry* function by transgenic activation of *Sox9*

In most mammals, including humans, *Sry* is the leading gene in the sex determination pathway, which acts in developing gonads and induces the development of testes (*21, 27*). *Sox9* (*Sry*-related HMG box gene 9), encoded on chromosome 11, is a direct target of SRY (*2*) and plays a pivotal role in male sexual development. In both mice and humans, ablation of *Sox9* causes male-to-female phenotypic sex reversal (*28-30*) while *Sox9* gain-of-function results in male gonad differentiation (*3, 31*). Transgenic overexpression of *Sox9* driven by *Wt1* (Wilms tumor 1) promoter leads to sex reversal, with XX mice developing as males; these XX*Sox9* males have testes devoid of germ cells due to a toxic dosage of X chromosome genes and the lack of the Y chromosome genes critical for spermatogonial proliferation (*3*). We placed the *Wt1-Sox9* transgene in the context of a single X chromosome carrying the *Eif2s3y* transgene (Fig. S1A) producing X^{E}O*Sox9* males (Table S1) with testis determination driven by transgenic overexpression of *Sox9* and the Y chromosome gene contribution limited to *Eif2s3y*. The analysis of testicular sections confirmed that spermatogenesis was ongoing allowing the development of germ cells with round spermatid-like morphology in all examined males (10/10). The numbers of spermatids were low and they arrested at step 7 of development (Fig. S2B). The quantitative analysis of spermatogenesis progression showed that spermatogonia/Sertoli cell and round spermatid/Sertoli cell ratios in X^{E}O*Sox9* males were similar to those observed in X^{E}O*Sry*, but 2- and 8-fold lower, respectively, than in XY (Fig. 1DE, Table S2). Round spermatids could be found in testicular cell suspensions from 8 out of 10 X^{E}O*Sox9* males (Table 1). Following round spermatid injection (ROSI) the oocytes were successfully fertilized, and when the resulting 2-cell embryos were transferred into the oviducts of recipient females, live offspring were obtained from 7 out of 8 males. ROSI efficiency, measured as a proportion of offspring developed from transferred embryos (15.7%, Table 1), was similar to that reported previously for X^{E}O*Sry* (9.1%, (*1*)) and that of XY controls (25%). We conclude that for the production of spermatids competent in assisted reproduction transgenic *Sox9* activation can substitute for the lack of *Sry* in mice without the Y chromosome but transgenic for *Eif2s3y*.

Replacement of *Eif2s3y* function by transgenic overexpression of *Eif2s3x*

Eif2s3y belongs to the eukaryotic translation initiation factor 2 subunit 3 protein family. It has an X chromosome encoded homologue, *Eif2s3x*, with which it retains 98% amino acid identity, despite only 86% nucleotide identity, an indication of strong selective constraint on both proteins (*32*). Both genes are ubiquitously expressed with strong expression in germ cells and represent a typical ancestral, single copy X-Y homologous gene pair, in which the X gene escapes X chromosome inactivation, and both X and Y copies are widely expressed in males (*13, 18*). We therefore hypothesized that if expression of *Eif2s3x* is high enough, it could substitute for *Eif2s3y* and replace its

function during the earliest phase of spermatogenesis. To test this hypothesis we produced mice transgenic for *Eif2s3x* using pronuclear microinjection and a bacterial artificial chromosome (BAC) clone encoding *Eif2s3x* and 21 kb upstream of its transcription site. In all 6 transmitting lines that were obtained (Tg1-6) the transgene incorporated on an autosome. The analysis of *Eif2s3x* expression in tissues from F1 and F2 transgenic XY males revealed that males of the Tg1,2,5&6 lines had elevated *Eif2s3x* levels (Fig. S2A). The *Eif2s3x* transgene was incorporated as a single copy in Tg2 and Tg6, as 2 copies in Tg5, and as 4 copies in Tg1 (Fig. S2B). The *Eif2s3x* transgene from the Tg1 line, which resulted in the strongest *Eif2s3x* overexpression, was placed in the context of XO*Sry* through selective breeding (Fig. S1B). The resulting XO*Sry*,*Eif2s3x* males (Table S1) had the Y chromosome contribution limited to one gene, *Sry*. Testis size in XO*Sry*,*Eif2s3x* males was similar to that of X^{E}O*Sox9* males but smaller than in XY or X^{E}O*Sry* (Fig. S3). In all males examined (13/13) the spermatogonial proliferation block was overcome and spermatogenesis progressed through meiosis up to step 7 of spermatid development (Fig. S4C). The spermatogonia/Sertoli cell ratio was similar to that observed in X^{E}O*Sry* (Fig. 1D, Table S2). Round spermatids, however, were dramatically depleted, with spermatid/Sertoli cell ratio 10- and 88-fold lower than in X^{E}O*Sry* and XY, respectively (Fig. 1E, Table S2). Out of 31 XO*Sry*,*Eif2s3x* males that provided testes for Assisted Reproductive Technology (ART) trials 17 had round spermatids identifiable in testicular cell suspension; these cells were used for injections followed by embryo transfer. Live offspring were obtained from 16 males, with an efficiency comparable to that obtained with XY control (22.5% vs. 25%, Table 1). We conclude that overexpression of *Eif2s3x* can substitute for the lack of *Eif2s3y* and initiate spermatogenesis in the XO*Sry* context. Moreover, our data suggest that the role of *Eif2s3x/y* during spermatogenesis is not limited to spermatogonial proliferation but extends to meiotic progression.

Spermatogenesis and germ cell function in males with no Y chromosome DNA.

Having demonstrated that functional substitutions of *Sry*-to-*Sox9* and *Eif2s3y*-to-*Eif2s3x* are possible, and males with single Y chromosome genes (either *Sry* or *Eif2s3y*) can produce haploid gametes and yield live offspring, we tested whether spermatogenesis can take place in males with a complete absence of Y chromosome genes. The testes from XO*Sox9*,*Eif2s3x* males were smaller than those from the other genotypes (Fig. S3) and were variable in morphology, with the minority of males (13/48, XO*Sox9*,*Eif2s3x*) having relatively normal testes, except for their reduced size and roundish rather than oval shape (Fig. S5B,D,F), and the remaining males (35/48, XO*Sox9*,*Eif2s3x*-alt) having severely disfigured testes with patchy discoloration throughout, an abundance of interstitial tissue, and a low number of seminiferous tubules essentially devoid of germ cells, except for a few spermatogonia (Fig. S5C,E,G). These latter testes likely developed from ovotestes as they showed an elevated expression of ovarian markers, *FoxL2*, *Rspo1*, and *Wnt4* (Fig. S5H-J). Spermatogonial proliferation arrest was overcome in testes from all examined XO*Sox9*,*Eif2s3x* males (10/10), and spermatogenesis progression was comparable to that of XO*Sry*,*Eif2s3x* males (compare Fig. S2C and Fig. 1C), with a spermatogonia/Sertoli cell ratio similar to that of X^{E}O*Sry*, and a very significant depletion of round spermatids (Fig. 1DE, Table S2). In ART trials, 13 males had round spermatids which were used for injections, and yielded zygotes with two well-developed

pronuclei, which subsequently cleaved to normal 2-cell embryos (Fig. S6A-C; Movie S1). Embryos from 11 males were used for transfer, 10 resulted in pregnancy, and 9 yielded offspring (20.7%, Table 1). Among the males that yielded progeny there were F1, F2, and F3 generation ROSI offspring derived from the first XO*Sry*,*Eif2s3x* male to produce round spermatids (Fig. S6D). The data support that males entirely devoid of the Y chromosome genes are able to produce haploid gametes and yield progeny when these cells are used for assisted fertilization. However, simultaneous *Sry*-to-*Sox9* and *Eif2s3y*-to-*Eif2s3x* substitutions lead to problems with both testis development and spermatogenesis initiation so that only a subset of XO*Sox9*,*Eif2s3x* males were able to sire offspring with the help of ART.

Progeny from males with a single or with no Y chromosome genes are healthy and normal.

Altogether, 18, 46, and 41 offspring were produced from X^{E}O*Sox9*, XO*Sry,Eif2s3x*, and XO*Sox9*,*Eif2s3x*, respectively. All ROSI offspring were genotyped and the genotypes and their frequency met expectancy (Fig. S7A-C). Some offspring from XO*Sox9*,*Eif2s3x* males were assayed for presence of Y chromosome genes, and none were present, as expected (Fig. S7D). ROSI progeny were delivered as normal and healthy pups (Fig. S8A). Some of them were maintained for more than 20 months and remained healthy into old age (Fig. S8B). All male progeny were expected to be infertile due to their sex chromosome gene content and thus were not bred. Female progeny were efficient and successful breeders (Fig. S8C-E). ROSI offspring derived from X^{E}O*Sox9*, XO*Sry,Eif2s3x* and XO*Sox9,Eif2s3x* males were weighed monthly from 3 to 8 months of age (Fig. S8F&S9AB). Two controls were 'XY mating' (B6D2F1 mice derived by mating) and 'XY ROSI' (ROSI offspring derived from wild-type males). Overall, ROSI offspring from X^{E}O*Sox9*, XO*Sry,Eif2s3x*, and XO*Sox9*,*Eif2s3x* males gained weight similarly to controls. The only exception was female offspring derived from X^{E}O*Sox9* males, which had significantly increased body weight. This group had the smallest number of mice analyzed which we suspect might have led to this result. Since it has been reported that X chromosome univalence is associated with decreased body weight in females (*33*), we defined the genotypes of all ROSI offspring subjected to body weight analysis (Table S3). To see if the number of X chromosomes influences our data, we compared body weight of XX vs. XO and XX*Eif2s3x* vs. XO*Eif2s3x* ROSI-derived females (Fig. S9CD). Although there was a trend for the average body weight of XX and XX*Eif2s3x* females being higher than that of XO and XO*Eif2s3x*, respectively, the differences between the groups at each given age were not statistically significant.

Relationship between spermatogenesis and *Eif2s3* transcript expression.

Males carrying the *Eif2s3x* transgene had significantly depleted round spermatid number when compared to the *Eif2s3y* transgenics (Fig. 1E, Table S2). This suggests that *Eif2s3y* and *Eif2s3x* genes may differ functionally and/or the observed variability of spermiogenic phenotypes is due to the differences in the global level of *Eif2s3* genes/transgenes. In order to relate spermatogenesis progression to *Eif2s3x/y* expression, we independently quantified *Eif2s3x*, *Eif2s3y*, and both, in mice of interest (Fig. 2A-C). Mice transgenic for *Eif2s3y* and for *Eif2s3x* had their respective transgenic transcript levels significantly elevated when compared to XY (Fig. 2A,B). The drop in fold

overexpression in males transgenic for *Eif2s3x* (compare Fig. 2B and C) was due to *Eif2s3y* transcripts being ~5-7-times more abundant than *Eif2s3x* in wild-type testes (*11*) and was not present when the data were normalized to XO*Sry* (Fig. S10). The higher global *Eif2s3x/y* expression in X^{E}O*Sry* and X^{E}O*Sox9* is consistent with the higher incidence of round spermatids in these males (relate Fig. 2C to Fig. 1E). However, such elevated *Eif2s3x/y* expression is clearly not essential for normal spermatogenesis since the levels of *Eif2s3x/y* in XY males were lower than in either transgenic model, raising the question of how spermatogenesis would progress in the XO context with fewer transgenic copies of either *Eif2s3y* or *Eif2s3x*. For the *Eif2s3y* transgenics, we had only one line available, with 10 copies of the transgene (*34*). However, among the *Eif2s3x* transgenics, mice from lines Tg2&6 carried a single copy of the transgene (Fig. S2B) and had lower *Eif2s3x* expression in the XY context (Fig. S2A). We therefore placed these *Eif2s3x* transgenes in the XO*Sry* context, and examined testes from the resulting XO*Sry,Eif2s3x* males (Tg2&6). No differences in spermatogonia/Sertoli cell ratio were observed but round spermatids were only found in Tg1 males in which *Eif2s3x* transcript levels are 2.4-2.9-fold higher than in Tg2&6 males (Fig. 2D-F,S4D-G). Altogether these data support that *Eif2s3x* can functionally replace *Eif2s3y* role during spermatogenesis but only if sufficiently overexpressed. We also conclude that in males lacking the Y chromosome spermatogonial proliferation and spermatogenesis initiation can efficiently take place within a broad window of *Eif2s3x/y* expression. However, progression through meiosis is more sensitive and requires higher *Eif2s3x/y* transcript levels.

One issue of concern was that the *Eif2s3x*-derived retroposons, *Gm2223* or *Eif2s3x-ps1*, might influence our analyses. These retroposons are described as pseudogenes in MGI, and there are no ESTs corresponding to them in the databases. Nevertheless, the X retroposon, *Gm2223*, is almost identical (2 nucleotide differences) to *Eif2s3x* and the primers used to quantify *Eif2s3x* and *Eif2s3x/y* expression (Fig. 2B,C&F, Fig. S10) would also amplify transcripts from Gm2223. To evaluate the contribution of *Gm2223* to transcript levels measured with our assays, we exploited the fact that neither *Gm2223* nor *Eif2s3x-ps1* includes the first coding exon of *Eif2s3x*, and used primers from the first exon of *Eif2s3x* to amplify *Eif2s3x* specifically (Fig. S11). We obtained very similar results to assays that would detect transcripts from *Eif2s3x* and *Gm2223* (compare Fig. 2B to Fig. S11A, Fig. S10B to Fig. S11B, and Fig. 2F to Fig. S11C); very slight differences were most likely due to the decreased number of males for certain genotypes. Because the expression levels did not vary between the two assay types, we conclude that *Gm2223* is either not expressed or is expressed at lower levels than *Eif2s3x* in testis. This conclusion was further strengthened by our sequence analysis across the nucleotide difference between *Eif2s3x* and *Gm2223* which revealed no traces of *Gm2223* (Fig. S12). We conclude that *Gm2223* transcripts can effectively be discounted in the interpretation of our expression data.

<u>*Sry*/*Sox9* and *Eif2s3y*/*Eif2s3x* gene pairs in humans</u>

Transgenic activation of *Sox9* is one of many examples of an alternative pathway driving sex determination. Manipulation of expression of other genes from the SOX family (*Sox3*, *Sox8*, and *Sox10*) as well as other genes (*e.g. Dax1*, *Dmrt1/2*, *Rspo1*, *Fgfr2*, *FoxL2*, *Cbx2*, *Map3k1/4*, *Igfr1*, and other) have been shown to result in the sex fate change (reviewed in (*6-8*)). Disorders of sex development (DSD) in humans involve

mutations of many of the aforementioned genes (reviewed in (*35*)) implying that sex determination pathways and players are for the most part conserved. However, in humans, these mutations are almost exclusively associated with sterility.

Eif2s3y is conserved on the Y chromosome in eutherians but is not present in simian primates, including humans (*32*). Considering its important role in spermatogenesis how do species lacking *Eif2s3y* handle the loss? Of potential interest in this regard the human Y chromosome retains EIF1AY whose homologue on the X chromosome is involved in the same aspect of translation initiation as EIF2S3X, the loading of the initiator ternary complex to the 40S ribosomal subunit. EIF1AY is lost, together with KDM5D, RPS4Y2 and RBMY1 from the Y chromosome in cases of *AZFb* deletion and its loss is associated with meiotic maturation arrest. Comparative gene mapping studies have provided evidence that Y gene loss can be compensated by gene transposition to an autosome, and that this mechanism is widespread among mammals (*14*). A *EIF2S3* copy with testis specific expression was identified in human (*32*) and in several primates, with the phylogenetic analysis indicating that the *EIF2S3* retrogenes originate from the X chromosome and arose independently at least 3 times during evolution indicating strong purifying selection (*14*). In the Japanese spiny rat, which has lost its Y chromosome, four ancestral Y chromosome genes, including *EIF2S3Y*, have been identified in the genome, transposed either to X or to an autosome (*36, 37*).

Future work

To further understand the roles of *Eif2s3y* and *Eif2s3x* in spermatogenesis and their functional equivalence the following strategies could be utilized: (1) generation of XO*Sry* mice expressing lower levels of *Eif2s3y* and higher levels of *Eif2s3x* so that both types of transgenics are more comparable; (2) transgenic rescue experiments in mice engineered to have *Eif2s3y* or *Eif2s3x* independently inactivated in male germ cells. The latter would involve replacement of *Eif2s3x* with *Eif2s3y*. In respect to sex determination, investigations of the processes taking place during the sex determination window in the genotypes used in this study would clarify the interplay between the sex determinants (*Sry* and *Sox9*) and the *Eif2s3x*/*y* genes, although the distinct transcript levels from the *Eif2s3y* and *Eif2s3x* transgenes might be a limitation.

Acknowledgements

The authors are grateful to numerous students who helped with mouse genotyping, to Guy Longepied for technical assistance with the *Eif2s3x* transgene construct, and to Paul Burgoyne for sharing unpublished data on spermatogenesis in XO*Sry* transgenic for *Eif2s3x*/*Klh15*, insightful discussions on the data generated in this project, and overall support. Histological sections were prepared by JABSOM Histology Core supported by NIH grants NNCRR 5 G12 RR003061-26 and NIMHHD 8 G12 MD007601-26. Pronuclear injections were done by IBR Transgenic Core supported by NIH P20GM103457.

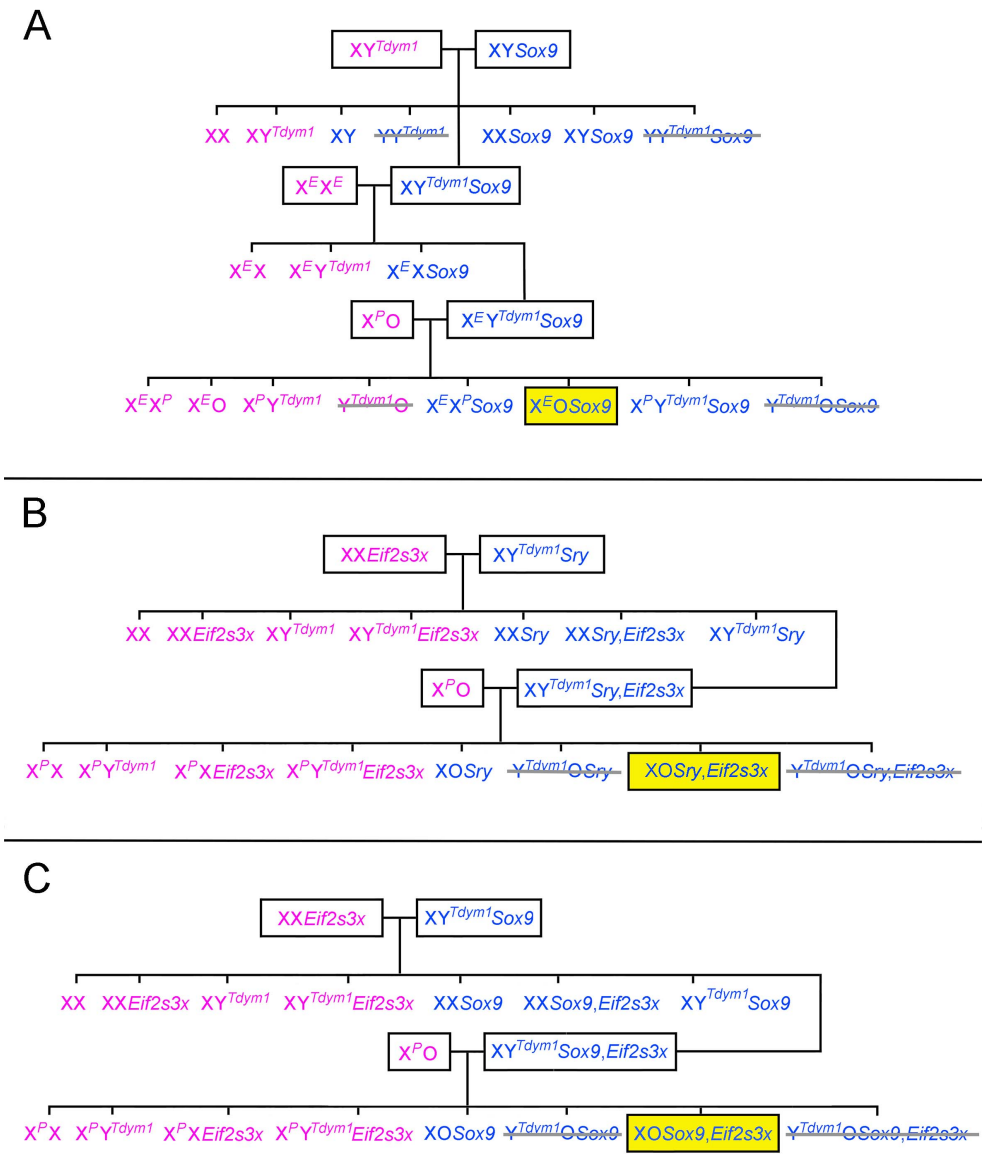


Fig. S1.

Breeding schemes. The breeding themes applied to produce males of interest, shown in yellow filled boxes: $X^{E}O$*Sox9* (**A**), XO*Sry*,*Eif2s3x* (**B**) and XO*Sox9*,*Eif2s3x* (**C**) are shown. Females are depicted in pink and males in blue font. X and Y represent normal sex chromosomes. X^{E} is an X chromosome carrying the *Eif2s3y* transgene. X^{P} is an X chromosome carrying the coat marker *Patchy-fur*. Y^{Tdym1} is a Y chromosome with *Sry* deleted. *Eif2s3x*, *Sry*, *Sox9* represent autosomally encoded transgenes. The offspring from the crosses were recognized by a combination of genotyping methods involving PCR, qPCR, testis size and fur appearance. Crossed genotypes do not survive to birth. For further explanation on mouse genotypes see Supplementary Material (Material and Methods; Animals).

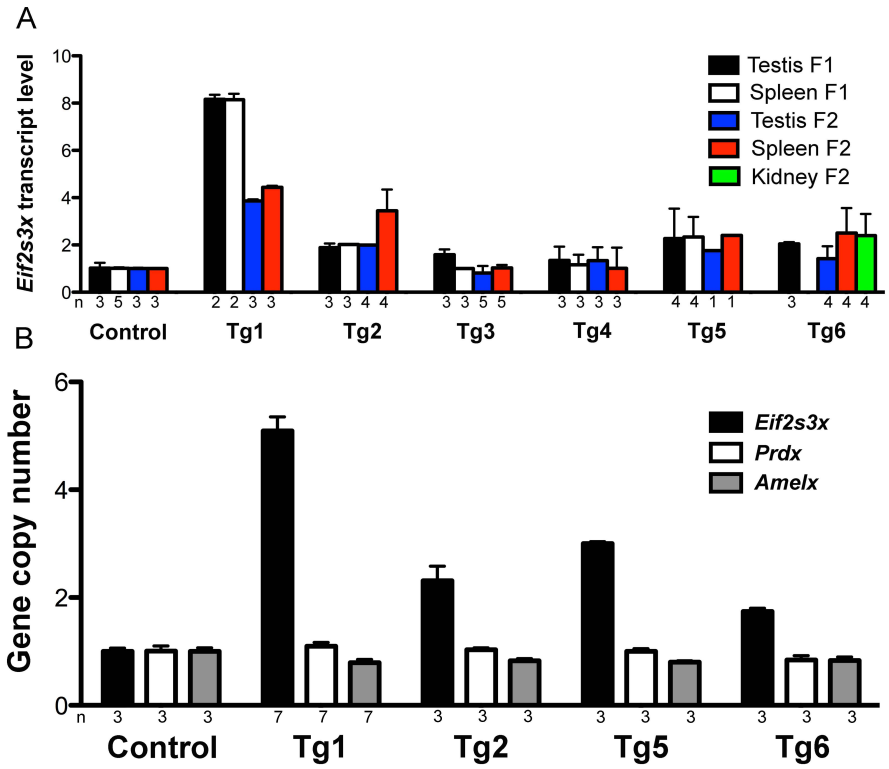


Fig. S2

***Eif2s3x* transcript levels and transgene copy number in XY*Eif2s3x* males.** (**A**) Pronuclear microinjection with *Eif2s3x* transgene resulted in 6 founders (F0, Tg1-6), which transmitted the transgene to next generations (F1 & F2). XY*Eif2s3x* transgenic males provided tissues (testis, spleen or kidney) for *Eif2s3x* transcript level quantification by real-time PCR, with *Actb* as a loading control, and primers recognizing both the endogenous and the transgenic *Eif2s3x*, and the *Eif2s3x* X-linked retroposon *Gm2223*. The controls were non-transgenic XY siblings. Values are mean ± SEM. The number of males examined is shown under each bar. *Eif2s3x* transcript levels remained at the level comparable to controls in lines Tg3&4, were slightly elevated in lines Tg2,5&6, and were strongly elevated in line Tg1. (**B**) *Eif2s3x* transgene copy number was assessed by quantitative PCR after amplification of three single copy X chromosome genes *Eif2s3x*, *Amelx* and *Prdx4*, with *Atr* used for normalization. The primers for detection of *Eif3s3x* were designed to recognize both endogenous and transgenic copies but did not recognize the *Gm2223*. Controls were non-transgenic XO females, in which only one copy of all genes was expected. Tg1, 2, 5 & 6 are XO*Sry* and XO mice transgenic for *Eif2s3x*. All transgenic mice had a single copy of *Prdx* and *Amelx*, as expected. Transgenic lines Tg2 and Tg6 were scored as having 2 copies (1 endogenous and 1 transgenic), Tg5 showed 3 copies (1 endogenous and 2 transgenic), and Tg1 showed 5 copies of *Eif2s3x* (1 endogenous and 4 transgenic). The final gene copy number was established using a typical mathematical rounding, i.e. readout <1.5 was scored as 1 and readout >/=1.5 as 2. Values are mean ± SEM, with number of males examined shown under each bar.

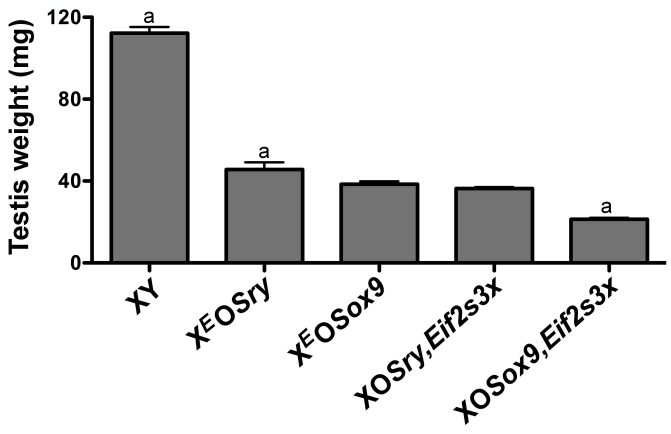


Fig. S3

Testis weight in males with limited or no Y gene contribution. Four types of males with limited or no Y gene complement (tested; X^{E}O*Sry*, X^{E}O*Sox9*, XO*Sry,Eif2s3x* and XO*Sox9,Eif2s3x*) were compared to wild-type XY males (control). Graph bars represent the average testis weight, with n=8, 6, 20, 80, 106 testes for XY, X^{E}O*Sry*, X^{E}O*Sox9*, XO*Sry,Eif2s3x* and XO*Sox9,Eif2s3x*, respectively. Statistical significance (t-test): [a] different than all others at $P<0.05$. Error = SEM

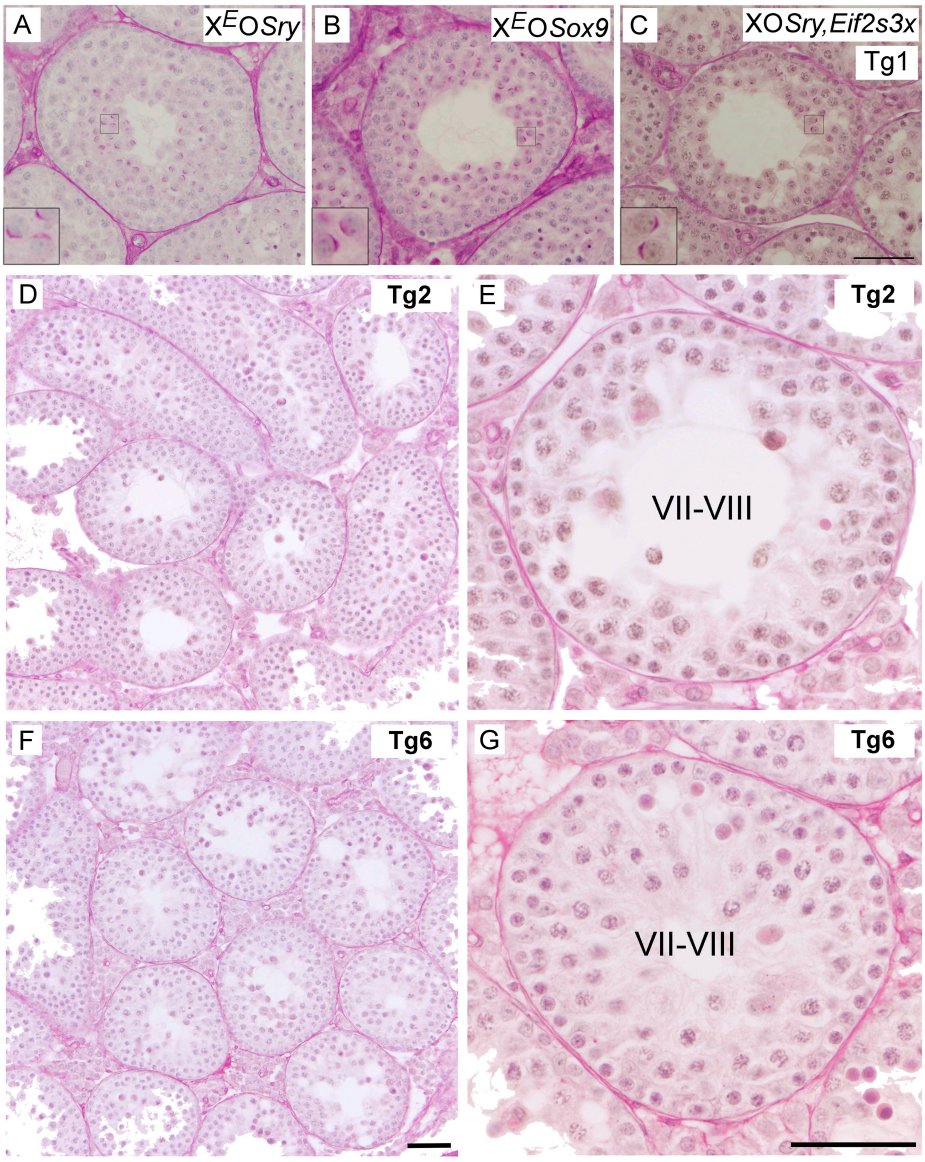


Fig. S4

Testis histology analysis. (**A-C**) Exemplary tubules of PAS-H stained sections of testis from males with limited Y gene complement (**A**) $X^{E}O$*Sry*, (**B**) $X^{E}O$*Sox9*, (**C**) XO*Sry,Eif2s3x*. All males have meiotic and postmeiotic arrests that occasionally allow formation of round spermatids (insets), arresting at step 7. All tubules are of stage VII-VIII. (**D-G**) Exemplary tubules of PAS-H stained sections of testis from XO*Sry*,*Eif2s3x* males with lower number of transgene copies, Tg2 (**D&E**) and line Tg6 (**F&G**). Spermatogonial proliferation arrest was overcome and spermatogonesis was initiated (**D&F**) but contradictory to line Tg1 (**C**), no round spermatids could be found (**E&G**). Scale bar, 50 µm, insets, x3 magnification.

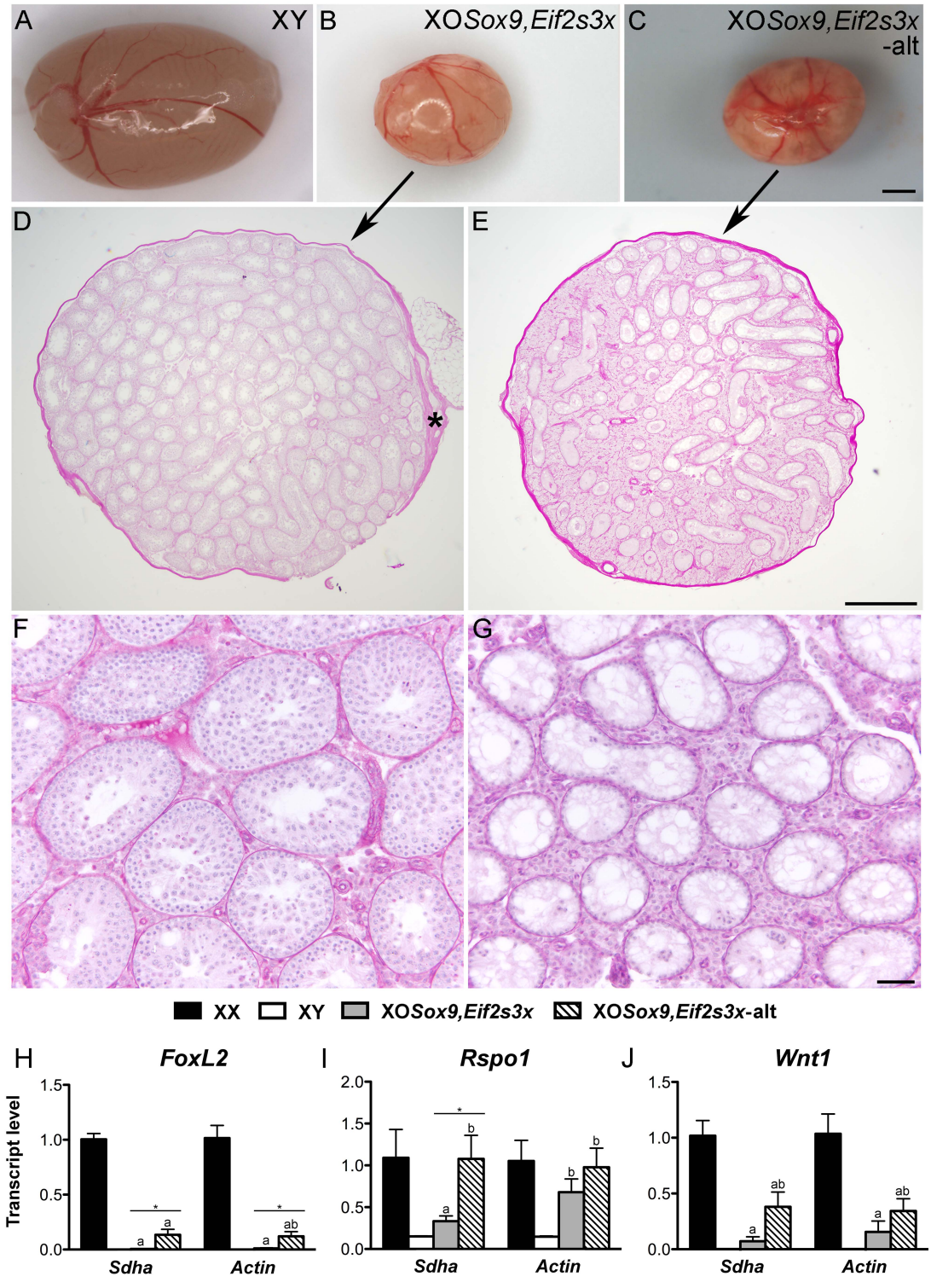


Fig. S5

Testis defects in XO*Sox9,Eif2s3x* males. Morphology of the testis from a wild-type male with an intact Y chromosome (XY; **A**) and males with no Y chromosome genes (XO*Sox9,Eif2s3x*; **B&C**). **D&E** are the panoramics of cross sections of testes shown in B

and C, respectively. * in D marks the region underlying and adjacent to coelomic artery, in which there is abundance of interstitial tissue and poor tubule formation. In E these defects are extremely severe and appear throughout the cross-section. The male whose testis is shown in B&D represents XO*Sox9,Eif2s3x* males, in which the seminiferous tubules contain germ cells indicative of ongoing spermatogenesis (**F**). The male whose testis is shown in C&E represents XO*Sox9,Eif2s3x*-alt males, in which the seminiferous tubules resemble those of XO*Sry*, with few spermatogonia present and no spermatogenesis (**G)**. Scale is 1 mm (A-C, shown in C) and 500 μm (D&E, shown in E), and 50 μm (F&G, shown in G). (**H-J**) Transcript levels of ovary-specific genes in testes from XO*Sox9,Eif2s3x* males. Testes were obtained from two groups of males with no Y gene complement, XO*Sox9,Eif2s3x* representing males with ongoing spermatogenesis (see B,D,F) and XO*Sox9,Eif2s3x*-alt with abnormal testes and no spermatogenesis (see C,E,G). Ovaries from wild-type females (XX) and testes from wild-type males (XY) served as controls. Transcript levels of *FoxL2* (**H**), *Rspo1* (**I**) and *Wnt4* (**J**) were quantified by real-time PCR, with *Actb* or *Sdha* as loading controls. Values are mean ± SEM, with n=3-4 males per group, Statistical significance (t-test, $P<0.05$): [a] different than XX, [b] different than XY, * $P<0.05$.

Fig. S6

Round spermatid injection (ROSI) with spermatids from males with a single or no Y chromosome genes. (**A**) Example of testicular cell suspension from XO*Sox9*,*Eif2s3x* male, with injection pipette visible on the right and inset (x3 magnification) showing a single round spermatids; (**B**) 6 hours after injection the oocytes developed two pronuclei (arrowheads) and extruded a second polar body (arrow). (**C**) 24 h after injection fertilized oocytes cleaved and became 2-cell embryos. Scale, 50 µm. (**D**) Three generations of XO*Sox9*,*Eif2s3x* males derived with ROSI. The albino male was the first XO*Sox9*,*Eif2s3x* male, generated by breeding, who successfully sired first ROSI offspring. His XO*Sox9*,*Eif2s3x* son (F1 ROSI) then sired XO*Sox9*,*Eif2s3x* (F2 ROSI), which then subsequently sired XO*Sox9*,*Eif2s3x* (F3 ROSI). All males had no Y chromosome genes.

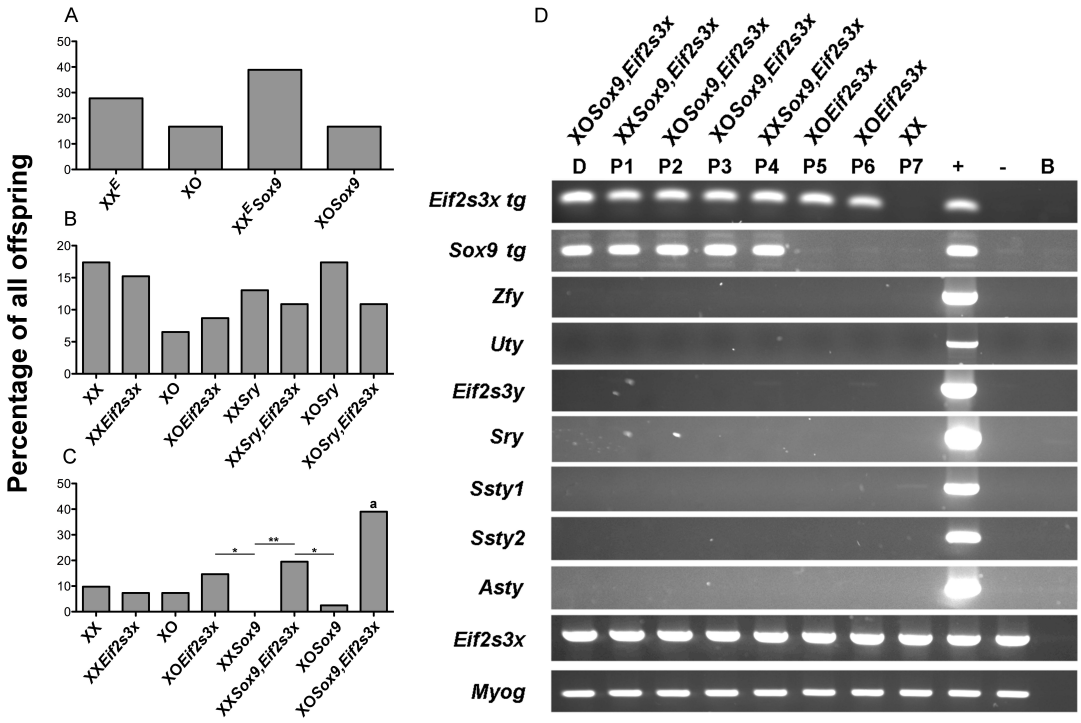
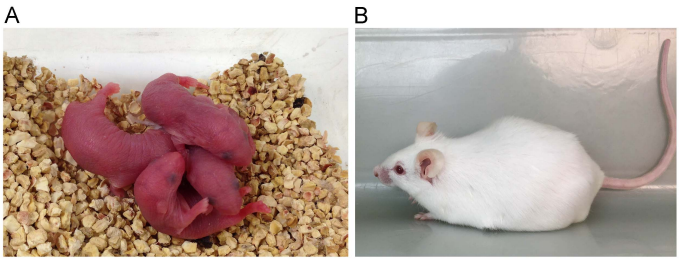


Fig. S7

Genotypes of progeny obtained after ROSI with spermatids from males with 1 or no Y genes. Progeny obtained after ROSI from X^{E}O*Sox9* (**A**), XO*Sry,Eif2s3x* (**B**), and XO*Sox9,Eif2s3x* (**C**) males were genotyped with PCR and qPCR. The expected progeny genotypes are 4, 8 and 8 for X^{E}O*Sox9*, XO*Sry,Eif2s3x*, and XO*Sox9,Eif2s3x* males, respectively. All expected progeny genotypes were obtained from X^{E}O*Sox9* and XO*Sry,Eif2s3x* males, and were evenly distributed. Among the progeny obtained from XO*Sox9,Eif2s3x* one expected genotype (XX*Sox9*) did not appear and the male progeny genotypes were not evenly distributed. Number of progeny genotyped was n=18, n=46, n=41 for A, B, and C, respectively. Statistical significance (Fisher's Exact Test): [a] different than all other except XX*Sox9,Eif2s3x*; * P<0.05; ** P<0.01 (**D**) The exemplary results of the genotyping of the progeny obtained after ROSI from XO*Sox9,Eif2s3x* male**.** D = XO*Sox9*,*Eif2s3x* male dad, which provided round spermatids for ROSI. P1-P7 = ROSI offspring derived from D. +, -, and B are positive, negative, and blank control, respectively. *Eif2s3x* tg and *Sox9* tg amplify transgenic *Eif2s3x* and *Sox9*, respectively, and not the endogenous copies of these genes. X-encoded *Eif2s3x* and autosomally encoded myogenin (*Myog*) are present in all mice while no Y chromosome genes are present in XO*Sox9*,*Eif2s3x* dad and its progeny.



C

Breeding of female progeny obtained after ROSI with spermatids from males with single or no Y chromosome genes.

Father's genotype	Father's Y gene contribution	ROSI daughter genotype	Breeding duration (months)	Number of litters obtained	Average number of pups/litter
$X^{E}O$*Sox9*	*Eif2s3y*	XX^{E}*	10	10	10
		XX^{E}	10	8	10
XO*Sry,Eif2s3x*	*Sry*	XX*	10	8	10
		XX	10	11	5
XO*Sox9,Eif2s3x*	None	XX	10	10	6
		XO*	8	7	7
		XO*Eif2s3x***	6	5	4
		XO*Eif2s3x***	6	4	13
		XO*Eif2s3x***	4	3	6

* Two male offspring derived from this female were bred and proven fertile
** This female was bred to XY^{Tdym1}*Sox9* male, rather than wild-type XY.

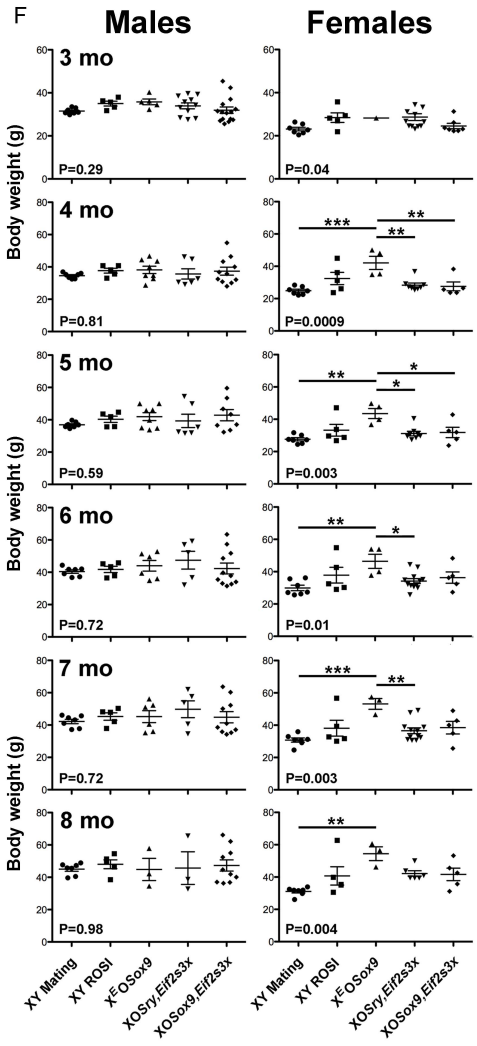


Fig. S8

Progeny from males with a single or no Y chromosome genes. (**A**) Newborn pups born after ROSI with spermatids from XO*Sox9*,*Eif2s3x* male. (**B**) 19 months old ROSI offspring derived from XO*Sox9*,*Eif2s3x* male. (**C**) The results of breeding of female ROSI offspring. (**D** &**E**). Female ROSI offspring derived from XO*Sox9*,*Eif2s3x* male with their own pups. (**F**) Offspring weights. ROSI offspring derived from $X^{E}O$*Sox9*, XO*Sry,Eif2s3x* and XO*Sox9,Eif2s3x* males were weighed monthly from 3 to 8 months of age. Two controls were 'XY mating' (B6D2F1 mice derived by mating) and 'XY ROSI' (ROSI offspring derived from wild-type males). P value in bottom left corner of each graph shows the result of one-way ANOVA analysis and horizontal lines show the differences between individual groups measured by post-hoc Tukey test * P<0.05; ** P<0.01 (GraphPad Prism). The only group that differed from others were female offspring derived from $X^{E}O$*Sox9* males; this group had the smallest number of mice analyzed.

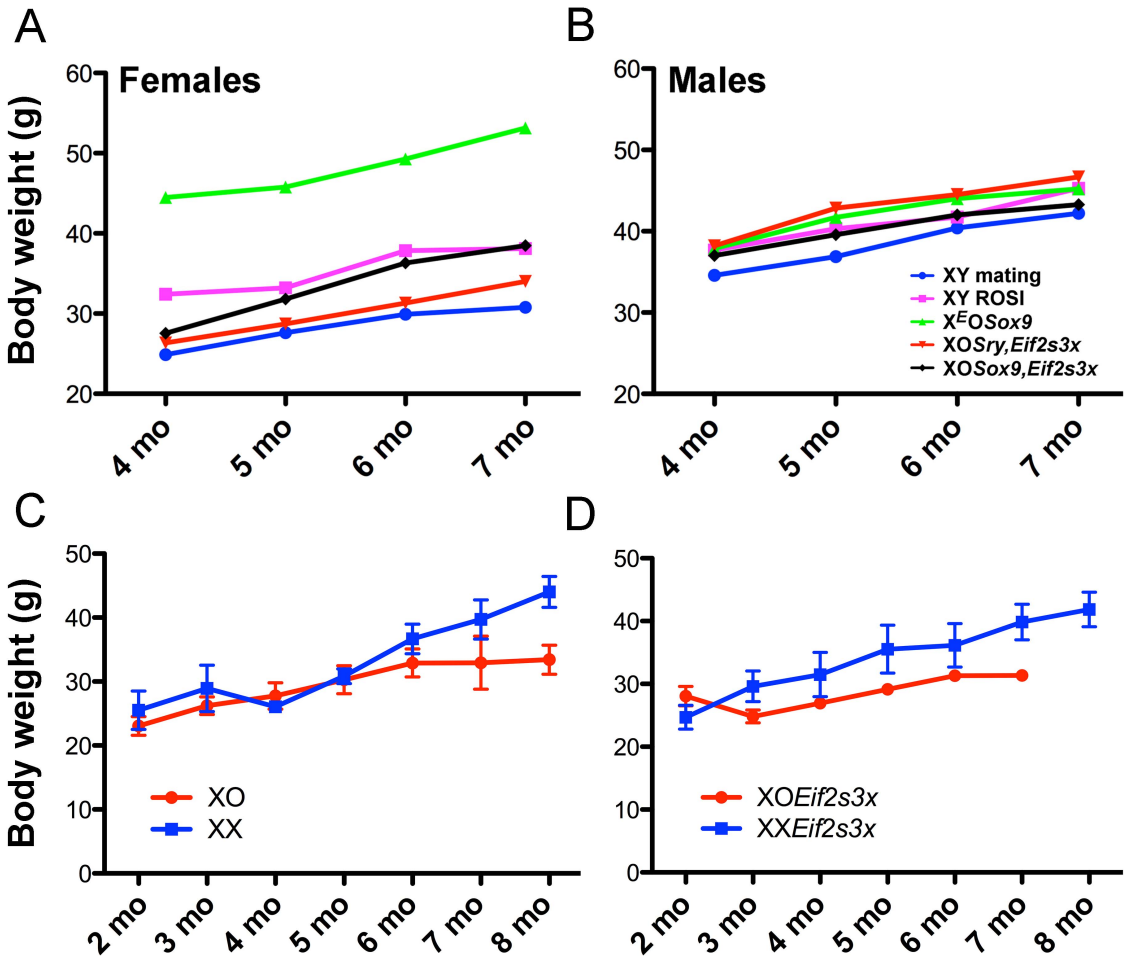


Fig. S9

Offspring growths measured as an increase in body weight over time. (**A-B**) ROSI offspring from X^{E}O*Sox9*, XO*Sry,Eif2s3x* and XO*Sox9,Eif2s3x* males were weighed monthly from 2 to 8 months of age. The data shown are from mice, for which weights were scored for all examined months (4, 5, 6, and 7). Two control groups were included in the analysis: 'XY mating' are B6D2F1 mice derived by mating. 'XY ROSI' are offspring derived after ROSI with wild-type males. The data were analyzed with two-way ANOVA with age and group as factors, and post-hoc Bonferroni test for multiple paired comparison. Males and females from all groups increased significantly in weight over the examined period (females, P=0.0003; males, P=0.003; ANOVA, age). There were no differences between the groups of male offspring at any age (P=0.345, ANOVA, group) but a difference was observed for females (P<0.0001, ANOVA, group). Post-hoc test demonstrated that female offspring derived from X^{E}O*Sox9* were different from female offspring from 'XY mating' group (P<0.001, P<0.01, and P<0.001, P<0.0001, for months 4,5,6,7 respectively), from XO*Sry,Eif2s3x* group (P<0.01, P<0.05, and P<0.01, P<0.01, for months 4,5,6,7 respectively) and from XO*Sox9,Eif2s3x* group (P<0.05, month 4). The number of mice examined for XY mating, XY ROSI, X^{E}O*Sox9*, XO*Sry,Eif2s3x* and XO*Sox9,Eif2s3x* groups was 7, 5, 3, 7, 5 (females) and 7, 5, 6, 4, 10 (males), respectively.

All ROSI offspring were derived after injection of spermatids from males on partial (transgenics) or full MF1 (XY) genetic background and oocytes from B6D2F1 females. Error bars are not shown for graph clarity. See Table S3 for information on the genotypes of offspring included in the body weight analyses. (**C-D**) Body weight comparison for female ROSI offspring with one and two X chromosomes. Females derived from XO*Sry,Eif2s3x* and XO*Sox9,Eif2s3x* males were weighed monthly between 2 to 8 months of age. The data shown represent the comparison of XO vs. XX (**C**) and XO*Eif2s3x* vs. XX*Eif2s3x* (**D**). The data were analyzed with two-way ANOVA with age and genotype as factors, and post-hoc Bonferroni test for multiple paired comparison. All mice increased significantly in weight over the examined period (C: $P<0.0001$; D: $P=0.027$, ANOVA, age), and the effect of genotype was observed (C: $P=0.022$; D: $P=0.021$, ANOVA, genotype). However, no differences between the genotypes were observed at any age with a post-hoc test, and with an independently performed t-test. Data are shown as mean ± SDev, with the number of mice examined:. XO: 5,6,5,5,4,3,2; XX: 5,5,2,2,5,5,4; XO*Eif2s3x*: 2,3,3,3,3,3,0; XX*Eif2s3x*: 4,4,4,4,6,6,6, for months 2,3,4,5,6,7,8, respectively.

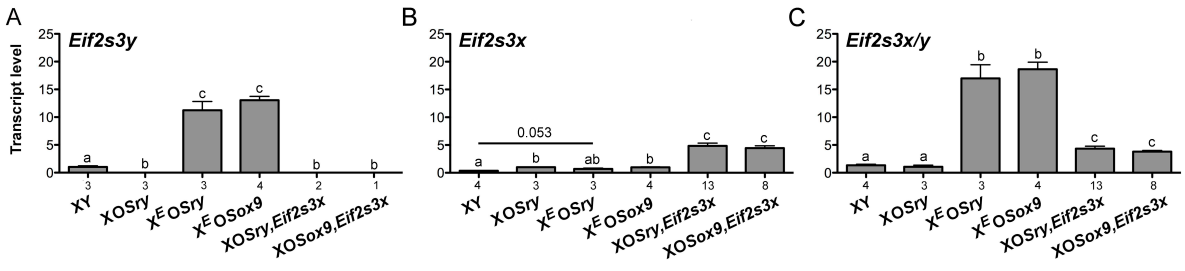


Fig. S10

***Eif2s3x/y* expression.** Transcript levels of endogenous and transgenic (**A**) *Eif2s3y*; (**B**) *Eif2s3x*; and (**C**) *Eif2s3x/y*. Transcripts were quantified by real-time PCR with *Actb* as a loading control, and XY (A) and XO*Sry* (B&C) males serving as reference controls. The data in (A) are the same as in Fig. 2A and is shown here for comparison. The data in (B) and (C) are the same as in Fig. 2BC but the normalization here is done with XO*Sry* instead of XY. These data are shown to explain the drop in fold overexpression in males transgenic for *Eif2s3x* (compare Fig. 2B and C), which was due to *Eif2s3y* transcripts being ~5-7-times more abundant than *Eif2s3x* in wild-type testes (*11*). This drop is not present when the data are normalized to XO*Sry* as shown here. Statistical significance (t-test, $P<0.05$): bars with different letters are different. Bars are mean ± SEM, with n shown under the X axis. The primers used to quantify *Eif2s3x* (B) and *Eif2s3x/y* (C) would amplify any transcripts from the *Eif2s3x* retroposon *Gm2223*.

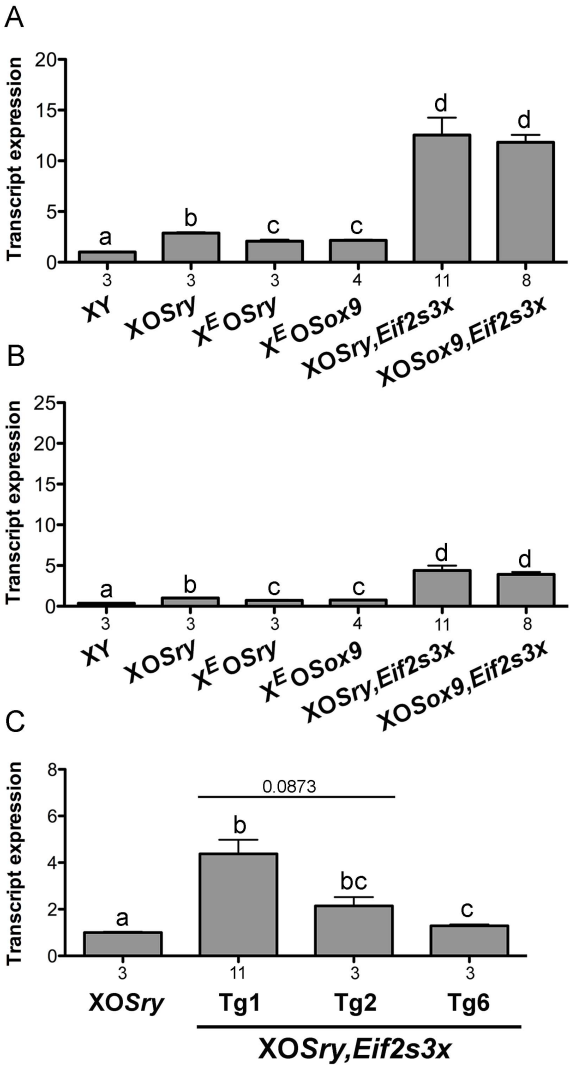


Fig. S11

***Eif2s3x* expression.** This analysis was performed with primers recognizing endogenous and transgenic *Eif2s3x* but not the *Eif2s3x* X-linked retroposon *Gm2223*, to estimate the contribution of *Gm2223* to the perceived *Eif2s3x* expression levels. Transcript levels of endogenous and transgenic *Eif2s3x* were quantified by real-time PCR with *Actb* as a loading control, and XY (A) and XO*Sry* (B&C) males serving as reference controls. The data shown in (A), (B), (C) were generated essentially by re-running samples used to generate data shown in Fig. 2B, Fig. S10B, and Fig. 2F, respectively, except that the number of males was decreased for XY (n=3 instead of n=4) and XO*Sry,Eif2s3x* Tg1 groups (n=11 instead of n=13 or n=12).

Statistical significance (t-test, $P<0.05$): bars with different letters are different. Bars are mean ± SEM, with n shown under the X axis.

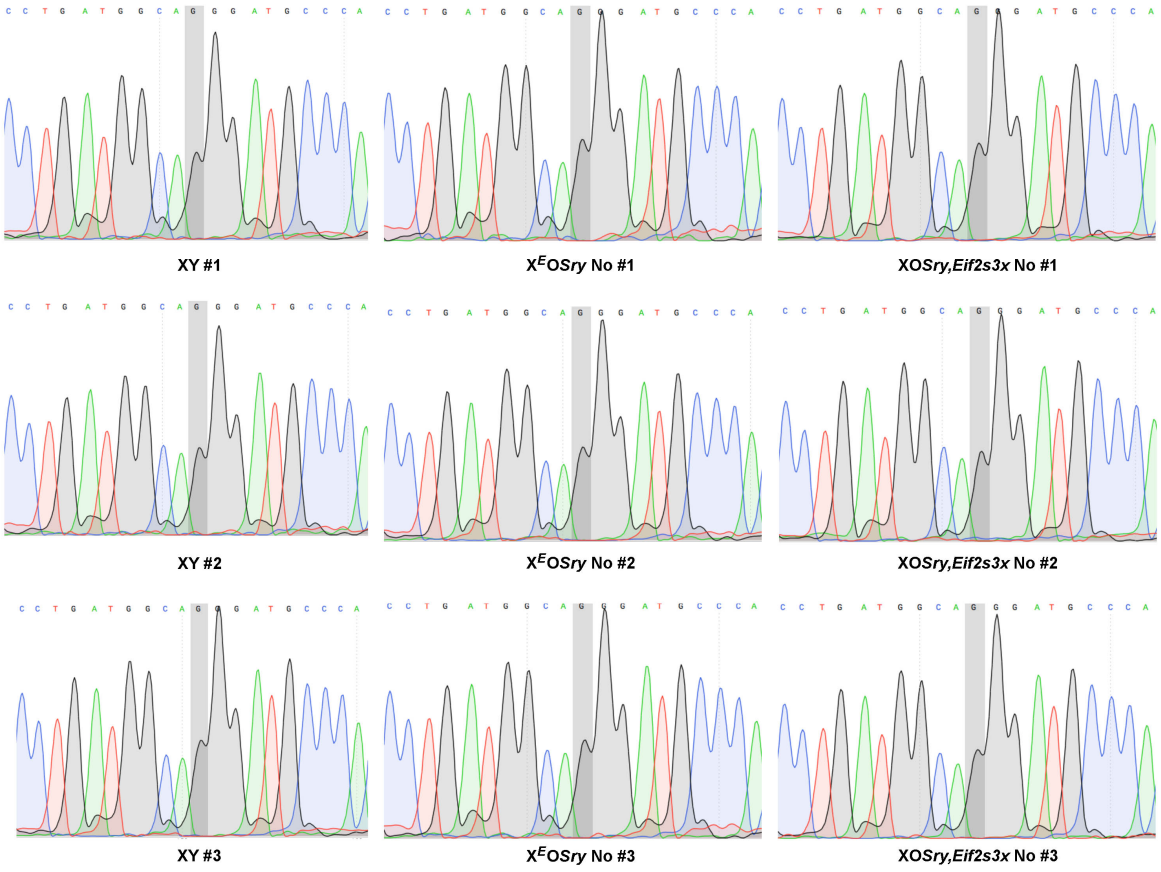


Fig. S12

Sequence analysis. Sequence analysis of the region encoding the nucleotide difference between *Eif2s3x* (chrX:94190069 G) and *Gm2223* (chrX:33507800 A) was performed on PCR products amplified from testis cDNA of XY, X^{E}O*Sry* and XO*Sry,Eif2s3x* males (n=3 each). No trace of the A nucleotide at the *Gm2223* genomic locus was observed (grey box). Genome coordinates are from GRCm38/mm10 assembly.

Table S1.
Summary characteristics of mice used in the study.

Male genotype	Y gene contribution	Sex determination driver	Spermatogenesis driver	Spermato genesis	Haploid round spermatids
XY	Intact Y	endogenous *Sry*	endogenous *Eif2s3y*	normal	frequent
XO*Sry*	*Sry*	*Sry* transgene	absent	absent	absent
X^{E}O*Sry*	*Eif2s3y* & *Sry*	*Sry* transgene	*Eif2s3y* transgene	impaired	rare
X^{E}O*Sox9*	*Eif2s3y*	*Sox9* transgene	*Eif2s3y* transgene	impaired	rare
XO*Sry,Eif2s3x*	*Sry*	*Sry* transgene	*Eif2s3x* transgene	impaired	extremely rare
XO*Sox9,Eif2s3x*	none	*Sox9* transgene	*Eif2s3x* transgene	impaired	extremely rare

Table S2.

Quantitative analysis of spermatogenesis progression.

Genotype	Spermatogonia/Sertoli cell ratio (mean±SEM)						
	XII-I	II-IV	V-VI	VII-VIII	IX-X	XI	**All stages**
XY	0.41±0.11	1.42±0.13$^{\&}$	2.38±0.61	0.11±0.02	0.20±0.01	0.27±0.02^{a}	**0.79±0.08$^{\&}$**
X^{E}O*Sry*	0.30±0.07	0.79±0.08	1.17±0.44	0.07±0.01^{a}	0.08±0.00*	0.10±0.01^{b}	**0.41±0.09**
X^{E}O*Sox9*	0.15±0.02$^{\&}$	0.70±0.06^{a}	1.15±0.12^{a}*	0.02±0.01^{b}*	0.07±0.02*	0.20±0.02^{a}	**0.39±0.03^{a}**
XO*Sry,Eif2s3x*	0.34±0.03	0.92±0.08^{b}	1.45±0.07^{b}*	0.05±0.01^{a}*	0.12±0.03	0.21±0.03	**0.52±0.03^{b}**
XO*Sox9,Eif2s3x*	0.28±0.03	0.94±0.08	1.52±0.13*	0.03±0.00^{b}*	0.07±0.01*	0.17±0.04	**0.51±0.03^{b}**
	Round spermatid/Sertoli cell ratio (mean±SEM)						
	XII-I	II-IV	V-VI	VII-VIII	IX-X	XI	**All stages**
XY	8.03±0.45$^{\&}$	14.72±2.04$^{\&}$	12.48±1.08$^{\&}$	13.21±0.95$^{\&}$	0	0	**7.88±0.50$^{\&}$**
X^{E}O*Sry*	0.40±0.02$^{\&}$	1.39±0.45^{a}	1.08±0.10^{a}	1.43±0.36^{a}	0.85±0.13^{a}*	0.34±0.07^{a}*	**0.92±0.15^{a}**
X^{E}O*Sox9*	0.27±0.03$^{\&}$	0.68±0.13^{a}	1.01±0.16^{a}	0.83±0.16^{a}	0.81±0.25^{a}	0.50±0.15^{a}	**0.67±0.12^{a}**
XO*Sry,Eif2s3x*	0.04±0.02	0.08±0.04^{b}	0.11± 0.04^{b}	0.16±0.10^{b}	0.06±0.02^{b}	0.05±0.02^{b}	**0.09±0.04^{b}**
XO*Sox9,Eif2s3x*	0.06±0.02	0.13±0.04^{b}	0.24±0.06^{b}	0.28±0.06^{b}	0.18±0.04^{c}	0.13±0.03^{c}	**0.16±0.04^{b}**

For each male 10 tubules were examined per stage and the numbers of spermatogonia, round spermatids, and Sertoli cells were counted. The data are expressed as germ cell/Sertoli cell ratios. In wild-type males no round spermatids are present in stages IX-XI so those observed in the remaining genotypes represent 'delayed spermatids'. Statistical significance (t-test): * different than XY; $^{\&}$ different than all other; ^{a,b,c} columns with different superscript letters are significantly different. The number of males per genotype was n=3, 3, 8, 13, 10 for XY, X^{E}O*Sry*, X^{E}O*Sox9*, XO*Sry,Eif2s3x*, XO*Sox9,Eif2s3x.*

Table S3.

ROSI offspring body weight data with genotypes of individual mice identified.

Father's genotype	ROSI offspring sex	ROSI offspring genotype	Body weight (g)						
			2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo
X^EO*Sox9*	Females	X^EX			48.10	47.68	53.89	55.52	56.48
		X^EX			50.12	49.77	53.95	57.41	60.64
		X^EX			35.27	39.87	40.01	46.56	46.20
		XO	24.90	28.20	34.87	36.72	37.77		
	Males	X^EX*Sox9*			44.44	45.80	51.41	56.17	57.82
		X^EX*Sox9*			34.68	39.80	39.41	40.48	42.15
		X^EX*Sox9*			28.67	33.80	34.87	35.26	34.55
		X^EX*Sox9*	33.05	40.26	46.70	50.01	52.82	52.32	
		X^EX*Sox9*	30.45	36.19	39.99	46.12	49.51	51.00	
		X^EX*Sox9*	29.98	32.27	32.75	34.77	36.06	36.04	
		X^EX*Sox9*	31.06	35.80	43.60	49.94			
		XO*Sox9*	31.16	34.47	34.70	35.05			
XO*Sry,Eif2s3x*	Females	XX*Eif2s3x*					25.70	36.35	39.50
		XX*Eif2s3x*					35.15	38.92	39.41
		XX					36.80	38.76	40.98
		XX					44.37	49.18	50.06
		XX					34.83	37.10	39.43
		XX*Eif2s3x*			36.80	40.41	42.85	47.75	43.75
		XO*Eif2s3x*		26.73	26.89	30.54	31.45	32.34	
		XO*Eif2s3x*		24.57	28.52	29.45	31.89	31.08	
		XX		24.30	26.53	29.70	30.02	30.73	
		XO*Eif2s3x*		23.18	25.38	27.40	30.63	30.65	
		XX*Eif2s3x*		24.38	26.05	28.57	32.17	32.72	
		XO		24.53	27.33	30.72	34.15	33.15	
		XO	23.63	26.49	29.13	32.48			
		XX	27.28	33.28					
		XX	34.40	41.14					
		XO	27.25	31.12					
		XX*Eif2s3x*	25.07	29.85					
		XX*Eif2s3x*	29.87	34.42					
	Males	XX*Sry,Eif2s3x*					59.20	62.02	65.59
		XX*Sry*			29.15	31.64	32.15	34.57	32.75
		XX*Sry,Eif2s3x*			32.73	35.45	36.67	40.62	38.69
		XX*Sry*		39.32	44.81	49.90	52.13	53.90	
		XO*Sry*		38.54	46.20	54.42	57.07	57.63	
		XX*Sry,Eif2s3x*	25.96	28.44	30.57	31.89			
		XO*Sry*	24.11	27.58	30.45	32.49			
		XO*Sry*	28.06	34.32					

		XX*Sry*	28.87	33.16					
		XO*Sry*	24.65	28.09					
		XX*Sry,Eif2s3x*	27.28	31.65					
		XX*Sry,Eif2s3x*	32.66	36.71					
		XO*Sry,Eif2s3x*	31.62	35.80					
		XX*Sry,Eif2s3x*	33.83	39.68					
XO*Sox9,Eif2s3x*	Females	XO*Eif2s3x*	26.51						
		XO*Eif2s3x*	29.58						
		XX	28.18						
		XO	20.11	22.23	23.70	27.71	32.43	40.03	35.68
		XX*Eif2s3x*	21.58	22.92	26.50	32.85	36.25	34.99	42.56
		XO	19.51	23.10	23.72	23.77	27.30	25.66	31.16
		XX	17.45	22.32	25.62	31.99	37.34	42.77	45.60
		XX*Eif2s3x*	22.26	31.29	38.27	42.83	48.32	48.95	53.20
		XX	20.29	23.66					
	Males	XX*Sox9,Eif2s3x*	32.05	42.39	46.61	53.42	57.34	60.3	62.16
		XO*Sox9,Eif2s3x*	33.80						
		XO*Sox9,Eif2s3x*	31.50						
		XX*Sox9,Eif2s3x*	32.61	45.43	54.93	59.59	63.41	63.7	66.18
		XO*Sox9,Eif2s3x*	25.89						
		XO*Sox9,Eif2s3x*	29.66						
		XO*Sox9,Eif2s3x*	26.4						
		XO*Sox9,Eif2s3x*	26.47						
		XO*Sox9,Eif2s3x*	25.34						
		XO*Sox9,Eif2s3x*	23.44						
		XO*Sox9,Eif2s3x*	24.31	26.58					
		XO*Sox9,Eif2s3x*	23.68	25.58	28.02	32.39	35.5	37.12	40.09
		XO*Sox9,Eif2s3x*	25.60	27.01	32.49	35.33	32.73	34.20	36.30
		XX*Sox9,Eif2s3x*	24.54	27.82	30.11	30.51	34.05	35.85	37.05
		XO*Sox9,Eif2s3x*	25.52	27.45	32.12	32.76	31.52		
		XO*Sox9,Eif2s3x*	29.80	31.15	35.77	36.54	39.70	40.98	44.73
		XO*Sox9,Eif2s3x*	28.67	31.53	36.75	37.01	37.84	38.54	41.92
		XO*Sox9,Eif2s3x*	29.23	34.65	40.01	43.37	48.63	50.07	52.46
		XO*Sox9*	31.02	37.01	43.31	46.69	51.74	52.21	55.05
		XX*Sox9,Eif2s3x*	29.29	30.61	30.82	33.60	33.12	35.29	36.84
		XX*Sox9,Eif2s3x*	20.01						
		XO*Sox9,Eif2s3x*	32.18	31.17					
		XX*Sox9,Eif2s3x*	30.81	32.41					
		XO*Sox9,Eif2s3x*	27.72	27.83					

The entire body weight data shown here are presented in Fig. S8F. The data shown here in blue font are presented in Fig. S9A.

Table S4.
Primers.

Gene	Primer ID	Primer sequence	Amplicon	Reference; Used in Figure
qPCR primers for sex chromosome copy number estimation				
Eif2s3x (sp1)*	*Eif2s3x-sp-CNAFP*	CAATGTGGCGAGATCCTGTC	87 bp	Current paper; Fig. S2B
	Eif2s3x-sp-CNARP	CTTTTCTCTCCGAGCAAGATG		
Prdx	*Prdx4*-F	CATGATATCCACTGAAAGCTAC	82 bp	(*38*); Fig. S2B
	Prdx4-R	GAGACAGTGTATCTATCCCTG		
Amelx	*Amelx*-F	GTTGGGTTGGAGTCATGGAG	162 bp	(*38*); Fig. S2B
	Amelx-R	GGCTGCACCACCAAATCATC		
Atr	*Atr*-WT L1	GGGATGTTTACAGCCAGCTC	143 bp	(*34*); Fig. S2B
	Atr-WT R1	AGCCGATTTGCCACAGTAAC		
Standard genotyping PCR primers				
Myog	*Om1a*	TTACGTCCATCGTGGACAGCAT	246 bp	(*39*); Fig. S7
	Om1b	TGGGCTGGGTGTTAGCCTTAT		
Zfy	*Zfyp1*	AAGATAAGCTTACATAATCACATGGA	600 bp	(*40*); Fig. S7
	Zfyp2	CCTATGAAATCCTTTGCTGCACATGT		
Uty	*oMJ274*	AACACAAGTTAAAGACTATTCAG	850 bp	(*41*); Fig. S7
	oMJ275	AAGCAGGAAGCTTTGTCAGC		
Eif2s3y	*gESYsF1*	ATAGATCCGGCAGAAGACAAAG	843 bp	Current paper; Fig. S7
	gESYsR1	GCCTCAACGAGGTAGAAGAATAG		
Sry (Y^{Tdym1})	*Muty3*	GTGTCTCAAAGCCTGCTCTTC	204 bp	(*42*); Fig. S7
	Mutyrp1	CATGTACTGCTAGCAGCTATC		
Ssty1	*Ymtfp1*	CTGGAGCTCTACAGTGATGA	342 bp	(*43*); Fig. S7
	Ymtrp1	CAGTTACCAATCAACACATCAC		
Ssty2	*Pc11fp2*	GTTTTTCCTCAGGTGAGGGA	237 bp	(*42*); Fig. S7
	Pc11rp2	CAGAGGGGTCTCTGGAATGT		
Asty	*Asty-F*	GGGGAGTAGAACTCATCATC	280 bp	(*44*); Fig. S7
	Asty-R	CAGGAGATGACTAACATAGCA		
*Eif2s3y tg***	*EyTspF8*	CAGCTCTGAGGGTGGGTAGTAG	782 bp	Current paper; Fig. S7
	EyTspR8	TGACCATGATTACGCCAAGCTAT		
*Sox9 tg***	*Sox9-sWTp*	CATCCGAGCCGCACCTCATG	300 bp	(*3*); Fig. S7
	Sox9-SS2	GCTGGAGCCGTTGACGCG		
*Eif2s3x tg***	*pTARBAF7*	GCCTGATGCGGTATTTTCTC	87 bp	(*34*); Fig. S7
	pTARBAR7	CGGCATCAGAGCAGATTGTA		
Real-time RT-PCR primers				
FoxL2	*FoxL2.F*	AGGGAGAGAATAAAACATTCATGG	63bp	(*45*); Fig. S5H-J
	FoxL2.R	GCAAACTCCAAGGCCATTAC		
Rspo1	*Rspo1_F*	CGACATGAACAAATGCATCA	82bp	(*46*); Fig. S5H-J
	Rspo1_R	CTCCTGACACTTGGTGCAGA		
Wnt4	*Wnt4_F*	CGAGGAGTGCCAATACCAGT	138bp	(*47*); Fig. S5H-J
	Wnt4_R	GCCACACCTGCTGAAGAGAT		

Actb	*Actb-F*	GGCACCACACCTTCTACAATG	352 bp	(*48*); Fig. S5H-J
	Actb-R	GTGGTGGTGAAGCTGTAGCC		
Sdha	*Sdha-F*	TGTTCAGTTCCACCCCAC	66 bp	(*49*); Fig. S5H-J
	Sdha-R	TCTCCACGACACCCTTCTGT		
*Eif2s3x****	*Eif2s3x-F2*	GGGACCAAAGGGAACTTCAAG	82 bp	(*50*); Fig. 2, S10
	Eif2s3x-R2	AGCATCGTAGCCATCAAAATATCA		
Eif2s3x (sp2) ****	FpESXsp	GACAGGATCTCGCCACATTG	108 bp	Current paper; Fig. S11
	RpESXsp	CATGAGCTACGTGACCAATTG		
Eif2s3y	*cESYsF2*	CGTTATGCCGAGCAGATAGAA	104bp	Current paper; Fig. 2, S10
	cESYsR2	CCGTCTCAGTAGGAAGTAGGA		
*Eif2s3x/y****	*cEIFsF2*	GACCAGAATGTTACAGAT	192bp	Current paper; Fig. 2, S10
	cEIFsR2	TACCAGCTATCAACAGAA		

* Recognize endogenous and transgenic *Eif2s3x* but do not recognize any of the *Eif2s3x* retrogenes or pseudogenes; ** Recognize specifically transgene; *** Recognize endogenous and transgenic *Eif2s3x* transcripts but will also pick up genomic *Eif2s3x* X-linked predicted pseudogene (MGI: *Gm2223* or VegaSanger: RP23-191G17.4), if it is expressed; **** Recognize endogenous and transgenic *Eif2s3x* transcripts but do not amplify *Gm2223* or other *Eif2s3x* pseudogenes.

Movie S1

Round spermatid injection with germ cells from XO*Sox9,Eif2s3x* male.

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