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A Genetic Basis for a Postmeiotic X Versus Y Chromosome Intragenomic Conflict in the Mouse

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Abstract

Intragenomic conflicts arise when a genetic element favours its own transmission to the detriment of others. Conflicts over sex chromosome transmission are expected to have influenced genome structure, gene regulation, and speciation. In the mouse, the existence of an intragenomic conflict between X- and Y-linked multicopy genes has long been suggested but never demonstrated. The Y-encoded multicopy gene Sly has been shown to have a predominant role in the epigenetic repression of post meiotic sex chromatin (PMSC) and, as such, represses X and Y genes, among which are its X-linked homologs Sxl and Slxl1. Here, we produced mice where TDs are deficient for both Sly and Sxl/Slk1 and observed that Sxl/Slk1 has an opposite role to that of Sly, in that it stimulates XY gene expression in spermatids. Sxl/Slk1 deficiency rescues the sperm differentiation defects and near sterility caused by Sly deficiency and vice versa. Sxl/Slk1 deficiency also causes a sex ratio distortion towards the production of male offspring that is corrected by Sly deficiency. All in all, our data show that Sxl/Slk1 and Sly have antagonistic effects during sperm differentiation and are involved in a postmeiotic intragenomic conflict that causes segregation distortion and male sterility. This is undoubtedly what drove the massive gene amplification on the mouse X and Y chromosomes. It may also be at the basis of cases of F1 male hybrid sterility where the balance between Sxl/Slk1 and Sly copy number, and therefore expression, is disrupted. To the best of our knowledge, our work is the first demonstration of a competition occurring between X and Y related genes in mammals. It also provides a biological basis for the concept that intragenomic conflict is an important evolutionary force which impacts on gene expression, genome structure, and speciation.


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Introduction

Transmission distorters (TDs), also known as segregation distorters or meiotic drivers, are genetic elements that are transmitted to the next generation with a higher frequency than the expected 1:1 Mendelian inheritance ratio. TDs have the tendency to accumulate in low recombination regions where tight linkage allows cooperation between TDs and responder genes to evolve, as seen in the mouse t-complex [1] [for recent reviews see [2,3]]. The non-recombining region of the heteromorphic sex chromosomes is the largest genomic example of recombination suppression [4], with the consequent potential for TDs to arise and distort the population sex ratio. Theory predicts that an unlinked suppressor of the sex ratio distortion (whether autosomal or on the other sex chromosome) would rapidly be selected for to restore the Fisherian 1:1 sex ratio [5]. A subsequent evolutionary arms race between the distorter and its suppressor may follow and lead to repeated bouts of amplification of the genes involved in this intragenomic conflict [6]. In Drosophila, the X- and Y-encoded multicopy genes Stellate and Suppressor of Stellate are believed to illustrate the genomic conflict theory since deletions of Stl/Sxl locus lead to a derepression of Stellate associated with a distorted sex ratio towards an excess of females; but to date it remains unclear whether or not Stellate is a transmission distorter [7,8]. Intragenomic conflicts over sex chromosome transmission are predicted to have influenced genome structure, gene expression and speciation [2,3]. Several cases of sex chromosome transmission distortion have been reported in the literature but they mostly concern Drosophila species [2,9–14] and remain poorly characterized in mammals. Sex ratio segregation distortion may be more frequent than observed as the distortion is often masked by the presence of a suppressor in wild-type (WT) populations [2,9–15].

In the mouse, the existence of an intragenomic conflict between X- and Y-linked genes has long been suggested: males with a partial deletion of the male specific region of the Y long arm (MSYq) produce offspring with a sex ratio skewed towards females [16], suggesting that MSYq encodes a factor(s) suppressing sex ratio distortion. MSYq consists of multicopy gene families, present in ~60 to 100 copies [17–24], many of which possess X-linked multicopy homologous genes [20,25,26]. This has been considered a manifestation of a conflict between an X-encoded TD and a Y-encoded suppressor that remain to be identified [16,20,26,27].
Author Summary

Both copies of a gene have normally an equal chance of being inherited; however, some genes can act “selfishly” to be transmitted to >50% of offspring: a phenomenon known as transmission distortion. Distorting genes on the X or Y chromosome leads to an excess of female/male offspring respectively. This then sets up a “genomic conflict” (arms race) between the sex chromosomes that can radically affect their gene content. Male mice that have lost part of their Y produce >50% female offspring and show over-activation of multiple genes on the X, providing strong circumstantial evidence for distortion. Here, we demonstrate the existence of a genomic conflict regulated by the genes Slx/Slxl1 and Sly, present in ~50 to 100 copies on the X and Y chromosomes respectively. SLX/SLXL1 and SLY proteins have antagonistic effects on sex chromosome expression in developing sperm and skew the offspring sex-ratio in favor of females/males. Interestingly, while deficiency of either gene alone leads to severe fertility problems, fertility is improved when both genes are deficient. We believe that the conflict in which Slx/Slxl1 and Sly are involved led to the amplification of X and Y genes and may have played an important role in mouse speciation.

We have previously shown that the MSYq-encoded multicopy gene Sly (Syo3-like Y-linked) represses the postmeiotic expression of X and Y genes [19]. Sly-deficient males – also known as shSLY males since they carry a short hairpin RNA-expressing transgene which triggers the specific degradation of Sly transcripts by RNA interference – present a remarkable up-regulation of sex chromosome genes in postmeiotic germ cells (spermatids) associated with a loss of repressive epigenetic marks, such as trimethylated histone H3 (H3K9me3) and CBX1 [19]. SLY therefore limits sex chromosome expression via the recruitment/maintenance of repressive epigenetic marks to post meiotic sex chromatin (PMSC) and has been proposed to associate with the sex chromosomes through its Corl domain – a domain thought to mediate chromatin interactions (Conserved Domain Database from the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=47120).

Interestingly, Slx and Slxl1, two multicopy X-linked genes related to Sly [25] have been co-amplified with Sly during the evolution of the mouse genome [18,20] and are among the genes that are up-regulated when Sly expression is reduced/absent [19]. Using a strategy of transgenically-delivered short hairpin RNA similar to the one previously used to disrupt the function of Sly, we have recently produced Slx/Slxl1-deficient mice (also known as shSLX mice). This study has shown that Slx/Slxl1 are indispensable for normal sperm differentiation, and that Slx/Slxl1 deficiency leads to the deregulation of a number of autosomal genes [28]. Moreover, both SLY and SLXL1 proteins have now been shown to interact with the acrosomal protein DKK1 [21,29].

In the present study we show that SLX/SLXL1 and SLY proteins have antagonistic effects on gene expression for both the sex chromosomal genes deregulated in shSLY and the set of autosomal genes deregulated in shSLX, and furthermore have antagonistic effects on offspring sex ratio. Our data demonstrate that Slx/Slxl1 and Sly are involved in a postmeiotic intragenomic conflict; we propose this phenomenon has had a strong impact on the structure and epigenetic regulation of the sex chromosomes, and may also have influenced the evolution of hybrid sterility in the mouse lineage.

Results

In Sly-deficient mice, SLX/SLXL1 proteins relocate to the nuclear sites vacated by SLY proteins

In normal males, SLX/SLXL1 proteins are located in the cytoplasmic compartment of spermatids [28], whereas SLY is additionally detected in the spermatid nucleus where it has been shown to colocalize with the X and Y chromosomes [19]. When performing immunofluorescence detection of SLX/SLXL1 proteins on spermatids devoid of SLY protein (i.e. on shSLY testicular sections), we observed an augmented SLX/SLXL1 signal in the cytoplasm compared to controls (WT) – confirming up-regulation at the protein level – and some signal in shSLXL1 round spermatid nuclei that was not visible in WT (Figure 1A). The presence of SLX/SLXL1 proteins in shSLY spermatid nuclei was confirmed by Western blot analyses of nuclear fractions (Figure 1B). We then investigated in more detail the nuclear localization of SLX/SLXL1 in the context of Sly deficiency. The vast majority of shSLY spermatid nuclei showed a strong SLX/SLXL1 signal (280/369, 76%) (Figure 1C and Figure S1). This signal colocalized with the postmeiotic sex chromatin (PMSC, i.e. the X or the Y chromosome since spermatids are haploid) in 96.5% of round spermatids (82/85; 32/34 for X-bearing and 50/51 for Y-bearing spermatids). In comparison, 84% of WT round spermatid nuclei (263/316) did not have any SLX/SLXL1 signal. The nuclear SLX/SLXL1 signal observed in the remaining ~16% of WT round spermatid was very weak when compared to the nuclear signal in shSLY round spermatids but appeared to colocalize with the PMMSC in the majority of the cases (Figure S1).

In addition to colocalizing with the PMMSC, foci of SLX/SLXL1 proteins were observed outside the sex chromatin, reminiscent of the SLY signal present in the nucleus of WT spermatids [19]. We have since established that these ‘ectopic’ SLY sites include a ~14 Mb cluster of 7 Spier genes on chromosome 5 that are up-regulated in shSLY spermatids. As a result, SLY immunofluorescence followed by fluorescent hybridization of a Spier DNA probe (DNA FISH) showed that, in the majority of WT round spermatids (107/136, 78.5%), SLY protein colocalized with the Spier DNA FISH signal (Figure 1D). We next looked at SLX/SLXL1 proteins in Sly-deficient round spermatids and observed that they colocalized with the Spier gene cluster in 73% of the cases (130/178) (Figure 1D). Thus, in the absence of SLY, SLX/SLXL1 proteins colocalize with the sex chromatin and with the autosomal Spier gene cluster, mimicking the pattern observed for SLY protein in WT spermatids.

Transgenic delivery of shSLX and shSLY short hairpin RNAs leads to a dramatic reduction in Slx/Slxl1 and Sly RNA and protein levels

We then wondered if the localization of SLX/SLXL1 proteins to the PMMSC in the absence of SLY also affects postmeiotic sex chromosome gene expression. To address this question, we generated males that were deficient for SLX/SLXL1 and SLY proteins: we produced males carrying shSLY (Sly specific short hairpin RNA) transgene [19] together with one or two shSLX (Slx/Slxl1 specific short hairpin RNA) transgenes, shSLX1 and/or shSLX2 [28]. Firstly, we checked the efficiency of Slx/Slxl1 and Sly knockdowns in round spermatids from males carrying shSLX1 and shSLY transgenes [hereafter named shSLX1shSLY males]. The reduction in Slx/Slxl1 transcript level was similar in shSLX1shSLY males and in shSLX1 siblings, while Sly knockdown was even stronger in shSLX1shSLY males compared to shSLY siblings (Figure 2A and 2B). Sly transcript quantification included both alternative splice variants (Sly1 and Sly2) [21] which
were knocked-down with the same efficiency [19]. No SLY1 protein could be detected in shSLX or in shSLX1shSLY tissues (Figure 2D–2E). To date it remains unclear whether Sly2 transcripts are translated since anti-SLY1 antibody cannot detect SLY2 protein [21]. The discrepancy between transcript and protein levels is likely due to the presence of non-coding Sly transcripts, as previously observed [19]. Reduction in SLX and SLXL1 proteins was similar in shSLX1shSLY males and in shSLX1 siblings (Figure 2C). We also produced shSLX1/2shSLY males that carry the two shSLX transgenes along with the shSLY transgene. As expected, shSLX1/2shSLY males showed a very efficient knockdown of Slx and Slxl1 (Figure S2A); Sly knockdown in these males was similar to that in shSLX1shSLY males (Figure S2B). Thus, the combination of shSLX and shSLY transgenes gives an efficient knockdown of Slx/Slxl1 and Sly genes; the resulting transgenic males are therefore deficient for Slx/Slxl1 and Sly transcripts and proteins (hereafter named Slx/y-deficient males).

Figure 1. SLX/SLXL1 proteins behave similarly to SLY in its absence. A) Immunofluorescence detection of SLX/SLXL1 protein (green) in wild-type (WT) and Sly-deficient (shSLY) testicular sections. DAPI (blue) was used to stain nuclei and lectin-PNA (red) was used to stain acrosomes in order to determine tubular stage. The inset represents a 3× magnification. Pictures were taken using the same image capture parameters. Scale bar indicates 10 μm. B) Western blot detection of SLX/SLXL1 proteins in nuclear extracts from shSLY and WT round spermatids. SLY1 antibody was used on the same extracts to confirm the absence of SLY protein in the shSLY nuclear fraction. Sly gene encodes two alternative splice variants (Sly1 and Sly2) which are predicted to be translated into a long and a short protein isoform (SLY1 and SLY2), but only SLY1 proteins have been detected so far and it remains unclear whether Sly2 transcripts are translated [21]. LAMIN-B1 detection was used as a loading control. C) Immunofluorescence detection of SLX/SLXL1 protein (green) in shSLY and WT round spermatid nuclei. DAPI (blue) was used to stain nuclei. X and Y chromosome painting were performed sequentially. A strong SLX/SLXL1 signal was observed in the majority of shSLY spermatid nuclei (76%). This signal colocalized with either sex chromosome in 96.5% of the cases. No signal could be detected in the majority of WT round spermatid nuclei (84%). D) Immunofluorescence detection of SLY1 (pink) or SLX/SLXL1 (green) protein in WT or shSLY round spermatids. Hybridization with a DNA probe detecting Speer gene cluster was subsequently performed, followed by Y chromosome painting. DAPI (white or blue) was used to stain nuclei. SLY1 protein colocalized with Speer gene cluster in 78.5% of WT spermatids while SLX/SLXL1 proteins colocalized with Speer gene cluster in 73% of shSLY spermatids.

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Figure 2. The combination of shSLX and shSLY transgenes produces an efficient knockdown of Slx/Slxl1 and Sly genes. A–B) Real time PCR quantification of Slx/Slxl1 (Slx-all primers) (A) and Sly (Sly1 and Sly2 variants) (B) transcript levels in WT, shSLX1, shSLY and shSLX1shSLY round spermatids. The y-axis indicates the level of expression compared to WT after normalization with Acrv1 (2^(-ΔΔCt) ± standard errors). The reduction in Slx/Slxl1 transcript level was similar in shSLX1shSLY males and in shSLX1 siblings. As observed before [19], Slx/Slxl1 transcript level was found increased in shSLY males. One asterisk indicates significant difference from WT (p=0.05; t test on ΔΔCt values). Sly knockdown was even stronger in shSLX1shSLY males compared to shSLY siblings (two asterisks indicate significant difference between shSLX1shSLY and shSLY (p=0.02; t test on ΔΔCt values)). C–E) Western blot detection of SLY1, SLX and SLXL1 proteins in nuclear and cytoplasmic fractions from WT, shSLY, shSLX1 and shSLX1shSLY round spermatids. LAMIN B1 and ACTIN detection were used as loading controls for nuclear and cytoplasmic fractions, respectively. No SLY1 protein could be detected in shSLY or in shSLX1shSLY samples.

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In Sfy-deficient spermatids, SLX/SLXL1 proteins increase sex chromosome gene expression associated with a reduction of H3K9me3 marks on PMSC.

We then performed microarray transcriptome analyses on Slx/y-deficient purified round spermatids and compared these results to those obtained from Sfy-deficient and from WT round spermatids (Figure 3 and Figure S3). The up-regulation of X- and Y-encoded spermatid transcripts was significantly less pronounced in Slx/y-deficient males than in Sfy-deficient males (Figure 3A–3C). Specifically, 222 genes showed a greater than 1.5 fold-increase in Slx/y-deficient spermatids relative to WT, and 196 of them were corrected to some degree by the additional Slx/Slxl1 deficiency (i.e. in Slx/y-deficient spermatids). As a Y-encoded gene, Sfy itself is affected by Slx/Slxl1 knockdown and thus is expressed at a lower level in Slx/y-deficient males than in Slx/y-deficient males (Figure 2B and Figure S3). The microarray findings were confirmed for several representative X and Y genes by real time PCR (Figure 3B and Figure S2C). These opposite effects of Sfy and Slx/Slxl1 deficiency show that, in the absence of SLY protein, SLX/SLXL1 proteins localize to PMSC where they increase sex chromosome gene expression; when both SLX/SLXL1 and SLY proteins are reduced/absent in PMSC (in Slx/y-deficient males), the level of X- and Y- encoded transcripts is closer to the WT value. It is worth noting that while Slx/Slxl1 deficiency significantly reduces the up-regulation of XY genes induced by Sfy deficiency, it does not bring expression all the way back down to WT levels. This may indicate that Slx/Slxl1 knockdown is not sufficient to fully compensate for the effect of Sfy deficiency; alternatively it may be that in the WT MF1 laboratory strain, the combined effect of the presence of both SLX/SLXL1 and SLY is a net reduction of XY expression level, thus leading to a net increase when both genes are deficient.

The up-regulation of X- and Y-encoded spermatid genes in Sfy-deficient spermatids has been shown to be concurrent with a diminution of the repressive epigenetic marks (such as H3K9me3) normally associated with PMSC [19]. We therefore decided to study these repressive marks in Slx/y-deficient spermatids, and observed that H3K9me3 staining on PMSC (as compared to H3K9me3 chromocenter staining) was significantly higher (p = 0.00003) in Slx/y-deficient spermatids than in Slx/y-deficient spermatids (average staining intensity: 0.59 and 0.51 respectively, and closer to but significantly different from the WT value (average staining intensity in WT: 0.63, p = 0.003) (Figure 3D and Figure S1 for quantification). Therefore Slx/Slxl1 deficiency partially compromises the loss of H3K9me3 marks induced by Sfy deficiency. These results correlate with the global effect of Slx/Slxl1 transcript knockdown on sex chromosome expression and suggest that SLX/SLXL1 and SLY proteins compete in spermatids for access to PMSC where they have activator and repressive effects respectively, at the whole-chromosome level.

We then compared the transcriptomes of WT, Slx/Slxl1-deficient and Slx/y-deficient spermatids. This revealed a 10% reduction in Y transcription in Slx/Slxl1-deficient spermatids compared to WT that was not seen in an earlier study [28] (Figure 3A–3B). This reduction is congruent with our observation of some SLX/SLXL1 proteins in a small number of WT spermatid nuclei (Figure 1B and Figure S1); this small fraction of SLX/SLXL1 proteins most likely increases sex chromosome gene expression in the nucleus of WT spermatids, while the loss of these proteins leads to a slight reduction of XY expression in Slx/Slxl1-deficient spermatids. A faint reduction of expression was observed for some X genes (for instance Actn1, see Figure 3B) but this did not significantly differ from the WT value.

Sfy knockdown corrects the gene deregulation induced by Slx/Slxl1 deficiency

We have previously shown that Slx/Slxl1 deficiency leads to delay in spermatid elongation and sperm release, associated with the deregulation (principally the up-regulation) of 115 genes, the majority of which are located on the autosomes. Given that SLX/SLXL1 proteins are almost entirely cytoplasmic in wild type, we proposed that these transcriptional changes were a manifestation of “cytoplasmic” defects, rather than a direct effect of SLX/SLXL1 proteins on autosomal gene expression; for instance, an as yet unidentified cytoplasmic partner of SLX/SLXL1 could mediate the transcriptional changes that are necessary for normal spermatid elongation, or it may be that the transcriptional changes seen reflect an altered cellular proportion of different spermatid forms in shSLX [28]. In the present study, we compared microarray results from Slx/Slxl1-deficient and Slx/y-deficient spermatids and, surprisingly, observed that most of the genes deregulated by Slx/Slxl1 deficiency were less affected in Slx/y-deficient spermatids (111/115 genes, Figure 3E and Figure S5). Therefore, Sfy knockdown corrects the deregulation of autosomal genes induced by Slx/Slxl1 with autosomal gene expression values close to WT levels in Slx/y-deficient spermatids; Figure 3E and Figure S5). These results show that SLX/Y proteins have opposite regulatory effects on autosomal gene expression as well as on sex chromosome gene expression.

Slx/y-deficient males have better reproductive parameters and overall fertility than males that are deficient for either Slx/Slxl1 or for Sfy

Our microarray results demonstrate that the deregulation of sex chromosome-linked or autosomal genes observed in Sfy-deficient or in Slx/Slxl1-deficient spermatids respectively, is corrected in Slx/y-deficient spermatids; we therefore compared the reproductive parameters of Slx/y-deficient males with those from males that are singly deficient for either Slx/Slxl1 or for Sfy. Firstly, Slx/y-deficient males had significantly improved sperm numbers (Table 1). This was particularly striking for the comparison between shSLX1/2 and shSLX1/2shSLY males: shSLX1/2 males had dramatically reduced spermatocaoa numbers but the addition of shSLX transgene to this genotype increased the number of sperm produced ~50-fold (Table 1). Low sperm count in shSLX males was attributed to the apoptosis of delayed elongating spermatids [28]. We therefore analyzed spermatid elongation delay and apoptosis in Slx/y-deficient males and in their Slx/Slxl1-deficient siblings. Remarkably, while Slx/Slxl1-deficient males presented a high number of delayed and apoptotic elongating spermatids, Slx/y-deficient models did not significantly differ from WT (Figure 4A–4B). The spermatocaoa morphology of Slx/y-deficient males was also much improved compared to that of Slx/Slxl1- or Sfy-deficient males (Figure 4C and Figure S6). Finally, we compared the fertility of Slx/y-deficient males with Slx/Slxl1- or Sfy-deficient siblings: Slx/y-deficient males had overall better fertility than males that are deficient for either Slx/Slxl1 or Sfy, with reproductive parameters close to WT values (Table 1). Strikingly, the addition of Sfy deficiency was able to reverse the sterility observed in Slx/Slxl1-deficient males (line shSLX1/2) (Table 1). All in all, males that were deficient for both Slx/Slxl1 and Sfy had considerably better reproductive parameters than males that were deficient for Slx/Slxl1 or Sfy alone.

These analyses show that Sfy deficiency almost completely rescues the defects and gene deregulation induced by Slx/Slxl1 deficiency, while Slx/Slxl1 knockdown only partially rescues those subsequent to Sfy deficiency. This may be due to a different
Figure 3. SLX/SLXL1 and SLY have opposite effects on gene expression and on the recruitment/maintenance of H3K9me3 on the sex chromatin. A) Representation of the microarray results obtained for Slx/Slxl1-deficient (shSLX1), Sly-deficient (shSLY) and Slx/y-deficient (shSLX1shSLY) compared to WT spermatids. B) Real time PCR quantification showed that transcript levels of X-encoded (Actrt1 and 1700008I05Rik) and of Y-encoded genes (Ssty1 and Zfy2) were lower in shSLX1shSLY than in shSLY spermatids. Transcript levels were also lower in shSLX1 spermatids compared to WT spermatids. The y-axis indicates the level of expression compared to WT after normalization with Acrv1 (2^ΔΔCt ± standard errors). One asterisk indicates significant difference from corresponding shSLY or WT value (t test on ΔΔCt values; p<0.05). C) Graphic representation of the expression ratio relative to WT for the 222 genes showing greater than 1.5 fold-change in shSLY. Most genes affected by shSLY are sex-linked and up-regulated [19]. The majority of them (196/222) were corrected to some degree by addition of the shSLX transgene (in shSLX1shSLY). For 46 of these genes, the difference between shSLY and shSLX1shSLY was itself statistically significant. D) Immunofluorescence detection of H3K9me3 (green) in WT, shSLY and shSLX1/2shSLY round spermatid nuclei. DAPI (white or blue) was used to stain nuclei. The round DAPI-dense structure is the chromocenter. The less DAPI-dense structure at the periphery of the chromocenter is the postmeiotic sex chromatin (PMSC) and is indicated by an arrow. Pictures were taken using the same image capture parameters. Note the decreased H3K9me3 signal on the sex chromatin of Sly-deficient spermatids; this is almost completely restored by Slx/Slxl1 deficiency (in shSLX1/2shSLY spermatids). See also Figure S4. E) Graphic representation of the expression ratio relative to WT for the 115 genes showing greater than 1.5 fold-change in shSLX1. Most genes affected by shSLX1 are autosomal and up-regulated [28]. Note that almost all of them (111/115) have a lower fold change in shSLX1shSLY than in shSLX1. For 91 of these genes, the difference between shSLX1 and shSLX1shSLY was itself statistically significant.

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Figure 4. Slx/y-deficient males have fewer spermiogenesis defects than males that are deficient for either Sly or Slx/Slxl1 alone. A) Bar graph representing the percentage of tubules containing apoptotic elongating spermatids measured by TUNEL assay (in grey) and the percentage of tubules containing delayed elongating spermatids (in black). Hatch symbol (#) indicates significant difference from WT (ANOVA, p<0.0001). One asterisk indicates significant difference between values obtained for Slx/y-deficient males (shSLX1shSLY or shSLX1/2shSLY) and Slx/Slxl1-deficient siblings (shSLX1 or shSLX1/2) (ANOVA, p<0.00001). B) Representative black and white TUNEL pictures of WT, shSLX1/2 and shSLX1/2shSLY testicular sections. Note the presence of apoptotic cells (TUNEL + cells in white) in most shSLX1/2 seminiferous tubules, while in WT and in shSLX1/2shSLY they are largely restricted to stage XII tubules (and correspond to metaphasic cells undergoing normal apoptosis). Scale bar represents 200 μm. C) Bar graph representing the percentage of sperm head abnormalities in Slx/Slxl1-deficient, Sly-deficient and Slx/y-deficient males (see also Figure S6). The percentage of total abnormal sperm heads is significantly lower in Slx/y-deficient males (shSLX1shSLY and shSLX1/2shSLY) than in Sly-deficient males (shSLY). In particular, the number of grossly flattened spermheads – which are specifically observed in Sly-deficient males – is reduced (ANOVA, p<0.001). ShSLX1/2shSLY males also show a significant decrease in this percentage compared to shSLX1shSLY males (ANOVA, p<0.03). The number of slightly abnormal sperm heads – which are specifically observed in Slx/Slxl1-deficient males – is also reduced in Slx/y-deficient males compared to Slx/Slxl1-deficient males (ANOVA, p<0.0008). For this category of sperm abnormalities, Slx/y-deficient males did not significantly differ from WT. doi:10.1371/journal.pgen.1002900.g004

Table 1. Analysis of the reproductive parameters of Slx/y-deficient mice compared to controls.

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<thead>
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<th>WT</th>
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<th>shSLX1</th>
<th>shSLX1/2</th>
<th>shSLX1shSLY</th>
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<td>Average number of litters per male ± standard errors</td>
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<td>1.9±0.7</td>
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<td>Average number of offspring per male ± standard errors</td>
<td>48.4±3.8</td>
<td>38.8±4.7</td>
<td>10.4±4.4</td>
<td>0±0.2</td>
<td>18.4±9.3</td>
<td>27.3±6.6</td>
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<tr>
<td>Average litter size ± standard errors</td>
<td>9.1±0.7</td>
<td>2.2±1.1</td>
<td>2.4±1.8</td>
<td>0±0.2</td>
<td>3.6±1.2</td>
<td>5.8±1.2</td>
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<tr>
<td>Sperm count/cauda (mean values x10^6 ± standard errors)</td>
<td>14.4±1.6</td>
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<td>2.1±0.6</td>
<td>0.2±0.1</td>
<td>11.7±0.9</td>
<td>8.8±0.5</td>
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*significant difference from WT (t-test,p<0.02);  
*significant difference from shSLY (t-test,p<0.03);  
*significant difference from shSLX1 (t-test,p<0.02);  
*significant difference from shSLX1/2 (t-test,p<0.03).  
doi:10.1371/journal.pgen.1002900.t001
knockdown efficiency: indeed, no SLY1 protein can be detected in Slx/y-deficient samples while some SLX/SLXL1 proteins remain (Figure 2C–2E).

Slx/Slxl1 deficiency causes a sex ratio distortion in favor of males that is restored by Sly deficiency

We previously reported a tendency of an excess of females in the progeny of Sly-deficient males (7.7% excess of females, Chi-square p = 0.0569) [19]. While analyzing the fertility of our transgenic lines, we observed that Slx/Slxl1-deficient males (i.e. shSLXL1) yielded an offspring sex ratio of 40% (74/187) female progeny, compared to a ratio of 51% (234/461) in WT siblings. This represents a statistically significant sex ratio distortion of 11% in favor of male offspring (Chi-square p = 0.006). Importantly, a normal sex ratio was restored by the addition of Sly deficiency: Slx/y-deficient males produced an offspring sex ratio of 50% (103/208) female progeny that did not differ from WT and was also significantly different from the offspring sex ratio of shSLXL1 males (Chi-square p = 0.03). These data show that both Slx/Slxl1 and Sly affect the transmission of X- and Y-bearing gametes, Slx/Slxl1 favouring X transmission while Sly favours Y transmission.

Discussion

SLX/SLXL1 and SLY proteins have antagonistic effects on the expression of two distinct sets of genes

In recent years, we have identified the transcriptional consequences of Sly and Slx/Slxl1 deficiency, and related these to the observed phenotypes in terms of spermatid development, sperm morphology and offspring sex ratio [19,28]. Remarkably, we now show that in dual shRNA knockdown models where both genes are deficient, the transcriptional and phenotypic consequences of the individual knockdown are dramatically ameliorated, correcting the X/Y/Spyr up-regulation and sperm shape abnormalities seen in Slx-deficient spermatids; the autosomal gene up-regulation, spermatid elongation delay and apoptosis, and sperm shape abnormalities seen in Slx/Slxl1-deficient spermatids; and improving fertility in both cases.

Strikingly, however, two different and almost entirely non-overlapping sets of genes are affected by the mutual antagonism of SLX/SLXL1 and SLY. In this discussion, we refer to “Group 1” genes as the set of X/Y/Spyr genes up-regulated in Slx-deficient spermatids and (partially) corrected in the dual knockdown, and “Group 2” genes as the set of metabolism-related autosomal genes up-regulated in Slx/Slxl1-deficient spermatids and (almost fully) corrected in the dual knockdown.

Group 1 genes: Nuclear consequences of antagonism between SLX/SLXL1 and SLY

Slx regulates the epigenetic repression of post meiotic sex chromatin (PMISC) and a few specific autosomal genes such as the Spyr cluster. In the nucleus, SLY appears to act via the recruitment/maintenance of the repressive heterochromatin marks H3K9me3 and H3K9me2, which consequently limits the expression of X and Y genes in spermatids, among which are its X-linked homologs Slx and Shil1 [19]. Here, we show that, in the absence of SLY, SLX/SLXL1 proteins relocate to the nuclear sites (both sex-linked and autosomal) vacated by SLY proteins. It is unlikely that SLX/SLXL1 nuclear localization in Slx-deficient spermatids is solely a consequence of increased SLX/SLXL1 protein abundance, since there is no clear enrichment in nuclear SLX/SLXL1 proteins in spermatids of transgenic mice overexpressing SLX or SLXL1 (our unpublished preliminary data).

Moreover, in the double transgenic model (Slx/y-deficient males) where SLX/SLXL1 family members are also reduced/absent, XY gene expression, Spyr expression and the intensity of H3K9me3 marks on the sex chromatin are closer to normal values. This indicates that SLX and/or SLXL1 have consequences both for transcriptional activity and for histone modification when present on sex chromatin, and that these are directly opposed to the effects of SLY. We therefore propose that SLX/SLXL1 and SLY proteins compete for access to nuclear sites in spermatids, where they act as positive and negative transcriptional regulators respectively. We cannot at this point say precisely where the competition occurs; it may be directly at the level of chromatin binding within the nucleus, or SLX/SLXL1 and SLY may compete for access to factors affecting nuclear import. We note that SLX and SLY1 proteins lack nuclear localization signals (NLS) while SLY NLS is mutated/truncated [22,25]; as such they probably depend on other interacting factors to enter the nucleus. It also remains possible that the competition is mediated indirectly; for example, SLY could affect SLX/SLXL1 intracellular localization via regulating the expression of a third factor controlling SLX/SLXL1 access to the nuclear sites.

Group 2 genes: Cytoplasmic consequences of antagonism between SLX/SLXL1 and SLY

Slx/Slxl1 deficiency has been shown to increase the level of ~100 autosomal transcripts which code for proteins of the cytoskeleton and the extracellular matrix, or are implicated in various cytoplastic processes (i.e. energy production, lipid metabolism, ubiquitin-mediated degradation, etc.) [28]. These transcriptional effects are corrected in Slx/y-deficient males, suggesting that these changes may also be manifestations of the same nuclear/chromatin regulatory antagonism exhibited by Group 1 genes, perhaps via relocation of repressive factors from sex chromatin to autosomal locations and vice versa. There are, however, three significant objections to this interpretation. Firstly, as noted previously, in WT spermatids SLX/SLXL1 are predominantly cytoplasmic proteins, and the levels in the nucleus are almost undetectable: it is hard therefore to see i) how Slx/Slxl1 knockdown could directly induce widespread transcriptional changes, ii) what would then be the function of the abundant SLX/SLXL1 proteins in the cytoplasm. Secondly, this interpretation would require not only that SLX/SLXL1 act simultaneously as transcriptional activators of Group 1 genes and as transcriptional repressors of Group 2 genes, but that SLY has the reverse effect in both cases: it is challenging to imagine a mechanism that could explain this. Thirdly, if both Group 1 and Group 2 gene effects are a manifestation of the changing balance of SLX/Y proteins in the nucleus and/or a relocation of repressive factors from the sex chromosomes to autosomes, then both groups of genes would be expected to change together. This is not the case: Group 1 genes are affected in shSLX but not in shSLXL1, and Group 2 genes vice versa.

For this reason, we favour our existing interpretation that Group 2 gene deregulation is a manifestation of the spermigenesis defects occasioned by cytoplasmic Slx/Slxl1 deficiency (i.e. spermatid elongation delay and apoptosis, reduced sperm count, abnormal head to tail connections of the spermatozoa and male infertility) [28], and is not a direct effect of SLX/SLXL1 proteins on autosomal gene transcription. Given that the (cytoplasmic) spermigenesis defects are corrected in the dual mutant, it stands to reason that the secondary expression changes follow the same pattern. We therefore propose that, in addition to the nuclear effects on Group 1 genes, SLY protein has a cytoplasmic role, opposing that of SLX/SLXL1. SLY proteins have been shown to...
be present in both the spermatid nucleus and cytoplasm [19,21]. Intriguingly, a recent report indicates that the acrosomal (cytoplasmic) protein DKKL1, which we previously identified as a binding partner of SLY1 [21], also interacts with SLXL1 [29]. We have performed additional experiments and observed that all SLX/Y family members (i.e. SLY1, SLY2, SLX and SLXL1) can interact with DKKL1 (Figure S7). Therefore, SLX/SLXL1 and SLY proteins could compete for interaction with (a) common partner(s) in the cytoplasm, and this competition could be at the basis of the opposite effects of SLX/SLXL1 and SLY on spermiogenesis and autosomal gene expression. A combined model proposing how SLX/SLXL1 and SLY proteins have antagonistic effects in both the spermatid nucleus and cytoplasm is presented in Figure 5.

We recognize that under our preferred model, it is difficult to explain the directionality of the expression changes seen in shSLX relative to WT, which was predominantly up-regulation of autosomal genes with comparatively few down-regulated genes [28]. A potential explanation for this lies in the spermatid developmental delay resulting in delayed spermatid elongation in shSLX. This could potentially skew the round spermatid population in shSLX tests towards earlier stages, i.e. proportionally more step 1 spermatids and fewer step 7–8 spermatids. Since there is a progressive transcriptional shutdown throughout spermatid development as chromatin is repackaged in preparation for nuclear condensation, this would thus manifest in shSLX as a selective up-regulation of those genes expressed specifically in early stage round spermatids (which in turn is plausible given the annotated functional categories for these Group 2 genes). Testing this interpretation will require further experiments on fractionated, staged sub-populations of round spermatids.

The mouse X and Y chromosomes are involved in an intragenomic conflict that is regulated by Slx/Slxl1 and Sly

Irrespective of the precise molecular mechanism(s) underlying the antagonistic effects of SLX/SLXL1 and SLY, our results demonstrate that both genes have an effect on offspring sex ratio. In particular, comparing shSLX (where Slx is still present) to the dual knockdown, there is a significant excess of males; and when comparing shSLY (where Slx/Slxl1 are still present) to the dual mutant, there is a trend towards excess of females. Thus, the net effect of these genes on inheritance is for X-linked family members to favour X chromosome transmission, and Y-linked members to favour Y chromosome transmission, constituting a prima facie genomic conflict. Such a conflict was first postulated in the 1990s following observations that male mice with a partial deletion of the Y long arm produce an excess of female offspring, however supporting evidence has not been forthcoming until recently [16,20,26,27]. The present study demonstrates that such a conflict exists between the sex chromosome-linked Sypβ-related genes. An intragenomic conflict is often not visible under normal conditions (i.e. in a WT population) [11,13] and here the positive effect of Slx/Slxl1 on sex chromosome transcription was uncovered by the production of mice that are deficient for both Sby and Slx/Slxl1; similarly, the effects of Slx/Slxl1 deficiency are also corrected in the dual mutant, although the molecular mechanisms involved are less clear.

Can sex ratio distortion be directly attributed to Slx/Slxl1 and Sly?

Under the distaster/responder model exemplified by the t complex [1], both Slx/Slxl1 and Sby are transmission distorters in that changes in their expression levels lead to a distortion of the sex ratio. However, it is unlikely that they are directly responsible for mediating the transmission skew (i.e. responder genes). Indeed, the physiological mechanism of the skew in the present model is an asymmetry in fertilizing ability between X and Y sperm [30]. This implies an underlying molecular functional asymmetry, namely the presence of a responder gene product which is not evenly shared between X and Y sperm. Both of the known mammalian examples of transmission ratio distortion depend on non-sharing of gene products (both transcript and protein) between sister spermatids: SpemI in the case of Rb(6,16) and Rb(6,15) translocation heterozygotes, and Tc10 mouse in the case of driving haplotypes [31–33]. We note that SLX/Y proteins appear to be similarly expressed in X- and Y-bearing spermatids. It therefore seems likely that the distortion in Yq deleted mice and in shSLXshSLY transgenic models is mediated by an as yet unidentified sex-linked gene or gene(s) (i.e. the responder), for which Slx/Slxl1 and Sby are competing regulators via their global effects on sex chromatin expression. Among the deregulated genes, a few appear as promising candidates, such as the X-encoded homolog of Tip11, which is one of the genes involved in the t-complex transmission distortion, albeit as a distaster rather than a responder [34], and Alkbh7, since another Alkbh gene has recently been found to cause sex ratio distortion [35]. However, there may be several linked genes involved, at least one of which is likely to evade transcript sharing. In view of this possibility, it is worth noting that both regulators of the conflict have a global effect on sex chromatin; this is an efficient way to control multiple sex chromosome-linked distorters and/or responders simultaneously. The ease of identifying the responder(s) will depend on how directly SLX/Y regulate them and how many there are. Finally, it is possible that autosomal factors also contribute to the regulation of sex-linked transmission distortion. We note that historically, Slx appeared on the X before Sby appeared on the Y, and its distorting effect on sex ratio may have subsequently been countered by a combination of Sby-mediated repression and other autosomal genes being selected to favour a balanced sex ratio [20].

The intragenomic conflict in which Slx/Slxl1 and Sby are involved has influenced the structure of the mouse sex chromosomes

In the mouse lineage, there has been a remarkable amplification of spermiatd-expressed sex chromosome genes (all of which fall into Group 1 identified above), and which has had a dramatic influence on the structure of the mouse sex chromosomes. This expansion occurred subsequent to the appearance of Sby, but was not accompanied by a matching increase in XY transcript levels [20]. It is therefore very likely that essential sex-linked spermiatd-expressed genes have become amplified in order to maintain a steady expression in the face of the enhancement of Sby-mediated repression and in a sense constitute a “collateral damage” arising from the conflict between Sby and Slx/Slxl1 that we unravel here. Interestingly, the Sper gene cluster is one of the autosomal gene families that have experienced the largest rodent-specific expansions [36] and is also repressed by SLY. Slx/Slxl1 and Sby competition may therefore have led to the amplification of reproductive genes outside the sex chromosomes as well as on them.

The intragenomic conflict in which Slx/Slxl1 and Sby are involved may have played an important role in mouse speciation

F1 hybrid sterile males produced by asymmetric crosses between M. m. musculus and M. m. domesticus display sperm...
Figure 5. Model presenting how SLX/SLXL1 and SLY proteins have antagonistic effects in the spermatid nucleus and cytoplasm. In
the spermatids, SLX/SLXL1 (yellow lozenges) and SLY (pink triangles) proteins have antagonistic effects i) in the nucleus, on the expression of XY
genes and of a few autosomal genes, such as Speer (group 1 genes); ii) in the cytoplasm, on the regulation of metabolic processes which secondarily
causes a deregulation of ~100 autosomal genes (group 2 genes). i) In WT, SLY proteins are located in both the nucleus and cytoplasm, while SLX/
SLXL1 proteins are almost exclusively in the cytoplasm. The nuclear fraction of SLY proteins colocalizes with the sex chromosomes and the autosomal
Speer gene cluster, and represses their expression. A very small fraction of SLX/SLXL1 proteins also appears to colocalize with the sex chromatin. In
SLy-deficient spermatids (shSLY), SLX/SLXL1 proteins relocate to the nuclear sites (both sex-linked and autosomal) vacated by SLY proteins; however,
SLX/SLXL1 proteins have an opposite effect to that of SLY, and activate XY gene expression. This is associated with a reduction in the repressive
epigeneic mark H3K9me3 on the sex chromatin (purple octagon), and produces sperm differentiation defects such as spermhead abnormalities,
shedding delay, motility defects and subsequent male infertility. In Slx/Slxl1-deficient spermatids (shSLX), the absence of SLX/SLXL1 nuclear proteins
has only minor effect on gene regulation, since it does not change SLY localization profile. There is only a slight reduction in XY transcription,
congruent with the idea that SLX/SLXL1 is a transcription activator sharing the targets of SLY when present in the nucleus. In the double knock-down
(shSLXshSLY), Slx/Slxl1 deficiency almost fully abrogates the effects of Sly knockdown: in shSLXshSLY spermatids, group 1 gene expression and
repressive epigenetic marks are close to WT values. This is correlated with a rescue of SLY-dependent sperm differentiation defects. In sum, in the
nucleus, the experimental observations indicate that SLX/SLXL1 competes with SLY at the level of sex chromatin regulation: SLY acts as a repressor
while SLX/SLXL1 acts as a positive regulator. ii) Slx/Slxl1 deficiency induces various spermiogenetic defects (such as spermatid elongation delay and
apoptosis, reduced sperm count, abnormal head to tail connections of the spermatozoa and subsequent male infertility) associated with an up-
regulation of ~100 autosomal genes which code for proteins of the cytoskeleton, the extracellular matrix, or implicated in various metabolic
processes (i.e. group 2 genes). Since SLX/SLXL1 proteins are predominantly cytoplasmic in WT spermatids, we propose that this gene deregulation is
a manifestation of the spermiogenesis defects occasioned by Slx/Slxl1 deficiency, and not a direct effect of SLX/SLXL1 proteins on autosomal gene
expression.
transcription. In the case of Sly deficiency, group 2 gene expression is unaffected; however, in the double knock-down, Sly deficiency corrects SLX/SXL1-dependent phenotypes which abrogates the subsequent group 2 gene up-regulation. This means that SLY protein has a cytoplasmic role, opposing that of SLX/SXL1. This antagonism could be mediated via interaction with (a) common partner(s) in the cytoplasm; the absence of competition between SLX/SXL1 and SLY proteins in the dual knockdown model would explain the absence of defects. In sum, SLX/SXL1 and SLY proteins apparently compete in the cytoplasm for the regulation of spermiogenic processes. The functional role of SLX/SXL1 could be to prevent the access of SLY to cytoplasmic proteins that are necessary for spermiogenesis. 

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differentiation defects and wide-spread overexpression of X-encoded spermiogenic genes [37]. Intriguingly, this only occurs in males with a M. m. musculus X chromosome and M. m. domesticus Y and autosomal chromosomes [38]. These males have an excess of Slx/Slxl1 copies compared to Sly copies, since the M. m. domesticus X and Y chromosomes carry ~40 to 60 copies of Slx/Slxl1 and Sly, while XY encoded Syp3-related genes have been more amplified in M. m. musculus, with >100 of Slx/Slxl1 and Sly on the X and Y [18,20]. Our data show that a balance between Slx/Slxl1 and Sly expression exists in wild-type populations and that disruption of this balance can cause male infertility. In light of these data, we propose that deficiency in the number of Sly copies compared to Slx/Slxl1 copies contributes to F1 male hybrid sterility (see Figure 6) in some of these crosses. This would explain the observed over-expression of X-encoded spermiogenic genes observed in some F1 hybrid males [37] and subsequent sperm differentiation defects and infertility. The observation that F1 males born from the reciprocal cross domesticus x musculus are reproductively normal [39] does not necessarily challenge this model. These males have an excess of Sly copies compared to Slx/Slxl1 copies and, according to our model, could be considered as Slx/Slxl1-deficient mice and thus display some spermiogenic defects. This however depends critically on the mechanism of the antagonistic effects of SLY and SLX/SXL1 in the cytoplasm, and on the threshold of copy number imbalance required to trigger abnormal spermatogenesis and/or sex ratio skewing. Given that autosomal genes will be selected to maintain a balanced sex ratio, the Slx/Sly conflict may well be “buffered” to some extent by epistatic interactions with autosomal genes.

We have observed that mice with a partial knockdown of Slx/Slxl1 (shSLX1 or shSLX2) have comparatively minor spermiogenic defects compared to mice with a severe knock-down (shSLX1/2) [28]. We also note that laboratory strain X chromosomes (including MF1 mice which were used in the present study) are predominantly derived from a domesticus background [40,41], yet are paired in these strains with a musculus

Figure 6. Model comparing Slx/Sly copy number imbalance in natural and laboratory mouse strains to Slx:Sly gene expression imbalance in shRNA knockdown models. A. A model for how Slx/Slxl1:Sly imbalance affects sperm shape, offspring sex ratio and fertility. B. Approximate copy number ratio of Slx/Slxl1 and Sly in the reciprocal crosses studied by Good et al. [37] based on an estimate of ~100 copies of each gene in musculus and ~50 in domesticus, in the WT laboratory strain MF1 Ynull which has a domesticus X and autosomes but a musculus-derived Y [40–42], and in the two natural mutants from the same background studied by us and others [18,20]. C. The relative magnitude of Group 1 and Group 2 transcriptional responses seen in the various shRNA/deletion models on the MF1 Ynull background. The double and triple shRNA models show a partial Group 1 response, but no Group 2 response. Importantly, in this model, the shSLX1 and shSLX1/2 phenotypes are expected to fall outside the range of variation seen in the natural mutant and reciprocal cross males, since they are on a background which has already a deficiency in Slx/Slxl1 copy number compared to Sly (50:100). We emphasise that the effects of Slx/Slxl1:Sly imbalance are only one contributor to hybrid sterility: sperm shape and testis size QTLs on the musculus X map to distinct locations and show different interactions with the domesticus autosomes and Y chromosome [30].

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Y chromosome YRH [42]. Thus laboratory strains are intrinsically comparable to the reciprocal cross. Our shSLX models therefore involve skewing the balance of SLX/SLXL1 and SLY even further, to pathogenic effect (see Figure 6). In this light it is intriguing that WT MF1 males have lower XY gene transcription than Slx/y deficient males: might this reflect the fact that laboratory strains are inherently “overdosed” for Sfy relative to Slx/Slxl by virtue of their hybrid origin?

Male hybrid sterility is a complex trait involving several X-linked loci (as demonstrated by the mapping of several quantitative trait loci – QTL – on the X chromosome [38,43,44]) as well as autosomal factors [43,46]. It is worth noting that among the four non-overlapping X-chromosome-linked QTL associated with abnormal spermheads and hybrid sterility, one encompasses Slx (0.37-1.1 Mb), the other, Slxl1 (47.9-81.8 Mb) [38]. Interestingly, it has been shown that one of the autosomal loci linked to hybrid sterility, Prdm9, encodes a histone H3 lysine 4 methyltransferase involved in the silencing of the sex chromosomes during meiosis (Meiotic Sex Chromosome Inactivation). It therefore epigenetically represses multiple X-chromosome loci, some of which part of the hybrid sterility gene network, and epistatic interactions between Prdm9 and multiple X and autosomal loci have been shown to cause asymmetric hybrid male sterility associated with a disruption of MSCI and thus a de-repression of the X chromosome [43,46]. However, Prdm9 does not appear to be involved in the X-chromosome up-regulation and sterility observed in F1 hybrid males studied by Good et al. [37].

Taken together, the genetic basis of reproductive isolation in mice is complex, and disruption of the transcriptional regulation of the X seems to contribute to the evolution of hybrid male sterility. The antagonistic effects of Slx/Slxl1 and Sfy at the transcriptional and phenotypic level, in particular the effects on postmeiotic XY gene regulation, may therefore be among the important elements contributing to the evolution of hybrid sterility between mouse species. The production of F1 males with a transgene-derived increased Sfy expression or with a knockdown of Slx/Slxl1 expression should help address this question.

In conclusion, we have demonstrated that the mouse X and Y chromosomes are involved in an intragenomic conflict that is regulated by the multicopy genes Slx/Slxl1 and Sfy. SLX/SLXL1 and SLY proteins compete during sperm differentiation, and notably have opposite effects on the regulation of sex chromosome gene expression. Disruption of Slx/y balance causes sex ratio distortion, sperm differentiation defects and male infertility. To the best of our knowledge, our work is the first characterization of a hybrid male sterility interval and suggests that MSCI pathogenicity might be the result of a conflict between sex chromosomes.

Materials and Methods

Generation and breeding of transgenic mice

shSLY (aka sh367), shSLX1 and shSLX1/2 males were produced and maintained as described before [19,28]. To produce shSLX1shSLY and shSLX1/2shSLY double transgenic mice, shSLX1 females were mated to shSLY or to shSLX1shSLX2 transgenic males. Double transgenic females were then mated to MF1 XYWT males [19] to maintain the stock, since shSLY males are subfertile and go progeny only rarely. Two-month-old males single or double transgenic for sh367 (shSLY), shSLX1 or shSLX1/2 transgenes, as well as their non-transgenic siblings (WT) were processed for all the analyses presented here. Animal procedures were in accordance with the United Kingdom Animal Scientific Procedures Act 1986 and were subject to local ethical review.

Elution of spermatids

Fractions enriched in round spermatids (>90%) were obtained from the above described transgenic and control (WT) males as described previously [19]. Each sample has been purified from a pool of testes obtained from 2 to 5 males.

Transfection

The coding sequence of mouse Slx1 and Slx cDNA were amplified by PCR and cloned into a C-terminal Myc-tagged pCMV vector; the coding sequence of mouse Slx, Slxl1, Sly and Syl2 cDNA were amplified by PCR and cloned into a N-terminal Flag tagged pCMV vector using EcoRI and NotI restriction sites (see Table S1 for a full list of primers). Co-transfections of HEK293 or COS cells were performed in 6-well plates using 1μg of each DNA and 5 μl of Lipofectamine (Invitrogen) following the manufacturer’s instructions. Proteins were extracted 24 hours post transfection in 200 μl of Lysis buffer (25 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 0.1%NP-40) and immunoprecipitated as described below.

Protein analyses

Nuclear and cytoplasmic protein extracts were obtained as described previously [19]. For immunoprecipitation experiments, proteins extracted from transfected cells were first pre-cleared with protein A/G sepharose for 1 hour at 4°C. They were then incubated overnight with Protein G- or Protein A-sepharose which had been covalently bound to MYC (Santa Cruz Biotechnology) or FLAG (Sigma) antibody beforehand (see [21] for a detailed protocol). Western blot experiments were performed as described previously [19]. Membranes were incubated overnight with anti-SLX/SLXL [28] diluted at 1/3000, anti-SLY1 [21] at 1/3000, anti–β-actin (Sigma) at 1/50000, or anti-LAMIN B1 (Santa Cruz Biotechnology) at 1/1000, anti-FLAG (Sigma) at 1/1000, or anti-MYC (Santa Cruz Biotechnology) at 1/1000. Detection by chemiluminescence was carried out after incubation with the corresponding secondary antibody coupled to peroxidase, as described by the manufacturer (Millipore).

Immunofluorescence and TUNEL

Immunofluorescence experiments were performed on testis material fixed in 4% buffered paraformaldehyde and sectioned as described before [25]. DAPI (4’6-diamidino-2-phenylindole) was used to stain nuclei (Vectorshied DAPI, Vectorlab). Alexa Fluor 594-conjugated peanut agglutinin lectin (Invitrogen), which stains...
the developing acrosome of spermatids, was used to stage the testis tubules. For the analysis of apoptotic elongating spermatids and delayed elongating spermatids, approximately 150 tubules were counted per individual (4 to 6 individuals per genotype). The percentage of tubules containing apoptotic elongating spermatids was determined on testis sections fluorescently stained using an in situ cell death detection kit (TUNEL, terminal deoxynucleotidyltransferase dUTP nick end labeling) as described by the manufacturer (Roche Diagnostics, Indianapolis, IN). The percentage of tubules containing delayed elongating spermatids (i.e. stage I to VIII tubules containing elongating spermatids) was assessed on testis sections fluorescently stained by H4K12Ac antibody (Millipore, Bedford, MA), a known marker of stage 9–12 elongating spermatids.

Antibody detection, chromosome painting, and DNA–FISH on surface-spread testicular cells

Antibody detection was performed on surface-spread testicular cells following a protocol described previously [19] adapted from Barlow et al. [47]. Incubation with the primary antibody (anti-SLY1 [17], anti-SLX/SLXL1 [28] or anti-H3K9me3 [Upstate]) diluted 1/100 was carried out overnight in a humid chamber at 37°C. DNA-FISH, then chromosome painting were performed after antibody detection as described previously [48]. Speer DNA-FISH was carried out using mouse BACs RP23-212A20 and RP24-310N20 (CHORI). As a control for specificity (see Figure S1), SLX/SLXL1 antibody was preabsorbed with 8 mg of SLX immunogenic peptide or with 5 mg of a noncompeting peptide (SLY peptide). For the quantification of H3K9me3 signal over the PMSC, the chromocenter domain was defined using the corresponding black and white DAPI picture. Then, H3K9me3 signal outside this chromocenter domain was measured and normalized to that of H3K9me3 signal over the chromocenter for each cell (100 cells per genotype), using Metaamorph and ImageJ (See Figure S4). Slides corresponding to 3 individuals per genotype were coded and randomized before the analysis; the analysis was therefore carried out blind as to genotype.

Analysis of sperm head morphology

For the quantification of spermhead abnormalities, sperm collected from the initial caput epididymis were suspended in phosphate-buffered saline. The suspension was smeared on slides (two slides per individual) and fixed in 3:1 methanol:acetic acid. The slides were then dipped in 0.4% Photoio for 2 min, air dried and stained on a plate heated at 60°C with one drop of 30% silver nitrate mixture with one drop of 2% gelatin (Sigma). The slides were coded and randomized. Sperm scoring was carried out blind as to genotype (4 to 6 individuals per genotype) and 100 sperm per slide were classified into 6 categories on the basis of the type and severity of abnormalities observed, using criteria described by Yamauchi et al. [49] and in Figure S6. In the text and figures, spermheads from category N were termed “normal”; category IS, “slightly abnormal”; category 2S, “slightly flattened”; category 3G, “abnormally thin”; category 4G, “grossly flattened” and categories 5G to 8G were pooled and named “other gross abnormalities” (cf. Figure S6).

Fertility testing and sex ratio of the offspring

To assess fertility and obtain sex ratio data from the offspring, five males of each genotype were mated with MF1 WT females over a period of six months.

Real-time PCR and microarray analyses

Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and microarray analyses were performed as previously described on RNA extracted from 2-month old testis or from round spermatids obtained after elutriation [19] (cf. Table S1 for a list of the primers used in this study). Real-time RT-PCR experiments were performed in parallel for all the genotypes described in this study, with between 3 to 5 individuals per genotype. For the microarray analysis, three shSLX1, three shSLY, three shSLX1shSLY and four wild type spermatid batches were analyzed (Illumina BeadChip, mouse whole-genome array, v2). These data thus include and extend our previously-reported results for shSLX1 round spermatids and for shSLY round spermatids in previous analyses [19,28], which collectively used two shSLY, two shSLX1 and four WT spermatid batches. Data normalization and calculation of FDR-adjusted p values was carried out in BeadStudio (Illumina) as previously described [19,28]. The full data set has been uploaded to GEO, accession number GSE39109.

Statistical analysis

For comparisons of the incidence of sperm head abnormalities, differences between genotypes were assessed by ANOVA after angular transformation of percentages, using the General Linear Models ANOVA provided by NCSS statistical data analysis software. The same test was applied to the frequency of abnormal head-tail connections, TUNEL positive elongating spermatids, delayed elongating spermatids (assessed by H4K12Ac staining) and H3K9me3 quantification. Student’s t test was used to compare the data obtained for fecundity, sperm number and real-time PCR (performed on the ΔCt values). A Chi-square test was used for sex ratio data. Microarray results were analyzed as described in Figure S3 and Figure S5.

Supporting Information

Figure S1 Immunofluorescence detection of SLX/SLXL1 proteins in spermatids. A) Representative pictures of the detection of SLX/SLXL1 proteins (green) by immunofluorescence in shSLY and WT round spermatid nuclei (surface spread technique). DAPI (blue) was used to stain nuclei. X and Y chromosome painting were performed sequentially. SLX/SLXL1 proteins are detected in shSLY spermatid nuclei in 76% of the cases. No signal could be detected in the majority of WT round spermatid nuclei (84%). The nuclear SLX/SLXL1 signal observed in the remaining ~16% of WT round spermatid is very weak compared to the nuclear signal in shSLY round spermatids. B) Control of the specificity of SLX/SLXL1 immunofluorescence signal (green) in surface-spread round spermatid nuclei. DAPI (in blue) was used to stain nuclei. Left Panel: SLX/SLXL1 proteins (in green) were observed in Syl-deficient (shSLY) round spermatid nuclei. Note in the picture the presence of a flattened sperm head, characteristic of shSLY testicular spread. When the antibody was preabsorbed with SLX/SLXL1 peptide, the signal disappeared. When the antibody was preabsorbed with a noncompeting peptide (SLY), SLX/SLXL1 signal was maintained. Right Panel: No signal was observed in the majority of WT round spermatids, in round spermatids deficient for SLX/SLXL1 proteins (shSLX1/2) and those deficient for both SLY and SLX/SLXL1 (shSLX1/2shSLY). All these controls demonstrate the specificity of the nuclear signal obtained with SLX/SLXL1 antibody in shSLY round spermatids.

Figure S2 Characterization of shSLX1/2shSLY males. We produced males carrying an shSLY transgene together with one
or two shSLX transgenes (i.e. shSLX1 and/or shSLX2). ShSLY males carry an shRNA-expressing transgene, which triggers the specific degradation of Slx transcripts via RNA interference [19]. Similarly, shSLX transgenic mice express Slx/Slx1-specific shRNA and display a decrease in whole testis Slx/Slx1 transcript levels estimated as ~60% and ~59% for transgenic lines shSLX1 and shSLX2, and ~93% for shSLX1/2 double transgenics [20]. A–B) Real-time PCR quantification of Slx/Slx1 (A) and Slx/Slx2 (B) transcript levels in WT, shSLY, shSLX1/2shSLY, shSLX1shSLY and shSLX1/2shSLY whole testes. The y-axis indicates the level of expression compared to WT (2^ΔΔCt ± standard errors). The combination of shSLY transgene with any shSLX transgene yielded an efficient knockdown of Slx and of Slx/Slx1 (one asterisk indicates significant difference from WT (p<0.02; t-test on ΔΔCt values)). The combination of shSLY transgene and two shSLX transgenes (i.e. shSLX1/2shSLY) produced a more pronounced decrease in Slx/Slx1 expression [two asterisks indicate significant difference between shSLX1/2shSLY and shSLX2shSLY or shSLX1shSLY (p<0.02; t test on ΔΔCt values)]. The average values obtained for WT, shSLY and shSLX1/2shSLY are respectively: 0.65, 0.51 and 0.59. B) Graph representing the H3K9me3 PSMC signal intensity in each category of sperm abnormalities, these abnormalities are specifically observed in Shy-deficient males. Hatch symbol (#) indicates significant improvement between other genotypes (ANOVA, p<0.04). One asterisk indicates significant improvement between other genotypes (ANOVA, p<0.02). Note the increase in the percentage of normal spermheads in Slx/y-deficient (shSLX1shSLY, shSLX2shSLY and shSLX1/2shSLY) compared to Shy-deficient male (shSLY). ShSLX1/2shSLY males also show a significant increase in the percentage of normal spermheads compared to shSLX1shSLY and shSLX2shSLY males. E) Quantification of grossly flattened spermhead abnormalities. These abnormalities are specifically observed in Shy-deficient males. Hatch symbol (#) indicates significant difference from WT (ANOVA, p<0.0001). One asterisk indicates significant improvement between other genotypes (ANOVA, p<0.03). Note the decrease in the percentage of grossly flattened spermheads in Slx/y-deficient (shSLX1shSLY, shSLX2shSLY and shSLX1/2shSLY) compared to Shy-deficient male (shSLY). ShSLX1/2shSLY males also show a significant decrease in this percentage compared to shSLX1shSLY and shSLX2shSLY males. According to those results, the genotypes can be classified in order of decreasing severity of their spermhead phenotype, as follow: shSLY>shSLX1shSLY=shSLX2shSLY>shSLX1/2shSLY. This is in clear correlation with the intensity of Slx/Slx1 knockdown and thus demonstrates that Slx/Slx1 knockdown rescues Shy-deficiency. F) Quantification of slightly abnormal spermhead abnormalities. These abnormalities are specifically observed in Slx/Slx1-deficient males. Hatch symbol (#) indicates significant difference from WT (ANOVA, p<0.04). One asterisk indicates significant difference between Slx/y-deficient and Slx/Slx1-deficient males (ANOVA, p<0.0000). Note the decrease in the percentage of slightly thin spermheads in Slx/y-deficient (shSLX1shSLY, shSLX2shSLY and shSLX1/2shSLY) compared to Slx/Slx1-deficient males (shSLX1 or shSLX2). For this category of sperm abnormalities, Slx/y-deficient males did not significantly differ from WT. This shows that Shy deficiency rescues the spermhead abnormalities observed in Slx/Slx1-deficient males.

Figure S7 SLX, SLX1, SLY1, and SLY2 proteins can interact with the cytoplasmic protein DKKL1. A) FLAG antibody detection of extracts from COS cells transfected with DKKL1-MYC and either FLAG-SLY1, FLAG-SLY2, FLAG-SLX, FLAG-SLX1, before [INPUT] and after immunoprecipitation [IP] with MYC antibody. A non-specific FLAG-tagged control was not immunoprecipitated by DKKL1-MYC (data not shown).