Mouse Y-Linked Zfy1 and Zfy2 Are Expressed during the Male-Specific Interphase between Meiosis I and Meiosis II and Promote the 2nd Meiotic Division

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Abstract

Mouse Zfy1 and Zfy2 encode zinc finger transcription factors that map to the short arm of the Y chromosome (Yp). They have previously been shown to promote meiotic quality control during pachytene (Zfy1 and Zfy2) and at the first meiotic metaphase (Zfy2). However, from these previous studies additional roles for genes encoded on Yp during meiotic progression were inferred. In order to identify these genes and investigate their function in later stages of meiosis, we created three models with diminishing Yp and Zfy gene complements (but lacking the Y-long-arm). Since the Y-long-arm mediates pairing and exchange with the X via their pseudoautosomal regions (PARs) we added a minute PAR-bearing X chromosome derivative to enable formation of a sex bivalent, thus avoiding Zfy2-mediated meiotic metaphase I (MI) checkpoint responses to the unpaired (univalent) X chromosome. Using these models we obtained definitive evidence that genetic information on Yp promotes meiosis II, and by transgene addition identified Zfy1 and Zfy2 as the genes responsible. Zfy2 was substantially more effective and proved to have a much more potent transactivation domain than Zfy1. We previously established that only Zfy2 is required for the robust apoptotic elimination of MI spermatocytes in response to a univalent X; the finding that both genes potentiate meiosis II led us to ask whether there was de novo Zfy1 and Zfy2 transcription in the interphase between meiosis I and meiosis II, and this proved to be the case. X-encoded Zfx was also expressed at this stage and Zfx over-expression also potentiated meiosis II. An interphase between the meiotic divisions is male-specific and we previously hypothesised that this allows meiosis II critical X and Y gene reactivation following sex chromosome silencing in meiotic prophase. The interphase transcription and meiosis II function of Zfx, Zfy1 and Zfy2 validate this hypothesis.

Introduction

Historically the realisation that there were spermatogenic factors on the human and mouse Y chromosomes distinct from the testis determinant came from the study of Y deletion variants [1,2]. However, it was not until the search for the testis determinant that Y-encoded genes began to be identified; amongst these were the human and mouse Y genes encoding zinc finger transcription factors cloned in the late 1980s [3–5]. Subsequent progress in assigning spermatogenic gene functions to mouse Y-encoded genes was thwarted by a failure to disrupt Y gene functions using the emerging gene targeting techniques that had proved successful in disrupting X and autosomal gene functions, compounded by the paucity of genomic sequence data for the mouse Y chromosome. To circumvent these problems the Mitchell and Burgoyne labs established a collaboration with the aim of identifying mouse Y gene functions using a Y ‘transgene rescue’ strategy whereby Y genes were added to Y deletion variants with defined spermatogenic failure. In the context of Y genes mapping to the short arm (Yp), three XO male mouse models with diminishing Yp gene complements were utilised (Figure 1): X;Syr/O in which the X carries the Yp-derived sex-reversal factor Tp(Y)1CtSyr− which provides an almost complete Yp gene complement [6], X;Syr/O males where the X carries an Sxr− derivative Tp(Y)1CtSyr− in which a 1.3Mb deletion (ΔSxrb) has removed the majority of the Yp gene complement [6,7], and X0Yy males in which the only Yp gene present is an autosomally located Sy transgene [8].

The latter two Yp-deficient models have a marked block in spermatogonial proliferation, and in 2001 we reported that this block could be circumvented by the addition of Eif2α3y; this Y-linked gene encodes a protein almost identical to that encoded by the X-linked gene Eif2α3x - a subunit of the essential translation initiation factor EIF2 [8]. Paradoxically, in both Eif2α3y rescue
models the majority of spermatocytes complete meiosis I, whereas in the XδXδ/o ‘control’ there is a very efficient apoptotic elimination of spermatocytes at the first meiotic metaphase (MI) [9–11]; this apoptosis is assumed to be triggered by an MI spindle assembly checkpoint (SAC) response to the univalent X at MI [12]. This suggested that a Yp gene that was deleted or inactivated in δXδ was necessary for an efficient apoptotic response to the univalent X, although a markedly reduced apoptotic response remained.

To identify the Yp gene that promoted the MI spermatocyte apoptosis, transgenes were tested by adding them to XO/δO males that carried an X-linked Eif2s3y transgene (here denoted as δXδO), but none of Yp genes completely removed by δδO (Figure 1C) reinstated the apoptotic response. Focus then shifted onto Zfy1 and Zfy2 because the δδO deletion breakpoints lie within these two genes, creating a transcribed Zfy1/Zfy1 fusion gene with the encoded protein almost identical to that encoded by δδO (Table S2). Introducing an X-linked Zfy2 transgene into δXδO was necessary for an efficient apoptotic response to the univalent X, although a markedly reduced apoptotic response remained.

Further studies of the Eif2s3y rescue models XδO/δO and XδδδO revealed that although most primary spermatocytes evaded the apoptotic response and completed meiosis I to form diploid secondary spermatocytes that entered interphase (“interphasic secondary spermatocytes”), very few spermatocytes recondensed their chromosomes and underwent meiosis II (Figure 1D); indeed Xδδ/O had a very depleted Yp gene complement so we next wanted to examine the rescue efficiency of the Yp genes responsible for meiosis II completion.

Yp-encoded genetic information promotes meiosis II

We will abbreviate the three models with YsX used in this study as Xδδδ/XδδO, XδδδδδδO and XδXδδδδδδO. Their Yp gene complements are as shown in Figure 1B–D, except for the addition of the Eif2s3y transgene to the X of the latter two models (denoted Xδδδδδδ). For comparison with the published data on ploidy frequency of post-meiotic cells in the XδδδδδδO and XδXδδδδδδO models [14] we have processed the YsX complemented males at 6 weeks of age. We used a combination of centromere (CREST) and chromosome axial element (SYCP3) immunostaining, which allows PAR-PAR by the univalent Yp genes to be distinguished from associations between the X-derived YsX centromere and the X centromere (Figure 2). This revealed an average of 74.9% PAR-PAR synapsis with no significant difference between the three YsX complemented models and the remainder of cells either having the X and YsX PARs unpaired, or lacking an identifiable YsX (Table 1). In the latter case it is likely that the tiny YsX chromosome was lost during cell spreading.

To assess the efficiency of the meiotic divisions, we analyzed the ploidy of spermatids in SYCP3 and DAPI-stained spermatogenic cell spreads. The DAPI nuclear morphology of diploid spermatids is indistinguishable from that of interphasic secondary spermatocytes, but the latter have a characteristic SYCP3 staining pattern [11] and were excluded. It is important to bear in mind when assessing the consequences of the YsX addition that in an average of 25.1% of MI cells from all three models the X fails to achieve PAR-PAR synapsis to be distinguished from associations between the X-derived YsX centromere and the X centromere (Figure 2). This revealed an average of 74.9% PAR-PAR synapsis with no significant difference between the three YsX complemented models and the remainder of cells either having the X and YsX PARs unpaired, or lacking an identifiable YsX (Table 1). In the latter case it is likely that the tiny YsX chromosome was lost during cell spreading.

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minute X chromosome derivative (denoted YsX for historical reasons) comprising a complete PAR, an X PAR boundary, a very short X-specific region and an X centromere [13–19] (Figure 1E). In the majority of MI spermatocytes this YsX mini-chromosome and XδXδ had formed a sex bivalent (indicative of prior PAR synapsis and crossing over) and thus evaded the MI SAC apoptotic elimination [19]. In the present study we therefore added this chromosome to the XδXδ/O, XδδδδδδO and XδδδδδδO models in order to assess if the near complete Yp gene complement of Sxr promoted meiosis II more effectively than the two depleted Yp gene complements. This proved to be the case so we then proceeded to use Yp gene transgene addition to identify the Yp genes responsible for meiosis II completion.

Results

Yp-encoded genetic information promotes meiosis II

We will abbreviate the three models with YsX used in this study as Xδδδ/XδδO, XδδδδδδO and XδXδδδδδδO. Their Yp gene complements are as shown in Figure 1B–D, except for the addition of the Eif2s3y transgene to the X of the latter two models (denoted Xδδδδδδ). For comparison with the published data on ploidy frequency of post-meiotic cells in the XδδδδδδO and XδXδδδδδδO models [14] we have processed the YsX complemented males at 6 weeks of age. We used a combination of centromere (CREST) and chromosome axial element (SYCP3) immunostaining, which allows PAR-PAR synapsis to be distinguished from associations between the X-derived YsX centromere and the X centromere (Figure 2). This revealed an average of 74.9% PAR-PAR synapsis with no significant difference between the three YsX complemented models and the remainder of cells either having the X and YsX PARs unpaired, or lacking an identifiable YsX (Table 1). In the latter case it is likely that the tiny YsX chromosome was lost during cell spreading.

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Strikingly, there was no significant increase in haploid frequency in Xδδδ/XδδO (17.4%) relative to XδδO (11.4%); in marked contrast the haploid frequency had significantly increased (P = 0.00059) in XδδδδδδO/δδδδδδO (54.4%) relative to XδδδδδδO (5.2%) (Figure 3A). This was an unexpected result because there was no indication from the two XO models that XδδO had a lower haploid spermatid frequency than XδδO. We conclude that meiosis II is not potenitated by the formation of a sex bivalent per se, but there is genetic information in XδδO that in the context of a sex bivalent promotes the completion of meiosis II.

Sxrδ has a very depleted Yp gene complement so we next wanted to assess the consequences of the YsX addition in the context of XδδO, which provides a near complete Yp gene complement. This
proved to have a much more potent effect than in the Sxrb context with the haploid frequency increasing to 96% (P = 0.00082) (Figure 3A). We conclude that there is genetic information on mouse Yp that promotes meiosis II when a sex bivalent is formed, and this is provided more effectively by Sxra than Sxrb.

Addition of Zfy1 and/or Zfy2 to XEY*Xsry promotes meiosis II

The protein-coding gene content of Sxrb is thought to be limited to a few copies of Rbmy, two copies of H2a12y, Sry and a Zfy2/1 fusion gene spanning the Sxrb deletion breakpoint [7,20,21]. Because interphasic secondary spermatocytes are a very transient cell type in normal testes, there is no published information on expression of these genes at this stage. However, by RNA in situ analysis Rbmy transcripts are not detected beyond early pachytene [22] and H2a12y does not appear until step 6 round spermatids (Figure S1), so they are unlikely to be transcribed in interphasic secondary spermatocytes. The Sry transcripts present in the adult mouse testis are circular transcripts that are thought to be untranslated [23,24]. Our initial focus was therefore on the Zfy2/1 fusion gene, which is known to be transcribed during early

Figure 1. The XO and XY*X mouse models. A. XY. The Y short arm (Yp) gene complement of an XY male (represented to scale in the magnified view) comprises seven single copy genes, two duplicated genes and one multi copy gene. The pseudoautosomal region (PAR) located distally on the Y long arm mediates pairing and crossing over with the X PAR during meiosis to generate the XY sex bivalent. B–D. The diminishing Yp gene complements for the three XO male mouse models that lack the Y long arm. B. X SxraO. The Yp-derived Sxra sex-reversal factor, attached distal to the X PAR provides an almost complete Yp gene complement. C. X SxrbO. The Sxra-derived deletion variant Sxrb has a 1.3 Mb deletion (ΔSxrb) removing 6 single copy genes and creating a Zfy2/1 fusion gene spanning the deletion breakpoint (†). D. XO Sry. This model has only one Y chromosome gene, namely the testis determinant Sry provided as an autosomally located transgene. E. Y*X. This mini sex-chromosome is an X chromosome with a deletion from just proximal to Amelx to within the DXHXF34 repeat adjacent to the X centromere. † represents the deletion breakpoint. This X chromosome derivative has a complete PAR that can pair with the PAR of X Sxra, X Sxrb or X to form a ‘minimal sex bivalent’. Scale bar for magnified views is 150 kb. doi:10.1371/journal.pgen.1004444.g001
Figure 2. Efficiency of XY synopsis in the XY*Y males with varying Yp complements. Spread pachytene spermatocytes from 6 week old XY and X*Y*Sry testes stained with antibodies against SYCP3 (green) and CREST (red). Frames show PAR-PAR sex chromosome synopsis in XY and X*Y*Sry males; the arrow points to an unsynapped Y* chromosome in an X*Y*Sry male. doi:10.1371/journal.pgen.1004444.g002

prophase, is presumed to be silenced by meiotic sex chromosome inactivation [MSCI, [25]] at the beginning of pachytene, as are Zfy1 and Zfy2 in normal males, but is transcribed post-meiotically [7,26]. The Sxr^ deletion breakpoint is located within a 95 bp region of sequence identity between intron 5 of Zfy1 and Zfy2 in normal males, but is transcribed post-meiotically [7,26]. The Sxr^ deletion breakpoint is located within a 95 bp region of sequence identity between intron 5 of Zfy2 and Zfy1, and the protein encoded by the fusion gene is predicted to be identical to that encoded by Zfy1 except for the 16th amino acid where a leucine replaces a phenylalanine [7,11].

Because the Zfy2/1 fusion gene encodes a protein nearly identical to that of Zfy1 we first added a Zfy1 transgene to X*Y*Sry to see if this mimicked the effect of Sxr^ in promoting the second meiotic division in the presence of Y*X. This proved to be the case in that the proportion of haploid spermatids increased significantly (P = 0.01219) from 17.4% to 47.2% (Figure 3B).

Based on their DNA sequences, Zfy1 and Zfy2 are expected to produce transcription factors that will bind to the same target genes. We therefore also generated X*Y*Sry males that were transgenic for Zfy2, and this addition increased the haploid frequency from 17.4% to 78.2% (P = 0.00011), which is significantly higher (P = 0.00065) than that achieved with the Zfy1 transgene (47.2%). Thus the Zfy2 transgene promotes meiosis II more effectively than the Zfy1 transgene. Both transgenes are single copy and inserted on the X chromosome, but we cannot assess relative transcript levels in interphase secondary spermatocytes because of our inability to adequately purify this rare cell type. However we have previously established by qRT-PCR that the transcript level for the Zfy1 transgene was higher than that for the Zfy2 transgene in testes from 17.5 day-old X*O*Sry carriers [11], so we would expect a similar excess of Zfy1 transcripts in interphase secondary spermatocytes. We were therefore surprised that the Zfy2 transgene had a markedly greater effect. With the addition of both transgenes the frequency of haploid spermatids increased to 87.6% (Figure 3B; Table S2C). These results point to the combined activity of Zfy1 and Zfy2 as important for promoting meiosis II.

Zfy1, Zfy2 and Zfx are transcribed during the interphase prior to meiosis II

Transcription of Zfy1 and Zfy2 is reportedly testis specific, at least post-natally [27–29]. Recently we have shown that in the adult testis this transcription is limited to germ cells, starting in leptotene spermatocytes, with more robust transcription in zygote spermatocytes, followed by silencing in pachytene spermatocytes as a consequence MSCI; there was no resumption of transcription prior to MI, but transcription was shown to have resumed in Y-bearing round spermatids [7]. However, no data are available for interphase secondary spermatocytes [14]. To assess transcription of Zfy1 and Zfy2 in these cells we used Zfy1 and Zfy2 DNA-FISH on DAPI- and SYCP3-stained testis cell spreads from XY males and confirmed the presence of the Y chromosome using Zfy1 and Zfy2 DNA-FISH. Zfy1 and Zfy2 transcription was detected in 45% and 27%, respectively, of the Y-bearing secondary spermatocytes (Figure 4; Table 2). As expected, Zfy1

Table 1. X-Y pairing efficiency in pachytene spermatocytes.

| Genotype | PAR-synapsis (n) | Unsynapped (n) | Missing Y or Y*X (n)* | PAR-synapsis (%)b |
|----------|-----------------|----------------|---------------------|-------------------|
| XY       | Average: 94.9%  |                |                     |                   |
| 1        | 48              | 2              | 0                   | 96.0              |
| 2        | 47              | 3              | 0                   | 94.0              |
| 3        | 49              | 1              | 0                   | 98.0              |
| 4        | 75              | 1              | 2                   | 96.2              |
| 5        | 67              | 3              | 2                   | 93.1              |
| 6        | 97              | 5              | 3                   | 92.4              |
| X*Y*Sry  | Average: 73.3%  |                |                     |                   |
| 1        | 68              | 29             | 3                   | 68.0              |
| 2        | 68              | 27             | 5                   | 68.0              |
| 3        | 40              | 5              | 10                  | 72.7              |
| 4        | 42              | 13             | 1                   | 75.0              |
| 5        | 32              | 5              | 4                   | 78.0              |
| 6        | 42              | 8              | 4                   | 77.8              |
| X*Y*Sry a | Average: 73.0%  |                |                     |                   |
| 1        | 72              | 28             | 0                   | 72.0              |
| 2        | 28              | 6              | 6                   | 70.0              |
| 3        | 82              | 21             | 4                   | 76.6              |
| 4        | 73              | 28             | 1                   | 75.0              |
| 5        | 73              | 27             | 2                   | 71.6              |
| XY*Y*Sry a | Average: 79.8%  |                |                     |                   |
| 1        | 81              | 11             | 2                   | 86.2              |
| 2        | 74              | 11             | 6                   | 81.3              |
| 3        | 49              | 18             | 2                   | 71.0              |
| 4        | 71              | 13             | 4                   | 80.7              |

*In a few cells, the Y or the Y*X chromosome appear to be missing. Some of them might have achieved a centromere pairing with the X chromosome or have been lost during cell spreading.

bThe average PAR-synapsis for all Y*X bearing males (n = 15) is 74.9%. doi:10.1371/journal.pgen.1004444.t001
and Zfy2 DNA-FISH signals were not observed in half of the secondary spermatocytes (X-bearing) and these also lacked Zfy1 and Zfy2 RNA-FISH signals. However, 92% of these X-bearing spermatocytes were transcribing the related X-linked gene Zfx (Figure 4; Table 2).

Our finding that Zfx is also expressed in interphase secondary spermatocytes raised the question as to whether Zfx also promotes the second meiotic division. We had available a Zfx transgenic line with 7 copies of a Zfx genomic BAC inserted on an autosome. As expected for an autosomally located X-chromosome-derived transgene it was exempt from MSCI, and was expressed in pachytene cells (Figure S2A,B); like the endogenous Zfx gene it was expressed in interphase secondary spermatocytes (Figure S2C). We added this transgene to X/Y<sup>Y<sub>Sry</sub></sup> males and the proportion of haploid spermatids increased from 15.0% (in non-transgenic secondary spermatocytes) to 79.9%, showing that Zfx also has a minor role in promoting meiosis II.

The Zfy2 transactivation domain is much more potent than that of Zfy1

We were struck by the much more potent effect of Zfy2 as compared to Zfy1 in promoting meiosis II. Based on an in vitro assay it was previously reported that Zfy2 encodes a protein with a much more potent transactivation (TA) domain than that of Zfy1, but Zfy1 was not assayed at that time [30]. We have therefore used a similar in vitro assay to compare the transactivation domains of mouse Zfa, Zfy1, Zfy2 and the autosomal Zfa (originating from a retrosped X transcript [31–35]), and have also compared these with the transactivation domains of human ZFX and ZFY (Figure 6). We confirmed the expression of all the ZF-Gal4 fusion proteins by western blot analysis (Figure S3). The assay revealed that the TA domain of mouse Zfy1 has a similar activity to human ZFX and ZFY. Strikingly, the mouse Zfy2 TA domain is 5.5-fold more active than that of mouse Zfy1, and is ~10-fold more active than that of mouse Zfx. The TA domain of the putative ZFA protein proved to have a very weak TA activity. A single nucleotide deletion near the beginning of the ZFY/ZFX open reading frame of Zfa actually makes it very unlikely to translate a protein that includes the zinc finger DNA binding domain, which would preclude binding to target genes. Zfa is now flagged as a pseudogene in Genbank (accession no. NR_037920).

Discussion

Our previous study of meiotic progression in the three XO male models with varying Yp gene complements revealed that the majority of spermatocytes in the Yp gene deficient models X<sup>O</sup>Sry and X<sup>Sry</sup>O reached the interphase that precedes meiosis II; however, they failed to recondense their chromosomes to enable completion of meiosis II and instead formed diploid round spermatids [14]. Formally, the failure to undergo meiosis II could be a consequence of the prior triggering of the MI SAC by the univalent X, the reduced apoptotic response due to the absence of Zfy2, or the lack of a Yp gene or genes that promotes meiosis II (Figure 7A). The aim of the present study was to check specifically for a Yp gene requirement by circumventing the MI SAC and apoptotic responses; for this we added a minute PAR-bearing X chromosome derivative (Y<sup>Y</sup>Y<sup>Sry</sup>) to all three XO models to enable completion of meiosis II and instead formed diploid round spermatids [14].

We attribute the difference in potency between S<sup>Y</sup> and S<sup>E</sup> to the presence of Zfy1 and Zfy2 in S<sup>E</sup> whereas S<sup>Y</sup> only has the Zfy2/Zfy1 fusion gene that encodes a protein almost identical to Zfy1 [7,11]. However, we were struck by the fact that S<sup>E</sup> did not promote meiosis II in the absence of Y<sup>Y</sup>Y<sup>Sry</sup>; this implies that the...
triggering of the MI SAC, and/or the reduced apoptotic response, impairs progression through meiosis II. In order to distinguish between these possibilities it is informative to consider what happens in XO females where there is an MI SAC response but no apoptotic response. XO female mice are fertile and produce XO (and XX) daughters, so that some XO oocytes must complete meiosis I and meiosis II. Furthermore, although some X univalents achieve bipolar attachment to the spindle (which is expected to satisfy the MI SAC), this is not a prerequisite for the completion of meiosis I [36]. This is in agreement with accumulating data for female mice showing that the MI SAC does not maintain arrest until all kinetochores have achieved appropriate attachments to the spindle - anaphase can proceed in the presence of one (or a few) univalents [37–41]. Thus XO oocytes can complete meiosis I to generate MII oocytes with either an XX sex chromosome complement (i.e. two X chromatids) or lacking an X chromosome, both of which should be able to complete meiosis II without triggering an MII SAC response. This suggests that in the three XO male models the triggering of the MI SAC per se would not impair meiosis II. We therefore favour the view that the addition of the Y*X to the XESxrbO model increases the haploid spermatid frequency from 5.2% to 54.4% because the formation of an XSyrb/Y*X bivalent avoids the reduced apoptotic response. We envisage that the reduced DNA damage at MI as a consequence of

Figure 4. The mouse Zfy and Zfx genes are transcribed in interphasic secondary spermatocytes. Representative images of interphasic secondary spermatocyte nuclei are shown hybridized with RNA FISH probes specific for Zfy1, Zfy2 or Zfx (arrows, top panels). Interphasic secondary spermatocytes were distinguished from diploid spermatids by staining spread spermatogenic cells from 6-week old XY males with an antibody against SYCP3 (red, top panels). The appropriate localization of the RNA FISH probe to the encoding genes was confirmed by DNA FISH (arrows, bottom panels). Nuclei are stained with DAPI (blue). X- or Y-bearing secondary spermatocytes are respectively represented by an X or a Y next to the cell. doi:10.1371/journal.pgen.1004444.g004

| Probe | DNA FISH Negative | DNA FISH Positive |
|-------|-------------------|-------------------|
|       | Total | RNA FISH Negative | RNA FISH Positive | Total | RNA FISH Negative | RNA FISH Positive |
| Zfy1  | 39    | 39                | 0                 | 40    | 22                | 18 (45%)           |
| Zfy2  | 46    | 46                | 0                 | 45    | 33                | 12 (27%)           |
| Zfx   | 25    | 25                | 0                 | 26    | 2                 | 24 (92%)           |

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the reduced apoptotic response is usually insufficient to trigger elimination at MI, but is sufficient to trigger a G2/M DNA damage checkpoint (reviewed in [42]) at the post meiosis I interphase and block progression to MII. The arrested interphase cells then enter spermiogenesis as diploid spermatids. Unfortunately we cannot use these models to assess the ultimate fate of the diploid spermatids, because in the absence of the Y long arm there is marked over-expression of X and Y genes due to the absence of \(Sly\) (50 copies on the Y long arm [43,44], and this (together with Yp gene deficiency) results in severely perturbed spermiogenesis [14]. Figure 8 summarises how we see these MI and G2/MII checkpoint responses operating in males with a normal ‘\(Zf\)’ gene complement (X\(Sxra\)O, XY\(X\)\(Sxra\) and XY).

The finding that both \(Zfy2\) and \(Zfy1\) promoted meiosis II was surprising because at MI only \(Zfy2\) promotes the robust apoptotic elimination of spermatocytes with a univalent X chromosome [11]. What is the basis for the resurrection of \(Zfy1\) function in the short interval between MI and meiosis II? Our previous RNA FISH analyses of nascent nuclear transcripts on spread spermatogenic cells from normal XY males [7] have established that \(Zfy1\) and \(Zfy2\) are transcribed in all mid-late zygotene nuclei, but this ceased in pachytene nuclei – an expected consequence of meiotic sex chromosome inactivation (MSCI – reviewed by [25]), and remained undetectable right through MI. The role of \(Zfy2\) in the apoptotic response to univalence at MI must therefore be a consequence of transcriptional changes mediated by ZFY2 translated from these zygotene transcripts.

\(Zfy1\) and \(Zfy2\) have the same predicted DNA target sequences, so if both are robustly expressed during zygotene, why is the apoptotic role limited to \(Zfy2\)? A plausible explanation is provided by our finding that during the pre-pachytene phase of transcription, alternative splicing of \(Zfy\) transcripts leads to \(~81\%\) of \(Zfy\) transcripts lacking exon 6 with the encoded protein lacking transactivation (TA) activity, whereas \(~96\%\) of \(Zfy2\) transcripts have exon 6 and thus a functional TA domain [7]. Our current TA domain analysis further demonstrates that the few full length \(Zfy\) transcripts that are produced during zygotene generate a protein with a much less potent TA domain than that of \(Zfy2\). In view of this it is reasonable to conclude that \(Zfy1\) function in meiosis II is based on the \(de\ nvo\) transcription in interphasic secondary spermatocytes (and that this

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**Figure 5. \(Zfx\) over-expression promotes meiosis II.** A. Spread cells found in testis of 6-week old \(X^EY*X\) males without or with \(Zfx\) transgene. Pachytene (Pa), diploid spermatid (St d) and haploid spermatids (St h) nuclei are stained with DAPI (top panel) and higher magnifications are shown additionally labelled with \(\gamma\)H2AFX, and SYCP3 antibodies (bottom panel). B. Percentage of haploid round spermatids found in mice from panel A (see also Table S2D). Key: in black, \(X^EY*X\) mice have a robust apoptotic elimination of the \(~25\%) of MI spermatocytes that have an X univalent (see Figure S5); striped, \(X^EY*X\) males have a markedly reduced apoptotic response so the frequency was adjusted as detailed in Table S1. The addition of the \(Zfx\) transgene significantly increases (\(**p\leq0.00001\)) the proportion of haploid round spermatids.

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**Figure 6. \(Zfy2\) acidic domain is a much more potent transactivator than other \(Zf\) acidic domains.** Levels of \(\beta\)-galactosidase induced by the Gal4-DNA-binding domain on its own (pGB-CEN6; negative control) or fused to an acidic domain from one of six different \(Zf\) isoforms from human (hs) or mouse (mm). Among the mouse sex-linked genes, \(Zfy2\) has a substantially more potent activation domain than \(Zfy1\), and \(Zfx\) is significantly less potent than \(Zfy1\). mm ZFA derives from the autosomal \(Zfa\) gene that originated from a retroposed X transcript. \(*p\leq0.05; **p\leq0.01.\)

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\(Y-Linked Zfy1\) and \(Zfy2\) Function during Meiosis II

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**Figure 7.** A summary of the meiotic outcome in **XO** and **XY** males with varying **Yp** gene content. Throughout this figure the thickness of the arrows indicates the proportion of cells progressing from one step to the next and the cheeses at the bottom represent the proportion of haploid and diploid spermatids. Size of the cheese indicates the relative success of the different models in meiosis completion. A. **XO** models. In **XO** males, the majority of spermatocytes complete meiosis I because of the reduced apoptotic response at MI due to the absence of **Zfy2** [11]. **Zfx** expression is likely responsible for the residual apoptotic response (see Figure S5). The majority of spermatocytes then arrest at the Y-Linked **Zfy1** and **Zfy2** Function during Meiosis II.
interphase between meiosis I and meiosis II. This could be a consequence of the prior triggering of the MI SAC by the univalent X, the reduced apoptotic response due to the absence of Zfy2, or the lack of a Yp gene or genes that promotes meiosis II. In XSexrO males there is a very efficient apoptotic elimination of spermatocytes at MI so that very few complete meiosis I and this results in a 97% reduction in the number of spermatids. This precludes any firm conclusion as to a role for Yp genes for completion of meiosis II because the apoptotic elimination may have had a bias towards removing MI cells that were otherwise destined to arrest at the following interphase. B. XY$k^a$ models. In these models the spermatocytes that form a sex bivalent circumvent the MI SAC/apoptotic response and complete meiosis I. This reveals that spermatocytes at MI so that very few complete meiosis I and this results in a 97% reduction in the number of spermatids.

Figure 8. A combined MI SAC and G2/MII checkpoint model to explain the consequences of the male-specific apoptotic response to spermatocytes with univalent chromosomes at MI. To illustrate the model we consider the consequences of these two checkpoint responses in SexrO, XY$k^a$SexrO and XY males, all of which have a complete ZF gene complement. Red arrows denote DNA damage. The chevrons at the bottom represent the proportion of haploid and diploid spermatids. A. In SexrO males each MI spermatocyte will have a univalent X that is expected to trigger the MI SAC and cause a brief delay in MI progression. We propose that this delay is detected by the surrounding Sertoli cell, which initiates a robust Zfy2-dependent apoptotic response. In order to explain the mix of diploid and haploid spermatids originating from the very few surviving MI spermatocytes we propose: 1) Rare MI spermatocytes complete meiosis I with apoptotic DNA damage that triggers a G2/MII checkpoint in the subsequent interphase and blocks progression to MI – these interphase secondary spermatocytes then enter spermiogenesis to form diploid spermatids. (The number of such cells was elevated in the XO models lacking Zfy2 in Figure 7); 2) In rare cases some MI spermatocytes evade the MI SAC and apoptotic death by achieving bipolar attachment to the spindle – these complete meiosis I and meiosis II to form haploid spermatids. B. In XY$k^a$SexrO males (25% of MI spermatocytes with univalent X and Y$k^a$) and XY males (5% of MI spermatocytes with univalent X and Y) the MI cells with univalents will follow the pathways 1) and 2) above, although the likelihood of both univalents achieving bipolar attachment to the spindle will be much lower. The MI cells that have formed a sex bivalent will progress through both divisions to form haploid spermatids (unless they have a pair of autosomal univalents in which case pathways 1 and 2 apply).

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X univalence at MI (Figure S5), which clearly demonstrates that Zfx is able to contribute to these functions. The marked promotion of these functions in both cases is unsurprising given that the transgene is present in 7 copies and that its autosomal location is associated with extension of transcription through to just prior to MI, together with exemption from the MSCI-dependent repression that affects the X and Y chromatin of interphase secondary spermatocytes (see below). Thus it is reasonable to conclude that the endogenous Zfx also contributes to these functions; indeed, in the X^O/Y^O model there is some MI apoptosis [11] and in the X^O/Y^O model there is some progression through meiosis II (17.4% haploid spermatids, Figure 3A). A role for the endogenous Zfx in spermatogenesis has also been suggested based on the reduced sperm count in Zfx knockout males, although this effect is confounded with severe growth deficiency [45].

Although Monesi reported transcription in interphase secondary spermatocytes in the 1960s [46,47], other than our finding that there is de novo transcription of the multi-copy mouse Y gene Sfy in interphase secondary spermatocytes [48], we are not aware of any published data giving information on which genes are actively transcribed at this stage. It has previously been concluded based on Cot1 RNA FISH assessments of global transcription in interphase secondary spermatocytes that the autosomal chromatin is actively transcribed, whereas the X and Y chromatin remains substantially repressed; the repression of the sex chromosomes has been shown to be dependent on the prior MSCI, and is carried through into round spermatids (‘post-meiotic sex chromosome repression’) [49–51]. This raises the possibility that the de novo transcription of the ‘Y’ gene family represents a selective reactivation. As a first look at this issue we assessed de novo transcription of Mtm1 (X-linked) and Uty (Y-linked) in interphase secondary spermatocytes and round spermatids, with Zfx serving as a positive control. This revealed that these two genes are also transcribed in interphase secondary spermatocytes, but it is noteworthy that for all three genes the frequency of RNA FISH positive cells was higher in interphase secondary spermatocytes (Zfx 90%; Mtm1 67%; Uty 88%) than in round spermatids (Zfx 32%; Mtm1 36%; Uty 60%) (Table S3). These preliminary data are consistent with: (1) there being a partial relaxation of sex chromosome silencing during the late diplotene-MI period, counterbalanced by the global transcriptional repression associated with the condensation of the metaphase chromosomes, (2) the decondensation of the chromosomes in interphase secondary spermatocytes allowing strong transcription from the autosomes, but weaker transcription from the sex chromosomes because of the MSCI carry-over effect; and (3) further repression of the sex chromosomes in round spermatids due to the repressive chromatin changes driven by the multi-copy Y gene Sfy [44].

The marked increase in transactivation activity of Zfy2 relative to Zfx and Zfy1 (Figure 6 and [30]) raises some interesting questions in an evolutionary context. The autosomal ‘Zf’ precursor of Zfy and Zfx is thought to have been added to the PAR after the separation of the eutherian and marsupial lineages 193–186 million years ago, and that with further PAR additions and rearrangements it became located in the non-recombining regions of the X-Y pair [32,33]. In eutherian mammals the X-linked genes with retained Y-linked homologues are typically exempt from X dosage compensation, suggesting a constraining dosage requirement in somatic tissues, and in most eutherian mammals this is known or is presumed to be true for Zfx and Zfy [54,55]. However, around 40–70 million years ago in the myomorph rodent lineage, Zfx became subject to X-dosage compensation and the Zfy-encoded proteins diverged [30,55–59]. Furthermore, the divergence in Zfy protein sequence is more marked in the highly acidic amino terminal TA domain that activates target genes, than in the carboxy terminal zinc finger domain that mediates binding to DNA. Here we have shown that in Mus musculus this divergence is associated with increased TA activity and that this is much more marked in Zfy2 than in Zfy1. Given that in mature male mice expression of the Zfy genes has only been detected in testes [27–29], specifically in the germ-line [7], this implies that there was a strong selective force in spermatogenic cells for improved TA activity. This male germ-line specific selective force is likely to have been MSCI, which will have affected Zfx as well as Zfy1 and Zfy2. For a zinc finger transcription factor needed for meiosis II that is dependent on transcription during the brief interphase between meiosis I and meiosis increasing the transactivation activity would be a major advantage. The TA domain of Zfx is likely precluded from responding to the selection because of a dosage sensitive role in somatic cells. On the other hand, the spermatogenic cell specific expression of the Y-encoded genes in the post natal testis allowed their TA domains to increase in activity, but the TA domain of Zfy2 has responded much more than that of Zfy1.

Given the importance of ‘Zf’ gene transcription during the interphase between meiosis I and meiosis II in male meiosis, it is intriguing that female mice (and female mammals generally) have no interphase between the two meiotic divisions. We previously hypothesized that the presence of an interphase between the two meiotic divisions in male mammals would be essential if there are meiosis II critical genes on the sex chromosomes, because they would have been transcriptionally silenced (MSCI) during the preceding ~8 days [14]. However, the two meiotic divisions in females are dependent on RNAs produced and stored during oocyte growth [60], and it may be this dependence on stored RNAs that has enabled female meiosis to dispense with the interphase.

In conclusion, our present findings provide evidence for a specific requirement for Zfy1 and Zfy2 expression in the interphase between meiosis I and meiosis II, for meiosis II to be efficiently completed. We have also provided additional evidence for a marked divergence in the functionality of the three ‘Zf’-encoded transcription factors, with Zfx providing a dosage constrained somatic role with only a minor contribution to sex-linked ‘Zf’ gene function in spermatogenesis, Zfy1 developing a dual role in spermatogenesis via alternative splicing to produce activatory and repressive proteins (see also [7]), and Zfy2 becoming a super-activating transcription factor (see also [30]) to enable it to function in the face of the repressive effects of MSCI and the linked post-meiotic sex chromosome repression. There are undoubtedly further sex-linked ‘Zf’ gene functions to be discovered so the identification of the direct targets of the ‘Zf’-encoded transcription factors is a high priority. This has been thwarted by a failure to obtain specific antibodies for chromatin immunoprecipitation analyses, but transgenes encoding tagged versions of the proteins should provide a way forward. We have also presented a case for their being a G2/M DNA damage checkpoint operating in the interphase between meiosis I and meiosis II that prevents progression to MII if there is unrepaired DNA damage present; our XO mouse models together with the recent first report of a successful method for the targeted disruption of a single copy Y gene [61], will be invaluable for investigating this further. Some intriguing recent data obtained with the X^O/Y^O model suggested that following the injection of diploid spermatids (almost certainly together with interphase secondary spermatocytes) into eggs (‘ROSIs’), a proportion of the cells completed the second meiotic division in the egg, thus avoiding triploidy which is lethal in early pregnancy [62]. The egg provides the cellular machinery for DNA damage
repair by non-homologous end joining (NHEJ) [63], which would be expected to release the proposed G2/M DNA damage checkpoint arrest in the X<sup>2</sup>S<sup>x</sup>0O model. However, this pathway of repair is inherently mutagenic [64], which may have important ramifications for the use of ROSI with cells harboring such DNA damage if they were unintentionally used when treating human male infertility.

**Materials and Methods**

**Ethics statement**

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

**Mice**

Aside from the mice with S<sup>x</sup>α or S<sup>x</sup>β attached to the Y<sup>α</sup>* chromosome (see section (1) below), the mice in this study have an outbred MF1 (NIMR colony) background. The XY<sup>α</sup>* males with varying Yp gene complements (Figure 1) were produced by either 1 or 2 below, and the Zf<sub>y</sub> and Zf<sub>x</sub> transgene additions to X<sup>Yα</sup>*S<sup>y</sup> males are described in 3.

1. (i) Mating XX females to X<sup>Yα</sup>S<sup>x</sup>Y* males, or (ii) Mating XX females homozygous for the X-linked Eif2s3y<sup>e</sup> transgene to X<sup>Yα</sup>S<sup>x</sup>Y* males. The fathers used in these crosses are unique genotypes generated specifically for this study to enable a more efficient production of males carrying the X chromosome derivative (Y<sup>α</sup>*; see below) and the Yp-derived sex reversal (S<sup>x</sup>) factors. These unique genotypes are males [15,16,18] with either the Tp(Y)[Ct]<sup>x</sup>α sex-reversal factor [65], or the Tp(Y)[Ct]<sup>x</sup>β sex-reversal factor [6,13], attached distal to the X PAR (denoted X<sup>Yα</sup>S<sup>x</sup>Y* and X<sup>Yα</sup>S<sup>x</sup>Y<sup>x</sup>* respectively). X<sup>Yα</sup>S<sup>x</sup>Y* males have a Y chromosome 'hijacked' by an X centromere attached distal to a rearranged PAR. One of the recombinant sex chromosomes generated is the minute Y<sup>α</sup>*X chromosome comprising a complete PAR with an X PAR boundary, a very limited amount of X-specific DNA, and an X centromere (Figure 1E). In these X<sup>Yα</sup>S<sup>x</sup>Y* males the recombination event generating the Y<sup>α</sup>* adds the S<sup>x</sup>α or S<sup>x</sup>β factor distal to the Y<sup>α</sup>* PAR. In producing the X<sup>Yα</sup>S<sup>x</sup>Y* fathers the S<sup>x</sup>α factors were passed through females heterozygous for the X-autosome translocation T(X;16)16H1 as previously described [66,67], and this introduced some 'non-MF1' genetic background. The resulting X<sup>Yα</sup>S<sup>x</sup>Y*<sup>x</sup>α and X<sup>Yα</sup>S<sup>x</sup>Y*<sup>x</sup>β offspring were 67.5% MF1. A detailed description of the production and characteristics of these X<sup>Yα</sup>S<sup>x</sup>Y* and X<sup>Yα</sup>S<sup>x</sup>Y<sup>x</sup>* males is in preparation.

2. Mating XY<sup>α</sup>* females [15,16,18] carrying an X-linked GFP transgene marker [68] to: (i) X<sup>Yα</sup>S<sup>x</sup>Y<sup>x</sup>*S<sup>y</sup> males that have the X chromosome carrying an Eif2s3y<sup>e</sup> Y-genomic BAC transgene [8]; a Y-chromosome with an 11 kb deletion removing S<sup>y</sup>r [dil1RbH] [69,70], and an autosomally located S<sup>y</sup>r transgene [Tg(Sry)2Ei] [71]; this cross produces X<sup>Yα</sup>S<sup>x</sup>Y<sup>x</sup>*S<sup>y</sup> males that do not exhibit GFP florescence when examined using GFP goggles and allowed to stand for one hour in a humid chamber at room temperature; (ii) XY<sup>Yα</sup>S<sup>x</sup>Y<sup>x</sup>* males that have a Y-chromosome with S<sup>x</sup>β attached distal to the PAR; this cross produces GFP-negative X<sup>Yα</sup>S<sup>x</sup>Y<sup>x</sup>* males with S<sup>x</sup>β attached to the X PAR. (iii) XY<sup>Yα</sup>S<sup>x</sup>Y<sup>x</sup>* males that have a Y-chromosome with S<sup>x</sup>α attached distal to the PAR; this cross produces GFP-negative X<sup>Yα</sup>S<sup>x</sup>Y<sup>x</sup>* males with S<sup>x</sup>α attached to the X PAR.

MF1 XY<sup>Yα</sup>S<sup>x</sup>Y<sup>x</sup>* males were used as normal controls; XY<sup>Yα</sup>S<sup>x</sup>Y<sup>x</sup>* is the strain of Y chromosome from which S<sup>x</sup>α and S<sup>x</sup>β derive.

3. Zf<sub>y</sub> or Zf<sub>x</sub> transgenes inserted on autosomes are expressed during pachytene, which results in pachytene stage IV apoptosis and consequent sterility [26], so we have used X-located single copy Zf<sub>y</sub>1 or Zf<sub>x</sub>2 transgenes that are silenced along with the endogenous X and Y genes at the beginning of pachytene. The Zf<sub>y</sub>1 and Zf<sub>x</sub> transgene additions involved cross 2(i) except that the X<sup>Yα</sup>S<sup>x</sup>Y<sup>x</sup>* male also carried either (i) 1 copy of an X-located Zf<sub>y</sub>1/Uba1y BAC (RP24-327G6) transgene [26], (ii) 1 copy of a Zf<sub>x</sub>2 BAC inserted by cassette mediated exchange (CME) into the Hprt locus on the X chromosome [11,26], or (iii) 7 copies of an autosomal located Zf<sub>x</sub> BAC (RP23-269L6) transgene.

**Genotyping and copy number estimation**

Crosses 1(i)-2(iii) above generate XO males as well as the XY<sup>α</sup>* males with varying Yp complements. As a guide to the presence of Y<sup>α</sup>* we utilised PCRs for X-linked Pdcd4 (absent in Y<sup>α</sup>*, Amels (present in Y<sup>α</sup>*), and Myog (on chromosome 1) for normalisation. Two PCR reactions were used to detect the presence of Y<sup>α</sup>* and the number of X-chromosomes. An 82-bp Pdcd4 and a 162-bp Amels fragment were amplified using primers Pdcd4-F and Pdcd4-R together with primers Amels-F and Amels-R. The 162-bp Amels fragment and a 246-bp Myog fragment were amplified using Amels primers together with Myog primers Om1a and Om1b [72]. Primer sequences are described in Table S4. The following conditions were used: 95°C for 5 min, followed by 28 cycles of 95°C for 30 sec, 60°C for 20 sec and 72°C for 30 sec, with a final extension at 72°C for 5 min. Products were separated on a 3.5% (w/v) agarose gel and the genotype inferred from the relative intensities of the PCR products: XO 1 Pdcd4 + 1 Amels + 2 Myog, XY<sup>α</sup>* 1 Pdcd4 + 2 Amels + 2 Myog, XX 2 Pdcd4 + 2 Amels + 2 Myog and XY<sup>α</sup>* 2 Pdcd4 + 3 Amels + 2 Myog.

For mice typed as Y<sup>α</sup>* positive that provided material for the present study the presence of the Y<sup>α</sup>* was confirmed either by examination of Giemsa-stained bone marrow chromosome spreads to check for the presence of the very small Y<sup>α</sup>* chromosome, by SYCP3 and CENT immunostaining of testis cell spreads (see below), or by quantitative PCR using Pdcd4-F, Pdcd4-R, Amels-F and Amels-R primers with the following genotypes as controls: XX, XO and XY<sup>α</sup>*. Om1a and Om1b primers were used for normalisation.

Copy number estimation was done by quantitative PCR as previously described in Royo et al 2010 with slight modification. A SacBII ampiclon obtained using SacBII-F and SacBII-R primers (Table S4), match the backbone of the Zf<sub>x</sub>-bearing vector. A X<sup>2</sup>Uba1<sup>Yα</sup>* sample was used as a reference, because it bears a known Y-linked copy number of one (Zf<sub>x</sub>l-7; [26]) and the backbone of the Zf<sub>x</sub>1/Uba1y-bearing vector contains SacBII ORF. Reactions were normalised against amplification of the <i>Atr</i> gene. The difference in PCR cycles with respect to <i>Avr</i> (ΔCt) for a given experimental sample was subtracted from the mean ΔCt of the reference samples (X<sup>2</sup>Uba1<sup>Yα</sup>*). The transgene copy number was calculated as the mean of the power 2 (ΔΔCt).

**Pairing efficiency**

Pairing efficiency between X and Y<sup>α</sup>* was assessed on surface-spread spermatogenic cells preparation from 6-week-old testes. Briefly, a portion of frozen testicular tissue (approximately 10 mg) was defrosted and macerated in 0.2 ml RPMI 1640 solution (Invitrogen Corporation, Gibco) to produce a thin cell suspension. One drop of cell suspension was applied on a pre-boiled microscope slide, mixed with five drops of 4.5% sucrose solution and allowed to stand for one hour in a humid chamber at room temperature for 30 sec, 60°C for 20 sec and 30 sec, with a final extension at 72°C for 5 min. Products were separated on a 3.5% (w/v) agarose gel and the genotype inferred from the relative intensities of the PCR products: XO 1 Pdcd4 + 1 Amels + 2 Myog, XY<sup>α</sup>* 1 Pdcd4 + 2 Amels + 2 Myog, XX 2 Pdcd4 + 2 Amels + 2 Myog and XY<sup>α</sup>* 2 Pdcd4 + 3 Amels + 2 Myog.
temperature. The cells were permeabilized by adding three drops of 0.05% Triton X-100 solution for 10 min, after which ten drops of 2% formaldehyde solution (TAAB) containing 0.02% SDS pH 8.4 were added for 30 min. The slides were then dipped briefly in distilled water and air-dried. After hydration in PBS the slides were soaked in PBST-BSA (PBS containing 0.1% Tween 20 and 0.15% BSA) for 1 hour and incubated overnight at 37°C with rabbit polyclonal anti-SYCP3 (1:300; Abcam) and an anti-centromere (CREST) antibody (1:500; Antibodies Inc.) diluted in PBST-BSA. Slides were washed in PBST, incubated with chicken anti-rabbit Alexa 488 (1:500; Molecular Probes) and goat anti-human Alexa 594 (1:500; Molecular Probes) diluted in PBS for 1 h at 37°C and washed in PBST. Pairing efficiency was evaluated on a Leica microscope after staining the cell nuclei with 4',6-diamidino-2-phenylindole (DAPI) diluted in the mounting medium (Vectashield with DAPI; Vector).

At least four mice per genotype were used and pairing efficiency was assessed for ~50 pachytene spermatocytes that were identified based on their DAPI nuclear morphology and their full autosomal synopsis identified by the synaptonemal complex (SYCP3) staining pattern. We classified the pairing of the Y\(^N\) in three categories: (i) clear PAR-PAR pairing of the X-chromosome with the Y\(^N\) chromosome, (ii) the Y\(^N\) chromosome clearly identifiable as a univalent chromosome, and (iii) no Y\(^N\) chromosome could be identified (most likely lost during cell spreading).

**Ploidy analysis on testis cell spreads**

Nuclear DNA content was measured on surface-spread spermatogenic cells from 6 week old testes as described previously [11,14] using SYCP3 staining and DAPI fluorescence intensity measurements. Antibody against γH2AFX (1:500; Upstate) was used to identify the sex body of pachytene spermatocytes [73].

**Fluorescence in situ hybridization (FISH)**

RNA-FISH for nascent nuclear transcripts from Zfy1, Zfy2 and Zfx was performed as previously described [7,74] using spread testis cells from adult MF1 male mice. Zfx RNA FISH was also carried out on spread testis cells from XY, ZFX, ZFY and mouse Zfx osteocytes in the mounting medium (Vectashield with DAPI; Vector). The Zfy2-specific probe was BAC CTTB-283D7 (Research Genetics), the Zfy1-specific probe was a modified version of BAC RP24-49K8 (CHORI) from which we had removed the entire UbA1y gene by recombineering, the Zfx-specific probe was BAC BMQ-372M23 (CHORI), the Mrm-specific probe was BAC RP24-287E17 (CHORI) and the Uty-specific probe was BAC CTTB-246A22 (Research Genetics). Zfy1, Zfy2 and Zfx RNA FISH signals were confirmed with DNA FISH as described previously [74]. Antibody against SYCP3 (1:100; Abcam) was used to identify secondary spermatocytes as previously describe [11].

**In vitro transactivation assay**

ZF TA domain-Gal4 fusion-protein constructs were made by inserting cDNA segments encoding the different acidic domains into the Ncol and SalI, or NdeI and SalI, restriction sites of the vector pGBK-CEN6, a single-copy version of pGBKTK7 (Clontech), downstream of the Gal4 DNA-binding domain and the c-myc epitope tag of the vector, pGBK-CEN6 without an insert was included as a negative control. We used a low-copy origin because we had previously noticed that the expression of an acidic domain that strongly transactivates (Zfy2 and Gal4) inhibits yeast growth [7], and this has been described for the overexpression of Gal4 [75]. Validating our strategy, yeast transformed with the different acidic domain constructs, including Zfy2, all showed similar growth rates (as did the Gal4 acidic domain — data not shown). To create pGBK-CEN6, we replaced the 2 μ high-copy origin of pGBKTK7 with the ARS4/CEN6 low-copy origin from pDEST22 (Invitrogen), by recombineering in the EcoRI strain DY380 [76].

Acidic domains from human ZFY and mouse Zfy1 and Zfy2 were transferred to pGBK-CEN6 from pGBKTK7 constructs as described previously [7]. The acidic domains from human ZFX and mouse Zfx were amplified from testis cDNAs and mouse Zfx was amplified from genomic DNA. PCR-amplified inserts were shown to be without error by sequencing primers. Primers used were Zfx: o4472/o4109, with respectively Nol and SalI adaptors, and ZFX: o4473/o4109 and Zfx: o4471/o4109, with respectively NdeI and SalI adaptors (Table S4). One recombinant was selected for each construct and transformed into the S. cerevisiae strain Y187, in which the β-galactosidase gene is under the control of the Gal4-responsive Gal1 promoter. Three single transformed colonies were picked from SD/-trp agar plates and grown separately in liquid culture to an OD\(_{600}\) of 0.9–1.26 in SD/-trp liquid minimal medium. The β-galactosidase assay was performed on 1 OD\(_{600}\) unit of the culture using the permeabilized cell assay [77].

**Statistical analysis**

For ploidy frequency differences between genotypes were assessed by one tail student t-test assuming unequal variances after angular transformation of percentages, using Excel (Microsoft) software. For the transactivation assay one tail student t-test assuming unequal variances was performed on the β-Galactosidase activity.

**Supporting Information**

Figure S1 Distribution of Pml1, H2al2y and H2ald transcripts in testis of 2-month old wild type mouse. In situ hybridisation using antisense probes for Pml1, H2al2y or H2ald on serial sections of a testis (see Text S1 for experimental procedures). Bottom panel indicates epithelium stages of the corresponding seminiferous tubules (identified using Lectin PNA antibody detection and DAPI staining that are not represented). On the right side of each bright field picture is reported a diagrammatic representation of the expression patterns of each gene with the specified colour code indicative of the relative signal intensity of the probe; ranging from very faint, faint, moderate to strong expression. The scale bar represents 160 μm. (TIF)

Figure S2 Transcription of the autosomal-located Zfx transgene assessed by RNA FISH for nascent nuclear transcripts. Representative images of pachytene spermatocyte (Pa) and secondary spermatocyte (Sertoli) nuclei from a 6-week old male bearing an autosomally-located Zfx transgene are shown hybridized with RNA FISH probes specific for Zfx (arrows) and stained with an antibody against SYCP3 as indicated. X- or Y-bearing cells were differentiated using X-paint labelling. A. A pachytene spermatocyte expressing the Zfx transgene next to a Zfx expressing Sertoli cell (Sert). B. To confirm autosomally-located Zfx transgene expression in pachytene spermatocytes, staining of sex body with γH2AFX antibody was also used. C. Representative images of a Y-bearing secondary spermatocyte (no X-paint labelling) expressing the Zfx transgene. D. Numbers of X- or Y-bearing secondary spermatocytes scored as positive or negative for Zfx expression. 4 out of 17 secondary spermatocytes were expressing only the autosomally-located Zfx transgene (see Text S1 for detailed experimental procedures). (TIF)
Figure S3  Western blot analysis of proteins extracted from yeast cells transformed with the seven constructs used to assess the transactivation activity of ZF protein acidic domains. Western blot analysis with anti c-myc antibody (Text S1) shows the presence of the ZF fusion proteins from the six different ZF isoforms from humans (h) or mouse (mm). Series 1–3 are the three transformed colonies used for each construct. The few observed differences in fusion protein concentration between transformants carrying the same construct did not correlate with β-galactosidase activity (Figure 6). However, the mm ZFX and mm ZFY1 fusion protein concentrations were higher than that of mm ZFY2 in the three series. We conclude from this that the fusion protein concentration is probably not limiting for the transactivation in any transformant and that it would therefore be inappropriate to normalise β-galactosidase activity to fusion gene concentration. mm ZFA is encoded by an autosomal gene derived from a retroverted X transcript. Molecular weights (MW) based on the size standard are shown in the first lane. The expected sizes of fusion proteins range from about 52 kDa for mm ZFA to 60 kDa for hs ZFX, hs ZFY and mm ZFX. The retarded migration of the ZF fusion proteins is most likely a consequence of the large positively charged acidic domain [7].

Figure S4  Zfx transcription in mid/late zygotene spermatocytes. The two pictures are of the same mid/late zygotene spermatocyte nucleus, showing the robust RNA FISH signal (green) obtained with the Zfx probe. The staining for phospho-H2AFX (left) followed by staining for SYCP3 (right) enables a confident assessment of meiotic stage. All 25 mid/late zygotene cells analyzed had robust Zfx RNA FISH signals.

Figure S5  Markedly increased MI apoptosis in 30 day old XO/Sry male transgenic for Zfcs. A. TUNEL-positive (green) first meiotic metaphases (Mls) and healthy phospho histone H3 (pH3, red) positive Mls were identified by their position away from the basal cell layers. As previously shown [11] there are relatively few apoptotic cells in XO/Sry testes, and these are predominantly spermatagonia located at the periphery of the tubules. With the addition of the Zfcs transgene there are now abundant more centrally-located apoptotic cells, which are apoptotic MI spermatocytes in stage XII tubules. DAPI (blue) was used as a nuclear stain (see Text S1 for detailed experimental procedures). The scale bar represents 200 μm. B. Quantitation of MI apoptosis was carried out on entire testis sections (16 to 46 seminiferous tubules with MI) from X0/Osry and X0/Osry;Zfcs mice as previously described [11]. Zfcs transgene addition is effective in promoting the apoptotic response at MI when added to X0/Osry males. *p≤0.05, **p≤0.01, ***p≤0.001. (TIF)

Table S1  Strategy for adjusting the haplode frequencies of the Ya- bearing males to remove the products of the MI cells that did not achieve PAR-PAR synopsis. (XLS)

Table S2  Haplode spermatid frequencies in XY mice, and in XO and XYa+ mice with varying Yp gene complements. (DOC)

Table S3  X- and Y-linked gene expression by RNA-FISH in spermatogenic cells from adult XY male. (DOC)

Table S4  List of primers used to amplify the acidic domains from human and mouse ZF proteins. (DOC)

Text S1  Supplemental experimental procedures. (DOCX)

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Author Contributions

Conceived and designed the experiments: NV SMY FD MJM MAW. Performed the experiments: NV SMY FD MJM. Analyzed the data: NIMR. Contributed reagents/materials/analysis tools: MJM PSB. Wrote the paper: NV MJM PSB.

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