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Regulation of Sex Determination in Mice by a Non-coding Genomic Region

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ABSTRACT To identify novel genomic regions that regulate sex determination, we utilized the powerful C57BL/6J-Y^{POS} (B6-Y^{POS}) model of XY sex reversal where mice with autosomes from the B6 strain and a Y chromosome from a wild-derived strain, *Mus domesticus poschiavinus* (Y^{POS}), show complete sex reversal. In B6-Y^{POS}, the presence of a 55-Mb congenic region on chromosome 11 protects from sex reversal in a dose-dependent manner. Using mouse genetic backcross designs and high-density SNP arrays, we narrowed the congenic region to a 1.62-Mb genomic region on chromosome 11 that confers 80% protection from B6-Y^{POS} sex reversal when one copy is present and complete protection when two copies are present. It was previously believed that the protective congenic region originated from the 129S1/SviMJ (129) strain. However, genomic analysis revealed that this region is not derived from 129 and most likely is derived from the semi-inbred strain POSA. We show that the small 1.62-Mb congenic region that protects against B6-Y^{POS} sex reversal is located within the *Sox9* promoter and promotes the expression of *Sox9*, thereby driving testis development within the B6-Y^{POS} background. Through 30 years of backcrossing, this congenic region was maintained, as it promoted male sex determination and fertility despite the female-promoting B6-Y^{POS} genetic background. Our findings demonstrate that long-range enhancer regions are critical to developmental processes and can be used to identify the complex interplay between genome variants, epigenetics, and developmental gene regulation.

THE formation of a testis or an ovary from an undifferentiated bipotential gonad relies on the expression of a single Y-chromosome gene, *SRY*, to trigger male sex determination (Sinclair *et al.* 1990; Koopman *et al.* 1991). In mammals, SRY expression is required for development of the testis, secondary sex characteristics, and, ultimately, fertility. In both humans and mice, mutations and deletions of *SRY* result in XY sex reversal (Berta *et al.* 1990; Sinclair *et al.* 1990), while translocations of *SRY* onto the X chromosome or an autosome result in XX males (Berkovitz *et al.* 1992). Similarly, overexpression of various *SOX* (***S**ry*-related HMG b**ox**) gene family members within the bipotential gonad results in XX males (Bishop *et al.* 2000; O'Bryan *et al.* 2008; Polanco *et al.* 2010), indicating that increased *SOX* gene expression during critical periods in fetal sex determination activates the male sex determination pathway. Disorders of sex development (DSD) are among the most common genetic disorders, occuring in ~1/100 births when patients with hypospadias are included (Lee *et al.* 2006).

One of the most powerful models in mammalian sex determination is a mouse strain in which XY males are sensitized to XY sex reversal. The strain's autosomes and X chromosome are from the *Mus m. musculus* C57BL/6J (B6) strain while the Y chromosome is from one of the *Mus m. domesticus* (*DOM*) substrains. B6 males with their native Y chromosome, Y^{B6}, develop as normal fertile males. In contrast, B6-Y^{DOM} strains show a range of sex determination phenotypes from full sex reversal (female phenotype) to delayed testicular development and incompletely masculinized males. Placing the *M. m. domesticus poschiavinus* Y chromosome (Y^{POS}), named after the wild-derived strain from the Poschiavo valley in Switzerland, on the B6 background results in a nearly 100% adult XY female phenotype. At the morphologic and molecular levels, all of the B6-Y^{POS} animals have abnormal early testis development and

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a significant delay in *Sry* expression (Bullejos and Koopman 2005).

The interaction between genetic background and Sry expression is critical to initiation of testis development *in utero*. Yet, on other genetic backgrounds, such as 129S1/SvIMJ, DBA/2J, C58/J, or BALB/cBy, Y^{POS} does not result in XY sex reversal (Eicher 1988). The altered testis phenotype detected during early gonadal development in Y^{POS} is specific to the B6 background.

Genetic differences in the *Sry* gene from *musculus vs. domesticus* strains do not explain the B6-Y^{DOM} sex reversal (Albrecht and Eicher 1997). For example, the *M. musculus* isoform is nearly twice as large (395 aa) as the *M. domesticus Sry* (230 aa) due to a premature termination codon located in the glutamine repeat region of the *M. domesticus* Sry. Additionally, the size of Sry glutamine repeat alone does not correlate with propensity toward B6-Y^{DOM} sex reversal.

Full testis development requires both spatiotemporally appropriate Sry levels and a receptive microenvironment within the somatic cells on which Sry acts to converge in a short 6-hr window (Hiramatsu *et al.* 2009). *Sry* expression is required within a specific proportion of somatic cells of the bipotential gonad and at a certain level to initiate and propagate male sex determination (Bullejos and Koopman 2005; Kashimada and Koopman 2010). In humans, genetic background modulates the phenotypic expression of *SRY* gene mutations and results in variable gonadal and genital phenotypes among family members who share the same *SRY* mutation (Maier *et al.* 2003). In mouse models, the interaction between the B6 background and the Y^{POS} chromosome results in a 4-hr delay in peak *Sry* expression in B6-Y^{POS} compared to B6-Y^{B6} embryos (Bullejos and Koopman 2005). While the genetic underpinnings of the spatiotemporal regulation of *SRY* remain to be fully elucidated, recent work has shown that upregulation of Sry is coordinated by MAP kinase signaling (Bogani *et al.* 2009; Gierl *et al.* 2012; Warr *et al.* 2012), as well as chromatin-modifying factors such as *CBX2* (Katoh-Fukui *et al.* 1998; Katoh-Fukui *et al.* 2012) and *Jmjd1a* (Kuroki *et al.* 2013). The multiple layers of genetic and epigenetic regulation underscore the tight regulation of developmental gene expression.

In addition to the intricate regulation of SRY expression, the receptiveness of the downstream genetic environment is equally important, yet poorly understood. Of the downstream genes, the best studied is the regulatory region of *SOX9*, which spans >2.5 Mb upstream and downstream of the *SOX9* ORF (Wagner *et al.* 1994; Pfeifer *et al.* 1999). Autosomal dominant mutations in *SOX9* result in campomelic dysplasia with XY sex reversal (Online Mendelian Inheritance in Man #114290) due to the importance of *SOX9* in both cartilage and testis determination (Wright *et al.* 1995). Point mutations within the human syntenic region of the testis-specific enhancer located 5′ to the *Sox9* open reading frame, TESCO, have not been found (Sekido and Lovell-Badge 2008; Georg *et al.* 2010). However, duplications and deletions limited to the *SOX9* regulatory regions have recently been identified in several familial and isolated cases of 46,XY DSD or 46,XX DSD (Benko *et al.* 2011; Cox *et al.* 2011; White *et al.* 2011). These deletions and duplications cause dysregulation of *SOX9* expression with either loss of testis-specific SOX9 expression or abnormal ectopic expression in an XX individual resulting in either 46,XY gonadal dysgenesis or 46,XX testicular DSD, respectively. Furthermore, these copy-number changes indicate that there are testis-specific regulatory regions of human *SOX9*. In addition to mutations, transgenic insertions in mice disrupting regulation of *Sox9* have been shown to result in aberrant activation of *Sox9* and XX males (Bishop *et al.* 2000).

In the congenic B6-Y^{POS} mouse model, the incompatibility between the gonadal microenvironment created by the B6 genetic background and the low levels of Sry^{POS} prevents timely activation of male sex determination (Bullejos and Koopman 2005). Early genetic linkage studies using B6 and a non-sex-reversing strain, DBA2/J, to identify genetic regions contributing to the formation of XY^{POS} hermaphrodites, identified three autosomal testis-determining loci on mouse chromosomes 2, 4, and 5, respectively, and demonstrated that homozygosity for the B6 allele is one of the major contributing factors in B6-Y^{POS} sex reversal (Eicher *et al.* 1996).

More recently, comparative studies of gene expression within B6 and another non-sex-reversing strain, 129S1, in developing testis showed that, compared to 129-Y^{129} gonads, B6-Y^{B6} gonads had a higher average expression of "female"-related genes and a lower expression of "male" genes (Munger *et al.* 2009, 2013). On the B6 genetic background, *Sox9* expression was elevated by nearly twofold, which may be compensation for the B6 strain's "pro-female" gene expression profile. Therefore, to develop as a phenotypic male, the strength of the interaction between the Y chromosome and autosomal loci must overcome the "pro-female" balance intrinsic to the B6 genetic background. These studies highlight the complex networks that control gonadal development and indicate that there exists a normal flux of gene expression between various genetic backgrounds. The maintenance of testis and ovary simply requires that the equilibrium is driven toward the correct developmental program and is evolutionarily maintained by fertility. Genetic mutations within the majority of genes implicated in either male or female mouse sex determination do not result in complete sex reversal, but instead in variable degrees of sex reversal, the severity of which can be modified by genetic background (Eicher 1988; Eicher *et al.* 1996; Bogani *et al.* 2009). Therefore, an independent model appears to be flawed as the interacting networks of genes with some prominent driver genes have large effects on gonadal developmental programs.

In this study, we utilized a congenic strain, previously developed by multiple backcross generations, in which a homozygous non-B6 region on chromosome 11 was identified that conferred 100% protection from B6-Y^{POS} sex reversal (Whitney *et al.* 2000; Nikolova *et al.* 2008).

To fully understand the genetic origin of the protection from B6-Y^{POS} sex reversal, we retraced the 30-year history of this congenic strain (Figure 1). The initial discovery of B6-Y^{POS} sex reversal occurred after backcrossing semi-inbred *POSA* males (cross between XY wild-derived *M. m. domesticus poschiavinus* and Naval Medical Research Institute Swiss XX mice) that carried the $Hba^{th\text{-}J}$ trait on chromosome 11 for α-thalassemia to the C57BL6/J background (Eicher *et al.* 1982). After 5 generations, the male-to-female sex ratio was highly skewed in favor of females, and half of the phenotypic females had an XY karyotype. Subsequent studies showed that the $Hba^{th\text{-}J}$ trait was not responsible for the sex reversal and that the sex reversal trait was Y-linked (Eicher *et al.* 1982). In the breeding of the protected congenic strain, fertile B6-Y^{POS} males were backcrossed onto 129S1 for 13 generations, resulting in a normal sex ratio in the consomic strain 129-Y^{POS}. At this point, it was hypothesized that the 129 genetic background protected from B6-Y^{POS} sex reversal, and therefore 129-Y^{POS} was backcrossed to B6 to initiate the current congenic strain. At each generation, only the most masculinized males were mated to B6 females, with the goal of selecting for autosomal regions that protect from B6-Y^{POS} sex reversal (Whitney *et al.* 2000). In a previous publication, we used SNP markers polymorphic between B6 and 129 and identified large regions on chromosome 11 that resulted in dose-dependent protection from B6-Y^{POS} sex reversal (Nikolova *et al.* 2008). While previous publications called this strain B6.129-Y^{POS}, we show in this article that the origins of the congenic region are unlikely to be from the 129 background and, based on our analysis, most likely are derived from POSA. Therefore, we will refer to the B6.129-Y^{POS} strain more accurately as B6.POSA-Y^{POS} (see Figure 1 and Figure 2).

To narrow down the genomic element important in protection from B6-Y^{POS} sex reversal, we backcrossed heterozygous B6.POSA-Y^{POS} males to B6 XX females and, at each generation, performed SNP genotyping to identify recombinants within the congenic region. Animals with recombination events within the congenic region were used to found 12 subcongenic lines. In this study, we narrowed the large 60-Mb region originally identified in Nikolova *et al.* (2008) to a small 1.62-Mb noncoding region that is responsible for protection from B6-Y^{POS} sex reversal. These results correct some longstanding assumptions about B6-Y^{POS} and the origins of previously reported protection from sex reversal phenotype. Additionally, they reveal the importance of the promoter of *Sox9* in the protection from B6-Y^{POS} sex reversal.

Materials and Methods

Mouse strains

Animals were housed according to the guidelines of the University of California at Los Angeles (UCLA) Division of Laboratory Animal Medicine. All experiments were approved by the Institutional Animal Care and Use Committees of UCLA. C57BL/6J females and males used for breeding and subcongenic line generation were obtained from the Jackson Laboratory. The Jackson Laboratory is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Construction of the C57BL/6J.POSA-Y^{POS} subcongenic strain

The original C57BL/6J.POSA-Y^{POS} (a.k.a. C57BL/6J.129-Y^{POS} in previous publication) strain was produced as described previously (Whitney *et al.* 2000; Nikolova *et al.* 2008). To generate the subcongenic strains, fertile heterozygous C57BL/6J.POSA-Y^{POS} males were backcrossed to B6-XX females from the Jackson Laboratory (Figure 1). At each generation, offspring were genotyped with SNP markers polymorphic between B6 and 129 on chromosome 11 identified in the SNP scan published in Nikolova *et al.* (2008) (Supporting Information, Table S1). A region surrounding each SNP was PCR-amplified from genomic DNA and then sequenced to determine genotype at each informative SNP. Animals in which a meiotic recombination occurred within the congenic region were used to generate new subcongenic chromosomes (Figure 2). Subcongenic founder Y^{POS} males were bred to B6-XX females, and all offspring were genotyped to determine the presence of the subcongenic region. Heterozygous brothers and sisters were mated together to generate animals that were homozygous for each subcongenic region.

Phenotyping of adults

A mouse was classified (1) as a female, if female external genitalia, yellow mammary-associated hair pigmentation, bilateral uterine horns, and normal-appearing ovaries were present; (2) as a hermaphrodite, if ambiguous genitalia, some yellow mammary-associated hair pigmentation, and an ovary and a contralateral ovotestis or testis were present; or (3) as a male, if normal-appearing male genitalia, no yellow mammary-associated hair pigmentation, and two testes were present.

Classification of fetuses

Midday of the day of a vaginal plug was considered embryonic day 0.5 (E0.5), and the fetal stage of development was verified by limb morphology (Theiler 1989). Individual gonads from E14.5–E16 fetuses were classified as an ovary, an ovotestis, or a testis (Eicher *et al.* 1982). Finally, the approximate amount of testicular tissue in each ovotestis was estimated at the time of dissection and confirmed using a captured image at 25× under a dissection microscope (Nikolova *et al.* 2008). If 50% or greater testicular tissue was present, the ovotestis was designated as "oT." If <50% testicular tissue was present, the ovotestis was designated as "Ot."

Sex chromosome genotyping

Chromosomal sex was determined with a PCR-based assay using a single primer pair, which detects the X-linked *Smc-x*

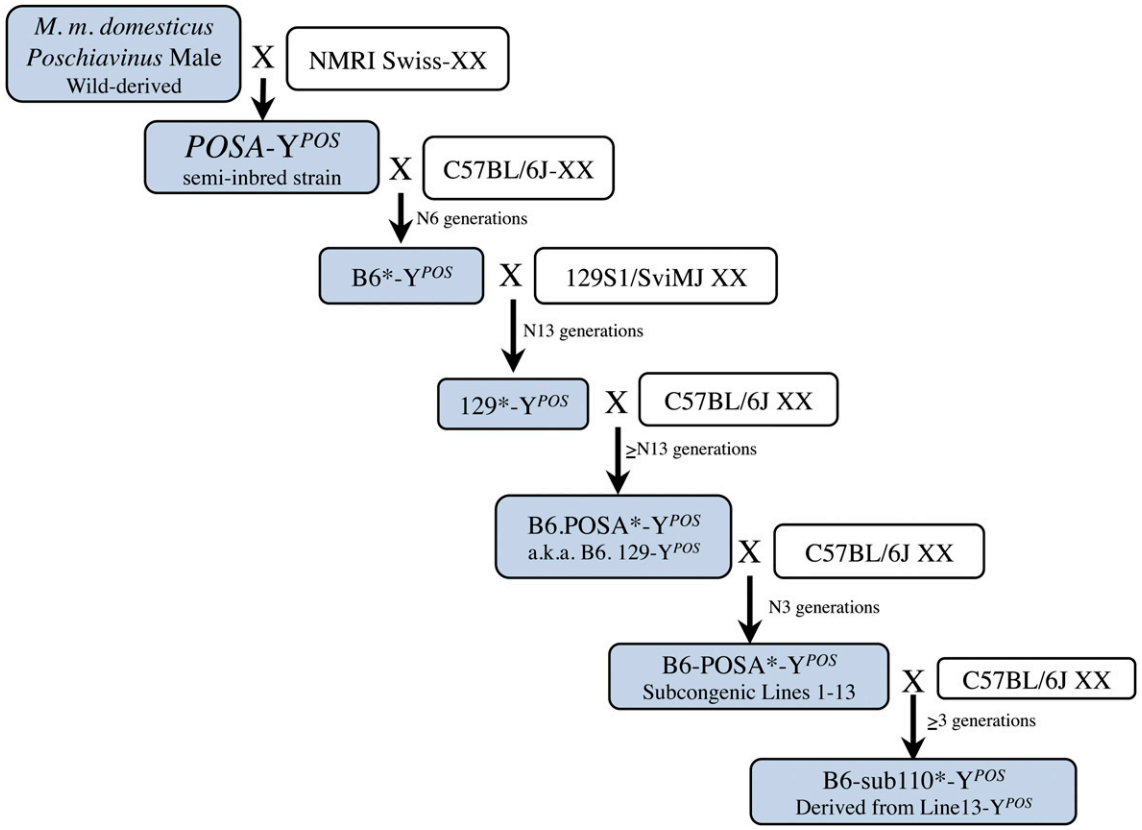


Figure 1 History of the protected B6-POSA-Y^{POS} strains. The original POSA strain was a semi-inbred strain created when Naval Medical Research Institute (NMRI) Swiss females were intercrossed to *M. musculus domesticus poschiavinus* wild-derived males. A POSA male was backcrossed to B6 over 6 generations, and one of the B6-Y^{POS} males that was not sex-reversed was backcrossed onto the 129 background for 13 generations. Finally, a 129-Y^{POS} was backcrossed over >13 generations to B6, and at each generation only the most masculinized males were selected for breeding, creating the B6.POSA-Y^{POS} congenic line (previous studies referred to this line as B6.129), which was then further crossed to B6 females to create subcongenic chromosomes 1–13. In each backcrossing experiment, a region of the POSA genome remained, undetected, conferring protection from sex reversal (asterisk) on all breeding males. The shaded boxes follow the transmission of the Y^{POS} chromosome throughout the generations.

gene (330 bp) and the Y-linked *Smc-y* gene (301 bp) (Bullejos and Koopman 2005). Immomix Red 2× (Bioline, London) was used for the PCR amplification as per the manufacturer's guidelines with an annealing temperature of 57°. Samples were resolved by electrophoresis on a 2% agarose gel.

Statistical analysis

All data analyses were conducted using Stata (version 11, StataCorp). Categorical and ordinal data analyses for the genotype–phenotype correlations were assessed using Fisher's exact tests or likelihood-ratio tests for the ordinal treatment of the data. The dose-effect hypothesis was tested using polytomous logistic regression (categorical outcomes) or ordered logistic regression (ordinal outcomes) and allowed for inclusion of covariates.

Mouse diversity genotyping array (Affymetrix)

Genomic DNA from 10 founder homozygous males from the subcongenic lines was extracted using the Gentra Puregene Mouse Tail Kit (Qiagen) and analyzed at JAX Mouse Diversity Genotyping Array Service (Bar Harbor, ME) according to the manufacturer's protocol. SNP and copy number variation (CNV) analyses were performed by the Jackson Laboratory Bioinformatics Core (Yang *et al.* 2009) using the R MouseDivGeno software package (http://cgd.jax.org/tools/MouseDivGeno/). The MouseDivGeno package assumes three possible genotypes—homozygous for allele A, homozygous for allele B, or heterozygous for alleles AB—and can also declare missing (N) calls and genomic location information using SNPs annotated on Build 37 mm9 mouse genome. In total, this array contains 623,000 SNPs and 916,000 CNV probes. On chromosome 11, there are a total of 28,000 SNPs with a mean distance between SNPs of 24 kb. The additions to chromosome 11 in the mouse build 39 (mm10) released in 2011 did not directly affect the 110 region or our interpretation of the SNP array results. For CNV analysis, the SimpleCNV function within the R MouseDivGeno package was used and normalized against a subset of 249 high-quality control arrays generated with the Diversity Array by the Jackson Laboratory and CEL files generated from this experiment.

Immunofluorescence

The time course of Sox9 expression was analyzed by whole-mount immunofluorescence (WM-IF) following the experimental

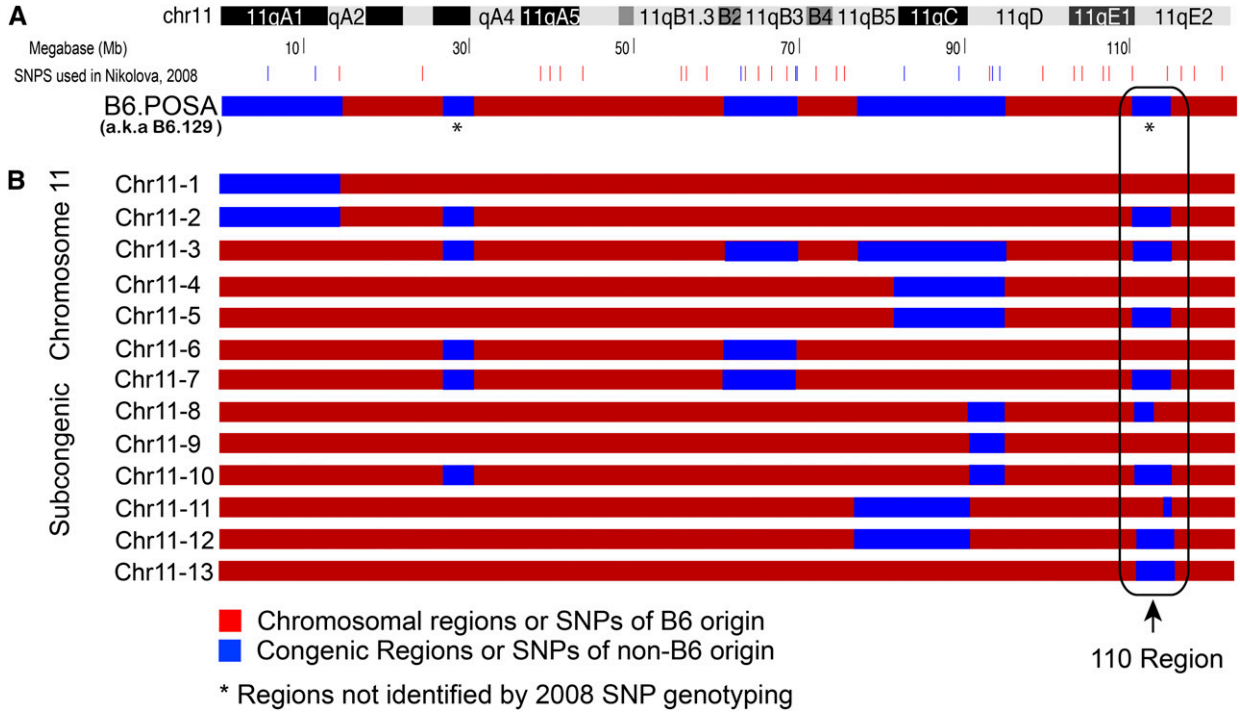


Figure 2 One congenic and 13 subcongenic chromosomes generated through breeding. (A) Location of SNPs polymorphic between the B6 (red) and non-B6 (blue) strains used to identify the original congenic region are shown next to a representation of mouse chromosome 11. The non-B6 markers are polymorphic between B6 and 129 and chosen because of the previous hypothesis that the protection was derived from the 129 strain. Two regions, one located at 30 Mb and the other located at 110 Mb, were not identified by the 2008 SNP scan (asterisk). (B) Thirteen different subcongenic configurations of chromosome 11 (Chr11-1 through Chr11-13), with varying degrees of non-B6 DNA, were created by backcrossing the B6.POSA- Y^{POS} with B6-XX females. Region 110 is circled.

design of Wilhelm *et al.* (2009) and using standard WM-IF protocols previously established in our laboratory (described in Fleming *et al.* 2012). The number of tail somites was used to assess the precise developmental stage of each embryo (Hacker *et al.* 1995; Bullejos and Koopman 2001). We examined B6-Y^{B6}, B6-Y^{POS}, LineB6/11-13-Y^{POS}, and B6-XX gonads at 17–18, 19–20, 21–22, 23–24, and 27–30 tail somites, roughly corresponding to E11.5–E12.5. Sox9 (rabbit polyclonal, SC-20095, Santa Cruz Lot#G0709) and FoxL2 (rabbit polyclonal, supplied by Dagmar Wilhelm, Monash University, Clayton, VIC, Australia) antibodies were used. ToproRed (Invitrogen) was used to counterstain nuclei. All images were taken on a Zeiss LSM 510 Meta confocal microscope. All genotypes and time points had n = 3–4, and the gonad shown is representative of the set.

Database use

The National Center for Biotechnology Information (http:/www.ncbi.nlm.nih.gov/), University of California at Santa Cruz Genome Bioinformatics (http:/genome.ucsc.edu/), and Mouse Genome Informatics (http:/www.informatics.jax.org/) websites were used to retrieve gene, marker, and sequence information. MatInspector Version 8.0.4 was used to identify predicted transcription-factor-binding sites within the promoter of *Sox9*. The genomic location information is based on Mouse Build 37 (July 2007).

Results

Generation of subcongenic lines

The original congenic strain, B6.POSA-Y^{POS}, generated as described in *Materials and Methods* (Figure 1), contained three major congenic regions on chromosome 11 located between the following base pairs: 1–14,267,131, 58,783, 266–71,981,592, and 76,380,940–94,191,519, respectively (Figure 2A). Identification of these three large regions relied on 149 microsatellite markers and 404 SNPs polymorphic between the B6 and 129 strains, which is equivalent to approximately one marker per 2 Mb (Nikolova *et al.* 2008). Over three generations, we generated subcongenic lines through backcrossing to B6-XX females. Animals with recombination within the congenic region were identified by genotyping of SNP markers from Nikolova *et al.* (2008) (Table S1A). Subcongenic lines were bred to homozygosity over two generations.

Our previous study had concluded that protection from B6-Y^{POS} sex reversal was 100% in animals that were homozygous for the chromosome 11 congenic regions (Figure 2A). However, since that study we have noted that, compared to our published data, the same congenic B6.POSA-Y^{POS} line began to show decreased rates, approaching 50%, of protection from sex reversal after 1.5 years of sibling matings. This loss of protection from B6-Y^{POS} sex reversal

was present only in offspring from specific fathers. All breeding males were confirmed to have Sry^{POS} and regenotyped with markers from the original article, which confirmed the presence of a homozygous congenic region (Figure 2A and Table S1A). We hypothesized that there was another non-B6 genomic region affecting protection that had not been identified due to either the choice of markers being specifically polymorphic between B6 and 129, the low density of SNP markers, or an undetected *de novo* event in one of our founder males. To address these hypotheses, we performed whole-genome SNP array analysis in the founder males to identify all of the non-B6 regions and their genetic origin.

Genomic analysis of the subcongenic lines

The Affymetrix Mouse SNP Diversity Array developed at the Jackson Laboratory (Yang *et al.* 2009) was performed on genomic DNA from homozygous, breeding, subcongenic males and two of our phenotypically male B6-Y^{POS} animals. This array was designed to identify regions of non-B6 origin and to determine from which inbred strain the region was derived. Call rates were >97% for all arrays, and the congenic regions on chromosome 11 had a large concentration of non-B6-SNPs (>100 SNPs/Mb) compared with all other chromosomes. All subcongenic regions identified through SNP genotyping were present, and no CNV regions were identified in more than one of the founder males (Table S2 and Table S3).

Our analysis confirmed the presence of the three previously identified regions and identified two new congenic regions on chromosome 11 that had a high density of non-B6 SNPs: the first spanning base pairs 20,652,664–29,502,320 and the second spanning base pairs 110,023,548–114,635,519 (Figure 2B and Figure 3). The region spanning base pairs 20,652,664–29,502,320 was present as heterozygous or homozygous in only half of the genotyped males, and thus its presence was not required for protection from B6-Y^{POS} sex reversal.

The second region from megabases 110 to 114.5, which we will refer to as the "110 region," was present as either heterozygous or homozygous in all founder XY^{POS} males that were protected from sex reversal (Figure 3A). Even the animals that we originally believed to be "B6-Y^{POS}" males were heterozygous for this 4.5-Mb region and were renamed B6/Line11-13-Y^{POS} (Figure 3A). We hypothesized that this newly identified 110 region resulted in the originally observed dose-dependent protection from sex reversal. The 110 region was present in every male protected from B6-Y^{POS} sex reversal and used for breeding. Furthermore, the 110 region contains a well-described sex determination gene, *Sox9*, as well as the entirety of its promoter region. The presence of the 110 region in all of our phenotypically male and breeding mice and the presence of *Sox9* within the 110 region strongly suggested that the 110 region was associated with protection from B6-Y^{POS} sex reversal.

Chr11-13, which has only the 110-region of non-B6 origin, protects from B6-Y^{POS} sex reversal

Based on our new genotypic data, we reanalyzed our subcongenic lines at adult and embryonic stages to determine the role of the 110 region in protection from B6-Y^{POS} sex reversal. Adult phenotypic analysis of animals that were heterozygous (LineB6/11-13-Y^{POS}) or homozygous (Line11-13/11-13-Y^{POS}) for Chr11-13 (Figure 2B), whose only non-B6 region is the 110 region, showed significant (Fisher exact test, P-value < 0.001) protection from B6-Y^{POS} sex reversal (Figure 4A). Adult animals homozygous for the 110 region were 100% protected from sex reversal. All embryonic gonads heterozygous or homozygous for the 110 region had some degree of testicular tissue (Figure 4B, Fisher exact test, P-value < 0.001). Thus, heterozygosity for the 110 region was sufficient to prevent the formation of complete embryonic B6-Y^{POS} ovaries. These data show that the 110 region alone is both necessary and sufficient to direct testis determination above the spontaneous rate in the B6-Y^{POS} strain.

To confirm that the 110 region was the only region responsible for the protection from B6-Y^{POS} sex reversal, we also performed analyses for each of the subcongenic lines in both embryos and adults. Our data showed that the subcongenic lines that did not contain the 110 region (Chr11-1, 4, 6, 8, 9, and 11) did not confer significant protection from Y^{POS} sex reversal compared to B6-Y^{POS} animals (Figure S1). Lines heterozygous or homozygous for the 110 region were associated with a significant protection from B6-Y^{POS} sex reversal ($P < 0.001$). When all subcongenic lines in which the 110 region is present (Chr11-2, 3, 5, 7, 10, 12, and 13) were grouped together, heterozygosity for the 110 region resulted in 80% (102/127) developing as adult phenotypic Y^{POS} males, and homozygosity for the 110 region resulted in 98.9% (109/111) Y^{POS} males. In the absence of the 110 region, only 6.9% (5/72) animals developed as XY^{POS} males. Embryonic gonad analysis corroborated our adult data analysis, with significantly more testis formation conferred by heterozygosity (~60–70% of gonads were oT or T) for the 110 region compared to B6-Y^{POS} gonads (0% were oT or T) (P-value < 0.001). However, heterozygosity for the 110 region does not result in fully normal embryonic testis, with the majority of gonads with some degree of ovarian formation (either oT or Ot) (Figure S2). Our data prove that the 110 region is required to drive testis formation in the majority of the gonad in the presence of the B6-Y^{POS} genetic background.

Sox9 protein expression in B6-Y^{POS} and B6-Y^{B6}

The 110 region spans 4.5 Mb and includes a well-studied sex determination gene, *Sox9*, and the entirety of its >2-Mb regulatory region. *Sox9* belongs to the SOX gene family and, along with *Sry*, plays a major role in male sex determination. *Sox9* is part of the initial genetic cascade that pushes the bipotential gonad toward the male pathway. A loss of

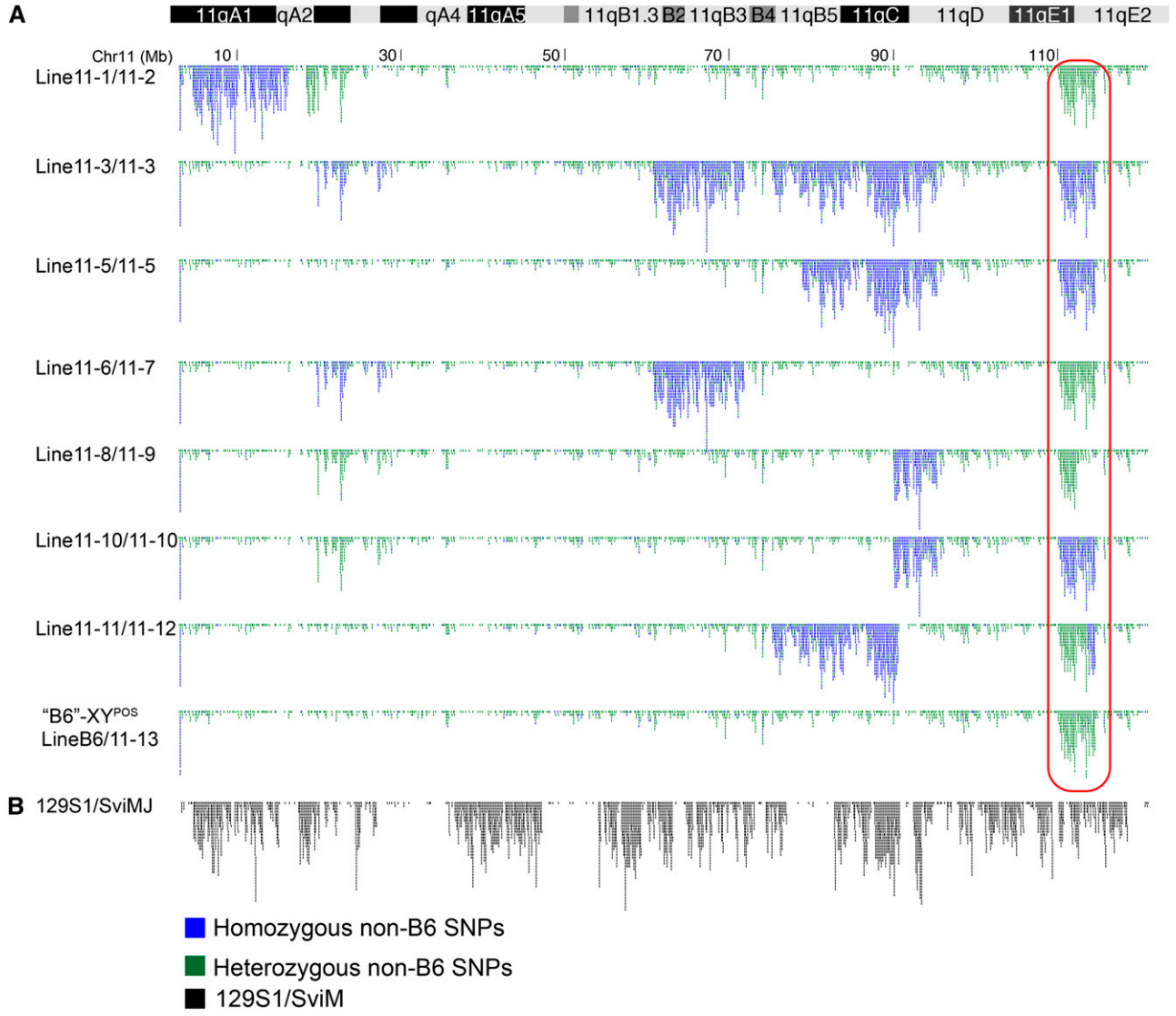
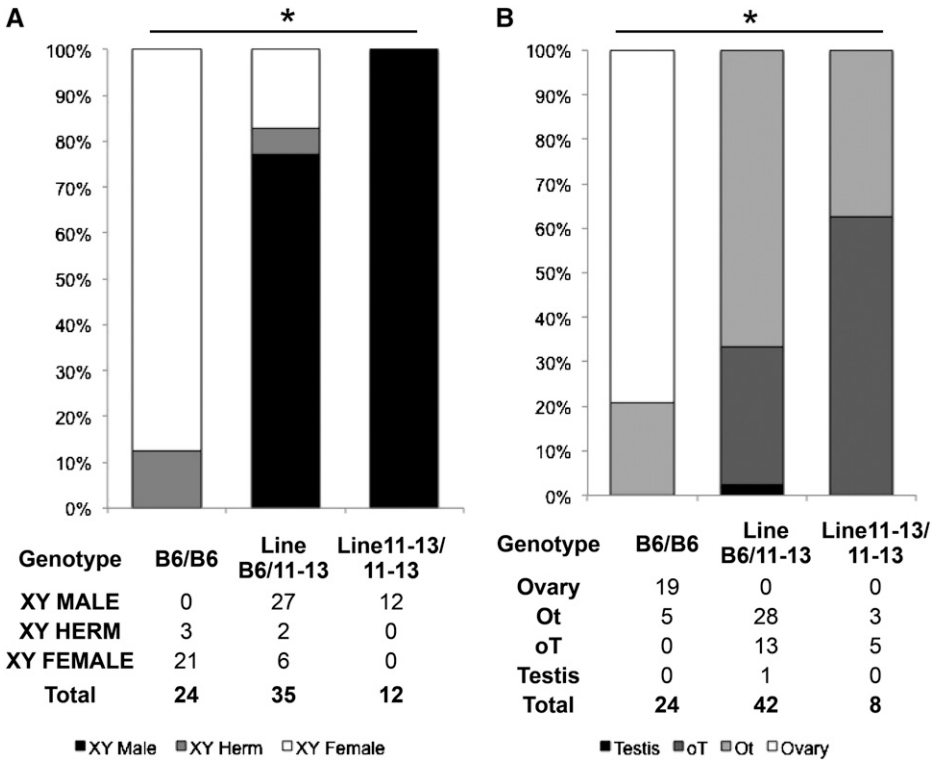


Figure 3 All subcongenic founder, non-sex-reversed B6-Y^{POS} male mice had a non-B6 region encompassing *Sox9* and its promoter that is not of B6 or 129 origin. (A) Each schematic represents a Y^{POS} male used for breeding and generation of subcongenic lines and is labeled for each version of chromosome 11 that the animal has (*e.g.*, Line11-1/11-2 is an animal with two different copies of chromosome 11, Chr11-1 and Chr11-2 (see Figure 2B for notations). All of the subcongenic XY^{POS} breeding males were either heterozygous (green SNPs) or homozygous (blue SNPs) for a large 4.5-Mb region, which encompasses *Sox9* and its promoter (red box). (Top) Ideogram of mouse chromosome 11. (B) Diversity array analysis of the 129S1 inbred strain has a SNP profile (black) that has a different pattern from the SNPs in the subcongenic protected males (compare to A). This shows that the congenic regions were not derived from the 129S1 strain, as previously described.

either *Sry* or *Sox9* results in XY sex reversal in humans and mice (Berta *et al.* 1990; Foster *et al.* 1994). The importance of *Sox9* in sex determination led us to further explore the protein expression levels of Sox9 in B6-Y^{B6}, B6-Y^{POS}, LineB6/11-13-Y^{POS}, and B6-XX embryonic gonads early in sex differentiation: at 17–18, 19–20, 21–22, 23–24, and 27–30 tail somites (ts) (E11.5–E12.5). Whole-mount immunofluorescence was performed with antibodies to Sox9 to assess if protein expression was delayed or decreased in the B6-Y^{POS} gonads and to determine whether Sox9 expression was rescued in animals that were heterozygous for Chr11-13.

Compared to the wild-type B6-Y^{B6} gonads, B6-Y^{POS} gonads showed decreased levels and fewer somatic cells expressing Sox9 at all time points (Figure 5). By E12.5, or 27–30 ts, when Sox9 is normally highly expressed and seminiferous tubules have begun to form in B6-Y^{B6} gonads, expression in B6-Y^{POS} is limited to a few scattered cells within the gonad. In contrast, a rescue of the testis molecular phenotype was observed in heterozygous LineB6/11-13-Y^{POS} animals with a significantly greater number of cells actively expressing high levels of Sox9 throughout the length of the gonad. However, heterozygous LineB6/11-13-Y^{POS} animals (Figure 5, row 3) were delayed in the tubule formation as compared to the wild-type B6-Y^{B6} gonads.

FoxL2 is a female somatic cell marker that is upregulated in female gonads at 27–30 ts. Studies of ovotestes have



Genotype	B6/B6	Line B6/11-13	Line11-13/ 11-13
XY MALE	0	27	12
XY HERM	3	2	0
XY FEMALE	21	6	0
Total	24	35	12

Genotype	B6/B6	Line B6/11-13	Line11-13/ 11-13
Ovary	19	0	0
Ot	5	28	3
oT	0	13	5
Testis	0	1	0
Total	24	42	8

Figure 4 Genotype–phenotype correlation show that heterozygous and homozygous presence of Chr11-13, which has only the 110 region of non-B6 origin, provides significant protection from B6-Y^{POS} sex reversal in adult and embryonic gonads. (A) B6-Y^{POS} adult animals, none of the animals are Y^{POS} males with testes. For adult animals that are heterozygous or homozygous for Chr11-13, there is significantly increased protection from B6-Y^{POS} sex reversal. (B) In B6-Y^{POS} animals, the majority of gonads in XY^{POS} animals are Ot (>50% ovary) or complete ovary. Embryonic gonad analysis showed that all LineB6/11-13 and Line11-13/Line11-13 gonads have some degree of testicular cord development and that none are XY^{POS} ovaries. *Fisher exact P-value < 0.001 for the three-way comparison.

demonstrated that expression of Sox9 and FoxL2 is mutually exclusive at the cellular level and that FoxL2 expression suppresses Sox9 (Wilhelm *et al.* 2009). At 27–30 ts, B6-Y^{POS} gonads had a female-similar phenotype with more FoxL2 and decreased Sox9 expression compared to both wild-type B6-Y^{B6} and protected LineB6/11-13-Y^{POS} animals. Probably because of the remaining minimal Sox9 expression, the number of cells expressing FoxL2 in the B6-Y^{POS} group was still lower than in the B6-XX female control (Figure 5; compare right-most panels in first and third rows). In the protected LineB6/11-13-Y^{POS}, female-specific FoxL2 expression is not completely suppressed, as it is in the wild-type B6-Y^{B6}. Therefore, the LineB6/11-13-Y^{POS} gonads are incompletely masculinized. Although the temporal and masculinized expression of Sox9 matches that of wild-type B6-Y^{B6} gonads, the female-specific marker FoxL2 was not extinguished.

These molecular phenotypes are concordant with the phenotypic findings above in which B6/11-13-Y^{POS} gonads at later stages in development have more testicular tissue at E15.5 and a male phenotype in adulthood (Figure 4).

Narrowing down the 110 region

To confirm that *Sox9* was solely responsible for protection from B6-Y^{POS} sex reversal, we aimed to narrow the Chr11-13 region through backcrossing to B6-XX females. We identified two mice with recombination events within the 110 region, creating two smaller subcongenic regions. The first animal had sub110-1, which spanned base pairs 110,111,305–112,514,446 with a boundary 120 kb 5′ to the Sox9 open reading frame. The second mouse had a subcongenic region, sub110-2, which spanned base pairs 110,887,739–113, 856,611 including *Sox9* and several telomeric genes (Figure 6A). Sub110-1 and sub110-2 had an overlap of 1.62 Mb, which is localized entirely within the upstream *Sox9* regulatory region. This overlapping region has no known open reading frames and does not include the only described testis-specific enhancer of *Sox9*, TESCO (Sekido and Lovell-Badge 2008). We performed embryonic analysis on both sub110-1 and sub110-2 to determine whether one or both lines was sufficient to confer protection from Y^{POS} sex reversal. Phenotype analysis showed that both sub110-1 and sub110-2 regions independently protect from B6-Y^{POS} sex reversal, producing both testicular tissue in embryonic gonads (Figure 6B, Fisher exact P-value < 0.001 for all comparisons to B6/B6) and fertile Y^{POS} adult males. These data further narrow the smallest congenic region required for protection from B6-Y^{POS} sex reversal to a minimal 1.62-Mb region encompassing the 5′ *Sox9* regulatory region. Our results contribute to the evidence of additional testis enhancers regulating *Sox9* expression in male sex determination.

The protective subcongenic region is not of 129S1 origin

Inspection of the SNPs in the congenic strains as compared to the 129S1 genotyping data (Figure 3B) showed marked differences in the SNP distribution. Thus, we suspected that the origin of the protective congenic regions was not from 129S1, as originally hypothesized, but instead from the original wild-type strain from which the Y^{POS} chromosome was derived >30 years ago (Figure 1). Further analysis comparing our strain to diversity array genotyping of 60 wild-derived strains (Yang *et al.* 2009, 2011) demonstrates that the protective region is most closely related to *M. musculus*

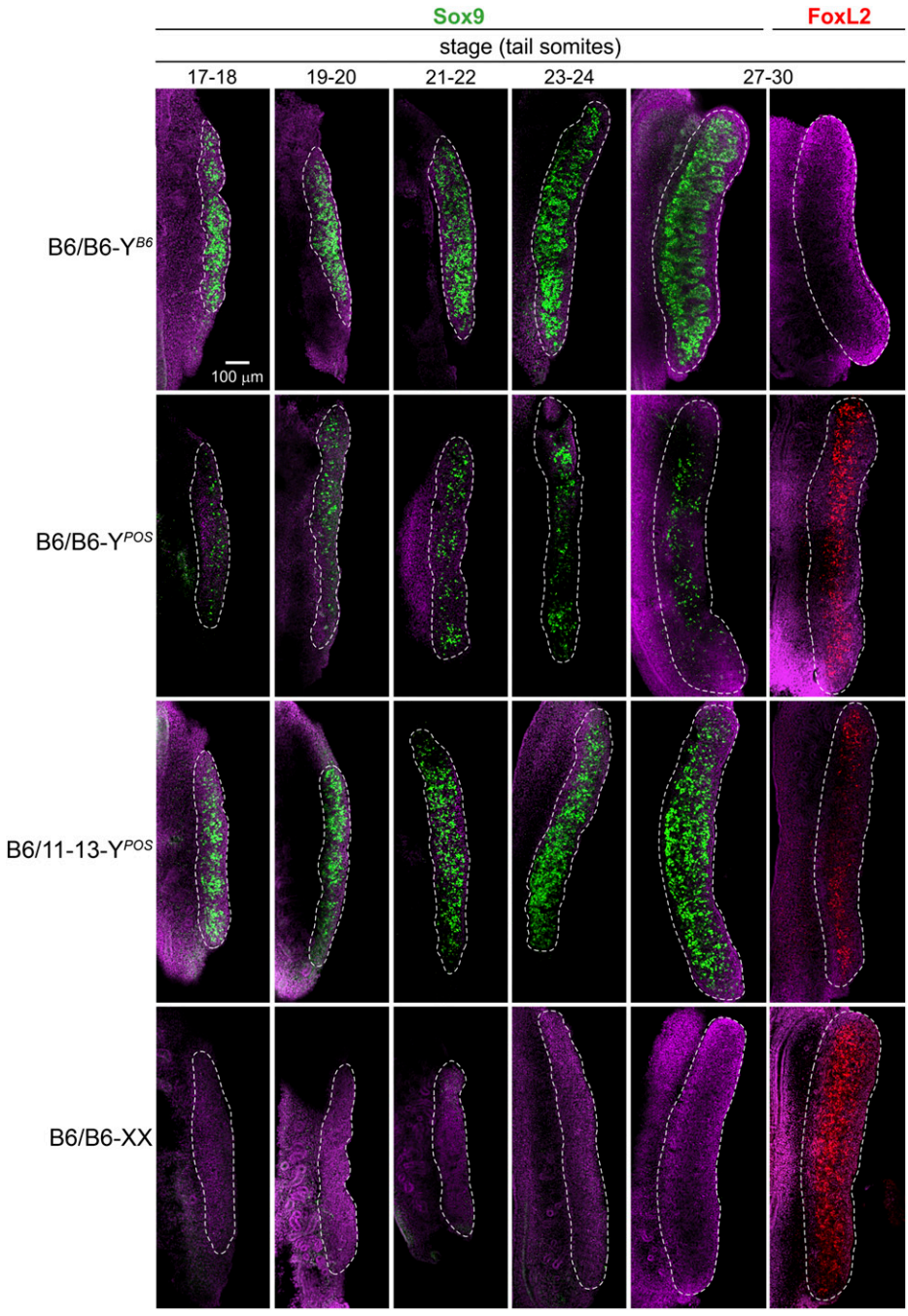


Figure 5 Timing and expression levels of the male marker Sox9 is rescued in the B6/Line11-13-Y^{POS} across critical stages of early gonadogenesis, but female marker FoxL2 is not fully extinguished. Using fluorescence immunohistochemistry, we stained embryonic gonads from B6-Y^{B6}, B6-Y^{POS}, LineB6/11-13-Y^{POS}, and B6-XX animals during sex determination (E11.5–E12.5). B6-Y^{POS} gonads showed diminished expression of Sox9 protein (green) compared to B6-Y^{B6} at all time points assayed and had significant expression of FoxL2 (red). Both timing and qualitative expression levels of Sox9 were rescued in the heterozygous LineB6/11-13-Y^{POS} embryonic gonads. FoxL2 expression in LineB6/11-13-Y^{POS} gonads was strongly diminished to much lower levels than in the B6-Y^{POS} gonads.

domesticus poschiavinus (Figure S3). Therefore, based on comparison of genotyping data from our congenic strains with that of the 129 inbred strain as well as wild-derived strains, we conclude that the entire congenic region is not of 129 origin, as originally hypothesized, and that the origin of the protective genomic elements is from the wild-derived *M. musculus domesticus poschiavinus* (Figure 1).

Discussion

The congenic approaches that we used to identify genetic loci that protect from B6-Y^{POS} sex reversal have highlighted the effect of interaction between the *poschiavinus* Y chromosome and a small, 1.62-Mb, noncoding regulatory region upstream of *Sox9* on male sex determination. This study redefined and narrowed the minimal protective region from >60 to 1.62 Mb, a nearly 37-fold decrease in size. The homozygous presence of the 1.62-Mb protective region rescues both the embryonic and the adult B6-Y^{POS} sex reversal phenotype. At the molecular level, embryonic gonads that are heterozygous for the protected region show increased protein expression of the male marker Sox9 and decreased expression of female marker FoxL2, compared to B6-Y^{POS} gonads.

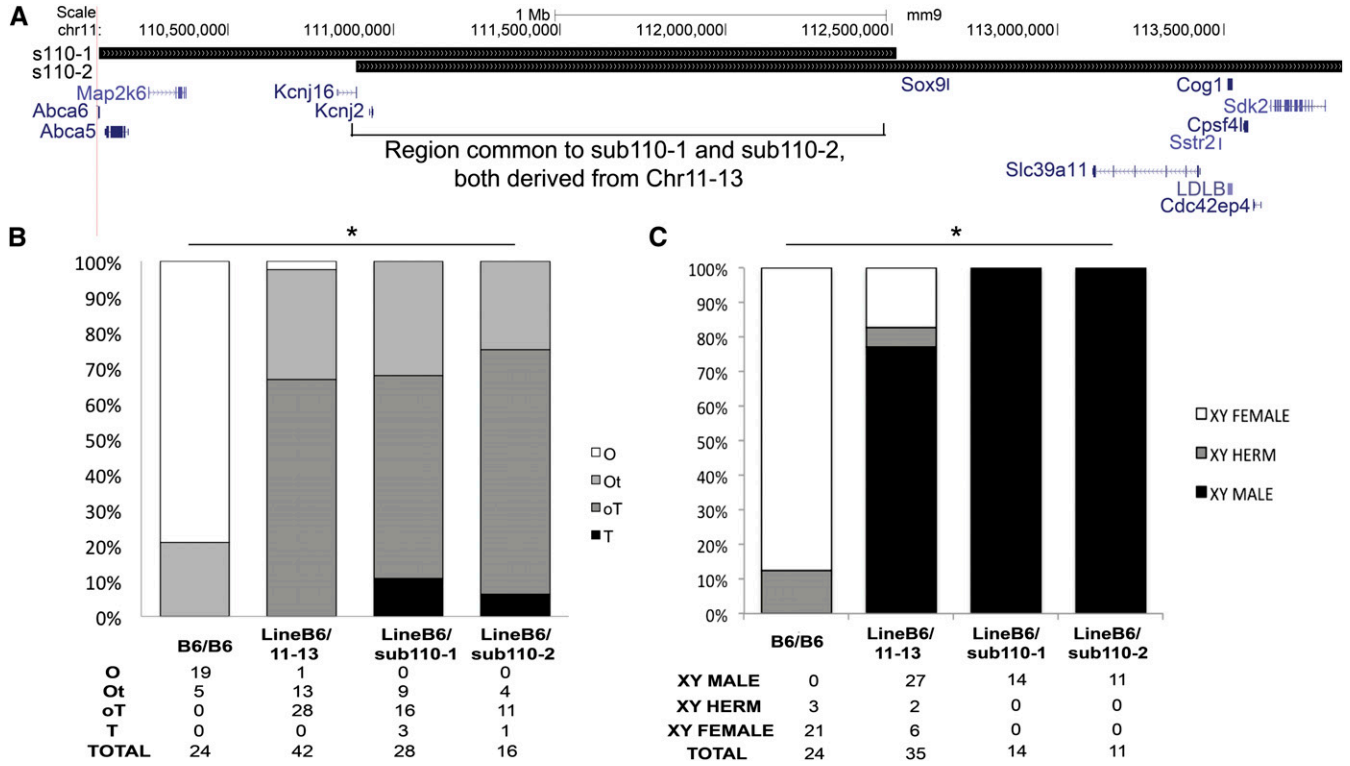


Figure 6 The 5′ regulatory region of *Sox9* is responsible for protection from B6-Y^{POS} sex reversal. (A) The region displayed here shows the entirety of the non-B6 region spanning megabases 110–114 on chromosome 11, and all known genes in this region are shown below in blue. Two smaller subcongenic lines, sub110-1 and sub110-2, were generated through breeding of LineB6/11-13 animals. Sub110-1 and sub110-2 overlap only over the 5′ regulatory region of *Sox9* (black box). Within the overlap region, there are no known genes. (B) Embryonic gonad analysis of the heterozygous animal lines showed significant protection compared to B6/B6 animals. The levels of protection between heterozygous animals LineB6/11-13, LineB6/sub110-1, and LineB6/sub110-2 were not significantly different. *Fisher exact *P*-value < 0.001 for the three way comparison. (C) Adult analysis of the heterozygous subcongenic lines showed significant protection from XY^{POS} sex reversal compared to B6/B6-Y^{POS} animals. The levels of protection between heterozygous animals LineB6/11-13, LineB6/sub110-1, and LineB6/sub110-2 were not significantly different. *Fisher exact *P*-value < 0.001 for the three-way comparison.

Our study also reexamines the history of this congenic strain. Previously, it was hypothesized that the protection from B6-Y^{POS} sex reversal was conferred by genetic elements of the 129S1/SviM (129) strain. However, analysis of several of our breeding males with the SNP Diversity Array proved that the protective congenic region on chromosome 11 was not of 129 origin but rather closely related to wild-derived strains of *M. m. domesticus poschiavinus* genotyped by the Jackson Laboratory. Therefore, we conclude that the genetic origins of the protection likely came from the semi-inbred strain POSA (Figure 1).

Revised history of the B6-Y^{POS} strain

In 1988, prior to the discovery of *SRY* as the testis-determining gene, mouse geneticist Eva Eicher postulated that, if there existed a single autosomal locus in the heterozygous state from the original POSA strain that allowed for some testicular development and fertility in B6-Y^{POS}, it would be "kept in a forced heterozygous state to perpetuate the B6-Y^{POS} strain" (Eicher 1988). This is exactly what we believe happened in our protected B6-Y^{POS} strain. Twenty-five years ago, Eicher hypothesized the scenario by which the wild-derived strain was able to maintain fertility and breeding through the presence of a heterozygous protective region.

The B6-Y^{POS} strain examined in this article diverged from existing colonies of B6-Y^{POS} animals in the mid-1980s, shortly after the initial publication of B6-Y^{POS} sex reversal (Eicher *et al.* 1982). Unlike our animals, most other colonies of B6-Y^{POS} are maintained in the presence of a transgenic *Sry* (Bullejos and Koopman 2005; Correa *et al.* 2012). It is highly unlikely that the protective genomic element identified in our animals continues to exist in other B6-Y^{POS} colonies because, in the presence of transgenic *Sry*, the selective pressure to maintain POSA-protective genomic elements would have been lost.

This minimal 1.62-Mb region located 5′ to *Sox9*, which we show protects from B6-Y^{POS} sex reversal, initially came as a surprise to us as this region was not identified by our SNP scan published in Nikolova *et al.* (2008) This region remained unidentified because we were limited by our then-incomplete knowledge about the genetic diversity among mouse strains. By testing only SNPs polymorphic between B6 and 129, we could not reliably pick up regions that were present in other strains, such as *M. m. domesticus*

poschiavinus, unless the SNP was, by chance, also polymorphic between B6 and *M. m. domesticus poschiavinus*. Similarly, previous linkage studies focused on identifying genomic regions important for promoting sex reversal were limited by genotyping of only SNPs polymorphic between B6 and DBA2 (Eicher *et al.* 1996). In both studies, strain-specific SNPs were limiting as they did not account for the above hypothesis that a surreptitious heterozygous autosomal genomic element derived from the original *POSA* strain may be responsible for protection from the Y^{POS} sex-reversal phenotype.

Improved genetic technologies have allowed for deeper genomic analyses of the ancestral diversity of current laboratory strains and paved the way for our greater genetic understanding of the congenic B6.POSA-Y^{POS} strain. Throughout the 30-year breeding history of these congenic mice, the assumption that one had achieved a pure consomic B6-Y^{POS} was based on statistical evidence, which holds true unless, such as in our case, there is a strong selective pressure to maintain specific genomic elements for fertility and breeding (Silver 1995). Reanalysis of our animals and close examination of the background history of the B6-Y^{POS} strain identified POSA-specific genomic elements required for B6-Y^{POS} male fertility. This region likely was important for propagation of the B6-Y^{POS} strain prior to the introduction of transgenic *Sry* into other existing colonies of B6-Y^{POS}. This region confers significant protection from B6-Y^{POS} sex reversal, but heterozygosity for the protective region is necessary but not sufficient to confer full protection from B6-Y^{POS} sex reversal. We do observe a dose-dependent protection, and homozygosity is sufficient for protection from B6-Y^{POS} sex reversal.

The protective effect of the POSA Sox9 promoter on B6-Y^{POS} sex reversal

The mechanisms underlying the protective effect of the POSA *Sox9* promoter region remain unclear. The entire mouse *Sox9* regulatory region spans nearly 2.4 Mb, both centromeric and telomeric of the *Sox9* open reading frame. *Sox9* is involved in development not only of the gonad (Sekido and Lovell-Badge 2008), but also of the neuroectoderm, brain, gut (Bagheri-Fam *et al.* 2006), skeletal (Bi *et al.* 1999), and craniofacial tissues (Benko *et al.* 2011). As such, *Sox9* has many tissue- and temporal-specific promoters that regulate its expression. Point mutations, deletions, and duplications limited to the *Sox9* regulatory regions can result in abnormal expression of Sox9, resulting in a variety of phenotypes, ranging from XX males (Bishop *et al.* 2000; Cox *et al.* 2011) to isolated craniofacial dysmorphism with Pierre Robin Sequence (Benko *et al.* 2009) to skeletal anomalies (Foster *et al.* 1994).

Developmentally important genes, such as *SHH*, *FOXL2*, and *SOX9*, are known to have long-range enhancers that function from >1 Mb away and from introns of neighboring genes (Beysen *et al.* 2005; Gurnett *et al.* 2007; Benko *et al.* 2009). Further studies will be required to precisely identify enhancers of *Sox9* and how the small region that we have identified here results in protection from B6-Y^{POS} sex reversal. TESCO, the only testis-specific enhancer identified in mouse (Sekido and Lovell-Badge 2008), does not cause any known cases of human 46,XY gonadal dysgenesis. Therefore, other testis-specific enhancers of *Sox9* may work in synergy with the TESCO enhancer to promote continued *Sox9* expression within the developing testis. Attempts to narrow down the location for a second testis-specific *SOX9* enhancer from human data with small deletions in the *SOX9* regulatory region have identified a minimal overlap of a 67-kb noncoding region 584 kb upstream of *SOX9* (Benko *et al.* 2011; Xiao *et al.* 2013). Both the 67-kb region and the entirety of the *SOX9* regulatory region are active lines of investigation within the field of sex determination. Our narrowed minimal protective region encompasses the mouse region syntenic to the smallest 67-kb region identified in human studies, but not TESCO.

Using a predictive transcription-factor-binding algorithm (Cartharius *et al.* 2005), we have identified putative binding sites for Nr5a1/Sf-1, Sry, and Sox9 transcription factors within the 67-kb minimal region and its flanking genomic region (Figure S4). Potential Sry- and Sf1-binding sites appear to be well represented within this region (approximately one binding site per 2 kb), while Sox9 transcription-factor-binding sites are more sparse (approximately one binding site per 7.5 kb). Although several sites fall into regions of the genome that are under selective constraint (Davydov *et al.* 2010), further interpretation of these data would require evidence to show that any of these transcription-factor-binding sites play a role in human sex development or are causative in our B6-Y^{POS} protective model as many mechanisms outside of transcription factor binding work within the noncoding genome to regulate transcription, including long noncoding RNAs, methylation, and chromatin conformation, to name a few.

Our SNP array studies show that the congenic strain's 110 region is very similar, but not identical, to *M. m. domesicus poschiavinus*. Therefore, further studies to characterize the base-pair sequence, CNV, presence of epigenetic marks, and noncoding RNA transcripts may shed light on the complex regulation of this developmentally important region.

Following *Sry* up-regulation, *Sox9* up-regulation in the somatic cells of the bipotential gonad is one of the initial and critical steps in testis sex determination. Previous studies have demonstrated that, within B6-Y^{POS} gonads, *Sry* messenger RNA expression is delayed beyond the critical window to initiate testis sex determination (Bullejos and Koopman 2005; Hiramatsu *et al.* 2009). While that might be the primary cause of B6-Y^{POS} sex reversal, the protection conferred by the 110 region rescues the sex reversal phenotype farther downstream, through up-regulating Sox9 expression despite decreased Sry expression. High levels of Sox9 have been shown in both humans and mice to be both necessary and sufficient for testis formation in both XX and XY individuals (Huang *et al.* 1999; Vidal *et al.* 2001).

What is remarkable is the strength of the interaction between Y^{POS} and the concomitant POSA regulatory region

of *Sox9*. Only co-inheritance of the Sry^{POS} and the POSA regulatory region of *Sox9* allowed for male sex determination, fertility, and breeding. Inheritance of Y^{POS} but not the POSA-protective elements results in line extinction through sex reversal and infertility of the male mice. Fertility in XX mice is unaffected by this genomic element. Only B6-Y^{POS} mice possessing this region were capable of reproducing, and thus we hypothesize that Y^{POS} and promoter regions co-evolved with Y^{POS} depending on or requiring the *POSA* promoter of *Sox9* to perpetuate the testis-specific gene network within the spermatic cords of an XY^{POS} mouse.

Our findings highlight the complex network of genes that can work to promote the key pathways within male sex determination. Mice harboring Y^{POS} require the *POSA* region to maintain the male-specific network equilibrium and ensure the up-regulation of *Sox9*, testis development, and fertility. Y^{POS} in the presence of the B6 genetic background shifts the balance toward female sex determination, and our findings indicate that this is due to decreased activation of *Sox9*. The co-evolution of the *POSA Sox9* promoter occurred because the presence of both these elements (Y^{POS} and *POSA* promoter) maintained male fertility; hence, it is likely that Sry^{POS}, in addition to other factors, is specifically geared to activate the *POSA* promoter of *Sox9*. While *Sry* from other mouse strains may be able to activate *POSA*, if the genetic background is not inherently "pro-female" as the B6 strain is, the presence of the *POSA* promoter may simply be a redundant mechanism pushing the network equilibrium further toward testis development and providing a buffer for minor genetic alleles that promote ovary formation. Unraveling the nature of these interactions will require evaluation of the entire *Sox9* promoter region to identify critical regions and to dissect how SNPs, DNAse hypersensitivity sites, and transcription-factor-binding sites specifically interact with Sry^{POS} and the entire gene network within the developing gonad.

Finally, these types of studies may provide a glimpse into the genetic heterogeneity that we may expect to find in human cases of disorders of sex development. To date, it is estimated that only 30–40% of all cases of 46,XY gonadal dysgenesis can be explained by single gene mutations, deletions, or duplications. The remaining 60% may involve more complex inheritance of multiple alleles required to promote sex determination. Thus, in the absence of one gene, the entire sex determination program may be delayed or, if the effect of the gene is large, may result in sex reversal. Early studies in humans have began to unravel these coexisting mutations and modifiers that influence early developmental processes (Sykiotis *et al.* 2010) and the intricate web in which cell-intrinsic processes as well as the external environment must come together in a delicate balance to promote proper sex determination.

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Supporting Information
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Regulation of Sex Determination in Mice by a Non-coding Genomic Region

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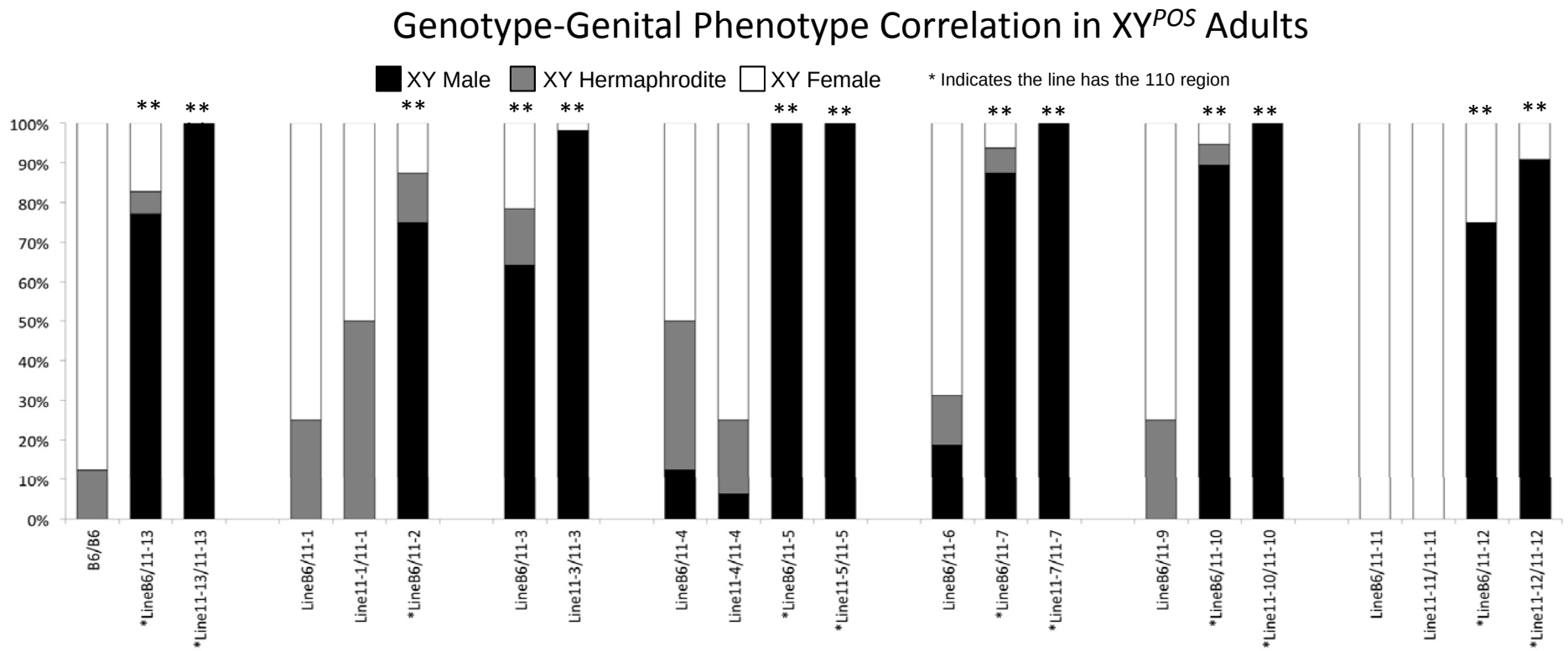
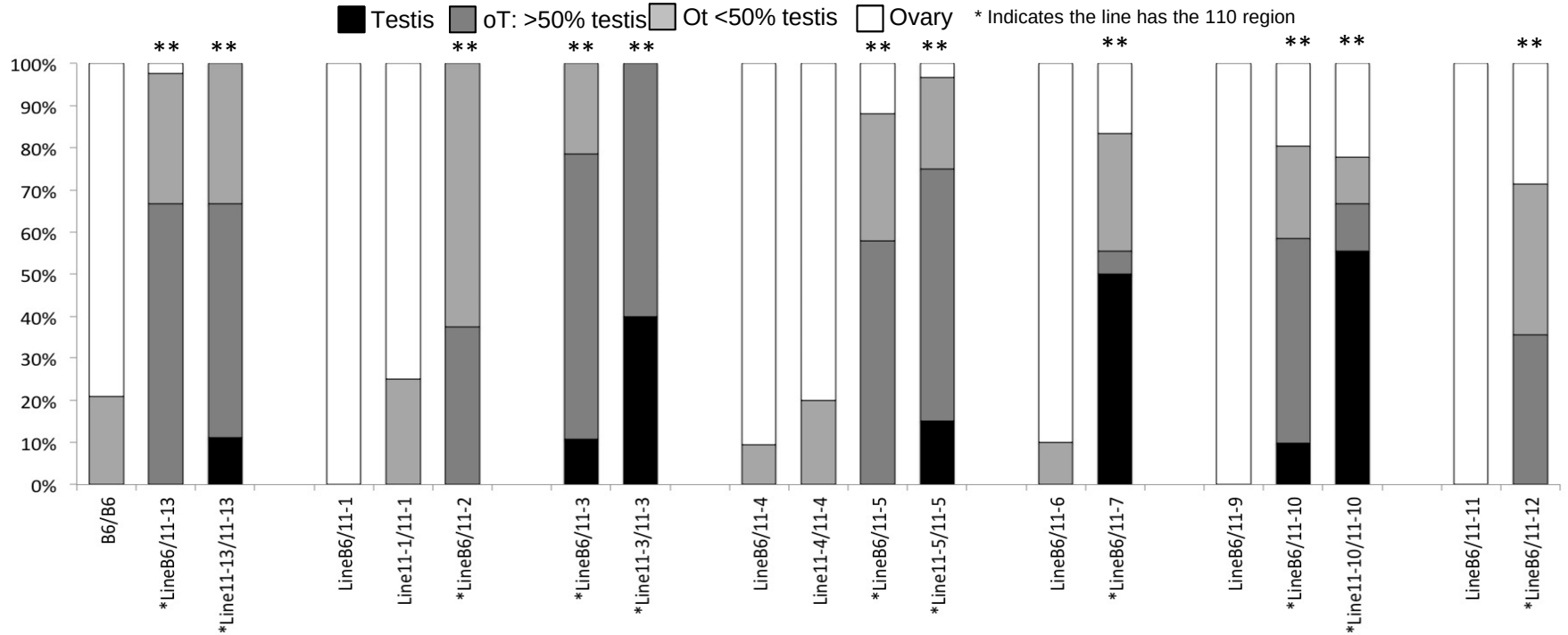


XY FEMALE	21	6	0		12	2	1		6	1		4	12	0	0		11	1	0		3	1	0		5	3	1	1
XY HERM	3	2	0		4	2	1		4	0		3	3	0	0		2	1	0		1	1	0		0	0	0	0
XY MALE	0	27	12		0	0	6		18	53		1	1	17	20		3	14	1		0	17	13		0	0	3	10
TOTAL	24	35	12		16	4	8		28	54		8	16	17	20		16	16	1		4	19	13		5	3	4	11

** Fisher exact test p-value <0.001 compared to B6/B6

Figure S1 Genotype/genital phenotype correlation in XY^{POS} adults shows that the 110 region promotes male sex determination on a B6-Y^{POS} background. Adult XY^{POS} animals that were heterozygous or homozygous for different lines were analyzed for their genital phenotype. All strains were compared to B6/B6-Y^{POS} and animals that were heterozygous for congenic regions, which did not contain the 110 region. An asterisk (*) refers to the presence of the 110 region within the congenic line and a double asterisk (**) refers to Fisher exact test p-value < 0.01. In all strains, only lines that carried the 110 region were significantly protected against sex reversal. Only a total of 5 animals were found to be phenotypically male, even in the absence of the 110 region (see LineB6/11-4, Line11-4/11-4, and LineB6/11-6). The number of animals analyzed in each group is shown below the graph, and lines that are either heterozygous or homozygous for the 110 region are highlighted in grey.

Genotype/Gonad Correlation in XY^{POS} Embryos



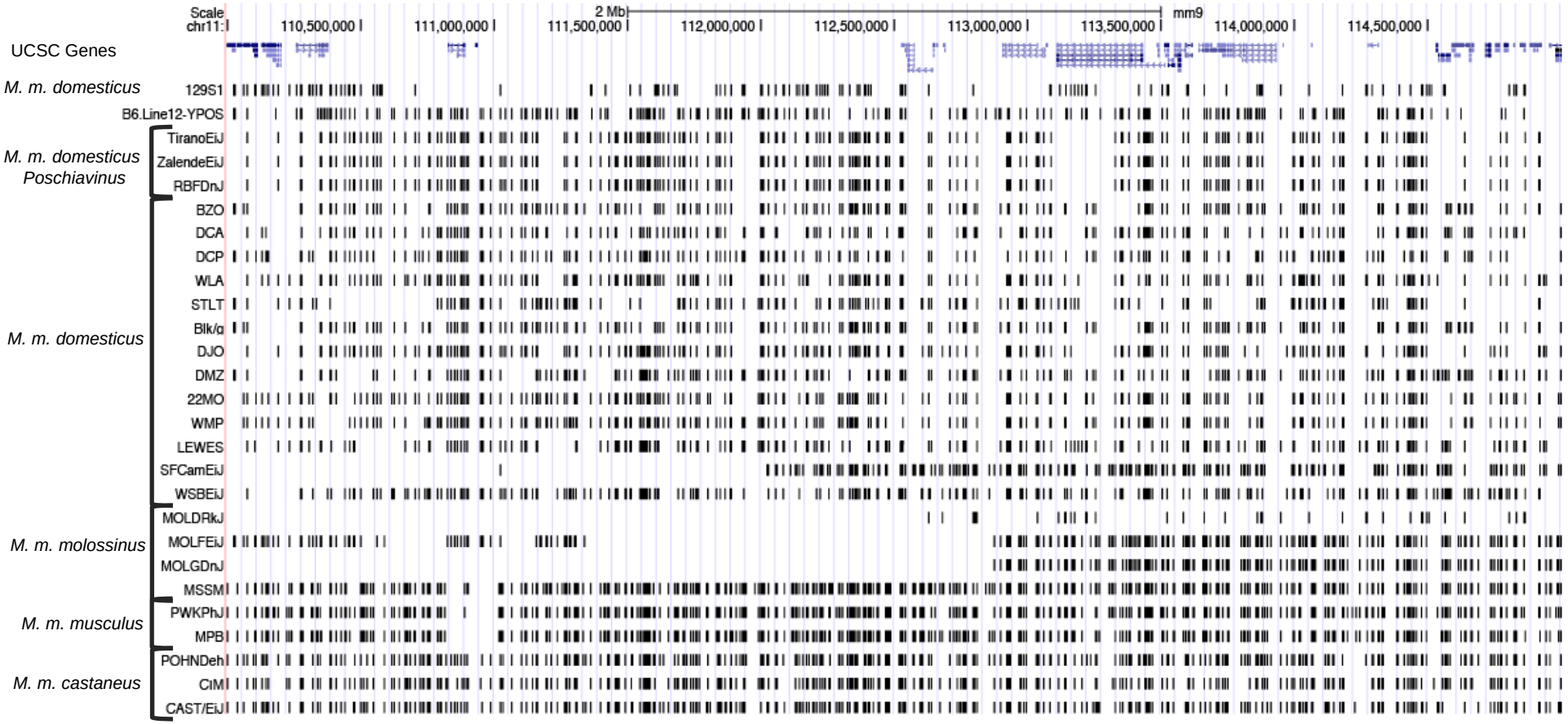
Ovary	19	1	0		6	3	0		0	0		29	8	6	2		9	3		12	8	4		16	4
Ot	5	13	3		0	1	5		6	0		3	2	15	13		1	5		0	9	2		0	5
oT	0	28	5		0	0	3		19	6		0	0	29	36		0	1		0	20	2		0	5
Testis	0	0	1		0	0	0		3	4		0	0	0	9		0	9		0	4	10		0	0
Total	24	42	9		6	4	8		28	10		32	10	50	60		10	18		12	41	18		16	14

** Fisher exact test p-value <0.001 compared to B6/B6

Figure S2 Genotype-genital phenotype correlation in XY^{POS} embryonic gonads shows that the presence of the 110 region results in a shift towards testis formation. The embryonic phenotype is a qualitative assessment closer to the critical timepoint of embryonic sex determination, which occurs between E10.5 and E12. Embryonic XY^{POS} gonads were dissected immediately after sex determination at E14.5-E15.5 and the proportion of testis formation was qualitatively assessed. All lines were compared to B6/B6-Y^{POS} and animals that were heterozygous for congenic regions containing the 110 region. An asterisk (*) refers to the presence of the 110 region within the congenic line and double asterisk (**) refers to a Fisher exact test p-value <0.01. The homozygous presence of the congenic region with 110 resulted in no Y^{POS} ovaries. Heterozygosity for a congenic region with the 110 region resulted in a shift towards increased testis formation with less formation of full ovaries.

A

Mouse Diversity Array Analysis of B6.Line12-Y^{POS} versus wild-derived strains



B

Mouse Diversity Array Analysis of B6.Line12-Y^{POS} vs other *M. m. domesticus poschiavinus strains*

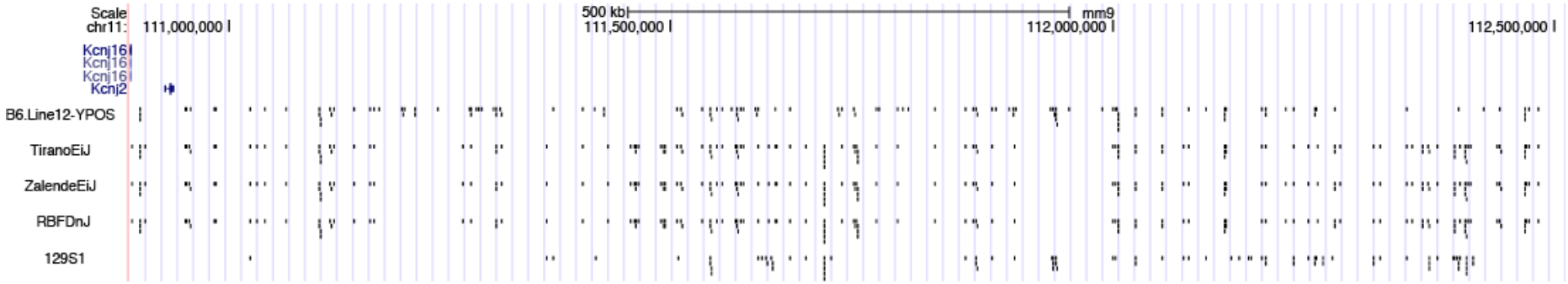


Figure S3 Mouse Diversity array analysis of B6.Line12-Y^{POS} versus wild-derived strains. A) To identify the potential origins of the protective region, we compared Diversity Array data from the data from the 129 strain (top line) to our LineB6/11-13-Y^{POS} protective congenic strain (2nd line), which contains only the 110 region, to and found that the Chromosome 11 110 region (shown here Chr11:110000000-115000000) is not derived from the 129 strain. Further analysis with array data from wild-derived strains of the 4 major subspecies of M. musculus: *domesticus, musculus, molossinus, and castaneus* identified significant similarity between 3 other typed *M. m. domesticus Poschiavinus* wild strains *Tirano*, *Zalende*, and *RBFDnJ* grouped under *M. m. domesticus Poschiavinus*. SNP data for the 129 and wild derived strains is obtained from http://cgd.jax.org/datasets/popgen/diversityarray/yang2011.shtml. B) In-depth analysis of the minimal overlap protective region derived from sub110-1 and sub110-2 (chr11:110,887,739-112,514,446) of the protected strain LineB6/11-13 and 3 genotyped animals from the *M. m. domesticus Poschiavinus* subspecies show significant similarity over the span of this non-coding region.

Predicted Transcription Factor Binding Sites in the mouse syntenic region of a second minimal region identified in cases of human DSD

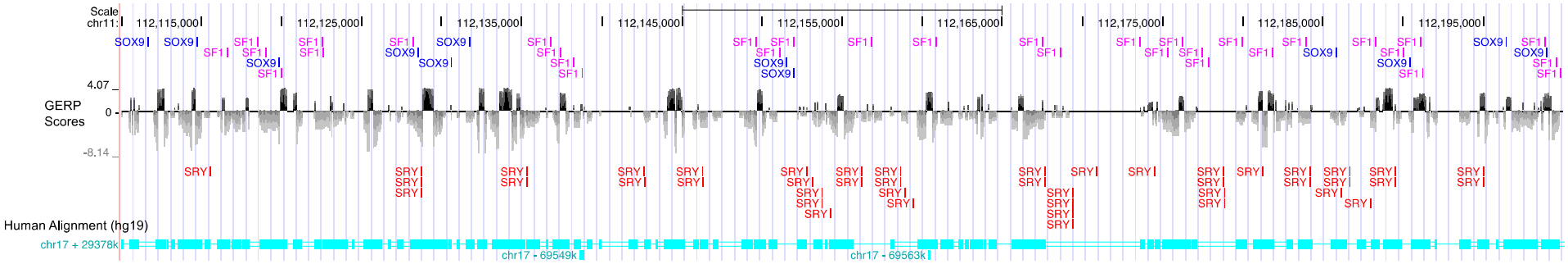


Figure S4 Sry, Sox9 and Sf1 binding sites in mouse region syntenic to the human minimal 90kb protective region identified from human cases of 46,XX and 46,XY DSD. Using MatInspector, we identified predicted transcription factor binding sites for Sry(red), Sox9(blue), and Sf1(pink) in the mouse genomie region spanning 112,110,000 to 112,200,000bp on chromosome 11. Here, 25bp regions representing predicted transcription factor binding sites regions are indicated by the vertical lines and colored to represent one of three factors, as stated above. Additionally, GERP scores were used to identify regions that are undergoing positive selection (GERP score > 0) or neutral selection (GERP score < 0). We show that the majority of predicted binding sites did not fall into highly conserved regions, based on GERP scores. At the bottom in turquoise, we show the alignment to the human genome hg19. The solid boxes indicate regions where there alignment between mouse and human reference sequence. Single lines represent gaps between the mouse and human alignment due to either deletion in the human reference or insertion in the mouse assembly. Double lines represent more complex regions that have gaps in either mouse, humans, or both. Scale bar (at top) = 20kb

Tables S1-S3
Available for download at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.160259/-/DC1

Table S1 PCR primers for SNP genotyping of Congenic Lines.

Table S2 Chromosome 11 results for strains genotyped by the Mouse Diversity Array at the Jackson Laboratories. Each sheet represents an individual mouse from Figure 3. Chromosome 11 position (build 37) is provided in columns B and C. Column D shows dbSNP or JAX lab identifier, if identifier known. The final column is the SNP call as performed at the Jackson laboratories. 0 = B6, 1= heterozygous, 2= homozygous for alternate non-B6 allele, -1= no call.

Table S3 Copy Number Variation (CNV) Analysis performed on a subset of animals did not identify and consistent CNVs in our congenic mice. CNV analysis was performed on the genotyped founders from Animals Line11-4/11-5, Line11-6/Line11-7, Line11-8/11-9, Line11-10/Line11-10 (see Figure 3). Results from the CNV analysis identified between 5 and 9 CNVs in each of these samples. These CNVs were classified as either a gain or a loss, but the number of copies was not quantitatively determined. Four of the CNVs recurred in most of the samples and had the same boundaries. These consisted of CNVs encompassing the variable antibody region on chromosome 12, the histocompatibility locus, erythropoietin-4 immediate early response gene, the intron of Neuroligin-1, and Lrmp. The locations of other CNVs identified in individuals are described in Table 3. Only two copy number variants were identified on chromosome 11, in the within the congenic region of the Line11-6/11-7 animal.