Zfy genes are required for efficient meiotic sex chromosome inactivation (MSCI) in spermatocytes

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Abstract

During spermatogenesis, germ cells that fail to synapse their chromosomes or fail to undergo meiotic sex chromosome inactivation (MSCI) are eliminated via apoptosis during mid-pachytene. Previous work showed that Y-linked genes Zfy1 and Zfy2 act as 'executioners' for this checkpoint, and that wrongful expression of either gene during pachytene triggers germ cell death. Here, we show that in mice, Zfy genes are also necessary for efficient MSCI and the sex chromosomes are not correctly silenced in Zfy-deficient spermatocytes. This unexpectedly reveals a triple role for Zfy at the mid-pachytene checkpoint in which Zfy genes first promote MSCI, then monitor its progress (since if MSCI is achieved, Zfy genes will be silenced), and finally execute cells with MSCI failure. This potentially constitutes a negative feedback loop governing this critical checkpoint mechanism.

Introduction

In mammals, quality control during gametogenesis is far more stringent in males than in females, and consequently the vast majority of aneuploidies arise via non-disjunction during oogenesis, rather than spermatogenesis (1,2). The proximate mechanism for this difference is the efficient apoptotic elimination of developing gametes carrying univalent (unpaired) chromosomes that occur during male, but not female meiosis (3,4). This elimination involves two separate checkpoints: a checkpoint operating during mid-pachytene that monitors both the synapsis of homologous chromosomes and meiotic sex chromosome inactivation (MSCI) (5,6), and a spindle assembly checkpoint (SAC) operating at metaphase of the first meiotic division (7). Zfy genes have been implicated in the control of apoptosis at both of these checkpoints (8,9).

Zfy genes are Y-linked transcription factors conserved throughout eutherian mammals (10,11). In humans, a single ZFY gene expresses two major splice variants: a full length version which shows transactivation ability in a yeast reporter system (12), and a short version which lacks a key acidic domain.

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and has no detectable transactivation activity. In the mouse, there are two paralogous copies, Zfy1 and Zfy2: Zfy1 preferentially expresses the short (inactive) isoform, while Zfy2 predominantly expresses the long (active) isoform (13). Moreover, the transactivation activity of full-length Zfy2 is substantially higher than full-length Zfy1 (14), suggesting that the bulk of Zfy transactivation activity in the mouse is supplied by Zfy2.

Like all protein-coding genes in the non-pairing regions of the sex chromosomes, Zfy genes are subject to meiotic sex chromosome inactivation (MSCI) in pachytenic cells (15). During MSCI, the unsynapsed axes of the sex chromosomes become condensed and transcriptionally inactive, forming the ‘sex body’ marked by the phosphorylated form of histone H2AX (γH2AX) (16). When present as autosomal transgenes, Zfy/y genes evade MSCI and are expressed ectopically during pachytene. In the cases of both Zfy1 and Zfy2 this triggers germ cell apoptosis during stage IV of the spermatogenic cycle (8). Extensive germ cell apoptosis at the same tubule stage is seen in other models where MSCI, and in particular Y chromosome silencing is impaired (e.g. XY males, H2afx −/−, Hormad2 −/−) (8,17,18), indicating that successful silencing of Zfy1 and Zfy2 is an absolute requirement for pachytene progression. This has therefore been termed an ‘MSCI checkpoint’ mediated in part by overexpression of Zfy genes during pachytene (6,19). In many model systems with autosomal synapsis failure (e.g. Dmc −/−, Hormad1 −/−, Msh5 −/−, Spo11 −/−) there is substantial apoptosis during tubule stage IV which was initially interpreted as a ‘synapsis checkpoint’ paralleling the mid-pachytene synapsis checkpoint known to operate in yeast (5,20–23). However, it is now known that in these mouse models, the failure of synapsis leads secondarily to failure of MSCI (24), and hence the MSCI checkpoint and the synapsis checkpoint are now widely believed to reflect different aspects of the same process.

Turning to the spindle assembly checkpoint (SAC), a convenient model system to investigate the underlying mechanisms is the response to the univalent X chromosome in sex-reversed males at 17.5 dpp. At this age, the most advanced spermatocytes in the testes are in late pachytene or early diplotene, just prior to meiosis I and activation of the SAC checkpoint. Thus, it is before both the MI arrest and apoptosis seen in X(Y)O,Sry and the accumulation of arrested secondary spermatocytes in X(Y)O,Sry.

The microarray results unexpectedly showed a preferential up-regulation of X-linked transcripts and down-regulation of autosomal transcripts in response to Zfy deficiency (Fig. 1A). This held true regardless of whether we focused on statistically significant transcripts, or on all transcripts changing by more than 1.5 fold regardless of significance (Supplementary Material, Table S1).

To test whether the observed difference in X expression was related to the absence of Zfy genes in X(Y)O,Sry, or an off-target effect of the Zfy2 transgene in X(Y)O,Sry, we compared the expression data to previously-obtained data from 17.5 dpp normal XY males (Fig. 1B) (26). Although there was marked variation in average X expression between individual replicates at this age, there was a highly significant upregulation of the X chromosome in X(Y)O,Sry males relative to wild type XY males, and this was largely corrected in X(Y)Sry. There was a slight but significant residual X upregulation seen in X(Y)Sry relative to wild type XY males (p = 0.0013), which may be due to the additional presence of Zfy1 in wild type males.

To determine the normal cellular distribution of the transcripts altered by Zfy deficiency, we compared our results to published data from separated germ cell datasets [GSE4193] (27). A scatter plot of X(Y)O,Sry: X(Y)Sry log2 ratio (our new data) versus the pachytene: B spermatogonia log2 ratio (calculated from the Namekawa et al. dataset) shows that there is a good correlation between these measures (Fig. 3C, r² = 0.5373). That is, transcripts which in wild type males are more strongly expressed in pachytene spermatocytes than in B spermatogonia (high P:B ratio) are downregulated in response to Zfy deficiency, and vice versa. We conclude that in Zfy-deficient testes, there is a global upregulation of the X chromosome that is associated with a deficiency of pachytene-specific spermatocyte transcripts as a proportion of total testis RNA, and a corresponding excess of spermatogonia-specific transcripts.

MSCI ‘leakage’ in X(Y)O,Sry spermatocytes at 17.5 dpp is associated with apoptosis during pachytene

Since X-linked genes are in general transcriptionally active in spermatogonia and inactive in spermatocytes, we considered
whether the upregulation of X transcripts in X^E\text{O}, Sry could be entirely explained by the shift in the balance between spermato
gonial and spermatocyte transcriptomes. Importantly, X-linked
spermatogonia-specific transcripts were more strongly upregu
lated than autosomal spermatogonia-specific transcripts
(Supplementary Material, Fig. S1). This indicates that the in
crease in X-linked transcript abundance cannot be explained
solely by an increase in the proportion of spermatogonia in the
testis, since this would affect all spermatogonia-specific genes
irrespective of chromosomal location.

The combination of upregulation of X-linked genes and a
shift in the proportion of spermatogonia-specific vs pachytene-
specific transcripts was therefore strongly suggestive of MSCI
failure (5), since MSCI failure is expected to lead to germ cell

Figure 1. (see also Supplementary Material, Table S1). (A) Histogram showing the log2 expression ratio (+/- s.e.m) per chromosome for X^E\text{O}, Sry versus X^E,Z^2\text{O}, Sry, averaged across all genes expressed in testis at 17.5 dpp. * In the X^E\text{O}, Sry: X^E,Z^2\text{O}, Sry comparison, the X chromosome is significantly upregulated relative to the autosomes, \( P = 1.94 \times 10^{-12} \). (B) Line chart of log2 expression ratio per chromosome for X^E\text{O}, Sry and X^E,Z^2\text{O}, Sry relative to wild type XY, averaged across all genes expressed in testis at 17.5 dpp. Values are shown for each genotype as a whole, and for individual replicates within each genotype. Grey = autosomes, black = X chromosome. ** In the X^E\text{O}, Sry: XY comparison, the X chromosome is significantly upregulated relative to the autosomes, \( P = 2.59 \times 10^{-76} \). *** In the X^E,Z^2\text{O}, Sry: XY comparison, the X chromo
some is significantly upregulated relative to the autosomes, \( P = 0.0013 \). (C) Scatter plot showing the log2 ratio of pachytene spermatocyte transcription to spermatogonial transcription (X axis) versus the log2 expression ratio of X\text{E}\text{O}, Sry and X\text{E},Z\text{2}\text{O}, Sry at 17dpp (Y axis). (D) Quantitative RT-PCR data showing \Delta \Delta C_t values for three X-linked genes (Rhox3a, Atp7a and Zfx) and three autosomal pachytene-specific genes (Tdrd6, Piwil2 and Mad2l2) at 13, 15 and 17 dpp. Bars represent the expression lev
els in X\text{E}\text{O}, Sry relative to X\text{E},Z\text{2}\text{O}, Sry, normalized to beta actin. Upregulation of Rhox3a is significant from 13dpp onwards and upregulation of Atp7a from 15 dpp onwards. Downregulation of Tdrd6 and Piwil2 is significant from 15dpp onwards, and downregulation of Mad2l2 at 17dpp only. (E) RNA FISH staining data for spread pachytene cells at 16-18 dpp. Immunostaining for \gamma\text{H2AX} was used to identify pachytene spermatocytes. Detection of an RNA FISH signal for Scml2 indicates failure of sex chromo
some silencing.
loss during pachytene. We therefore predicted that the upregulation of X genes and downregulation of autosomal pachytene-specific genes should initiate around 13 dpp when pachytene cells first appear in the testis, and the autosomal downregulation should increase in severity over subsequent days as the proportion of pachytene cells increases in normal testes, and this was confirmed by qPCR (Fig. 1D).

As a second measure of MSCI leakage during the first wave of spermatogenesis, we performed RNA FISH experiments to determine whether the X-linked gene Scml2 was correctly silenced. Scml2 is an X-linked gene near the pseudoautosomal region (PAR) boundary, is one of the last genes to become fully silenced during normal meiosis, and is therefore a sensitive indicator of MSCI failure. Initial experiments showed that a YH2AX-containing sex body was formed in spermatocytes of all genotypes including X0, Sry, and so in these analyses we identified spermatocytes by immunostaining for YH2AX together with DAPI staining of DNA. It is possible that some spermatocytes failed to form an identifi-able YH2AX-positive sex body and were excluded from our analysis. Since failure to form a YH2AX-positive sex body is universally associated with MSCI failure, this means that our results will therefore be a minimum estimate of the proportion of spermatocytes with MSCI leakage in each genotype. Leaky transcription of Scml2 was observed in 12–42% of X0, Sry pachytene spermatocytes at 16-18 dpp, compared to 5-6% in X0Z0, Sry spermatocytes and 2–3% in XY wild type spermatocytes.

MSCI leakage is expected to lead to spermatocyte apoptosis at the mid-pachytene stage IV checkpoint. We therefore used hematoxylin and eosin (H&E) staining of testis sections to quantify the level of apoptosis in X0, Sry testes at 15 dpp (Fig. 2 upper panels and Table 1), this being the earliest time point at which autosomal pachytene-specific gene downregulation was observed by qPCR. Consistent with the gene expression data, there was a borderline significant increase in the number of tubules containing apoptotic cells in X0, Sry, together with a highly significant increase in the number of apoptotic cells per tubule. Although most of the tubules with high levels of apoptosis in X0, Sry were judged to be in stage IV, this did not constitute a complete stage IV block and both surviving and apoptotic pachytene spermatocytes were observed throughout subsequent stages. We also noticed a population of cells in which the chromatin was homogeneous (suggestive of prophase exit) but which were not yet judged to be apoptotic (Supplementary Material, Fig. S2). Owing to the difficulty of generating these genotypes, we were unable to generate enough sample material to quantitate the percentage of apoptosis in each individual tubule stage.

### MSCI ‘leakage’ in X0, Sry spermatocytes is also present at later ages

The finding of leaky MSCI and pachytene cell death in X0, Sry testes during the first wave of spermatogenesis presents a paradox in that our previous work has documented a marked accumulation of diploid spermatids in X0, Sry males at 30-31 dpp (9). This accumulation indicates that many cells survive up to and beyond the meiotic cell divisions. We considered it possible therefore that the MSCI leakage might be restricted to the first wave of spermatogenesis. To test this, we extended our investigation of the MSCI leakage to the 30-31 dpp timepoint used in our studies of the SAC. Direct expression analysis of these genotypes is not feasible at this age, because expression profiles from whole testes are confounded by the accumulation of the arrested cells in X0, Sry that are removed at the SAC checkpoint by apoptosis in X0Z0, Sry. Therefore, in these older animals we assayed MSCI leakage using RNA FISH only (Fig. 3 and Supplementary Material, Table S2). As with the 16-18 dpp males, spermatocytes were identified by YH2AX and DAPI staining. In some experiments, spermatocytes were further divided into early and late stages by RNA FISH for Adam3, an autosomal gene expressed exclusively in late pachytene/diplotene spermatocytes.

RNA FISH for Scml2 at 30-31 dpp confirmed that there is MSCI leakage in X0, Sry at this age relative to WT testes (n = 3 per genotype). Transgenic replacement of either Zfy1 or Zfy2 greatly reduces the MSCI leakage, (n = 3 per genotype). X0Z0 males (n = 3), which have both Zfy1 and Zfy2 (28, 29), also showed very little MSCI leakage. Aggregating all our experiments together, the proportion of late pachytene spermatocytes (i.e. cells positive for Adam3) with MSCI leakage (i.e. where the cells were also positive for Scml2) was 66/766 cells in X0, Sry males, 15/665 cells in males transgenic for Zfy2, 2/403 cells in males transgenic for Zfy1 and 2/403 cells in X0Z0 males (Supplementary Material, Table S2). All three ‘rescued’ genotypes are significantly different from X0, Sry (P < 0.01 by chi-squared test in all cases), however the ‘rescued’ genotypes are not significantly different from each other after multiple testing correction. We therefore conclude that both Zfy genes are able to promote MSCI at 30-31 dpp, but our data do not show which gene copy is more effective.

Finally, we examined young adult testes (6 weeks to 2 months post partum, Fig. 4) by RNA FISH. Again, we observed MSCI leakage in early pachytene Adam3-negative spermatocytes in X0, Sry, which was reduced but not absent in late pachytene/diplotene Adam3-positive spermatocytes. Transgenic replacement of Zfy2 abolished the MSCI leakage in both early and late spermatocytes at this age. In this experiment we also investigated three further X-linked genes; Zfx, GM773 and Magea2a + 5. There was a strong per-cell correlation between X-linked genes in terms of MSCI escape: e.g. all cells positive for Magea2a/5 were also positive for Scml2 leakage. This indicates that in young adult X0, Sry spermatocytes the leakage X genes are not independent of each other. Together with the fact that Scml2 - which showed the highest levels of MSCI leakage - is known to be slow to achieve MSCI in normal males, this suggests that the MSCI leakage in young adult X0, Sry testes may represent a delayed imposition of MSCI, rather than the stochastic breakthrough activity of individual X genes.

### The MSCI leakage in X0, Sry at 30-31 dpp does not lead to apoptosis of pachytene spermatocytes

The finding of ongoing MSCI leakage in older X0, Sry testes was surprising (as noted above) since we know that at this age germ cells are able to survive up to and beyond the first meiotic division. Moreover, in our RNA FISH data, at both 30-31 dpp and at two months old, for every X gene examined we were able to find cells positive for both Adam3 and the X gene of interest, indicating that at least some X0, Sry spermatocytes are able to survive past the stage IV checkpoint despite ongoing MSCI leakage. We therefore used three independent methods to quantitate apoptosis during pachytene in males at 30-31 dpp (see Methods). We found no significant difference between genotypes either in the proportion of tubules with apoptotic pachytene cells or in the number of apoptotic cells per tubule, by H&E staining or by TUNEL staining. In stage XII tubules at this age, while the number of apoptotic MI metaphase spermatocytes per tubule was significantly increased in X0Z0, Sry relative to X0, Sry due to the reinstatement of the SAC by the Zfy2 transgene, there was no difference between the genotypes in the total number of cells entering metaphase I (Table 1).
We conclude that in X\textsuperscript{E}O, Sry testes, the MSCI leakage observed by gene expression analysis of juvenile males is associated with increased pachytene apoptosis during the first wave of spermato genesis, while the MSCI leakage observed by RNA FISH analysis at 30-31dpp does not lead to a detectable increase in apoptosis.

X\textsuperscript{E}O, Sry males show no alteration either in early meiotic progression or in the overall length of meiotic prophase.

Since the cells with MSCI leakage do not appear to undergo apoptosis in older X\textsuperscript{E}O, Sry males, we sought to determine whether Zfy deficiency and MSCI leakage had any other effects on either early meiotic progression or on the overall length of meiotic prophase.

The first of these was assayed by immunostaining for SCP3, HORMAD2 and 3H2AX in order to define different stages of synaptonemal complex assembly and sex body formation (Supplementary Material, Table S4). This showed no change between X\textsuperscript{E}O,Sry and X\textsuperscript{E,Z2}O,Sry males. The SAC is functional and the majority of dividing spermatocytes are undergoing apoptosis at metaphase of meiosis I.

The second was assayed by BrdU staining of developing testes (Fig. 5 and Supplementary Material, Table S5). Following a single injection of BrdU at 18-21 dpp, BrdU incorporated during early spermatogonial divisions is diluted in subsequent rounds of DNA replication and is not detectable. This protocol thus traces the fate of those germ cells undergoing the final two...
rounds of spermatogonial DNA replication and division at the time of injection. In the normal XY testes, at 12 days after the BrdU injection, these germ cells have completed one full round of the spermatogenic cycle, and are detected as pachytene spermatocytes of stage IX to XII. The stage distribution of BrdU-positive tubules present in wild type XY males, X\(^O\),Sry males and X\(^E,Z_2\)O,Sry males at 12 days post injection revealed a unimodal distribution in all cases, with no delay in meiotic

Table 1. Quantification of apoptosis in juvenile and older X\(^E,O\),Sry and X\(^E,Z_2\)O,Sry males. An extended version of this table including raw tubule and counts is available as Supplementary Material, Table S3

| Time Points               | Genotype          | Proportion of tubules with at least 1 apoptotic cell | Number of apoptotic cells per tubule with apoptosis | Total number of cells per tubule |
|---------------------------|-------------------|----------------------------------------------------|---------------------------------------------------|---------------------------------|
| 15 dpp, H&E staining, all tubule stages combined | X\(^O\),Sry       | 77.0% +/- 5.3%                                      | 8.92 +/- 0.61                                      | 32.15 +/- 2.20                  |
|                           | X\(^E,Z_2\)O,Sry  | 56.9% +/- 5.0%                                      | 3.63 +/- 0.26                                      | 33.00 +/- 1.63                  |
| 30-31 dpp, H&E staining, stage IV tubules only  | X\(^O\),Sry       | 31.9% +/- 0.7%                                      | 2.62 +/- 0.63                                      | n/d                            |
|                           | X\(^E,Z_2\)O,Sry  | 29.3% +/- 7.6%                                      | 1.62 +/- 0.36                                      | n/d                            |
| 30-31 dpp, TUNEL staining, tubule stages I - XI combined | X\(^O\),Sry       | 39.7% +/- 4.5%                                      | 2.71 +/- 0.1                                       | n/d                            |
|                           | X\(^E,Z_2\)O,Sry  | 28.9% +/- 3.1%                                      | 2.48 +/- 0.12                                      | n/d                            |
| 30-31 dpp, TUNEL staining, tubule stage XII, metaphase cells only | X\(^O\),Sry       | 87.0% +/- 7.6%                                      | 4.72 +/- 0.55                                      | n/d                            |
|                           | X\(^E,Z_2\)O,Sry  | 81.6% +/- 5.4%                                      | 7.45 +/- 0.53                                      | n/d                            |

aBorderline significant increase in proportion of tubules with apoptosis in X\(^E,O\),Sry, t test P value = 0.0629 after arcsine transformation of percentages.
bSignificant increase in number of apoptotic cells per tubule in X\(^E,O\),Sry, t test P value = 0.0059.
cSignificant increase in number of apoptotic metaphase cells per tubule in X\(^E,Z_2\)O,Sry due to reinstatement of the SAC, t test P value = 0.023.
dNo significant difference between genotypes in the total number of metaphase cells per tubule, t test P value = 0.677.

Figure 3. (see also Supplementary Material, Table S2). Histogram showing the percentage of cells with a positive RNA FISH signal for Scm2 in males at 29 dpp and older, i.e. after the first wave of spermatogenesis is complete. Each bar represents a measurement of ~100 cells from a single individual, with genotypes and ages as given on the X axis. Experiments focusing on early pachytene cells, mixed pachytene cells or late pachytene cells are presented in separate groups. Different genotypes are coloured as a visual aid. The Zfy gene complements of each genotype are as follows: Wild type XY—both Zfy1 and Zfy2 are present; X\(^O\),Sry—no Zfy genes present (9); X\(^E,Z_2\)O,Sry—Zfy2 only is present, in the form of an X-linked transgene (9); X\(^E,Z_1,U\)O,Sry—Zfy1 only is present, in the form an X-linked integrant of a BAC containing both Zfy1and Ube1y (Royo et al. 2010); X\(^E,O\),O—both Zfy1 and Zfy2 are present (29;28).
progression either between X\textsubscript{O},Sry and X\textsuperscript{E,E}\textsubscript{O},Sry or relative to XY wild type.

Discussion

Zfy genes are required for efficient meiotic sex chromosome inactivation

In this study, we investigated the transcriptional and phenotypic consequences of Zfy gene deficiency during pachytene in the context of X\textsubscript{O},Sry sex-reversed male mice. These are males that completely lack Y chromosomal gene content other than transgenes for Sry (promoting maleness), Eif2s3y (promoting spermatogonial proliferation) and the Zfy transgenes under investigation. While the lack of the remaining Y chromosome gene content is known to have consequences during late spermiogenesis, previous work by us and others has shown that no other Y genes are necessary for progression through to and past the meiotic divisions, and therefore these genotypes are appropriate to investigate the effects of Zfy genes during the prophase of meiosis I (9,14,30).

At 17.5 dpp we observed widespread expression changes in Zfy-deficient testes that proved to be a combination of (a) MSCI leakage in early pachytene cells, and (b) loss of later-stage pachytene transcripts due to cell death during pachytene. We subsequently confirmed that the MSCI leakage is sustained beyond the first wave of juvenile spermatogenesis, and showed that transgenic restoration of Zfy genes corrected the MSCI leakage.

A parallel can be drawn with the situation in females, where unsynapsed chromatin is also silenced during oogenesis. In females, meiotic silencing is slower and leakier than in males (31-33): our results suggest that this may be due to the absence of Zfy genes in females. The genes involved in promoting meiotic silencing in females, and in promoting the delayed MSCI that occurs in X\textsubscript{O},Sry remain to be ascertained. Transgenic overexpression of Zfx, the X-linked homolog of Zfy, is able to compensate for Zfy deficiency in promoting the second meiotic division, and therefore these genes are functionally interchangeable to at least some extent (14). Zfx is therefore an attractive candidate for a role in the less stringent MSCI observed in females and X\textsuperscript{E,O},Sry males.

Zfy’s triple role at the MSCI checkpoint is suggestive of negative feedback regulation

Previous work (8) established a role for Zfy genes as 'executors' at the MSCI checkpoint—to which we can now add a further role in the initial imposition of MSCI. A third role is implicit in their genomic location: since Zfy genes are Y-linked, Zfy transcription acts as a sensor for the success or failure of MSCI. Also implicit in the genomic location of Zfy is a negative feedback loop (which may be direct or indirect) in which Zfy genes repress their own expression at the transition into pachytene. The mechanism by which Zfy genes exert their effects remain to be elucidated, and it will be particularly important to establish the directness of the link between Zfy activity and the onset of meiosis, i.e. does it have a 'preparatory' role prior to the onset of meiosis, or does it act in real-time to directly regulate its own expression?

If a real-time negative feedback loop can be demonstrated, this then suggests an elegant model in which in normal males, cells that are slow to achieve MSCI will continue to express Zfy genes, leading to one of two options. If the prolonged Zfy stimulus enables the lagging cells to complete MSCI, Zfy gene transcription ceases and prophase proceeds as normal. If MSCI remains incomplete even with prolonged Zfy gene activity, as in the various models with MSCI failure, then the cells undergo apoptosis. The same occurs if Zfy activity is artificially prolonged past the imposition of MSCI, as in the models with autosomal Zfy transgenes.

The apoptotic response to MSCI leakage in X\textsuperscript{O},Sry males is incomplete and age-dependent

In this study, we find that during the first wave of spermatogenesis in X\textsuperscript{O},Sry males, the apoptosis seen in association with MSCI leakage is incomplete and not restricted to stage IV, while in X\textsuperscript{O},Sry males at 30-31 dpp there is no excess apoptosis of pachytene cells at any tubule stage despite ongoing MSCI leakage. The first of these findings is unusual in that many models with MSCI failure show a strong stage IV block with virtually no spermatocytes surviving past stage IV. However, there are two other model systems that show MSCI leakage together with an incomplete stage IV block, these being Ubr2 -/- males (34) and Trip13mod/mod males (35,36). In both of these models, a variable proportion of spermatocytes survives past stage IV before succumbing to apoptosis, similar to the situation during the first wave of spermatogenesis in X\textsuperscript{O},Sry males. The leakiness of the block in these males may be due to a low severity of MSCI disruption. In the H2AX null model, where MSCI is completely lost and there is complete apoptosis at stage IV, there is a twofold upregulation of X chromosome expression at both 16.5dpp and 18.5 dpp (17). In our data, we observe an average log\textsubscript{2} ratio of 0.4 for X-linked genes at 17.5 dpp, i.e. only a 30% increase in X chromosome expression. Consistent with this hypothesis, the Ubr2 knockout model (which also has an incomplete stage IV block) shows an average log\textsubscript{2} ratio of 0.4 for X-linked genes at 17dpp (37). Expression profiling has not yet been performed on the
Trip13<sup>mod/mod</sup> males at this age and so the level of MSCI leakage cannot be directly compared.

The findings in older X<sup>O</sup>Sry males—of ongoing MSCI leakage without a detectable increase in apoptosis during pachytene—are to our knowledge unprecedented. Logically, there are two potential explanations for the difference between the first wave phenotype versus later ages: either there is less MSCI leakage in the spermatocytes of older animals, or the spermatocytes of older animals are better able to tolerate MSCI leakage without becoming apoptotic. It is not possible to distinguish these possibilities with current technology, since it would require tubule stage specific expression profiling of spermatocytes at each age.

**What can the models with MSCI ‘leakage’ and an incomplete stage IV block tell us about MSCI mechanisms?**

Mechanistically, the MSCI leakage we observed in X<sup>O</sup>Sry spermatocytes was distinct from most other models with MSCI failure in that the cells nevertheless formed a morphologically normal sex body positive for γH2AX (Fig. 4 inset). Intriguingly, this also applies to both Trip13<sup>mod/mod</sup> males and Ubr2 -/- males, consistent with the low level of X upregulation in the latter and the incomplete stage IV apoptosis in both models. It is possible that the ‘leaky’ genes in each case are contained within pockets of γH2AX-negative chromatin, however, an alternative possibility is that the leakage reflects an alteration in silencing pathways operating downstream of, or independently to the standard BRCA1/ATR/γH2AX pathway of MSCI. Regardless of the precise mode of MSCI escape, these observations highlight that formation of a γH2AX-positive sex body must not be taken as proof of successful MSCI, and the only conclusive way to demonstrate sex chromosome silencing is by direct RNA FISH measurement of transcription.

**Evidence for multiple ‘executioner’ genes at the stage IV checkpoint**

Four independent lines of evidence have led to the current view that there is an MSCI checkpoint acting during spermatogenesis. Firstly, many models with synapsis failure also show failure of MSCI, continued expression of Zfy genes during pachytene, and stage IV apoptosis (5). Secondly, in T(X;16)16H males (Searle’s translocation), those cells which fail to silence the X-derived portion of the X;16 translocation product undergo apoptosis during pachytene despite the fact that Zfy is correctly silenced in these cells (38). Thirdly, transgenic expression of either Zfy1 or Zfy2 during pachytene has been shown to trigger apoptosis of spermatocytes at tubule stage IV (8). Finally, transgenic expression of selected X-linked miRNA genes during pachytene leads to apoptosis at tubule stages IV and XII (39).

Collectively, these data suggest that both X-and Y-linked genes are independently capable of triggering apoptosis and acting as ‘executioner’ genes for the MSCI checkpoint. Our data
corroborate this and provide further evidence for the existence of multiple sex-linked executioner genes acting during pachytene, since the X^O,Sry males showed increased apoptosis of pachytene cells during the first wave of spermatogenesis in conjunction with MSCI leakage despite a complete absence of Zfy genes.

We note however that in T(X;16)16H males the stage at which aberrantly synapsed cells are eliminated is not known (38), and that overexpression of X-linked miRNAs triggered apoptosis at two different tubule stages (39). It is therefore not proven whether the apoptosis seen in response to wrongful X gene expression in these two models acts via the same mechanism as the Zfy-driven apoptosis occurring at tubule stage IV in other models of MSCI failure. Moreover, the extent and stage-specificity of the apoptosis in X^O,Sry males differs from that seen in most other models of MSCI failure. It may be that different executioner genes at the MSCI checkpoint trigger apoptosis via different downstream pathways and/or at different tubule stages, with Zfy simply being an early-acting executioner. Under this hypothesis, the timing of cell death during pachytene would depend not only on the degree of MSCI failure, but also on the specifics of which genes escape silencing in each cell. Like Tolstoy's families, each dying spermatocyte in these males may be unhappy in its own way.

Materials and Methods

All animal procedures were in accordance with the United Kingdom Animal Scientific Procedures Act 1986 and were subject to local ethical review.

Chromosome spreads

Testes were frozen in liquid nitrogen and stored at -80 prior to thawing in RPMI medium. Surface spreads were prepared as described by Barlow et al (40) and RNA FISH was performed as described previously (8).

Fluorescence in situ hybridization (FISH)

Surface spreads and fluorescence in situ hybridization (FISH) RNA-FISH for nascent nuclear transcripts from Scml2, Zfx, GM773 and Magea 2/5 was performed as previously described (41) using spread testis cells from 6 week old X^O,Sry, X^E,Z2,O,Sry and XY MF1 male mice.

Histological analysis and immunohistochemistry

For standard histological analysis testes were fixed in Bouin’s fluid for 24 h, embedded in paraffin, sectioned at 5 µm on glass slides and then stained with Hematoxylin and Eosin (H & E). At least three males per genotype and age group were analysed. For immunohistochemistry, testes were fixed overnight in 4% buffered paraformaldehyde at 4 degrees. Tissues were washed in 70% ethanol, dehydrated, and embedded in paraffin. 5 µm sections on glass slides were dewaxed in xylene and hydrated in a graded series of alcohols. After washing in PBS, sections were blocked for an hour at room temperature in PBT (PBS, 0.1% Tween, 0.15% BSA) and incubated overnight at 37°C with primary antibody diluted in PBT. Slides were washed in PBS, incubated with appropriate fluorescently-conjugated secondary antibodies diluted in PBS for 1 h at 37°C, washed in PBS, and mounted in Vectashield containing 4’,6-diamidino-2-phenylindole (DAPI, Vector).

Further details of reagents used including BAC probe IDs and antibodies used for RNA FISH and immunostaining, and primers used for qPCR are given in Supplementary Methods.

BrdU and TUNEL assays

BrdU (Sigma), dissolved in phosphate-buffered saline, was injected intraperitoneally into males at 18-21 dpp, at 50 mg/kg of body weight. Males were killed 12 days after the injection, and testes were fixed in Bouin’s fluid for 24 hr, and then embedded in paraffin. BrdU incorporation was detected by immunofluorescence labelling as previously described (42). DAPI counterstaining, γH2AX immunostaining of spermatocytes, and PNA-lectin staining of the acrosomal cap (where present) were used to determine the stage of BrdU-positive seminiferous tubules. Phospho-histone H3 immuno-fluorescence and detection of apoptotic cells by TUNEL were performed as previously described (9).

Quantitation of apoptosis

At 15dpp apoptosis was quantified by hematoxylin and eosin (H&E) staining of histological sections. Since this age is prior to the MI arrest/apoptosis seen in X^E,Z2,O,Sry and the accumulation of arrested secondary spermatocytes in X^O,Sry, it therefore specifically measures cell death during meiotic prophase I. At 30-31 dpp, there are two confounding factors affecting the comparison of apoptosis between the two genotypes. Firstly, in stage XII tubules there is widespread apoptosis of cells at the spindle assembly checkpoint in X^E,Z2,O,Sry that is less pronounced in X^O,Sry (9). Secondly, in X^O,Sry, cells which fail to die at stage XII develop into diploid spermatids and subsequently undergo apoptosis throughout the following tubule stages, which will inflate the numbers of apoptotic cells per tubule in X^O,Sry (14). We therefore used three different methods to assay apoptosis in these males at d30-31.

1) Counting of apoptotic pachytene cells specifically in stage IV tubules in H&E sections. In these sections, tubules were staged by the hematoxylin staining pattern of the peripheral spermatagonia, and arrested diploid spermatids were distinguished by their nuclear morphology and excluded. This assay thus specifically measures cell death at the stage IV checkpoint.

2) Counting of TUNEL-positive cells in histological sections. In these preparations, the detailed tubule staging was not possible and we therefore counted dying cells in all tubules from stages I—XI (defined as all tubules lacking meiotic metaphase figures). This analysis thus excludes the apoptotic metaphase I cells found in X^E,Z2,O,Sry at stage XII, but does not exclude the dying diploid spermatids seen in other stages in X^O,Sry. Accordingly, this comparison represents a very stringent check of increased apoptosis in X^O,Sry.

3) Counting the total number of meiosis I metaphase figures per stage XII tubule. TUNEL staining and staining for phospho-histone H3 was used to classify the metaphases as healthy or apoptotic, as previously described (9). This assay measures the total number of cells surviving through to meiosis I, and thus is an indirect measure of apoptosis occurring at all stages of meiotic prophase I.
RNA extraction and expression analysis

Total RNA was extracted from frozen testis tissue using Trizol (Invitrogen) according to the manufacturer’s protocol. Microarray analyses were performed as previously described (26) using total testis RNA from males at 17.5 dpp. Briefly, single-color hybridization data (Illumina BeadChip, mouse whole-genome array, v2) was obtained for three X:O:Sry individuals and matched X:O,22:O:Sry controls, each individual being hybridized separately. Quantile normalization and differential expression testing was performed using BeadStudio (Illumina), and false discovery rate correction was performed using Benjamini and Hochberg FDR correction.

Quantitative RT-PCR experiments were performed as previously described (26). Briefly, two micrograms of total RNA were DNasel-treated (Invitrogen), and reverse transcription of polyadenylated RNA was performed with Superscript Reverse Transcriptase II according to the manufacturer’s protocols (Invitrogen). Samples from three 13.5, 15.5 and 17.5 day old mice for each genotype (X:O,Sry and X:O,22:O,Sry) were analysed. All reactions were carried out in triplicate and beta actin was included on every plate as a loading control. The difference in PCR cycles with respect to beta actin (ΔΔCt) for a given experimental sample (X:O,Sry) was subtracted from the mean ΔCt of the reference samples (X:O,22:O,Sry) (ΔΔCt).

Data Availability

Normalised array data and the processing pipelines for figure generation are available in Supplementary Material, Table S1. Raw array data is available through GEO, accession number GSE87598.

Supplementary Material

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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