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## Sexual differentiation in the developing mouse brain: contributions of sex chromosome genes

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### Abstract

Neural sexual differentiation begins during embryogenesis and continues after birth for a variable amount of time depending on the species and brain region. Because gonadal hormones were the first factors identified in neural sexual differentiation, their role in this process has eclipsed investigation of other factors. Here, we use a mouse with a spontaneous translocation that produces four different unique sets of sex chromosomes. Each genotype has one normal X-chromosome, and a unique second sex chromosome, creating the following genotypes: XY<sup>\*x</sup>, XX, XY<sup>\*</sup>, XX<sup>Y\*</sup>. This Y<sup>\*</sup> mouse line is used by several laboratories to study two human aneuploid conditions: Turner and Klinefelter syndromes. Since sex chromosome number affects behavior and brain morphology, we surveyed brain gene expression at embryonic days 11.5 and 18.5 to isolate X-chromosome dose effects in the developing brain as possible mechanistic changes underlying the phenotypes. We compared gene expression differences between gonadal males and females as well as individuals with one versus two X-chromosomes. We present data showing, in addition to genes reported to escape X-inactivation, a number of autosomal genes are differentially expressed between the sexes and in mice with different numbers of X-chromosomes. Based on our results, we can now identify the genes present in the region around the chromosomal break point that produces the Y<sup>\*</sup> model. Our results also indicate an interaction between gonadal development and sex chromosome number that could further elucidate the role of sex chromosome genes and hormones in the sexual differentiation of behavior.

### Keywords

sex chromosome; sex differences; X-inactivation; X-chromosome; Y-chromosome; Klinefelter syndrome; Turner syndrome; aneuploidy

### Introduction

Sex differences in the brain and behavior are widespread in animals. Neural development occurs before and just after birth, and during this period sex differences are organized. The powerful role of androgens on sexually dimorphic behaviors was described over 50 years ago (Phoenix *et al.* 1959). Since then, much attention has focused on hormonal modulation of brain sexual dimorphisms. Yet, in addition to sex differences in hormone secretions, males and females have differences in gene expression arising from genomic imprinting and sex chromosomes (Arnold 2009).

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Two of the mouse models used to examine these sex chromosome effects on brain and behavior include the Four Core Genotypes in which gonadal (testes vs. ovaries) and chromosomal (XX vs. XY) sex are uncoupled (De Vries *et al.* 2002), and the SF1 knock-out mouse that has normal sex chromosomes but lacks gonads (Majdic and Tobet 2011; Budefeld *et al.* 2012). Here, we use a third model, the Y\* mouse which offers a way to assess the roles of X-chromosome number (Eicher *et al.* 1991). In addition to this purpose, several laboratories have used these mice to model human aneuploid conditions, particularly Klinefelter syndrome (XXY). Data from all these models provide evidence that sex chromosome complement is involved in behavior (Gatewood *et al.* 2006; Quinn *et al.* 2007; Gioiosa *et al.* 2008; Grgurevic *et al.* 2008; Park *et al.* 2008; Liu *et al.* 2010; Cox and Rissman 2011; Bonthuis *et al.* 2012; Grgurevic *et al.* 2012).

To identify sexually dimorphic gene targets during neural development that may potentially underlie behaviors regulated by sex chromosome, we compared the four genotypes generated in the Y\* model. The Y\* mouse exhibits a rearrangement of the pseudoautosomal regions (PAR) which creates the unique Y\* chromosome. This chromosome contains Y-chromosome genes, an inverted duplication of a partial PAR, and a small amount of X-chromosome including the centromere (Eicher *et al.* 1992; Burgoyne *et al.* 1998). Mating the XY\* male with a normal XX female results in offspring with four genotypes: normal XX females (XX), 1X females, (XY\*X), 2X males (XXY\*) and 1X males (XY\*). In this cross, the XY\* chromosome consists of an X-chromosome, with an incomplete PAR, attached to the Y\* chromosome. The Y\*X contains the X centromere with X-chromosome genes close to PAR; it lacks any Y-chromosome genes. Comparisons between males and females with one or two X-chromosomes enable us to determine which genes are differentially represented in each genotype.

In this study, we collected whole heads at embryonic (E) day 11.5 prior to gonadal differentiation and whole brains from embryos on day E18.5, a time point after which gonads differentiate to become testes or ovaries. We used these time points because gonadal differentiation in mice occurs between E12-14 (Koopman *et al.* 1990; Vazquez *et al.* 1998) and by E18.5 testes are producing androgens (vom Saal and Bronson 1980). We determined gene expression differences using microarrays and confirmed interesting candidates with quantitative real time PCR. Through this analysis, we discovered another gene, *Msl3*, located on the Y\* chromosome.

## Materials and Methods

### Animals

Y\* mice (B6Ei.LT-Y(IsXPAR;Y)Ei/EiJ, strain# 002021) were purchased from Jackson Laboratories and bred in our facilities. All mice used in this study were in the C57BL/6JEiJ background and housed in constant conditions under a 12:12 light cycle (lights on at 0600 h). Food (Harlan Teklad diet #7912) and water were provided ad libitum. All experimental procedures were approved by University of Virginia Animal Use and Care Committee and performed according to the AALAC guidelines. For these studies, males from the XY\* line were mated with females from the same substrain to produce offspring with four genotypes (Table 1): gonadal females with two copies of the complete X-chromosome (XX), females with one complete X-chromosome and a PAR with a few X-chromosome genes (XY\*X), males with one sex chromosome containing all X-chromosome genes, the unique Y-chromosome genes, a small subset of X-genes, as well as two duplications and one deletion in the PAR (XY\*), and males with the sex chromosome just described plus one normal X-chromosome (XXY\*). Adult females (n=7) were paired with fertile males and checked daily for the presence of mating plugs. The day that a plug was found was designated as embryonic day 0.5 (E0.5). On E11.5 and E18.5 embryos were rapidly removed from deeply

anesthetized pregnant dams between 1000 and 1200h and kept in ice-cold Dulbecco's phosphate buffered saline. Four dams were used to harvest litters at E11.5 and three dams were sacrificed at E18.5. Whole heads were removed from E11.5 embryos (n=2–4/genotype) and whole brain was dissected from E18.5 fetuses (n=2–4/genotype) and immediately flash frozen and stored at –80°C until RNA extraction. Pups were selected based on intrauterine position to reduce any potential variation from hormones produced by neighboring embryos (vom Saal 1989). Only mice residing next to zero or one male *in utero* were used for our analysis.

To determine sex and genotype, RNA from embryonic mice was isolated from tail clippings as previously described (Park *et al.* 2008) and reverse transcribed into cDNA. We measured *Xist* mRNA as an indication for the number of X chromosomes since *Xist* is expressed only when an inactivated X chromosome is present and thus a PCR product only appears in samples containing two or more X chromosomes. The primers used for *Xist* genotyping were 5' TAAGGACTACTTAACGGGCT 3' (forward) and 5' TACTCAGACATTCCCTGGCA 3' (reverse) and we normalized the product to GAPDH as an endogenous control. The primers used for GAPDH were 5' ACCACAGTCCATGCCATCAC 3' (forward) and 5' TCCACCACCCTGTTGCTGTA 3' (reverse). To determine the gonadal sex of each embryo, we assayed for the presence or absence of the spermiogenesis specific transcript (*Ssty1*) which is only present on the Y-chromosome. The primers used for *Ssty1* were 5' CTGGAGCTCTACAGTGATGA 3' (forward) and 5' CAGTTACCAATCAACACATCAC 3' (reverse) and we normalized the product to myogenin as an endogenous control; 5' TTACGTCCATCGTGGACAGCAT 3' (forward) and 5' TGGGCTGGGTGTTAGTCTTAT 3' (reverse). In addition to this, we used gene expression profiles across chromosomes X and Y to confirm genotypes. As shown in Supplemental Figure 1, replicate samples clustered together according to age and genotype based on our principle component analysis of chromosome X- and Y-gene expression profiles.

### RNA isolation and microarray hybridization

Total RNA was extracted from individual embryonic heads (E11.5) or whole brain (E18.5) in Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration and quality was assessed by the Agilent 2100 Bioanalyzer (Agilent). Briefly, total RNA (1 µg) was transcribed into double-stranded cDNA using the One-cycle Targeting and Control Reagent kit (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA was synthesized from cDNA, purified and fragmented according to manufacturer's instructions. Labeled cRNA from individual animals (n = 2–4/group, each from a different litter) was hybridized to a Mouse Genome 430 2.0 array (Affymetrix) for each sample (n = 24 total microarrays).

### Statistical analysis of microarrays

Microarray data were processed using GeneChip Operating Software v4.1 (GCOS, Affymetrix). Quality control analyses were performed on the array data including intensity histogram plots, calculation of background levels, and fraction of probe sets detecting RNA. As shown in Supplemental Figures 2 and 3, we generated normalized unscaled standard error (NUSE) and relative log expression (RLE) plots, which are sensitive indicators of hybridization quality (Gentleman *et al.* 2005). The data were further checked for reproducibility by generating MvA plots which are pairwise scatter plots of M = difference of log intensities (y-axis) versus A = average log intensities (x-axis) for all replicates. All arrays were determined to be acceptable and further analyzed using GCRMA, a low-level analysis algorithm to arrive at estimates of relative gene expression levels. We performed modified t-tests within age groups to compare gene expression differences between genotypes as well as between age groups using the limma package in the Bioconductor

software suite (Smyth 2004). In order to arrive at robust differences in gene expression that can be detected by qPCR, we applied a 1.5 fold change and 20% false discovery rate (FDR) cutoff for each comparison. The FDR was calculated using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995). We also assessed reproducibility and confirmed genotypes by generating a principle component plot (Supplemental Figure 1) using the plotPCA function from the Affycoretools package within Bioconductor using normalized, log<sub>2</sub> GCRMA expression values for all probe sets on X- and Y-chromosomes.

### Quantitative real time PCR

Total RNA was isolated from the whole heads (E11.5) and the brain tissue (E18.5) from male and female embryos (n=2–4/group, each from a different litter). cDNA was generated from 1 µg of total RNA by reverse transcription with the iScript cDNA kit (Bio-Rad). Quantitative real time PCR was performed using the OneStep™ System (ABI) according to the manufacturer's instructions for SYBR Green based detection. Samples were run in triplicates on either one or two plates and the average values were analyzed. SYBR Green primers were designed for *BC022906*, *Eif2s3x*, *Hccs*, *Kmd5c*, *Kdm6a*, *Mid1*, *Xist*, *Msl3*, *Msl2*, *Msl1*, *Myst1*, *Dxh9* and *Ppib* (control gene). Primer sequences for these genes can be found in Supplemental Table 1. Quantification of candidate gene expression was calculated based on the threshold cycle ( $C_t$ ) for each well using the provided software and normalized to peptidylprolyl isomerase B (*Ppib*) as the endogenous control. Relative amount of gene expression was scaled to XX females as the reference sample. All data was analyzed using NCSS (2007). For data analysis, we used two-way ANOVA with gonadal sex and X-chromosome number as factors. Significant results were assessed by Tukey-Kramer's *post hoc* tests that adjust significance levels to take multiple comparisons into account.

### Copy number assay

Genomic DNA was extracted from adult cerebellum of the four Y\* genotypes: XX, XY\*X, XX<sup>Y\*</sup> and XY\*. For comparison, genomic DNA was isolated from whole brain of E18.5 wild type male and female C57BL/6J embryos. DNA was isolated using standard phenol:chloroform extraction procedures. Briefly, brain tissue was homogenized in TE buffer and protein was digested using proteinase K. DNA was extracted twice using phenol:chloroform:isoamyl alcohol, then precipitated with 3M sodium acetate in ethanol. Isolated DNA was washed in ethanol, resuspended in TE buffer and any remaining RNA was digested by RNase A. The amount and purity of DNA was assessed by spectrophotometer. 20ng of DNA was used in a PCR reaction to determine the relative amount of *Msl3*, *Hccs* and *BC022960* within each genotype and normalized to peptidylprolyl isomerase B (*Ppib*) as the endogenous control. The relative amount of each gene of interest was calculated using the  $\Delta\Delta C_t$  method (Livak and Schmittgen 2001). For copy number analysis, relative amount of genomic DNA was normalized to *Ppib* as an endogenous control and XY C57BL/6 males were used for the reference sample. Thus, the relative gene copy in C57BL/6 males was set to 1. For data analysis, we used two-way ANOVA with gonadal sex and X-chromosome number as factors. Significance was determined by Tukey-Kramer *post hoc* tests.

## Results

### Sex differences in Y\* mice prior to and after gonad differentiation

We compared E11.5 male and female embryos with one or two X-chromosomes to determine which genes were differentially expressed between males and females before gonadal differentiation (Figure 1); 32 transcripts were significantly altered (Table 2). Of these, four Y-chromosome genes and 16 autosomal genes differed between XY\*X and XY\*. Genes encoding kinases (*Frk* and *Mobk11k*) or a voltage-gated calcium channel that

regulates AMPA activation (*Cacng5*), as well as a few genes that likely play a role in cell growth and or immune response (*Nedd9*, *Cyr61*, *Wdr1*, *Defcr3* and *Ebi3*) were significantly different by sex in mice with one X-chromosome. *Frk* and *Nedd9* were also altered in the XY\*<sup>X</sup> and XX female comparison, thus this change is not related to gonadal sex and may be produced by non-gonadal effects of *Sry*, other Y-chromosome genes, or some other genetic differences between XY\* and XY\*<sup>X</sup>.

At E11.5, comparisons between XX<sup>Y\*</sup> and XX mice revealed 28 differentially expressed transcripts (Table 2); 13 autosomal genes were significantly different. In general, genes involved in signaling and transport within the cell (i.e. syntaxin 5a and dystonin) or involved in synaptic plasticity and myelination (neuronal pentraxin 2, *Nptx2*, and SH3 domain and tetratricopeptide repeat 2, *Sh3tc2*) differed in males and female with two X-chromosomes. Src homology2 domain (*She*), GTP-binding protein 8 (*Gtpbp8*) and Scm-like with four mbt domains (*Sfmbt1*), all genes that encode for proteins involved in tyrosine kinase signaling and transcriptional repression were decreased in XX<sup>Y\*</sup> males. Two genes with putative roles in melanin synthesis, *Trpm1*, transient receptor potential cation channel m1, and *Tyrp1*, tyrosinase-related protein 1 are also decreased in XX<sup>Y\*</sup> males as compared to XX females.

Four Y-chromosome genes represented by 7 probe sets were higher in both XY\* and XX<sup>Y\*</sup> males when compared to their female counterparts. *Kdm5d*, *Eif2s3y*, *Ddx3y*, and *Uty*, all have paralogs on the X chromosome that escape X-inactivation. One additional Y gene, RNA binding motif protein, Y chromosome, member A1, (*Rbmy1a1*) was elevated in XX<sup>Y\*</sup> vs. XX.

At embryonic day 18.5, following gonadal differentiation, 24 transcripts were differentially expressed between males and females with one X-chromosome (Figure 1). Two transcripts encode receptors: *Ht5b*, serotonin receptor 5b, and *Rorb*, a nuclear receptor with no currently defined function, but associated with neurological disease. One protein kinase A subunit, *Prkar1b*, was increased in XY\*<sup>X</sup> females as compared to XY\* males. As in the E11.5 comparisons, four Y-linked transcripts, *Kdm5d*, *Eif2s3y*, *Ddx3y* and *Uty*, were expressed in both XY\* and XX<sup>Y\*</sup> males, but not in females.

When XX<sup>Y\*</sup> males and XX females were compared, 45 transcripts were differentially expressed (Table 2). One of these genes, *Cux2*, cut-like homeobox 2, was higher in XX females when compared to XX<sup>Y\*</sup> males and recently this same sex difference was noted in female mouse liver (Conforto and Waxman 2012). In our study, *Cux2* was also increased in XX females when compared to XX<sup>Y\*</sup> males and XY\*<sup>X</sup> females. This gene is involved in dendritic spine morphology in brain and could be important for sex differences. A few receptors important for neuronal signaling were influenced by sex in this comparison as well. The ionotropic glutamate receptor, AMPA (*Grik2*), GABA A receptor gamma subunit (*Gabrg1*), the serotonin receptor (*Htr1a*), neuropeptide Y receptor 2 (*Npy2r*), and an inwardly rectifying potassium channel (*Kcnj3*) were increased in E18.5 XX<sup>Y\*</sup> as compared to XX brains. Tetracoid repeat domain 39B (*Tct39b*) was higher in XX females compared to XX<sup>Y\*</sup> males and is reported to be sensitive to estrogens, suggesting that estrogens could depress this mRNA in brain. Finally, *Cd99* is an interesting gene candidate; it encodes a surface glycoprotein, and in humans it resides within the pseudoautosomal region (PAR) thus escaping X-inactivation. In mouse, however, it resides on Chromosome 4, and is increased in females compared to males at both ages regardless of sex chromosome complement.

### Gene expression affected by number of X-chromosomes prior to gonad differentiation

At embryonic day 11.5, gonadal tissues have not yet differentiated into testes or ovaries. Presumably any differences in gene expression profiles would be from the contribution of

sex chromosomes. Within each sex, we identified genes affected by X-chromosome number (Figure 1 and Table 3). We identified 22 probe sets with significantly altered transcript levels between XY\*<sup>X</sup> and XX females. About half of the probe sets (9 out of 22) were located on the X-chromosome, and a few of these have been identified as genes that escape X-inactivation (Table 2 and Figure 2). Most of the autosomal genes we discovered are not well characterized. But two of these genes, tyrosinase-related protein (*Tyrp1*) and neural precursor (*Nedd9*) are binding partners. *Nedd9* is a scaffolding protein involved in tyrosine kinase activity and neuronal development (Vogel *et al.* 2010). Other annotated autosomal genes such as Fyn-related kinase (*Frk*), neuronal pentraxin 2 (*Nptx2*) and ORAI calcium release-activated calcium channel (*Orai1*), are kinases or receptors that are involved in neuronal development. All of these genes were elevated in XY\*<sup>X</sup> as compared to XX females. In male XY\* vs. XX<sup>Y\*</sup> mice, expression of 16 transcripts was significantly different. Surprisingly, only two autosomal genes were differentially expressed: PTPRF interacting protein (*Ppfibp1*, a tyrosine kinase involved in axonal guidance) and pleckstrin homology domain containing member 2 (*Plekhh2*) were higher in XY\* than XX<sup>Y\*</sup> males, but little is known about their function.

Four of the 13 mouse genes known to escape X-inactivation, *Xist*, *Tsix*, *Kdm6a*, and *Eif2s3x* (Yang *et al.* 2006; Reinius *et al.* 2010), were higher in tissues from mice with two X-chromosomes (Figure 2). Interestingly, we also observed X-chromosome genes with higher transcript levels in XY\*<sup>X</sup> and XY\* mice as compared with XX and XX<sup>Y\*</sup> mice: *Msl3*, *Hccs*, and *BC022960*. These genes tended to be located on the distal tip of the X-chromosome (Figures 3 and 5). One of these genes, *Msl3*, male-specific lethal-3, in *Drosophila* targets the dosage compensation complex (DCC) to the X-chromosome.

### Differential gene expression in mice with one versus two X-chromosomes following gonadal differentiation

When comparing gene expression profiles of whole brains at E18.5, XY\*<sup>X</sup> and XX females, had 32 unique transcripts that were significantly different. A majority of these genes reside on autosomes (21 transcripts out of 32). Of the differentially expressed X-linked genes, *Xist* and *Tsix*, as expected had much higher expression in tissues with two X-chromosomes (Figure 2). Genes located on the distal tip of the X-chromosome, *Msl3*, *BC022960*, *Arhgap6* and *Hccs* had higher expression in XY\*<sup>X</sup> and XY\* mice versus XX and XX<sup>Y\*</sup> mice (Table 3 and Figure 3). Many of the autosomal genes affected by X-chromosome number, in females, can be divided into three main categories: neurogenesis and neuronal differentiation, G-protein mediated cell signaling, and genes associated with mood disorders. Four genes were consistently increased in XY\*<sup>X</sup> females including a kinase, double-cortin like kinase (*Dclk1*) and a calmodulin regulator cAMP regulated phosphoprotein (*Arpp21*). Both play a role in neuronal migration and neurogenesis. The ionotropic AMPA glutamate receptor (*Grik2*) and neuronal leucine rich repeat transmembrane 2 (*Lrrtm2*) which regulates the surface expression of AMPA, were also higher in XY\*<sup>X</sup> females. Four genes may play a role in schizophrenia and bipolar disorders; serotonin receptor 3 (*Htr3a*), transcription factor cut-like 2 (*Cux2*), a phosphodiesterase (*Pde10a*), and a nuclear hormone receptor (*Rorb*). A final category of genes significantly affected by X-chromosome number in females included genes involved in G protein coupled signaling such as the catalytic subunit of PI3 kinase (*Pik3c*), membrane associated guanylate kinase (*Magi1*), an uncharacterized G-protein (*Gpr151*), and regulator of G-protein signaling binding protein (*Rgs7bp*).

In the male XY\* vs. XX<sup>Y\*</sup> comparison at E18.5, 9 genes were differentially expressed, similar to the E11.5 comparison where 16 genes were changed prior to the onset of gonad development. Of these 9 transcripts, five genes reside on the X-chromosome (*Xist*, *Msl3*, *Tsix*, *B022960*, and *Hccs*). These same genes were affected by X-chromosome number in females (XX and XY\*<sup>X</sup>). In addition, two genes that reside on the Y chromosome, ubiquitin

activating enzyme E1 (*Ube1y1*) and an uncharacterized cDNA clone, *4933439C20Rik*, were different in the male comparison. One autosomal gene, DEAD box polypeptide 4 (*Ddx4*) that in the gonad plays a role in germ cell development and sperm motility was higher in XY\* versus XX<sup>Y\*</sup> males. However, *Ube1y1* is increased 3-fold in XX<sup>Y\*</sup> males while *Ddx4* is decreased 1.5-fold. Further studies examining the actions of these genes in the brain are needed to understand these findings.

### Overview of gene expression differences between ages and genotypes

As shown in Figure 1, on the order of half the genes in the genome (i.e., ~15,000 probe sets) were differentially expressed on the development scale between E11.5 to E18.5 for each genotype. We observed about twice the number of differentially expressed genes between females (54 total) with the same number of X-chromosomes as compared to males (22 total). Because development had a larger effect on gene expression profiles than genotype, a principle component plot using all probe sets only revealed differences between the ages (data not shown). Therefore, we generated a principle component plot using the expression profiles of only X- and Y-chromosome genes (Supplemental Figure 1). As expected, the individual biological replicates grouped together. Of note, XX females and XY\* males, cluster together while XY\*<sup>X</sup> females and XX<sup>Y\*</sup> males are the furthest apart. Perhaps this is due to gene expression programs evolving to reduce the differences between males and females on the sex chromosomes. Finally, we observed higher variability in the E11.5 tissue as compared to the E18.5 brains. This may be due to differences in the source of the tissue, which could have introduced greater variability in the expression data from the E11.5 embryos.

### Confirmation of selected genes by RT qPCR

Microarray data were confirmed by quantitative RT PCR for several genes at both embryonic ages (Figures 2 and 3). In the microarray analysis, *Xist* mRNA expression was assessed by three different probe sets. Fold change differences between mice with one or two X-chromosomes ranged from 67 to more than 500 depending on the probe set, age and comparison (Table 3 and Supplemental Table 2). Using quantitative PCR on cDNA, we observed a similar range of 59 to 158-fold increase in *Xist* transcript in mice with two X-chromosomes at both developmental ages. Although, *Eif2s3x* and *Kdm5c* have been reported to escape X-inactivation, only *Eif2s3x* was significant for each comparison in the microarray analysis. Using qPCR, however, *Eif2s3x* and *Kdm5c* were significantly increased in 2X mice compared to 1X for both sexes and ages (i.e., *Eif2s3x* fold increase between 1.4 and 1.7, and *Kdm5c* fold increase between 1.3 and 2.1 in mice with two X-chromosomes at both ages). *Kdm6a* was only significantly increased in the XX and XX<sup>Y\*</sup> E11.5 embryos in the microarray dataset, but using qPCR it was significantly elevated at both ages. Genes located on the distal tip of the X-chromosome, *Msl3*, *Hccs* and *BC022960*, were increased on average about two-fold in embryos with one X-chromosome. These genes are increased two-fold in the confirmatory qPCR as well.

### Expression and copy number analysis of genes on the distal tip of the X-chromosome

In *Drosophila*, Msl-3 targets the DCC to the X-chromosome and is responsible for dosage compensation (Lucchesi 1998). Because the DCC deposits marks that lead to increased transcription of the X-chromosome in male *Drosophila*, we measured expression in adult brain for *Msl3* and the other members of the complex to determine if the whole complex was elevated in XY\*<sup>X</sup> and XY\* mouse brain. The DCC is comprised of 5 proteins, Msl1, Msl2, Msl3, Mof and Mle; but mRNA of these transcripts was not significantly altered by sex or genotype (Figure 4).

*Msl3* is present on the distal tip of the X-chromosome and lies very close to the region where X- and Y-chromosomes have a translocation event in the Y\* mouse model (Eicher *et al.* 1991; Isles *et al.* 2004). Two nearby X-genes, holocytochrome c synthase (*Hccs*) and *BC022960*, fit a similar profile for mRNA expression both in the microarray data and in confirming qPCR (Supplemental Table 2). In the Y\* model, *Hccs* and *BC022960* also lie within the small X-chromosome section on the Y\* chromosome (Isles *et al.* 2004). *BC022960* has previously been identified to escape X-inactivation. However, *BC022960* shows expression levels two-fold higher in mice with only one vs. two X-chromosomes, obviously not fitting an X-inactivation escapee profile in the Y\* model. *Hccs* has not previously been shown to be an X-inactivation escapee, and its relative transcript level changes are similar to that of *BC022960* and *Msl3*.

Using genomic DNA from adult cerebellar tissue, we asked if copy number for *Msl3*, *Hccs*, and *BC022960* was different in mice possessing one or two X-chromosomes. In normal C57BL/6 embryonic male and female brain tissue, which does not contain the unique Y\* chromosome, we expected all three genes would be present in one copy in males and two copies in females. Indeed, the relative copy numbers of *Msl3*, *Hccs*, and *BC022960* were twice as high in female C57BL/6 brains as compared to males (Supplemental Figure 3), as expected for genes residing on the X-chromosome.

The relative copy number of *Msl3* was not different between any of the Y\* genotypes ( $p > 0.05$ ), indicating two copies of *Msl3* in mice with one complete X-chromosome (XX), and mice with one complete X-chromosome and an additional few X-genes attached to a portion of the PAR (XY\*X). In the Y\* mice, mRNA expression and copy number data strongly suggest that all three genes, *Msl3*, *Hccs* and *BC022960* are present in equivalent amounts. One copy of each gene appears to be inactivated when two X-chromosomes are present (XX and XX<sup>Y\*</sup>). In the case of Y\* mice with only one X-chromosome (XY\* and XY\*X), these genes are present in two copies, one on the X-chromosome and one on the Y\* chromosome (see Figures 3 and 4). The copy on the Y\* chromosome is not subjected to X-inactivation, leading to higher mRNA expression in mice with one X-chromosome.

Transcript levels of *Mid1*, which is located distal to *Hccs*, warrant further investigation since the levels are almost 50% higher in XY\*x, XY\* and XX<sup>Y\*</sup> brains compared to XX at E18.5, but are the same across all four genotypes at E11.5. *Mid1* sits very close to the pseudo-autosomal region (PAR). At the regions interrogated by our probe sets, portions of *Mid1* may not be present on the small X-chromosome section of the Y\* chromosome, giving the observed expression levels.

## Discussion

Sex chromosome complement modulates a variety of behaviors (Gatewood *et al.* 2006; Quinn *et al.* 2007; Gioiosa *et al.* 2008; Grgurevic *et al.* 2008; Park *et al.* 2008; Liu *et al.* 2010; Cox and Rissman 2011; Bonthuis *et al.* 2012; Grgurevic *et al.* 2012). Using the Y\* mouse model, we identified genes that are sexually dimorphic and/or differed by X-chromosome number prior to and just after gonadal differentiation. Few autosomal genes were changed by X-chromosome dosage in males while 10 genes were altered in females. Following gonadal differentiation, many autosomal genes changed in females only. Three of these, *Rorb*, *Cux2* and *Htr3a* have been associated with mood disorders (Niesler *et al.* 2001; Glaser *et al.* 2005; Mansour *et al.* 2009; McGrath *et al.* 2009; Gatt *et al.* 2010), while the remaining genes primarily function in G-protein signaling and neuronal development. These targets may contribute to the behavioral phenotypes previously described.



About 15% of human genes escape X-inactivation (Carrel and Willard 2005) and it is believed that this contributes to sexual dimorphism within the brain and behavior. In mice, far fewer genes escape X-inactivation. The largest lists catalogue 13 potential escapees (Lopes *et al.* 2010; Yang *et al.* 2010) and most studies consistently validate five (Reinius *et al.* 2010; Werler *et al.* 2011). Many X-escapees have a paralog on the Y-chromosome (*Kdm5c-Kdm5d*, *Eif2s3x-Eif2s3y*, and *Kdm6a/Utx-Uty*) that in theory could compensate for the inactivated copy (Xu and Disteche 2006). In Y\* mice, regardless of age or X-chromosome complement, four Y-linked paralogs (*Kdm5d*, *Eif2s3y*, *Uty* and *Ddx3y*) were increased in males compared to females but were not differentially expressed in males at either age. Other X-inactivation escapees, such as *Shroom4* and *Car5b*, do not have expressed Y-paralogs and expression differences between males and females may contribute to sexual dimorphism.

An earlier study examining sexually dimorphic gene expression (Dewing *et al.* 2003) reported 51 altered genes in CD-1 mouse heads prior to gonad differentiation. Of these only three X-linked genes, *Xist*, *Eif2s3x*, and *Kdm6a* were in our study and were elevated in mice with two X-chromosomes. Two Y-chromosome genes, *Eif2s3y* and *Dby*, were increased in CD-1 males; we replicated only *Eif2s3y*. None of the autosomal genes reported in Dewing *et al.* were replicated in our dataset. Experimental differences such as mouse strain, embryo ages and an older microarray design, could account for these discrepancies. More importantly, both studies confirm the potential for sex differences in neural development prior to gonadal differentiation.

Before gonadal differentiation, six transcripts known to escape X-inactivation were detected at higher levels in mice with two X-chromosomes; *Xist*, *Kdm5c*, *Kdm6a*, *Eif2s3x*, *Tsix* and one uncharacterized transcript, *5530601H04Rik*. Following gonadal differentiation, only *Xist* and *Tsix* remained significantly different. If organizational gonadal hormones promote multiple changes in gene transcripts, this could result in region-specific changes in gene expression, reducing our power to detect genome-wide differences. Another limitation of our design is that we used whole head or brain and thus, we can only detect the most robust differences in gene expression (Reinius *et al.* 2010). Moreover, different regions in the developing brain may express different X-inactivation escaping genes (Xu *et al.* 2006) and/or steroid receptors that together create differential gene expression profiles in discrete brain regions. Additionally, E11.5 heads included various non-neural tissues whereas the E18.5 samples were brain only. Finally, the use of 2–4 samples per group may have limited our power to detect differentially expressed genes. Given these limitations, it is interesting that specific X-inactivation escaping genes are temporally segregated.

Three X-chromosome genes in our data set produce proteins that act on chromatin to regulate gene transcription. Two histone demethylases, *Kdm5c* and *Kdm6a*, escape X-inactivation and have Y-paralogs. Previous studies have suggested, as we do here, that Y-paralogs may not compensate for lower levels of transcript in 1X individuals (Xu *et al.* 2008; Xu *et al.* 2008). *Ms13* acts within the DCC to upregulate transcription of the X-chromosome in male *Drosophila* (Lucchesi 1998). Paradoxically, few autosomal genes/potential targets were changed in any genotype. Given that these genes regulate transcription, are differentially expressed in 1X vs. 2X mice and are not compensated by Y-paralogs, we suggest that the complex in which each functions does not form (e.g., no DCC in mice) or that other mechanisms act in a compensatory manner.

In fact, X-chromosome dosage significantly altered expression of few genes in the Y\* model. Less than 80 total transcripts were differentially expressed between mice with one or two X-chromosomes. Similarly, a study investigating sex differences in a cross between two inbred mouse strains (C57BL/6 and C3H) found that differences between sexually

dimorphic genes were modest in brain (Yang *et al.* 2006). In the Four Core Genotype, a transgene for *Sry* has been inserted to rescue the spontaneous deletion of this Y-chromosome gene, and may modulate autosomal gene expression (Wijchers *et al.* 2010). In the Y\* mouse, *Sry* is expressed from the endogenous gene in both male genotypes. Only three autosomal genes differed between XY\* and XX<sup>Y\*</sup> males, raising the possibility that the presence of *Sry* affects expression of autosomal genes which are responding to one versus two copies of the X-chromosome. Alternatively, the lack of large perturbations in gene profiles could indicate that addition or deletion of sex chromosomes does not globally disrupt gene regulation. Importantly, gonadal hormones are also a likely factor since more autosomal genes were affected in E11.5 than E18.5 brains.

Expression of 3 genes, *Msl3*, *Hccs* and *BC022960* was 2-fold higher in mice with one X-chromosome regardless of age. All three are located on the distal tip of the X-chromosome, near PAR and may be part of the X-chromosome region crossing over with the Y\* chromosome. *Hccs* and *BC022960* reside on the small Y\*<sup>X</sup> and Y\* chromosomes (Isles *et al.* 2004) which are not subject to X-inactivation (Davies *et al.* 2007). *Msl3* was not previously reported to be on the Y\*<sup>X</sup> chromosome (Burgoyne *et al.* 1998). Genomic DNA analysis confirmed that there are two copies of *Msl3* in each genotype. One copy is silenced on the inactivated X-chromosome, which explains the 2-fold higher expression in mice with only one X-chromosome. Our data suggest that *Msl3* resides on the Y\*<sup>X</sup> chromosome, since it is present in two copies and there is no X-inactivated chromosome in 1X mice. Thus, higher expression of *Msl3*, *Hccs*, and *BC022960* in the XY\* and XY\*<sup>X</sup> is likely unique to the Y\* model due to the contribution of the few near-PAR X-chromosome genes involved in the translocation.

*Msl3* is of particular interest because it is known to participate in the DCC, a complex responsible for dosage compensation in *Drosophila*. Male flies never have more than one X-chromosome. To compensate, X-genes are increased two-fold by the DCC (Akhtar and Becker 2000; Smith *et al.* 2000). The DCC is comprised of 5 proteins: Msl1, Msl2, Msl3, Mof and Mle (Lucchesi 1998). Notably, mice lack roX1 and roX2—two noncoding RNAs that target the complex to chromatin (Aleksyenko *et al.* 2008). None of the other members of the mammalian complex (*Msl1*, *Msl2*, *Mof* and *Mle*) were significantly affected by sex or genotype. Thus, we do not expect increased *Msl3* levels to have an effect on X-chromosome expression in 1X mice, consistent with our analysis.

Behavioral differences between animals with one versus two X-chromosomes have been found in Y\* mice. An extra X-chromosome in adult males tends to increase aggression and in mating tests XX<sup>Y\*</sup> mice are significantly faster to display most aspects of male sexual behavior (Bonthuis *et al.* 2012). In a novel object recognition task, XX<sup>Y\*</sup> mice fail to discriminate (Lewejohann *et al.* 2009). We observed few genes to be differentially regulated between XY\* and XX<sup>Y\*</sup> males at either age of development (i.e., only X-escapees and near-PAR X-chromosome genes). Several limitations of our study may account for this lack of robust differences. For example, whole head or whole brain was used for gene expression analysis that would preclude detection of difference in specific brain regions. Also, the ages used to measure gene expression may not be pertinent to these adult behaviors.

Female mice with only one X-chromosome are more anxious than normal females with two X-chromosomes (Isles *et al.* 2004). Our gene profiling identified a few autosomal genes (*Nptx2*, *Nedd9*, *Rorb*, *Cux2* and *Htr3a*; all genes involved in neuronal development or associated with neurological disease) that were differentially expressed between XY\*<sup>X</sup> and XX female embryos; these might be, at least in part, responsible for the differences in adult behavior. Prior to gonadal differentiation, genes altered by sex chromosome number in females are involved in neuronal differentiation and migration. It is reasonable to presume

that these genes play a role in brain organization. Following the onset of gonadal differentiation, three main classes of genes are altered in females: genes involved in G-protein coupled signaling, neurogenesis and mood disorders. All of these observed changes occur prior to activational hormones at puberty. Together, these findings add to the theory that sex chromosomes contribute to brain organization and could ultimately lead to changes in social, cognitive and sexual behavior.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

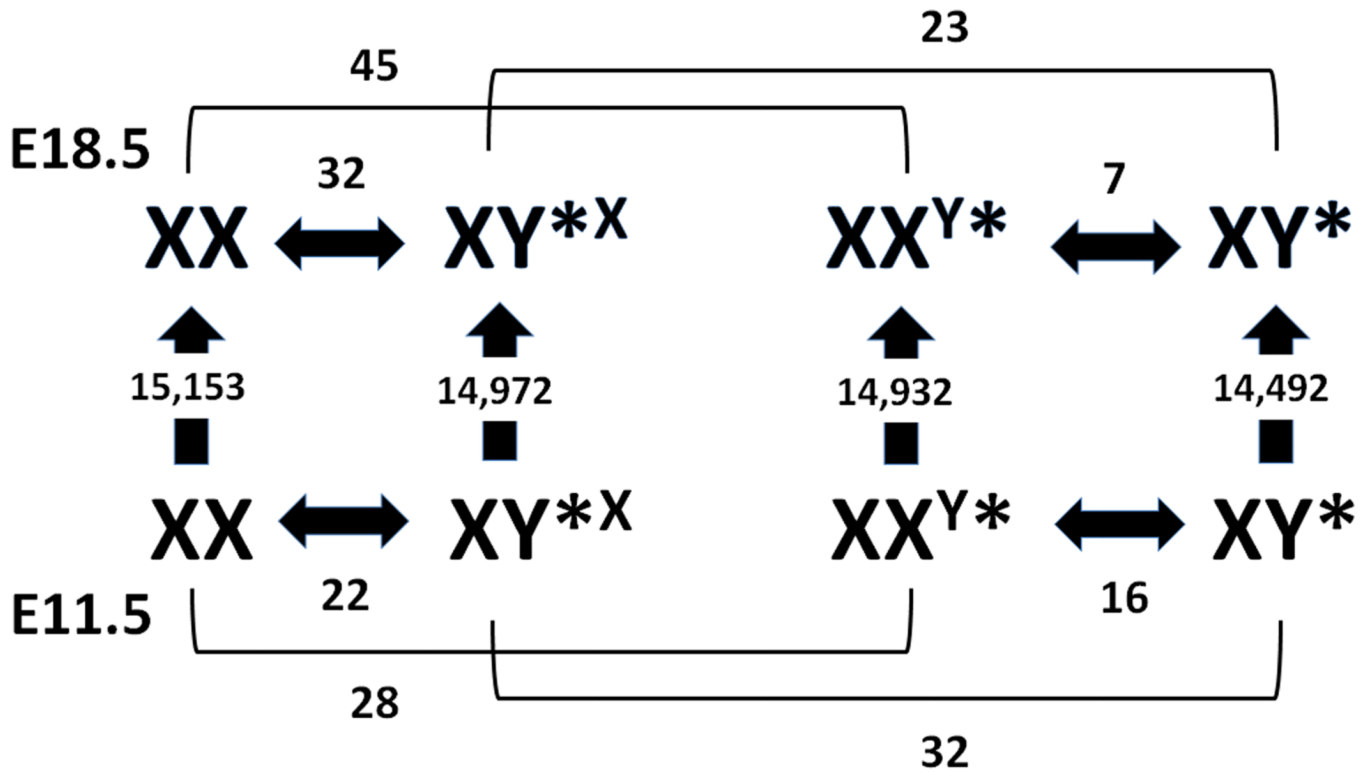
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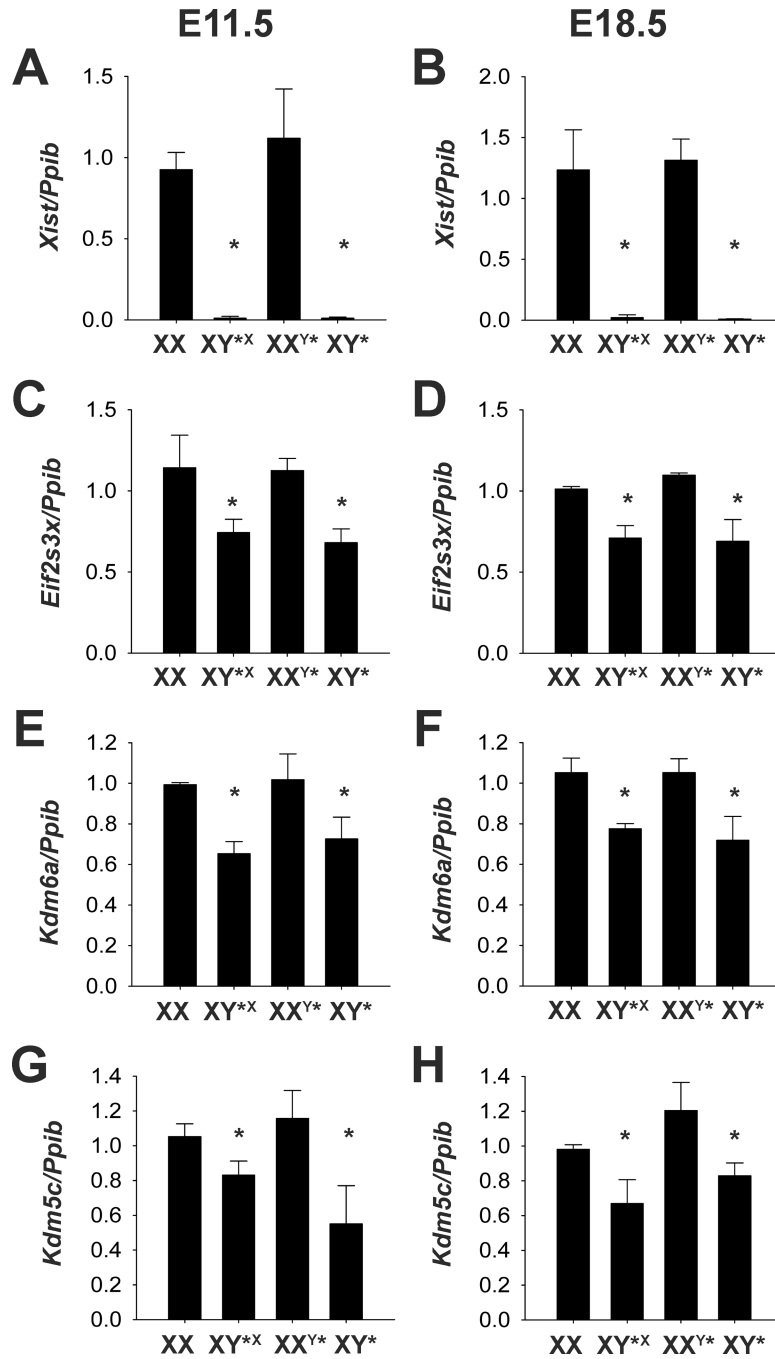
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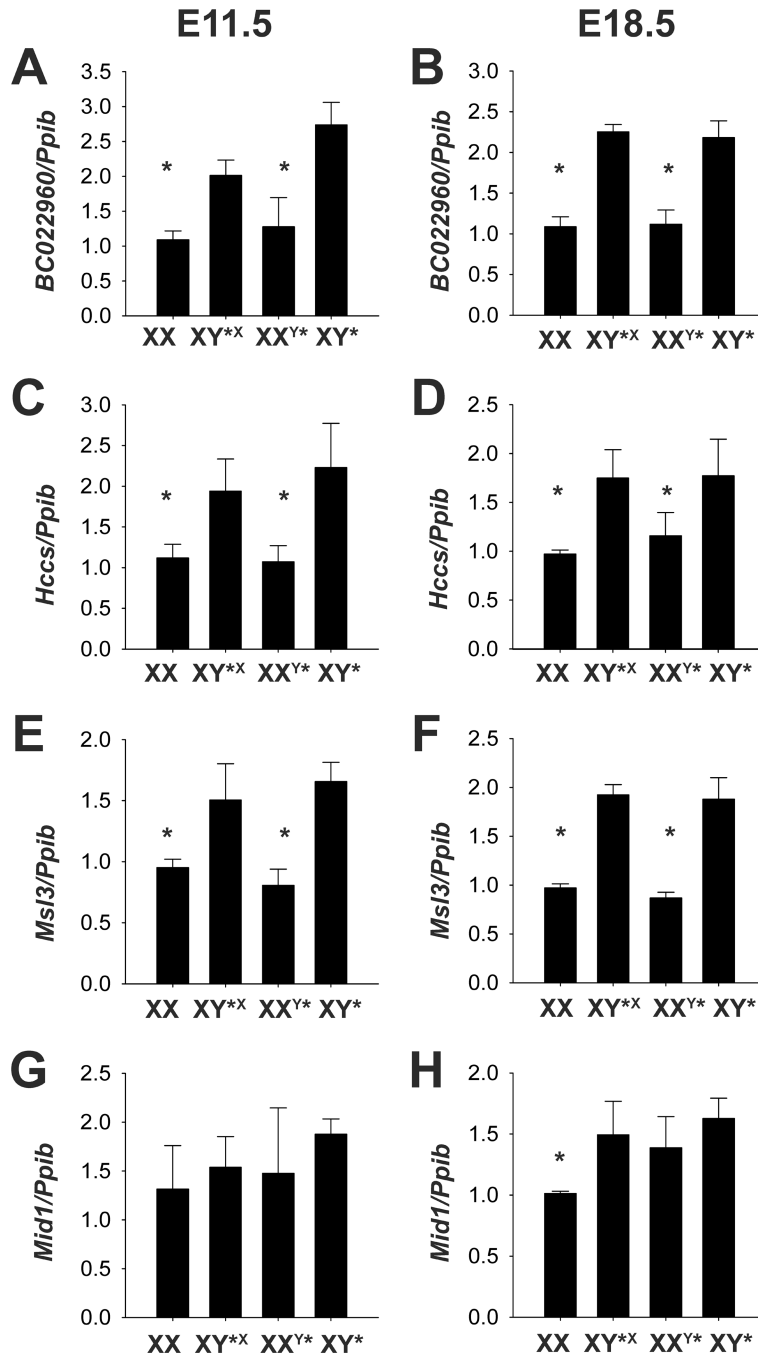
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**Figure 1. Numbers of genes significantly altered by age, sex or genotype in the Y\* mouse brain**  
 Numbers and associated arrows shown between age, sex or genotype indicate the number of differentially expressed genes called for a given comparison. The majority of gene expression changes occurred between embryonic day (E) 11.5 and 18.5 within each genotype. Far fewer genes were significantly altered between genotype or sex. XX = normal female, XY\*<sup>X</sup> = 1X female, XX<sup>Y\*</sup> = 2X male, XY\* = normal male.



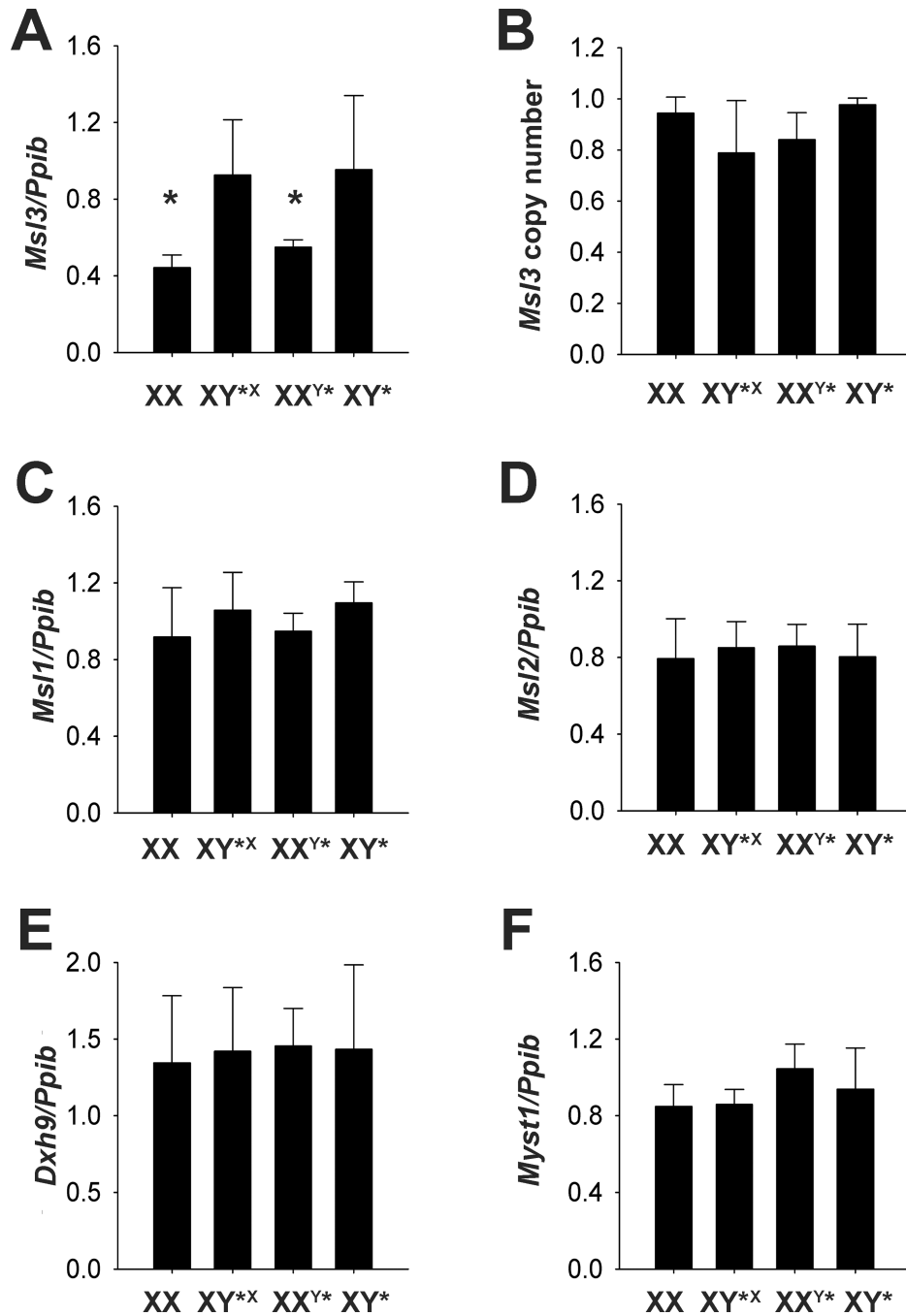
**Figure 2. Genes escaping X-inactivation at E11.5 and E18.5**  
 Quantitative PCR of relative gene expression for X-inactive specific transcript (*Xist*) at **A.** E11.5 and **B.** E18.5, eukaryotic translation initiation factor 2 subunit 3 X-linked (*Eif2s3x*) at **C.** E11.5 and **D.** E18.5, lysine(K)-specific demethylase 6a (*Kdm6a*) at **E.** E11.5 and **F.** E18.5 and lysine(K)-specific demethylase 6a (*Kdm5c*) at **G.** E11.5 and **H.** E18.5. Expression is relative to peptidylprolyl isomerase B (*Ppib*) and normalized to XX females. \*  $p < 0.05$  main effect of X-chromosome number.



**Figure 3. Genes located on distal tip of X-chromosome with expression higher in 1X mice at E11.5 and E18.5**

Quantitative PCR of relative gene expression for *BC022960*, an uncharacterized transcript at **A.** E11.5 and **B.** E18.5, holocytochrome c synthase (*Hccs*) at **C.** E11.5 and **D.** E18.5, male specific lethal 3 (*Msl3*) at **E.** E11.5 and **F.** E18.5, and midline-1 (*Mid1*) at **G.** E11.5 and **H.** E18.5. Expression is relative to peptidylprolyl isomerase B (*Ppib*) and normalized to XX females. \*  $p < 0.05$  main effect of X-chromosome number.





**Figure 4. Gene expression levels of the DCC complex in adult cerebellum**  
 Quantitative PCR of relative gene expression for **A.** male specific lethal 3, (*Msl3*), **B.** Relative copy number of genomic DNA for *Msl3*, **C.** male specific lethal 1 (*Msl1*), **D.** male specific lethal 2 (*Msl2*). **E.** dead box polypeptide 9 (*Dxh9*), **F.** Myst histone acetyltransferase 1 (*Myst1*). Expression is relative to peptidylprolyl isomerase B (*Ppib*) and normalized to XX females. \* p<0.05 main effect of X-chromosome number.



**Figure 5. Schematic of the mouse X-chromosome with an overlay of genes reported to escape X-inactivation**

Transcripts significantly altered in 1X vs. 2X mice at embryonic day 11.5 and 18.5 as described. Up arrows indicate gene expression levels are higher in 2X mice versus 1X mice and escape X-inactivation. Down arrows indicate decreased gene expression in 2X vs. 1X mice. On the right of the schematic, we show the name and location of genes previously reported to escape X-inactivation (Reinius *et al.* 2010; Yang *et al.* 2010).

**Table 1**

Genotype and sex chromosome complement in Y\* mice

Genotype	Gonads	Sry	Copies of Y	Copies of X
XX	ovary	0	0	2
XY*X	ovary	0	0	1
XX <sup>Y*</sup>	testes	1	1	2
XY*	testes	1	1	1

**Table 2**

Genes differentially expressed between males and females with the same number of X-chromosomes prior to (E11.5) and following (E18.5) the onset of gonadal hormones. P-values are corrected for multiple tests and represent the False Discovery Rate (FDR). Fold changes above and below 1 are represented differently. Those above 1 are the expression value of the first genotype divided by the second in the comparison. Those below 1 are negative the expression value of the second genotype divided by the first in the comparison.

Probe	Symbol	Gene Name	Chromosome	p-value	Fold Change
<b>E11.5 XY* vs. E11.5 XY*X</b>					
1424903_at	Jarid1d	jumonji, AT rich interactive domain ID (Rbp2 like)	Y	0	12.7
1417210_at	Eif2s3y	eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	Y	0	14.4
1426438_at	Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	Y	0	16.4
1457945_at				0	11.6
1452077_at	Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	Y	0	9.6
1426598_at	Uty	ubiquitously transcribed tetrapeptide repeat gene, Y-chromosome	Y	0	8.0
1426439_at	Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	Y	0	9.4
1442153_at				0	7.5
1457582_at	Uty	ubiquitously transcribed tetrapeptide repeat gene, Y-chromosome	Y	0	7.8
1421362_a_at	Frk	fyn-related kinase	10	0	-1.7
1438139_at	Arhgap28	Rho GTPase activating protein 28	17	0.002	-1.5
1446289_at				0.002	-0.5
1436320_at				0.003	-1.9
1430514_a_at	Cd99	CD99 antigen	4	0.007	-3.2
1428850_x_at	Cd99	CD99 antigen	4	0.008	-2.9
1450767_at	Nedd9	neural precursor cell expressed, developmentally down-regulated gene 9	13	0.009	-1.7
1426519_at	P4hal	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-	10	0.011	-1.5

Probe	Symbol	Gene Name	Chromosome	p-value	Fold Change
1422802_at	Defcr3	hydroxylase), alpha 1 polypeptide			
1449446_at	Ccdc59	defensin related cryptdin 3	8	0.012	-3.8
1459775_at		coiled-coil domain containing 59	10	0.012	1.5
1448057_at				0.012	1.6
1441715_at				0.014	-1.7
1444479_at				0.018	1.8
1437591_a_at	Wdr1	WD repeat domain 1	5	0.028	-1.9
1424484_at	Mobk11b	MOB1, Mps One Binder kinase activator-like 1B (yeast)	6	0.096	-2.3
1453556_x_at	Cd99	CD99 antigen	4	0.128	-2.2
1434785_at	Cacng5	calcium channel, voltage-dependent, gamma subunit 5	11	0.147	-1.8
1442398_at	LOC100047829	similar to ubiquitin-conjugating enzyme E2T (putative)	1	0.159	1.9
1438133_a_at	Cyr61	cysteine rich protein 61	3	0.167	-1.6
1439419_at	IRik	RIKEN cDNA 2310057M2	7	0.169	1.6
1449222_at	Ebi3	Epstein-Barr virus induced gene 3	17	0.183	2.1
1416213_x_at	Surf4	surf4 gene 4	2	0.194	-1.5
<b>E11.5 XX<sup>Y</sup>* vs. E11.5 XX</b>					
1424903_at	Jarid1d	jumonji, AT rich interactive domain ID (Rbp2 like)	Y	0	87.4
1417210_at	Eif2s3y	eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	Y	0	104.7
1457945_at				0	49.9
1426438_at	Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	Y	0	136.2
1452077_at	Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	Y	0	30.3
1426598_at	Uty	ubiquitously transcribed tetrapeptide repeat gene, Y-chromosome	Y	0	16.0
1422894_at	Sfmbt1	Scm-like with four mbt domains 1	14	0	-1.5

Probe	Symbol	Gene Name	Chromosome	p-value	Fold Change
1426439_at	Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	Y	0	17.6
1457582_at	Uty	ubiquitously transcribed tetratricopeptide repeat gene, Y-chromosome	Y	0	11.5
1442153_at				0	9.2
1419952_at	Stx5a	syntaxin 5A	19	0	-1.5
1422802_at	Defcr3	defensin related cryptdin 3	8	0.004	-4.7
1436320_at				0.005	-1.9
1448057_at				0.008	-1.8
1415861_at	Tytp1	tyrosinase-related protein 1	4	0.022	-1.6
1420720_at	Nptx2	neuronal pentraxin 2	5	0.022	2.8
1438227_at	She	src homology 2 domain-containing transforming protein E	3	0.029	-1.6
1445419_at	Gtpbp8	GTP-binding protein 8 (putative)	16	0.046	-1.9
1421117_at	Dst	dystonin	1	0.058	1.7
	C030015D1				
1459290_at	9Rik	RIKEN cDNA C030015D19 gene	2	0.071	1.6
1428850_x_at	Cd99	CD99 antigen	4	0.071	-2.4
		ubiquitously transcribed tetratricopeptide repeat gene, Y-chromosome		0.078	4.3
1422247_a_at	Uty		Y	0.085	-1.7
1458880_at					
1456020_at	Sh3tc2	SH3 domain and tetratricopeptide repeats 2	18	0.086	-1.6
1420786_a_at	Rbm1a1	RNA binding motif protein, Y-chromosome, family 1, member A1	Y	0.087	3.1
1453556_x_at	Cd99	CD99 antigen	4	0.099	-2.3
1437531_at	Trpm1	transient receptor potential cation channel, subfamily M, member 1	7	0.129	-2.0
1454447_at	5830434F19 Rik	RIKEN cDNA 5830434F19 gene	2	0.164	-1.6
<b>E18.5 XY* vs. E18.5 XY*X</b>					
1424903_at	Jarid1d	jumonji, AT rich interactive domain 1D (Rbp2 like)	Y	0	52.3
1417210_at	Eif2s3y	eukaryotic translation initiation	Y	0	226.0

Probe	Symbol	Gene Name	Chromosome	p-value	Fold Change
		factor 2, subunit 3, structural gene Y-linked			
1426438_at	Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	Y	0	296.1
1452077_at	Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	Y	0	32.4
1457945_at				0	22.2
1426439_at	Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	Y	0	47.8
1426598_at	Uty	ubiquitously transcribed tetratricopeptide repeat gene, Y-chromosome	Y	0	8.6
1425162_at	Rorb	RAR-related orphan receptor beta	19	0.001	-1.7
1442153_at				0.001	6.7
1422196_at	Htr5b	5-hydroxytryptamine (serotonin) receptor 5B	1	0.001	1.7
1436320_at				0.001	-2.1
1457582_at	Uty	ubiquitously transcribed tetratricopeptide repeat gene, Y-chromosome	Y	0.001	6.7
1431642_at	Eif2s3y	eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	Y	0.001	6.1
1448057_at				0.005	-1.8
1453556_x_at	Cd99	CD99 antigen	4	0.006	-2.9
1416754_at	Prkar1b	protein kinase, cAMP dependent regulatory, type I beta	5	0.011	-1.6
1430514_a_at	Cd99	CD99 antigen	4	0.017	-3.0
1437644_at	B3gal12	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	1	0.033	-1.9
1429730_at	1810009N0 2Rik	RIKEN cDNA 1810009N02 gene	12	0.047	-2.1
1441949_x_at	Slc39a6	solute carrier family 39 (metal ion transporter), member 6	18	0.098	-1.5
1428850_x_at	Cd99	CD99 antigen	4	0.116	-2.3
1453429_at	EG665787	predicted gene, EG665787	8	0.118	1.7

Probe	Symbol	Gene Name	Chromosome	p-value	Fold Change
1440716_at	6430604M1 IRik	RIKEN cDNA 6430604M11 gene	6	0.171	1.8
<b>E18.5 XX<sup>Y</sup>* vs. E18.5 XX</b>					
1424903_at	Jarid1d	jumonji, AT rich interactive domain 1D (Rbp2 like)	Y	0	51.3
1417210_at	Eif2s3y	eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	Y	0	196.7
1426438_at	Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	Y	0	195.4
1452077_at	Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	Y	0	27.9
1457945_at				0	21.7
1426439_at	Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	Y	0	33.1
1450285_at	Ube1y1	ubiquitin-activating enzyme E1, Chr Y 1	Y	0	4.9
1426598_at	Uty	ubiquitously transcribed tetraatricopeptide repeat gene, Y-chromosome	Y	0	7.8
1442153_at				0	8.2
1436320_at				0	-2.3
1440192_at	Ttc39b	tetraatricopeptide repeat domain 39B	4	0.001	-1.5
1448057_at				0.003	-1.9
1457582_at	Uty	ubiquitously transcribed tetraatricopeptide repeat gene, Y-chromosome	Y	0.004	5.7
1431642_at	Eif2s3y	eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	Y	0.004	5.2
1443545_at				0.004	-1.9
1450181_at	Cux2	cut-like homeobox 2	5	0.009	-1.6
1430206_at	4933407L21 Rik	RIKEN cDNA 4933407L21 gene	1	0.014	-1.9
1453556_x_at	Cd99	CD99 antigen	4	0.029	-2.6
1428575_at	Fcho1	FCH domain only 1	8	0.032	-1.9



Probe	Symbol	Gene Name	Chromosome	p-value	Fold Change
1457555_at	Gpr151	G protein-coupled receptor 151	18	0.034	-1.7
1440054_at	Pik3ca	phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	3	0.043	-1.7
1460030_at	Hecw1	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1	13	0.074	1.7
1428850_x_at	Cd99	CD99 antigen	4	0.075	-2.4
1444025_at	Kenj3	potassium inwardly-rectifying channel, subfamily J, member 3	2	0.078	2.2
1425790_a_at	Grik2	glutamate receptor, ionotropic, kainate 2 (beta 2)	10	0.085	1.9
1438685_at	Znym6	zinc finger, MYM-type 6	4	0.096	1.6
1417489_at	Npy2r	neuropeptide Y receptor Y2	3	0.114	2.1
1438710_at	Htr1a	5-hydroxytryptamine (serotonin) receptor 1A	13	0.127	1.6
1429249_at	4833424O1 5Rik	RIKEN cDNA 4833424O15 gene	3	0.134	1.7
1429730_at	1810009N0 2Rik	RIKEN cDNA 1810009N02 gene	12	0.134	-1.9
1421519_a_at	Zfp120	zinc finger protein 120	2	0.134	1.6
1457523_at	9530056K1 5Rik	RIKEN cDNA 9530056K15 gene	5	0.136	-1.6
1452623_at	Zfp759	zinc finger protein 759	13	0.146	1.7
1449634_a_at	Anks1b	ankyrin repeat and sterile alpha motif domain containing 1B	10	0.146	3.4
1432329_a_at	Matk	megakaryocyte-associated tyrosine kinase	10	0.146	2.2
1432836_at	4833410I11 Rik	RIKEN cDNA 4833410I11 gene		0.152	-1.6
1440457_at				0.152	1.6
1417852_x_at	C1cal	chloride channel calcium activated 1	3	0.154	1.9
1421606_a_at	Sult4a1	sulfotransferase family 4A, member 1	15	0.154	1.7
1427227_at	Gabrg1	gamma-aminobutyric acid (GABA-A) receptor, subunit gamma 1	5	0.164	1.8
1421138_a_at	Ptkib	protein kinase inhibitor beta, cAMP dependent, testis specific	10	0.168	-1.8

Probe	Symbol	Gene Name	Chromosome	p-value	Fold Change
1442956_at				0.182	1.5
	1110008L16				
1428775_at	Rik	RIKEN cDNA 1110008L16 gene	12	0.182	1.7
1458534_at	Rgs7bp	regulator of G-protein signalling 7 binding protein	13	0.183	2.4
1456637_at	Lrrtm2	leucine rich repeat transmembrane neuronal 2	18	0.195	1.5

Genes significantly altered in 1X vs. 2X mice at prior to (E11.5) and following (E18.5) the onset of gonadal hormones. P-values are corrected for multiple tests and represent the False Discovery Rate (FDR). Fold changes above and below 1 are represented differently. Those above 1 are the expression value of the first genotype divided by the second in the comparison. Those below 1 are negative the expression value of the second genotype divided by the first in the comparison.

Table 3

Probe	Symbol	Gene Name	Chromosome	P-value	Fold Change
<b>E11.5 XY*X vs. E11.5 XX</b>					
1427263_at	Xist	inactive X specific transcripts	X	0.00	-67.18
1427262_at	Xist	inactive X specific transcripts	X	0.00	-249.00
1448645_at	Msl3	male-specific lethal 3 homolog (Drosophila)	X	0.00	1.88
1421362_a_at	Frk	fyn-related kinase	10	0.00	1.62
1420087_at	Fzd3	frizzled homolog 3 (Drosophila)	14	0.00	-1.75
1420890_at	Hccs	holocytochrome c synthetase	X	0.01	2.07
1427198_at	BC022960	cDNA sequence BC022960	X	0.01	2.06
1436936_s_at	Tsix	X (inactive)-specific transcript, antisense	X	0.01	-167.73
1415861_at	Typ1	tyrosinase-related protein 1	4	0.02	-1.72
1444479_at				0.05	1.96
1457433_x_at	Zfp120	zinc finger protein 120	2	0.05	-1.51
1427672_a_at	Kdm6a	ubiquitously transcribed tetrapeptide repeat gene, X-chromosome	X	0.05	-1.83
1452750_at	5530601H04 Rik	RIKEN cDNA 5530601H04 gene	X	0.05	-1.87
1459290_at	C030015D19 Rik	RIKEN cDNA C030015D19 gene		0.05	1.69
1420720_at	Npx2	neuronal pentraxin 2	5	0.05	2.66
1450767_at	Nedd9	neural precursor cell expressed, developmentally down-regulated gene 9	13	0.07	1.57
1458880_at				0.10	-1.72
1423744_x_at	Eif2s3x	eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked	X	0.12	-1.55
1430116_at	Cyp4f41-ps	cytochrome P450, family 4,	17	0.13	-1.56

Probe	Symbol	Gene Name	Chromosome	p-value	Fold Change
1442245_at		subfamily f, polypeptide 41 pseudogene		0.13	1.55
1419712_at	Il3ra	interleukin 3 receptor, alpha chain	14	0.18	-1.51
1424990_at	Orai1	ORAI calcium release-activated calcium modulator 1	5	0.19	2.08
<b>E11.5 XY* vs. E11.5 XX<sup>Y</sup>*</b>					
1427263_at	Xist	inactive X specific transcripts	X	0.00	-118.60
1427262_at	Xist	inactive X specific transcripts	X	0.00	-254.23
1448645_at	Msl3	male-specific lethal 3 homolog (Drosophila)	X	0.00	2.01
1427198_at	BC022960	cDNA sequence BC022960	X	0.00	2.39
1420890_at	Hccs	holocytochrome c synthetase	X	0.00	2.25
1436936_s_at	Tsix	X (inactive)-specific transcript, antisense	X	0.00	-149.09
1459775_at				0.01	1.72
1441715_at				0.03	1.79
1443029_at	Ppfbp1	PTPRF interacting protein, binding protein 1 (liprin beta 1)	6	0.04	1.53
1446289_at				0.05	1.60
1427672_a_at	Kdm6a	ubiquitously transcribed tetrapeptide repeat gene, X-chromosome	X	0.06	-1.69
1423744_x_at	Eif2s3x	eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked	X	0.09	-1.51
1435348_at	D930009K15 Rik	RIKEN cDNA D930009K15 gene	X	0.09	-1.51
1452750_at	5530601H04 Rik	RIKEN cDNA 5530601H04 gene	X	0.09	-1.68
1451090_a_at	Eif2s3x	eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked	X	0.11	-1.52
1444817_at	Plekhh2	pleckstrin homology domain containing, family H (with MYTH4 domain) member 2	17	0.12	1.84

Probe	Symbol	Gene Name	Chromosome	p-value	Fold Change
<b>E18.5 XY*X vs. E18.5 XX</b>					
1427263_at	Xist	inactive X specific transcripts	X	0.00	-81.01
1427262_at	Xist	inactive X specific transcripts	X	0.00	-498.00
1427198_at	BC022960	cDNA sequence BC022960	X	0.00	3.46
1436936_s_at	Tsix	X (inactive)-specific transcript, antisense	X	0.00	-1209.34
1448645_at	Msl3	male-specific lethal 3 homolog (Drosophila)	X	0.00	2.04
1420890_at	Hccs	holocytochrome c synthetase	X	0.00	2.53
1425384_a_at	Ube4a	ubiquitination factor E4A, UFD2 homolog (S. cerevisiae)	9	0.00	1.74
1425162_at	Rorb	RAR-related orphan receptor beta	19	0.00	1.74
1430206_at	4933407L21R ik	RIKEN cDNA 4933407L21 gene	1	0.00	-2.41
1450181_at	Cux2	cut-like homeobox 2	5	0.00	-1.82
1443545_at				0.01	-1.84
1423125_at	Dclk1	doublecortin-like kinase 1	3	0.03	2.25
1458499_at	Pde10a	phosphodiesterase 10A	17	0.03	1.60
1457555_at	Gpr151	G protein-coupled receptor 151	18	0.04	-1.78
1443802_at				0.09	-1.58
1428775_at	1110008L16R ik	RIKEN cDNA 1110008L16 gene	12	0.10	1.92
1418268_at	Htr3a	5-hydroxytryptamine (serotonin) receptor 3A	9	0.11	2.20
1458534_at	Rgs7bp	regulator of G-protein signalling 7 binding protein	13	0.13	2.69
1429249_at	4833424O15 Rik	RIKEN cDNA 4833424O15 gene	3	0.13	1.73
1440429_at	Arpp21	cyclic AMP-regulated phosphoprotein, 21	9	0.14	1.99
1457523_at	9530056K15R ik	RIKEN cDNA 9530056K15 gene	5	0.15	-1.64
1440054_at	Pik3ca	phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	3	0.15	-1.64
1449634_a_at	Anks1b	ankyrin repeat and sterile alpha	10	0.17	3.68

Probe	Symbol	Gene Name	Chromosome	p-value	Fold Change
1440457_at		motif domain containing 1B			
1426246_at	Pros1	protein S (alpha)	16	0.17	1.69
1452597_at	2310061C15 Rik	RIKEN cDNA 2310061C15 gene	8	0.17	-1.51
1440871_at	Magi1	membrane associated guanylate kinase, WW and PDZ domain containing 1	6	0.17	1.60
1456637_at	Lrrtm2	leucine rich repeat transmembrane neuronal 2	18	0.18	1.60
1425790_a_at	Grik2	glutamate receptor, ionotropic, kainate 2 (beta 2)	10	0.18	1.85
1432836_at	483341011Ri k	RIKEN cDNA 483341011 gene		0.18	-1.58
1438344_at	4833424015 Rik	RIKEN cDNA 4833424015 gene	3	0.19	1.58
1451867_x_at	Arhgap6	Rho GTPase activating protein 6	X	0.20	1.88
<b>E18.5 XY* vs. E18.5 XX*</b>					
1427263_at	Xist	inactive X specific transcripts	X	0.00	-84.45
1427262_at	Xist	inactive X specific transcripts	X	0.00	-541.19
1448645_at	Msl3	male-specific lethal 3 homolog (Drosophila)	X	0.00	2.11
1436936_s_at	Tsix	X (inactive)-specific transcript, antisense	X	0.00	-935.76
1450285_at	Ubely1	ubiquitin-activating enzyme E1, Chr Y 1	Y	0.00	-3.05
1427198_at	BC022960	cDNA sequence BC022960	X	0.00	2.30
1420890_at	Hccs	holocytochrome c synthetase	X	0.00	2.04
1427242_at	Ddx4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4		0.15	1.54
1429452_x_at	4933439C20 Rik	RIKEN cDNA 4933439C20 gene	13	0.17	-1.83