Human Molecular Genetics, 2005, Vol. 14, No. 18 ***2705–2715***
doi:10.1093/hmg/ddi304
Advance Access published on August 8, 2005

Deletions on mouse Yq lead to upregulation of multiple X- and Y-linked transcripts in spermatids

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Received June 24, 2005; Revised July 22, 2005; Accepted August 2, 2005

Deletions on the mouse Y-chromosome long arm (MSYq) lead to teratozoospermia and in severe cases to infertility. We find that the downstream transcriptional changes in the testis resulting from the loss of MSYq-encoded transcripts involve upregulation of multiple X- and Y-linked spermatid-expressed genes, but not related autosomal genes. Therefore, this indicates that in normal males, there is a specific repression of X and Y (gonosomal) transcription in post-meiotic cells, which depends on MSYq-encoded transcripts. Together with the known sex ratio skew in favour of females in the offspring of fertile MSYqdel males, this strongly suggests the existence of an intragenomic conflict between X- and Y-linked genes. Two potential antagonists in this conflict are the X-linked multicopy gene *Xmr* and its multicopy MSYq-linked relative *Sly*, which are upregulated and downregulated, respectively, in the testes of MSYqdel males. *Xmr* is also expressed during meiotic sex chromosome inactivation (MSCI), indicating a link between the MSCI and the MSYq-dependent gonosomal repression in spermatids. We therefore propose that this repression and MSCI itself are evolutionary adaptations to maintain a normal sex ratio in the face of X/Y antagonism.

INTRODUCTION

Several naturally occurring deletion models exist with losses of part or all of the male-specific region of the Y-chromosome long arm (MSYq) in mouse. These models show a range of teratozoospermia and infertility phenotypes with severities correlated to the extent of the deletion (1–7). Intriguingly, the models with less severe deletions, which retain fertility, show a sex ratio skew in favour of females in the offspring of mutant males (2). The gonosomes are of great importance in spermatogenesis and are pre-disposed to accumulate genes with roles in this process. Enrichment for spermatogenic genes on the X is likely to be due to hemizygous uncovering of male-benefit recessive alleles (8), whereas enrichment on the Y is due to recruitment and retention of male-benefit genes against a background of ongoing genetic degeneration resulting from its non-recombining status (9–13). However, the X gene complement is also affected by the consequences of meiotic sex chromosome inactivation (MSCI), leading to a deficiency of meiosis-expressed genes on the X (14).

Previous work has identified some of the morphological and hormonal processes affected in the various MSYq-deleted phenotypes, notably abnormal vesicle formation in the developing acrosome (15), a flattened sperm head shape (1–4,6) and overexpression of aromatase in spermatids and Leydig cells (16). Progress has also been made in identifying the MSYq-linked transcripts, deficiency of which is likely to be the primary cause of the spermiogenic abnormalities (2,17–19). In the course of our microarray analysis that identified reduction or loss of MSYq-encoded testis transcripts in MSYq-deficient mice (Touré *et al.*, submitted for publication), we also identified a number of downstream transcriptional changes, which we have verified by northern blotting and real-time RT–PCR. Surprisingly, as we report here, the majority of these changes involve an increase rather than a decrease in transcript levels, the cellular and chromosomal distribution of these transcripts indicates a specific derepression of X- and Y-chromosome transcription in post-meiotic germ cells of the mutant mice.

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RESULTS

Our initial analysis of the data from the microarray screen of three different MSYqdel models (Fig. 1) served to provide a more comprehensive picture of the reduction in MSYq-encoded testis transcripts resulting from the deletions. Our further analysis of these models has focussed on the other (downstream) transcriptional changes. In order to distinguish the primary consequences of MSYq deletion from secondary degenerative changes in the mutant testes, we extended the study to include an earlier time point for the X*Sxr*^{a}Y^{*X} model, which has the most severe deletion phenotype. Microarray hybridizations and analysis were carried out as described previously (20), except that microarray data normalization was based on the global median signal for Cy3 and Cy5 channels rather than on a panel of control genes. This form of normalization is valid because the majority of genes do not vary between the mutant and control samples for any of the models (Fig. 2). Array data were then filtered to select genes showing a 1.5-fold change and 1% *t*-test relative to control in both the 1 month and 2 month X*Sxr*^{a}Y^{*X} samples.

Strikingly, these downstream changes predominantly comprise increases in transcript levels, 23 genes being upregulated compared with only two being downregulated. This thus signifies that in normal males, the main effect of MSYq-linked transcription is a repression in transcript levels of a set of downstream genes. Even more strikingly, most (15/23) of the upregulated transcripts are X- or Y-linked, whereas both downregulated transcripts are autosomal (Fig. 2; Table 1). Mapping to X, Y or autosome was established by southern blotting in the cases where a definitive mapping was not already assigned for a given transcript (Supplementary Material, Fig. S1). Comparison with previous array data (20) indicated that the upregulated genes were all likely to be expressed in spermatids in normal males on the basis of their profile across the first wave of spermatogenesis and level of expression in four germ cell depleted models. The expression distribution of several of the most highly affected genes was confirmed in normal testes by *in situ* hybridization (Fig. 3) (full staging diagrams available as Supplementary Material, Fig. S2), and their upregulation in the MSYqdel models was confirmed by northern blotting (Fig. 4). Interestingly, one of the X-linked genes examined by ISH (*AK006251*, Fig. 3E) proved to be expressed in late pachytene spermatocytes as well as in spermatids, indicating that this gene is not silenced during MSCI.

Further *in situ* analysis (Supplementary Material, Fig. S3) showed that the expression distribution was unchanged in testes of the nine-tenth MSYqdel and X*Sxr*^{a}Y^{*X} models, indicating that the observed upregulation was due to elevated levels of transcription in spermatids (spermatocytes in the case of *AK006251*), rather than expression in different cell types. Analysis of the chromosomal distribution of the arrayed libraries in conjunction with our prior data (20) showed that the sex chromosome bias of the upregulated clones was not due to bias in the underlying libraries or due to bias in the haploid-expressed gene clusters (data not shown). Many of the genes identified by the microarray screen are members of highly homologous gene families, and thus hybridization-based assays, such as northern blotting, microarray expression profiling and ISH, are insufficient to unpick the complexity of changes in transcript abundance. Therefore, we performed an extensive series of real-time PCR experiments to precisely distinguish between near relatives. The panel of genes investigated comprised:

- every gene found to be significantly upregulated in the microarray screen, along with all further known X-, Y- or autosomal homologues;
- all known MSYq-linked genes and X-homologues, and four further sets of X–Y homologous genes mapping to the short arm of the Y-chromosome (thus outside the extent of the deletion);
- *Ckt1* and relatives, which gave inconsistent results in the microarray screen and thus were not counted in the 23 genes found as significant by the array screen;
- a panel of control genes chosen to include autosomal housekeeping genes, X-linked non-spermatid genes and autosomal spermatid-specific genes;
- aromatase and the androgen receptor. The former has been reported to be upregulated in spermatids of B10.BR-Ydel males bearing a two-third MSYq deletion (16), and the latter is an X-linked component of the androgen response pathway.

Primer pairs for all genes are listed in Supplementary Material, Table S1. The results of the RT–PCR survey are shown in Table 2 and are plotted in Figure 5A, demonstrating the upregulation of multiple X- and Y-linked transcripts as well as downregulation of MSYq-linked transcripts. The 10 control genes included in the survey showed slight or no significant change in expression levels in the three MSYqdel models relative to normal mice; this was also true for aromatase and the X-linked androgen receptor. Eight of the genes indicated as upregulated by the array screen (*Testatin, AK086357, NM_198677, AK007033, BY366152, XM_146592, AK006132* and *Tm4sf6*) were not confirmed by RT–PCR. These false positives showed no significant chromosomal bias, seven being autosomal and only one being X-linked. Discounting these leaves an even higher degree (14/15 genes) of sex chromosome linkage among the genes confirmed as upregulated by both techniques. Figure 5B–G highlights further RT–PCR results of particular interest.

AK005922 and *XM_358268* are upregulated, whereas an autosomal homologue is not (Fig. 5B)

The most highly upregulated X-linked gene in both the initial microarray screen and the RT–PCR follow-up was *AK005922*, an intronless gene containing histone-fold TAFII domains, present in multiple copies mapping close to the X-centromere. BLAST searches revealed a single-copy autosomal relative (*AK018839*) and several Y-linked copies (e.g. *XM_358268*), all further family members also being intronless. According to contig NT_078925.3 from mouse genome build 34.1, the Y-members are located on Yp, 200 kb distal to the *Rbmy* cluster, between *Rbmy1a1* and *Sry*. Importantly, transcripts from the X- and Y-linked copies were upregulated, whereas those from the autosomal copy were not.

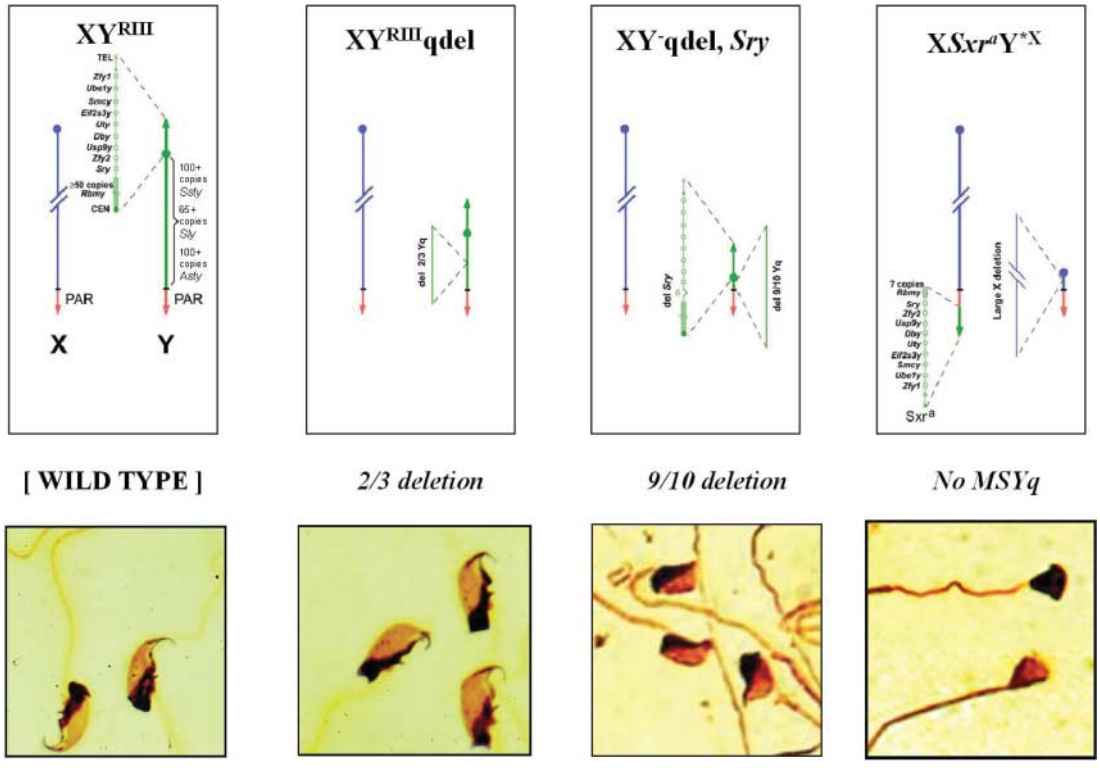

Figure 1. Diagrams illustrating the sex chromosome complements and sperm phenotypes of normal male mice and the mice with MSYq deficiencies. The short arm of the normal Y-chromosome (shown expanded) carries seven single copy genes, one duplicated gene (*Zfy*) and multiple copies of *Rbmy*. MSYq carries multiple copies of the *Ssty, Sly* and *Asty* gene families. Y^{-} denotes the fact that the Y in this MSYq deletion mutant also carries a Yp deletion removing the testis determinant *Sry*. This is complemented by an autosomally located *Sry* transgene. X*Sxr*^{a} denotes an X-chromosome bearing the Y short arm-derived factor Tp(Y)1Ct$^{Sxr\text{-}a}$ (hereafter *Sxr*^{a}) attached distal to the PAR. Further details of the genotype and phenotype of the deleted models are given in Materials and Methods. TEL, telomere; CEN, centromere and PAR, pseudoautosomal region.

Mgclh is upregulated, whereas the autosomal *mgcl-1* is not (Fig. 5C)

The second most highly upregulated X-linked gene in both the microarray and RT–PCR studies was *mgclh*, an intronless, multicopy, centromeric, homologue of *Drosophila gcl* (germ cell-less). *gcl* is a maternal-effect transcriptional repressor necessary for specifying germ plasm in the developing embryo (21–23). There is a second, autosomal homologue of *gcl* in the mouse, *mgcl-1* (24–27), an intron-containing gene located on chromosome 2, which shares 45% amino acid identity and 62% similarity with *mgclh* over the entire length of the coding region, suggesting that the two genes have a similar function. Once again, the autosomal homologue is not upregulated.

Xmr and *AK015913* are upregulated, whereas Sly is downregulated (Fig. 5D)

Xmr and relatives are a highly multicopy gene family with an estimated 50–75 copies on each of the X- and Y-chromosomes (28). The founder member of the family, *Xlr*, was identified as encoding a lymphocyte antigen (29) and another member of the family, *Xmr*, was subsequently shown to encode a germ cell antigen which binds to the nucleolus and the asynapsed arms of the X- and Y-chromosomes during MSCI (30–32). The Y-linked copies, founder member *Sly* [Mouse Chromosome Y Mapping Project (Jessica E. Alfoldi, Helen Skaletsky, Steve Rozen and David C. Page at the Whitehead Institute for Biomedical Research, Cambridge MA, USA and the Washington University Genome Sequencing Center, St Louis, MO, USA)], map to MSYq and produce a transcript expressed specifically in spermatids; this transcript is consequently reduced or absent in the MSYqdel models. We find that *Xmr* and the closely related X-linked *AK015913* are significantly upregulated in the MSYqdel models. *Xlr* was included in the panel for the sake of completeness, but is not thought to be expressed in any of the germ or supporting cell lineages. Absolute Ct values for this gene were high, and none of the ratios to control was significant. The high Ct values indicate very low transcript abundance, which may be derived from lymphocytes in testicular blood vessels.

Surprisingly, our ISH results indicate that *Xmr, AK015913* and *Sly* are strongly expressed in spermatids in a tubule stage-specific manner (Fig. 3). The primers used to assay *Xmr* expression in our study show a marked increase in transcript abundance between testes at day 15 and day 23 postpartum (data not shown), again consistent with spermatid expression. This runs counter to immunofluorescence-based reports (30,32) that *Xmr* expression is specific to meiotic stages. It may be that the *Xmr*-related transcripts in spermatids

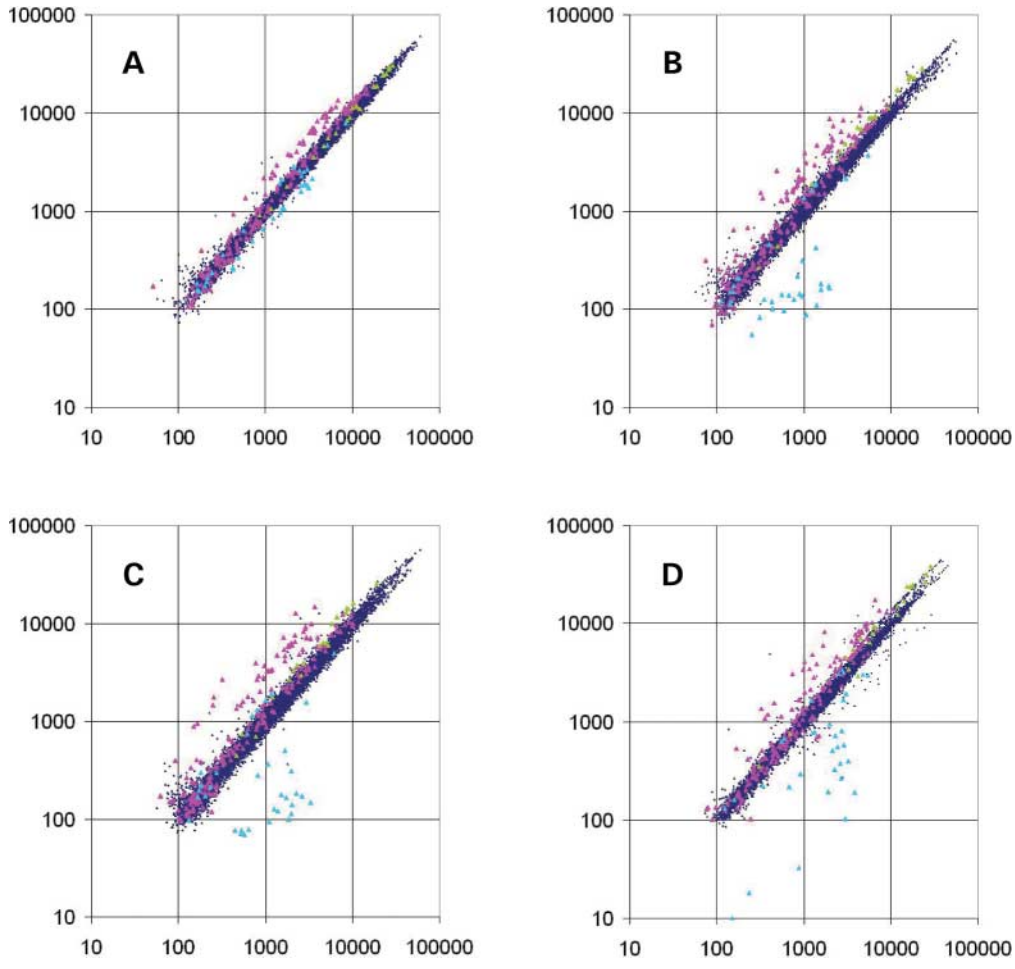

Figure 2. Array analysis shows a bias in the chromosomal location of regulated genes. Scatter plots show gene expression levels in the testis of mutant relative to control genotypes as measured by microarray hybridization. Following normalization and averaging of technical replicates, the measured fluorescence (arbitrary units) for the control genotype is plotted on the *x*-axis and for the mutant genotype on the *y*-axis in each panel. (**A**) XY^{RIII} versus XY^{RIII}qdel, (**B**) XY^{-} versus XY^{-}qdel, (**C**) XY^{RIII} versus $XSxr^{a}Y^{*X}$ and (**D**) XY^{RIII} versus $XSxr^{a}Y^{*X}$. (A–C) analysed at 2 months post-partum and (D) at 1 month post-partum. ▲, autosomal clones; ▲, X-linked clones; ▲, Y-linked clones and ▲, mitochondrial clones.

are not translated or that the epitope recognized by the antibody used in these studies is absent or inaccessible in the family members expressed in spermatids. It is clear from the northern blot data in the original article of Calenda *et al* (30) that a great increase in *Xmr*-related transcript abundance occurs in the testis between post-natal weeks 2 and 3 and 5, consistent with the majority of these transcripts being spermatid derived.

Ckt1 and X-linked relatives are upregulated, whereas the autosomal *Ckt1r2* is not (Fig. 5E)

Ckt1 and *Cktr1-3* are a family of closely related genes identified as differentially phosphorylated spermatid-specific targets of *Ck2* (X. Xu *et al.*, unpublished data, direct submission to GenBank) and have since been shown to be a component of the post-acrosomal perinuclear theca (33). *Ckt1r2* is the only autosomal member of the family and is intronless (thus most likely a retroposed copy), whereas the other X-linked family members all contain a single intron. Again, it is X-linked members of the family that are specifically upregulated.

X-linked members of a novel multigene family are selectively upregulated (Fig. 5F)

Expressed sequence tags, *AK005630, AK006132, AK005817* and *XM_141928*, together comprise a multigene family with two subfamilies. *AK006132* is intronless, appears to derive from a retroposed copy of *AK005630* and maps to chromosome 12. The other three family members are X-linked and may be present in multiple copies. All three X-linked transcripts are significantly upregulated in the MSYqdel models, whereas the autosomal relative is not. All four transcripts potentially encode proteins, but there were no significant motifs found when the putative protein sequences were searched against the PROSITE database. It may be significant that all family members except *AK005630* contain potential phosphorylation sites for *Ck2*.

Table 1. Transcripts found to be significantly altered in the initial microarray screen and their chromosomal location

Gene	Accession	Map
Upregulated transcripts		
	AK005922	X
Mgclh	*AB055854*	X
Xmr	*NM_009529*	X
	AK015913	X
	AK005630	X
	AK005817	X
	XM_141928	X
	AK006132	12
	AK015451	X
	AK006251	X
	AA183442	X
	AK015086	X
Tgifx1	*NM_153109*	X
	XM_141720	X
Tm4sf6	*NM_019656*	X
	AK006152	Y
Testatin	*NM_009979*	2
Grhpr	*NM_080289*	4
	XM_146592	Autosomal
	NM_198677	Autosomal
	AK007033	Autosomal
	AK086357	7
	BY366152	17
Downregulated transcripts		
	AK006749	10 C1
	AK010292	8 C2

This list focusses on downstream effects of MSYq deletion, thus the set of downregulated transcripts excludes genes known to be contained in the deletion intervals (*Ssty1, Ssty2, Sly, Asty and Rbmy1a1*).

Other X- and Y-linked genes show varying degrees of upregulation (Fig. 5G)

Upregulation of the Y-linked *AK006152* was confirmed. A further four Yp-linked genes were assayed along with their X- and autosomal homologues. Of these, *Rbmy1a1* showed significant downregulation in X*Sxr*^{a}Y^{*X}, confirming the partial deletion of this multicopy gene in this genotype. *Rbmx* and *Usp9x/y* showed no clear pattern of variation between genotypes; however, both *Ube1x* and *Ube1y* were upregulated, the extent of the upregulation increasing with deletion size and being significant in the X*Sxr*^{a}Y^{*X} model. None of *Zfy1, Zfy2, Zfx* or *Zfa* showed significant upregulation.

DISCUSSION

The aim of this study was to investigate the downstream transcriptional consequences of deletions on mouse MSYq. In addition to the loss of MSYq-linked transcripts, we found a moderate upregulation of several genes in the spermatids of MSYqdel males. These upregulated genes may contribute to the testicular phenotypes seen in the MSYq deletion models. In particular, *mgclh* is closely homologous to genes thought to function in sperm head development in mouse and in human (34,35), and the upregulated genes also include several known or potential substrates of the kinase *Ck2*, which is known to regulate sperm head morphogenesis (36).

We further observed that the upregulated genes were predominantly X- and Y-linked and confirmed this in three separate instances where there are closely homologous X and autosomal genes and in one instance where X, Y and autosomal copies exist. In every case, the X- and Y-genes were significantly upregulated, whereas the autosomal homologues were not. Therefore, this indicates that there is a specific derepression of gonosomal genes in mutant spermatids associated with a loss of MSYq-encoded transcripts. Among the genes known to lie within MSYq, the best candidate for a role in XY regulation in spermatids is *Sly*. *Sly* is the most highly transcribed of the known MSYq genes, all of which are spermatid specific. The putative *SLY* protein contains a COR1 chromatin-binding domain, and the X-linked relative *Xmr* produces a protein which associates with the transcriptionally repressed X- and Y-chromosomes in pachytene spermatocytes during MSCI (30,31). This suggests that *Sly* may be targeted to the gonosomes in spermatids and may regulate gonosomal chromatin conformation and expression.

Although *Sly* is downregulated in the MSYqdel models, its X-linked homologues, *Xmr* and *AK015913*, are upregulated in these models. Together with the highly expanded copy numbers of these genes and the sex ratio skew in the offspring of XY^{RIII}qdel males, this suggests that there is an intragenomic conflict between the X- and Y-members of this gene family. Further evidence in favour of this '*Sly*/*Xmr* conflict theory' is given by a recent study of hybrid breakdown in *Mus musculus* × *M. molossinus* crosses (37). Haldane's rule suggests that hybrid sterility in males of interspecific crosses is due to unbalancing of sex ratio distorter genes and their suppressors (38). The hybrid breakdown in the *musculus*/*molossinus* cross leads to male-specific infertility with sperm head abnormalities highly similar to those seen in the nine-tenth MSYq deletion model addressed in this article. The sperm head phenotype was mapped to three QTL regions on the mouse X-chromosome, named *Spha1–3* (37). Of these, *Spha1* and *Spha2* map closely to the two largest complexes of *Xmr* sequences, whereas *Spha3* may also contain *Xmr* copies.

The potential for conflict between the X and the Y has long been advanced; however, strong selective constraints on offspring sex ratio dictate that any such conflict must inevitably be concealed by the evolution of suppressor alleles (39). The effects of the distorter/suppressor complexes are thus only seen when the suppressor is deleted (e.g. the MSYqdel models) or when the 'wrong' sets of drivers and suppressors are combined, as in the inter-subspecific cross discussed earlier. Under our *Sly/Xmr* conflict hypothesis, one or more of the X-linked transcripts upregulated in the mutant models are responsible for the sex ratio distortion in XY^{RIII}qdel, and the gene complement of MSYq has evolved to oppose the effect of the X-transcript and maintain a normal sex ratio. One candidate gene for the sex ratio distorter is *Xmr* itself; however, an alternative possibility is that the distorter gene is among the other upregulated X-linked loci, with the competitors *Xmr* and *Sly* being opposing regulators of this gene via an antagonistic action on sex chromatin.

The involvement of *Xmr* homologues also indicates that there may be a link between the MSCI and the gonosomal derepression seen in the mutant models, for example, a

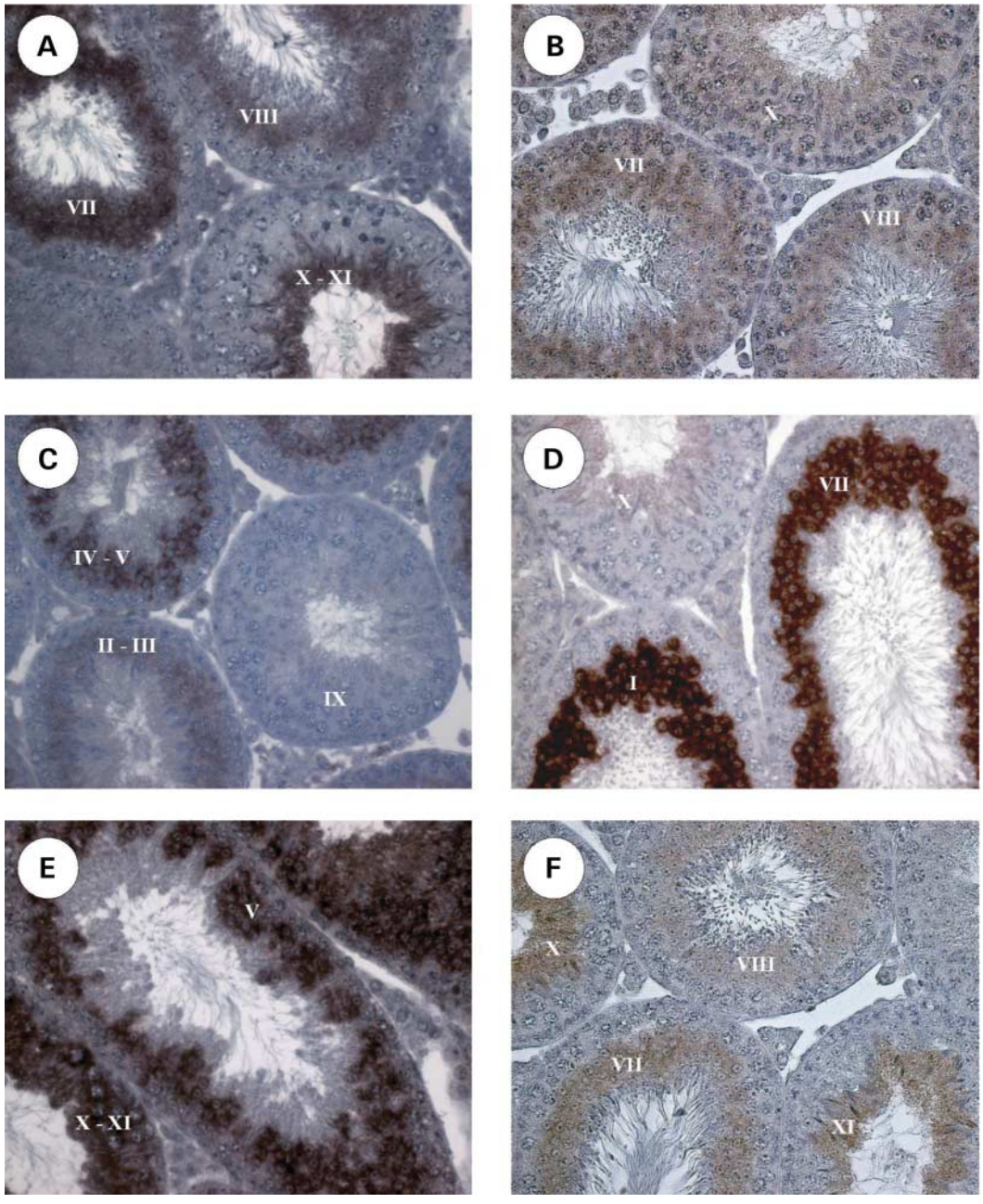

Figure 3. Expression of the upregulated X-linked transcripts is confirmed to be specific to spermatids and late stage spermatocytes. *In situ* localization of transcripts in testes of adult C57BL/6J mice. Roman numerals indicate tubule stage. **(A)** *AK005922* localizes to round and early elongating spermatids. **(B)** *mgclh* localizes to round and early elongating spermatids. **(C)** *AK015913* localizes to round spermatids. **(D)** *Xmr* localizes to round and early elongating spermatids. **(E)** *AK006251* localizes to pachytene spermatocytes, round and early elongating spermatids. **(F)** *Tm4sf6* localizes to round and early elongating spermatids.

persistence of the meiotically silenced state into spermatids, which is modulated by the action of *Xmr, Sly* and other family members. Such a persistence has been suggested by Huynh and Lee (40) as a means of providing a 'pre-inactivated' X for dosage compensation in the early embryo. More recently, it has been shown that the X- and Y-chromosomes and all non-pairing DNA are silenced during meiosis in mouse, and MSCI may be a special case or elaboration of this more general phenomenon (41–43). Many of the upregulated genes discussed in this article are present in multiple copy ampliconic regions (e.g. *AK005922, mgclh, Xmr* and *AK015913*) or multigene families (*Ckt* and relatives and *AK005630* and relatives), and some of those that are not multicopy are nevertheless contained within palindromes (e.g. *Tgifx1*) (44). Both the X- and Y-chromosomes have been shown to be enriched for such palindromic repeat regions (44–46). It is possible that these repetitive regions can self-synapse during pachytene, thus escaping recognition as unpaired DNA. DNase I hypersensitive regions (indicative of active chromatin conformation) have been observed on

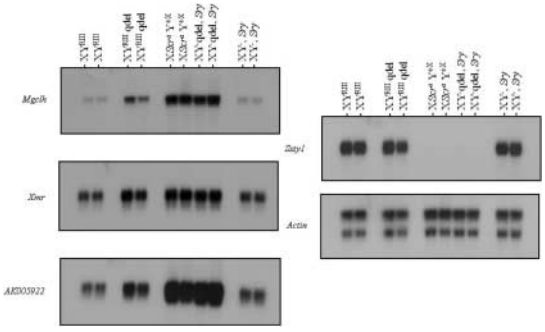

Figure 4. Upregulation of key X-linked genes in the MSYqdel models is confirmed. Northern blot of total testis RNA probed with clones for *AK005922*, *mgclh* and *Xmr*. An actin probe was used as a loading control, and a probe for *Ssty1* was used to demonstrate the loss of MSYq sequence in the deleted genotypes. Hybridization to *AK005922* and *mgclh* probes clearly shows a dose-dependent response with the degree of upregulation depending on the degree of MSYq deficiency. *Xmr* is more evenly upregulated in the MSYq-deleted models; however, the microarray and RT–PCR results again show a degree of dose dependency.

the otherwise silenced X and Y during pachytene, and this hypersensitivity persists through to at least metaphase 1 (47). The recent observation that *Xmr* also binds to the tandem repeat arrays of ribosomal genes in the nucleolus during MSCI may also have a bearing on this issue (32). If repetitive regions (whether palindromic or direct repeats) are processed differently during pachytene, this may explain why some X-genes (*AK006251, Xmr*) are expressed in spermatocytes, and also why some X-genes are able to activate expression in spermatids, whereas others remain silent following MSCI.

Irrespective of the precise mechanism involved, our observations demonstrate a link between breakdown of gonosomal silencing and sex ratio abnormalities, indicating that one function of this silencing is to prevent transmission distortion by selfish elements. We therefore propose that meiotic silencing of unpaired DNA is a general genomic defence mechanism against transmission distorters and other selfish elements, which acts in the form of MSCI to maintain a normal sex ratio in the face of X/Y antagonism. MSCI thus has the effect of 'brokering a truce' between warring X- and Y-genomes. However, the proposed involvement of the mouse-specific *Xmr* gene (and relatives) with gonosomal regulation means that caution must be exercised in generalizing observations in the mouse to other species, especially concerning gonosomal regulation in spermatids.

If silencing of unpaired DNA is indeed an adaptation for genomic defence, it is tempting to see the largely heterochromatic nature of the mouse Y-chromosome long arm as a more extreme manifestation of the same type of process. Recruitment of heterochromatin may provide a longer term means of achieving the silencing reiterated in each generation by MSCI, or it may be a secondary process occurring at sites where selfish elements and/or their repressors have become amplified. In this regard, the extensive heterochromatic blocks on other Y-chromosomes (including the human Y) may be viewed as representing 'genomic scars' left at the site of past intragenomic battles, the mouse Y long arm revealing an intermediate stage in the process of Y degeneration and heterochromatinization.

MATERIALS AND METHODS

Mice

All mice were produced on a random-bred albino MF1 strain (NIMR colony) background. The mice used to provide RNA for microarray analysis were the three MSYq-deficient genotypes that we have previously analysed with respect to *Ssty, Sly* and *Asty* expression and sperm abnormalities, together with appropriate age- and strain-matched controls.

(1) XY^{RIII}qdel males (approximately two-third MSYq deletion). These mice have an RIII strain Y-chromosome with a deletion removing approximately two-thirds of MSYq. The sperm has mild distortions of head shape; the mice are nevertheless fertile with an intriguing distortion of the sex ratio in favour of females (2). XY^{RIII} males are the appropriate controls.
(2) XY^{Tdym1}qdel*Sry* males (approximately nine-tenth MSYq deletion). These mice have a 129 strain Y-chromosome with a deletion removing approximately nine-tenths of MSYq and also a small deletion (Tdy^{m1}) removing the testis determinant *Sry* from the short arm, this deficiency being complemented by an *Sry* transgene. These males are sterile with virtually all the sperm having grossly distorted heads (6). XY^{Tdym1}*Sry* males are the appropriate controls. Y^{Tdym1} is referred earlier as Y^{-}.
(3) X*Sxr*$^{a}Y^{*X}$ males (no MSYq). In these mice, the only Y-specific material is provided by the Y short arm-derived factor *Sxr*^{a}; the Y^{*X}-chromosome provides a second PAR, thus allowing fulfilment of the requirement for PAR synapsis. These males lack the entire Y-specific (non-PAR) gene content of MSYq; they also have a 7.5-fold reduction in copies of the *Rbmy* gene family located on the short arm adjacent to the centromere. The males are sterile and have even more severe sperm head defects than XY^{-}qdel*Sry* males (1,6). As *Sxr*^{a} orginated from a Y^{RIII}-chromosome, the appropriate controls are again XY^{RIII}.

Sample collection

Testes were obtained from two 2-month-old XY^{RIII}qdel, XY^{-}qdel*Sry* and XSxr$^{a}Y^{*X}$ males, from three 1-month-old XSxr$^{a}Y^{*X}$ males and from equal numbers of age- and strain-matched controls. Total RNA was isolated using TRI reagent (Sigma) and cleaned using RNEasy columns (Qiagen), according to the manufacturers' protocols.

Array analysis

Microarray hybridizations and analysis were carried out as described (20), except that microarray data normalization was based on the global median signal for Cy3 and Cy5 channels, rather than on a panel of control genes, and intensity-dependent Lowess correction was applied. This form of normalization is valid because the majority of genes do not vary between the mutant and control samples for any of the models (Fig. 2). Normalization, *t*-tests and fold-change filtering were performed using GeneSpring version 4.2.1. Genes

Table 2. ΔΔCt values for control relative to mutant genotypes for all genes tested in the RT–PCR study

		ΔΔCt for control relative to mutant genotypes					ΔΔCt for control relative to mutant genotypes		
Gene	Map	XY^{RIII}qdel	XY^{-}qdel, *Sry*	$XSxr^{a}Y^{*X}$	Gene	Map	XY^{RIII}qdel	XY^{-}qdel, *Sry*	$XSxr^{a}Y^{*X}$
X–Y genes					Other regulated genes				
Ssty1[a]	Yq	−0.555	**−12.775**[b]	**−14.095**[b]	***AK015451***[a]	X	0.695	**2.325**[b]	**3.005**[b]
Ssty2[a]	Yq	**−1.905**[c]	**−10.725**[b]	**−15.595**[b]	***AK006251***[a]	X	**1.595**[c]	**2.025**[c]	**2.555**[b]
Sstx	X	−0.105	1.575	1.655	***AA183442***[a]	X	0.745	**1.625**[b]	**1.355**[b]
Sly[a]	Yq	**−1.505**[b]	**−7.175**[b]	**−16.895**[b]	***AK015086***[a]	X	**0.995**[c]	**2.075**[b]	**1.955**[b]
Xlr	X	−0.355	1.275	1.855	***Tgifx1***[d]	X	1.445	**2.075**[c]	**2.205**[c]
Xmr[d]	X	0.295	**2.225**[c]	1.655	***XM_141720***[d]	X	1.195	**2.075**[c]	**2.955**[c]
AK015913[d]	X	1.045	**1.875**[c]	**1.905**[c]	*Tm4sf6*	X	−0.055	1.225	0.155
Asty[a]	Yq	**−2.255**[b]	**−4.875**[b]	**−6.595**[b]	***AK006152***[d]	?	0.295	**1.475**[c]	**1.555**[c]
Astx[a]	X	−0.105	**0.775**[c]	−0.095	***Testatin***[a]	2	**−3.555**[b]	0.925	−0.145
Rbmy1a1[a]	Yp	0.045	0.575	**−2.395**[c]	***Grhpr***[d]	4	0.245	1.075	**1.605**[c]
Rbmx	X	−0.105	0.525	0.905	***AK086357***[d]	7	−0.405	−0.375	**−1.245**[c]
Usp9y[a]	Yp	−0.455	**−0.825**[c]	0.255	*XM_146592*	Auto	0.345	0.525	2.655
Usp9x	X	−1.255	0.725	0.055	*NM_198677*	Auto	0.595	0.525	−0.245
Ube1y[d]	Yp	−0.605	0.775	**1.255**[c]	*BY366152*	17	−0.055	0.175	0.505
Ube1x	X	0.795	1.325	**2.705**[c]	*AK007033*	Auto	−0.105	1.275	1.305
XM_358268[a]	Yp	−0.855	0.575	**2.005**[b]					
AK005922[a]	X	0.145	**2.675**[c]	**3.755**[b]					
AK018839[d]	2	−0.655	−0.575	0.705	Hormonal pathways				
Zfy1	Yp	−1.005	−0.725	−0.595	*AR*	X	−0.205	0.925	1.555
Zfy2	Yp	−0.405	1.725	1.455	*Aromatase*	9	0.495	−0.275	1.155
Zfx	X	−0.655	0.275	0.955					
Zfa	10	−0.355	−0.025	0.905					
X-autosomal genes					Controls				
Ckt1[d]	X	0.145	1.325	**1.505**[c]	*Rbm3*	X	−0.655	−0.525	0.055
Ckt1r1[d]	X	0.495	1.075	**1.855**[c]	*Taf7l*	X	−0.455	−0.025	−0.145
Ckt1r2	9	0.545	0.625	0.355	*HPRT*	X	−0.755	−0.325	0.055
Ckt1r3	X	0.945	0.825	1.805	*Odf1*	15	0.045	**−0.625**[c]	−0.295
AK005630[d]	X	0.945	**1.525**[c]	**3.105**[c]	*Shippo1 (Odf3)*	7	−0.505	**−0.725**[c]	−0.445
AK005817[d]	X	0.145	**1.875**[c]	1.405	*Tnp1*	1	0.845	0.325	−0.045
XM_141928[a]	X	0.295	**3.025**[b]	**2.805**[b]	*Prm2*	16	0.245	−0.225	−0.595
AK006132	12	0.545	0.925	1.505	***Ubb***[a]	11	**0.745**[b]	**0.325**[c]	−0.195
Mgclh[a]	X	0.995	**3.225**[c]	3.505	*Cyclophilin*	11	−0.005	1.025	0.555
mgcl-1	6	0.345	0.025	1.355	*Gapdh*	6	0.495	0.775	1.055

Positive values indicate overexpression in the mutant and negative values indicate underexpression in the mutant. Significant results are highlighted in bold.
[a]$P < 0.01$ following ANOVA of five genotypes.
[b]Dunnett *post hoc* test, significant at 99% CI.
[c]Dunnett *post hoc* test, significant at 95% CI.
[d]$P < 0.05$ following ANOVA of five genotypes.

were designated as significant if they showed a 1.5-fold change and a 1% *t*-test relative to control in both the 1 month and 2 month $XSxr^{a}Y^{*X}$ samples (Table 1). Full experimental protocols and raw data have been deposited in the public access ArrayExpress database, accession no. E-MEXP-251.

Real-time RT–PCR

Real-time RT–PCR was performed in 96-well plates using the QuantiTect SYBR Green RT–PCR kit (Qiagen) according to the manufacturer's protocols, quantifying the resulting fluorescence using an iCycler system (BioRad, UK). Thirty nanograms of total RNA was used for each RT–PCR reaction. RNA was DNase-treated before RT–PCR, and primer pairs (Supplementary Material, Table S1) were designed to span an intron boundary and to include at least one intron wherever possible. The identity of all RT–PCR products was confirmed by sequencing.

We analysed XY^{RIII}qdel, XY^{-}qdel, *Sry*, $XSxr^{a}Y^{*X}$, XY^{RIII} and XY^{-}, *Sry* genotypes, all at 2 months of age. Duplicate RT–PCR reactions for each gene were performed for each genotype, and *Ubb* was included on every plate as a loading control, along with appropriate negative controls. A 'crossing-point' Ct was obtained for each well, being the fractional cycle number at which the measured fluorescence crossed the threshold of 100 U, a value chosen such that all reactions in all plates were in log phase. Differential loading was controlled on a per-genotype per-plate basis by subtracting the mean Ct for the loading control *Ubb* to give ΔCt. ΔΔCt values were then calculated as the change in ΔCt for the MSYqdel genotypes relative to the appropriate control genotype. Finally, the complete expression profiles for each genotype were normalized to the mean ΔΔCt of the 10 control genes included in the panel. This last step was necessary as the fold changes in transcript abundance are small, and a panel of control genes forms a more robust basis for comparison than the single gene *Ubb*. Raw Ct values and all stages of

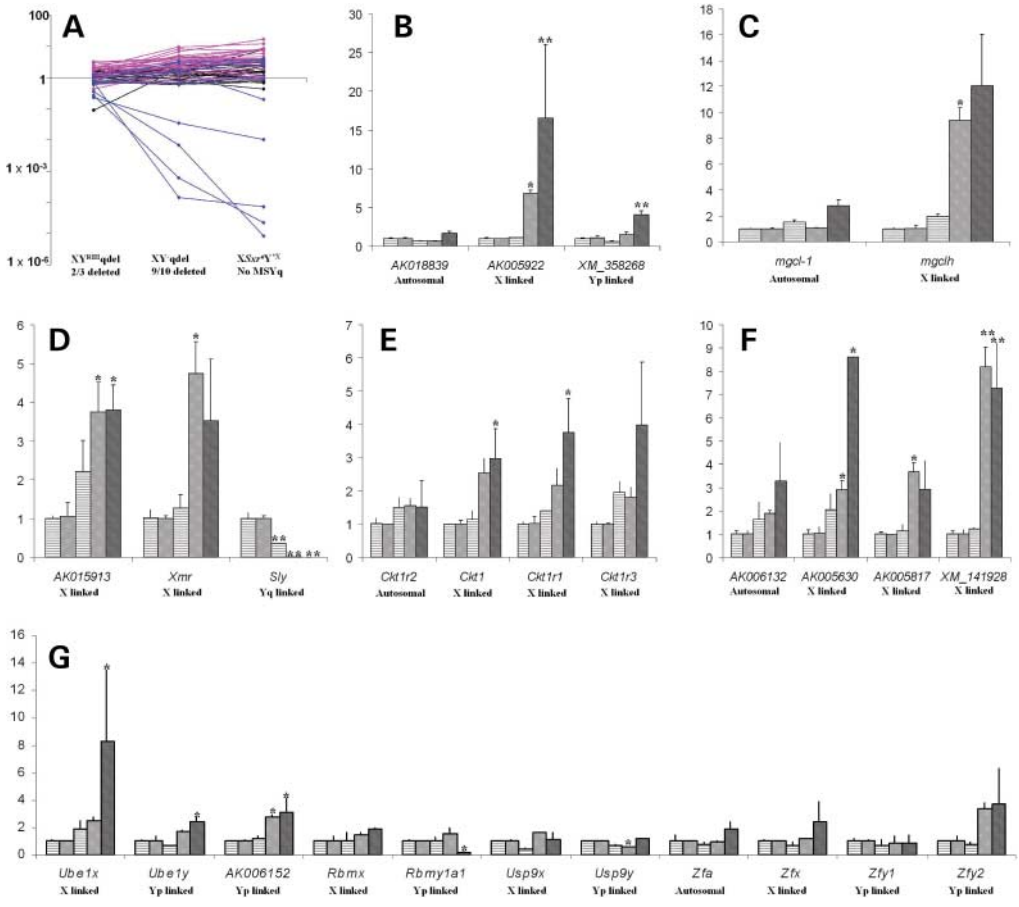

Figure 5. RT–PCR analysis shows specific upregulation of X- and Y-linked members of multigene families. Values plotted are $2^{-\Delta\Delta Ct}$. (**A**) Line plot showing all genes surveyed. A log axis is used to demonstrate the dramatic drop in expression level of MSYq-encoded transcripts. Blue, Y-linked transcripts; pink, X-linked transcripts and black, autosomal transcripts. Remaining panels are bar charts showing $\Delta\Delta Ct \pm SEM$ for (**B**) *AK005922* and relatives, (**C**) *mgclh* and *mgcl-1*, (**D**) *Xmr* and relatives, (**E**) *Ckt1* and relatives, (**F**) *AK005630* and relatives and (**G**) further X–Y homologous genes and the new Y-linked transcript *AK006152*. *Dunnett *post hoc* test, significant at 95% CI (**99%). ▨, XY^{RIII} expression (control for XY^{RIII}qdel and $XSxr^{a}Y^{*X}$); ▨, XY^{-}*Sry* expression control for XY^{-}qdel, *Sry*); ▨, XY^{RIII}qdel expression; ▨, XY^{-}qdel, *Sry* expression; ▨, $XSxr^{a}Y^{*X}$ expression.

normalization are shown in Supplementary Material, Table S2. Significance testing was performed using Analyse-It software to perform one-way analysis of variance of the five genotypes (three mutants and two controls) within Microsoft Excel, followed by Dunnett *post hoc* testing of the mutant/control comparisons.

RNA *in situ* analysis

In situ hybridization using digoxigenin-labelled cRNAs was used to localize each mRNA on Bouin's fixed paraffin-embedded mouse testis sections using procedures previously described (48) with hybridization and washing temperatures up to 55°C. Both antisense and sense (negative control) cRNAs were used on each sample, in every experiment, for each set of conditions tested.

Northern blot analysis

Clones for *AK005922, mgclh* and *Xmr* were used to perform northern blot analysis. An actin probe that recognizes α- and β-actin transcripts (49) served as a loading control. Total RNA (20 μg) was electrophoresed in a 1.4% formaldehyde/agarose gel and transferred to Hybond-N membrane (Amersham) using 20× SSC buffer. The RNA was cross-linked to the membrane with ultraviolet (StrataLinker™), the membrane fixed for 1 h at 80°C and hybridized overnight at 60°C with ^{32}P-labelled probes in hybridization buffer (6× SSC, 5× Denharts, 0.1% SDS, 50 mM sodium phosphate, 100 μg/ml salmon sperm DNA). After two 60°C washes (30 min 0.5× SSC, 0.1% SDS and 30 min 0.1× SSC, 0.1% SDS), the membrane was exposed to X-ray film for 5–6 h except for the actin filter which was exposed overnight.

Southern blot analysis

Southern blot analysis was performed for the unmapped transcripts *AK006152, XM_146592, NM_198677* and *AK007033*. Genomic DNA was extracted from normal XX and XY mouse livers and 12 μg aliquots of each sample digested to completion with three different restriction enzymes, *Eco* R1,

Taq$^{\alpha}$1 and *Sst*1 (NEB). Results for digestion with *Taq*$^{\alpha}$1 are shown in Supplementary Material, Figure S1. Digested samples were electrophoresed through 0.8% TAE agarose gel, depurinated, denatured and transferred to Hybond-N nylon membranes (Amersham). Membranes were baked at 80°C for 2 h to fix the DNA, followed by overnight hybridization of ^{32}P-labelled probes at 65°C in Church buffer. Wash step: 15 min at 65°C in 2× SSC, 0.1% SDS. Membranes were exposed to X-ray film at −80°C for times ranging from 3 h to 2 weeks.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank Aine Rattigan for PCR genotyping and Anthony Brown, David Carter and Rob Furlong at the Department of Pathology Centre for Microarray Resources for printing and QC of microarray slides. J.M.A.T. is an MRC Career Development Fellow. The study was funded partly by BBSRC and the Wellcome Trust (E.J.C. and P.J.I.E.), the NHMRC of Australia (K.L.L. no. 143792) and the ARC (Paul S. Burgoyne and Kate L. Loveland).

Conflict of Interest statement. There are no conflicts of interest in regard to this manuscript for any of the authors.

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