

Evidence that Meiotic Sex Chromosome Inactivation Is Essential for Male Fertility

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Summary

The mammalian X and Y chromosomes share little homology and are largely unsynapsed during normal meiosis. This asynapsis triggers inactivation of X- and Y-linked genes, or meiotic sex chromosome inactivation (MSCI) [1]. Whether MSCI is essential for male meiosis is unclear. Pachytene arrest and apoptosis is observed in mouse mutants in which MSCI fails, e.g., *Brca1*^{-/-}, *H2afx*^{-/-}, *Sycp1*^{-/-}, and *Msh5*^{-/-} [2, 3]. However, these also harbor defects in synapsis and/or recombination and as such may activate a putative pachytene checkpoint [4]. Here we present evidence that MSCI failure is sufficient to cause pachytene arrest. XYY males exhibit Y-Y synapsis and Y chromosomal escape from MSCI without accompanying synapsis/recombination defects [5]. We find that XYY males, like synapsis/recombination mutants, display pachytene arrest and that this can be circumvented by preventing Y-Y synapsis and associated Y gene expression. Pachytene expression of individual Y genes inserted as transgenes on autosomes shows that expression of the *Zfy1/2* paralogs in XY males is sufficient to phenocopy the pachytene arrest phenotype; insertion of *Zfy1/2* on the X chromosome where they are subject to MSCI prevents this response. Our findings show that MSCI is essential for male meiosis and, as such, provide insight into the differential severity of meiotic mutations' effects on male and female meiosis.

Results and Discussion

Successful progression through meiosis is dependent upon synapsis and recombination between homologs. In male mice, mutations in genes involved in synapsis, e.g., *Sycp1* and *Syce2*, or recombination, e.g., *Dmc1*, *Msh5*, *Brca1*, and *H2afx*, cause meiotic arrest at midpachytene, defined as stage IV of the spermatogenic cycle [2, 6]. In females, the effects are generally less severe and occur at a later stage than in males [3]. The mechanisms that trigger pachytene arrest are poorly

understood. In *Saccharomyces cerevisiae*, defects in synapsis or recombination are sensed by a "pachytene checkpoint" [4]. Whether an analogous checkpoint operates in mammals remains an open question.

Recent work has implicated a second, male-specific factor in pachytene arrest that may explain why males are more severely affected than females [7–10]. During early pachytene in males, the X and Y chromosomes become transcriptionally silent. This process, meiotic sex chromosome inactivation (MSCI), is conserved in the animal kingdom, yet its purpose remains unclear [1]. MSCI is a manifestation of a general silencing mechanism that targets any unsynapsed chromosome [11, 12]. Interestingly, MSCI fails to initiate in mice harboring mutations in synapsis or recombination genes, either because the affected gene encodes a protein that also functions in MSCI, e.g., in *H2afx*^{-/-} males [13], or because the mutation results in the formation of stalled recombination intermediates that sequester silencing proteins, making them unavailable for MSCI, e.g., in *Sycp1*^{-/-} and *Msh5*^{-/-} males [10, 14]. The discovery that these mutants all exhibit MSCI failure has led to speculation that it is sufficient to precipitate stage IV arrest [3]. However, none of these mutants can be used to discriminate the MSCI failure model of germ cell demise from the checkpoint model.

The XYY male mouse is useful in this context because it exhibits escape from MSCI without synapsis/recombination defects. During XYY meiosis, the two Y chromosomes often undergo synapsis, and the resulting YY bivalents escape H2AFX phosphorylation (γ H2AFX), a marker of silencing, and fail to inactivate Y genes [5]. We previously noted that cells exhibiting Y-Y synapsis are apparent at early but not at late pachytene, suggesting that they have been eliminated during midpachytene [5]. Although XYY mice are sterile [15], whether germ cell arrest occurs during stage IV, as it does in synapsis/recombination mutants, has not been determined. We therefore sought to define the stage of arrest in XYY males and to test the hypothesis that Y chromosomal escape from MSCI is responsible for the arrest.

Our conclusion that selection against cells with YY bivalents occurred between early and late pachytene in XYY males was based on an analysis of synaptic configurations [5]. Here, we wished to ascertain directly whether cells with MSCI escape were subject to negative selection. We carried out RNA fluorescence in situ hybridization (FISH) for the Y-linked gene *Uty*, which in XY males is silenced throughout pachytene as a result of MSCI [5], together with immunostaining for γ H2AFX to allow pachytene substaging (Figure 1). We detected *Uty* expression in 23% of early-pachytene XYY cells ($n = 100$) but in no late-pachytene XYY cells ($n = 100$; Figures 1A and 1E). A control autosomal gene, *Atr*, was expressed in 88% ($n = 100$) and 100% ($n = 100$) of early- and late-pachytene XYY cells, respectively (Figures 1B and 1E). These data support the model that XYY cells exhibiting MSCI escape are eliminated prior to late pachytene.

To further corroborate this, we then combined RNA FISH for *Uty* with that for *Adam3*, an autosomal gene expressed in mid- to late-pachytene spermatocytes (Figure 1C) [16]. In support of the selection model, we found *Uty* expression in 30% of

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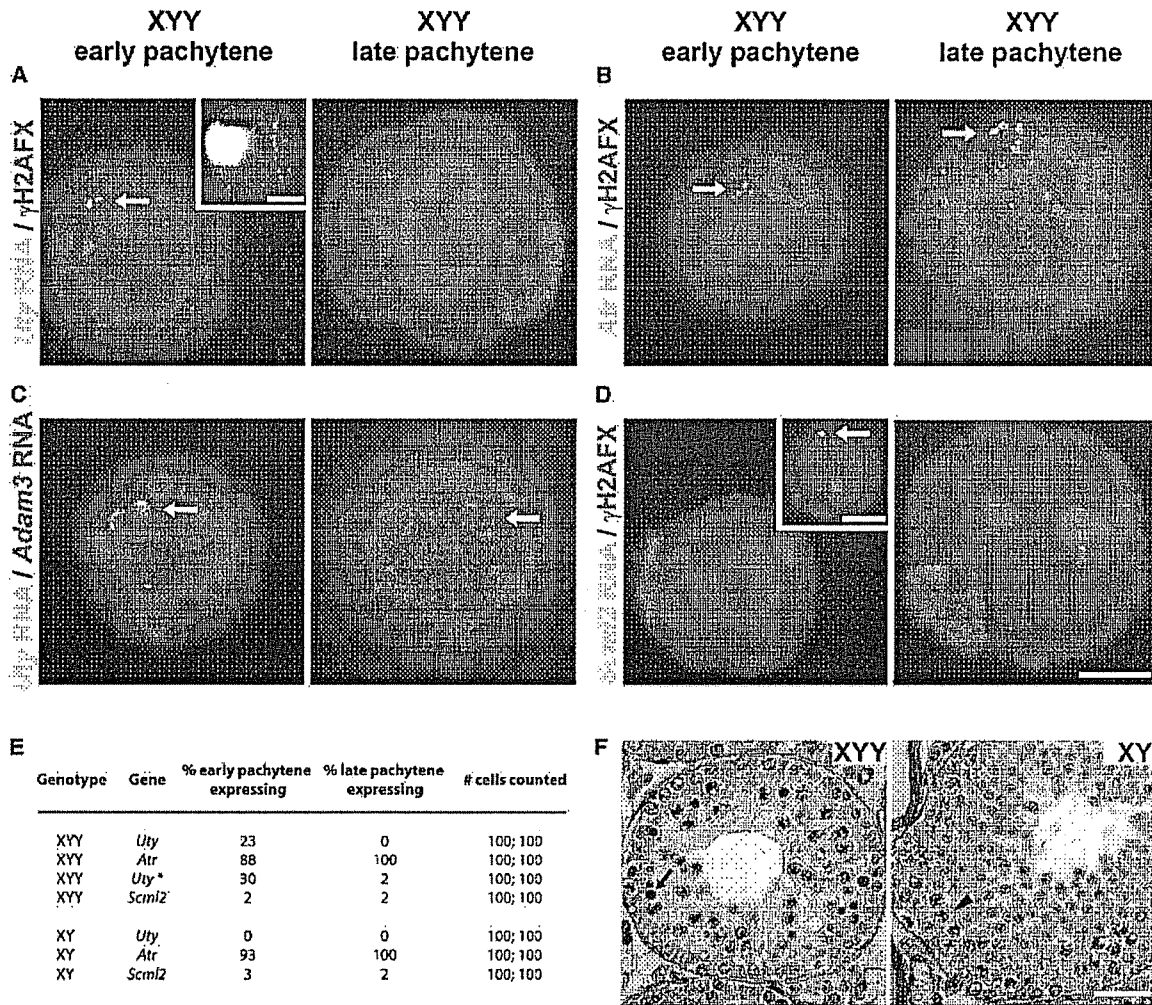


Figure 1. Misexpression of Y-Linked Genes in XYY Males Correlates with Midpachytene/Stage IV Germ Cell Loss

(A–D) RNA FISH images for early-pachytene (left panels) and late-pachytene (right panels) XYY cells, discriminated by their size (late-pachytene cells are bigger) and their staining pattern for γ H2AFX (red in A, B, and D; early-pachytene cells have immature sex bodies and γ H2AFX recombination foci). RNA signals are in green (A–D) and red (C); DAPI is in blue. Scale bars represent 5 μ m.

(A) *Uty* is expressed in early- but not late-pachytene spermatocytes. Inset shows γ H2AFX staining with increased brightness in order to see the recombination foci.

(B) A control autosomal gene, *Atr*, is expressed at both early and late pachytene.

(C) Double RNA FISH for *Uty* (green) and *Adam3* (red). *Uty* is expressed in the *Adam3*-negative (early pachytene) but not the *Adam3*-positive (late pachytene) cell.

(D) No expression is seen for the X-linked gene *Scml2* in the early- and late-pachytene spermatocyte. Inset shows spermatogonia as a positive control.

(E) Quantitative analysis of RNA FISH data for XYY and XY males, using γ H2AFX staining to discriminate early and late pachytene. *Early- and late-pachytene spermatocytes are discriminated in this case by the absence or presence of *Adam3* RNA FISH signal, not γ H2AX staining (only late-pachytene cells express *Adam3*).

(F) PAS-stained stage IV tubule sections showing arrest in XYY but not XY males. Arrow points to dying pachytene cell in XYY males; arrowhead points to healthy pachytene cell in XY males. Scale bar represents 20 μ m.

Adam3-negative, i.e., early-pachytene, cells but in only 2% of *Adam3*-positive ones ($n = 100$; Figures 1C and 1E). Finally, we wished to ensure that MSCI escape in XYY males affected the Y but not the X chromosome and therefore did not reflect a general retarding effect of excess sex chromatin on silencing. We detected expression of the X-linked gene *Scml2* in 2% of early-pachytene XYY cells ($n = 100$) compared with 3% of XY controls ($n = 100$; Figures 1D and 1E), consistent with normal X chromosome silencing. Thus, MSCI escape in XYY males affects only the Y chromosome.

Our RNA FISH analyses placed the timing of elimination of XYY cells with MSCI escape at some point during midpachytene. In mice, midpachytene lasts 45 hr, spanning spermatogenic stages II–V [17]. We wished to ascertain whether germ cell arrest in XYY males occurred specifically at stage IV, as in synapsis/recombination mutants. Histology on three XYY males revealed that this was indeed the case; each exhibited marked germ cell apoptosis at stage IV (Figure 1F), with some germ cells progressing to the meiotic divisions and early spermiogenesis (data not shown). We conclude that MSCI

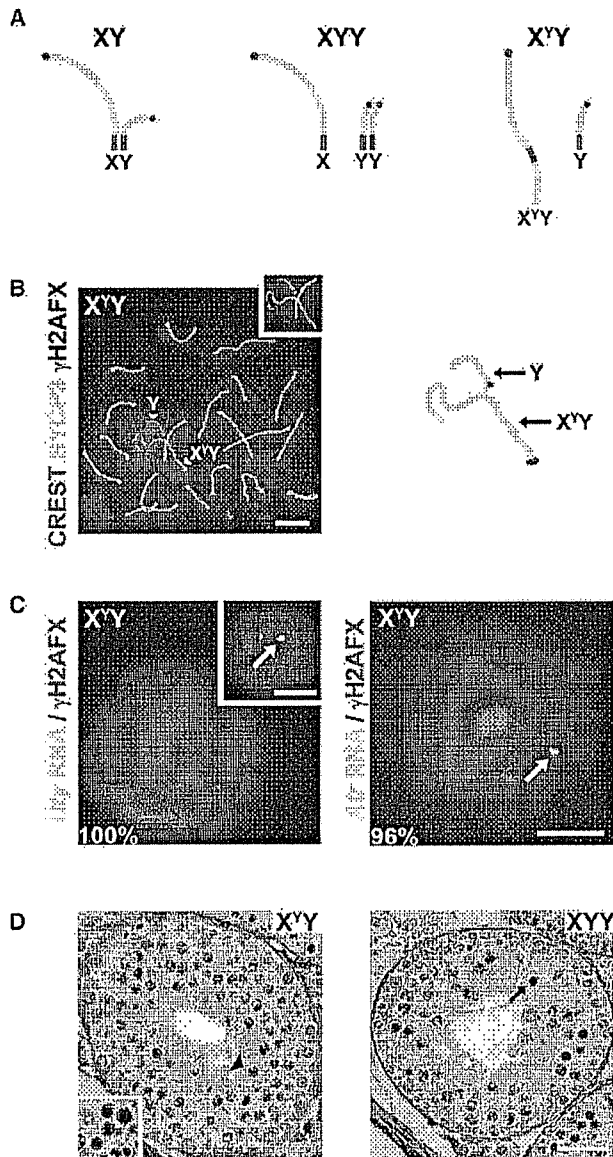


Figure 2. Midpachytene/Stage IV Germ Cell Loss in XYY Males Is Caused by Y-Y Synapsis

(A) Cartoon of sex chromosome configurations in early-pachytene XY, XYY, and X^Y cells. In XY males, the X and Y chromosomes synapse through the pseudoautosomal region (gray). In XYY males, a substantial proportion of cells have the two Y chromosomes fully synapsed. In X^Y males, Y-Y synapsis is prevented because the pseudoautosomal region of the X^Y is not at the chromosome extremity. Centromeres are shown in red.

(B) Surface spread image of a pachytene X^Y cell, showing failure in Y-Y synapsis. SYCP3 is green, centromeres (labeled with CREST) are red, and γH2AFX is blue. Inset shows X^Y and Y chromosome configurations without γH2AFX staining; a cartoon of this is shown to the right with the overlapping autosome omitted.

(C) RNA FISH images for early-pachytene X^Y cells. *Uty* is silent during early pachytene, whereas the control *Atr* gene is expressed. Inset shows *Uty*-expressing spermatogonia as a positive control. Scale bars in (B) and (C) represent 5 μm.

(D) PAS-stained stage IV tubule sections showing substantial arrest in XYY but not X^Y males. Arrowhead points to healthy pachytene cell in X^Y male; arrow points to dying pachytene cells in XYY male. Inset shows dying MI spermatocytes in X^Y males. Scale bar represents 20 μm.

escape in XYY males is associated with stage IV midpachytene arrest.

The results of our RNA FISH analyses were consistent with a model in which Y-Y synapsis drives stage IV arrest. Next, we asked whether preventing Y-Y synapsis allowed XYY germ cells to progress through pachytene. We produced a second type of XYY male with the same chromosomal content, but in which one of the two Y chromosomes is connected to the X chromosome at its pairing end (pseudoautosomal region; PAR), creating the long, "X-attached-Y" (X^Y) chromosome (Figure 2A) [18]. Because synapsis between sex chromosomes requires distal alignment of their PARs [19], we envisaged that the interstitial location of the X^Y chromosome PAR would abolish synapsis between the X^Y chromosome and the second Y chromosome (Figure 2A). As a consequence, all sex chromatin in X^Y pachytene cells should be silenced. Synapsis between the two Y chromosomes in X^Y males was indeed infrequent, occurring in only 2% of pachytene cells (n = 50), and within the cells with Y-Y asynapsis, all of the sex chromatin was γH2AFX positive (Figure 2B). Furthermore, *Uty* RNA FISH revealed normal MSCI in X^Y males: RNA signals were observed in no early-pachytene X^Y cells (n = 100), whereas the control autosomal gene *Atr* was expressed in 96% of cells at this stage (n = 100; Figure 2C).

To ascertain whether preventing Y-Y synapsis influenced meiotic progression in XYY males, we then compared testis histology of X^Y males with XYY brothers. This revealed a marked difference: in XYY males, pachytene spermatocytes underwent stage IV apoptosis, whereas in X^Y males, they progressed to stage XII, the time of the meiotic divisions, where they underwent apoptosis (Figure 2D). The later arrest is predicted to result from failure in crossover formation between the two sex chromosomes, which activates a spindle checkpoint [3]. We conclude that Y-Y synapsis drives stage IV losses in XYY males.

How might Y-Y synapsis cause stage IV arrest? We hypothesized that the arrest resulted from a toxic effect of sustained expression of one or more Y genes on midpachytene spermatocytes. We examined the expression of all 15 known mouse Y genes by reverse transcriptase-polymerase chain reaction (RT-PCR) in XYY testes at 15.5 days postpartum, when the first wave of germ cells reach midpachytene, to identify possible candidates (Figures 3A and 3B). We detected expression of nine of the genes, *Zfy1*, *Ube1y1*, *Kdm5d*, *Eif2s3y*, *Uty*, *Ddx3y*, *Usp9y*, *Zfy2*, and *Rbmy1a1*, at this stage (Figure 3B). No expression was seen for the remaining genes, *H2al2y*, *Sry*, *Ssty1*, *Ssty2*, *Sly*, and *Asty*, consistent with studies showing that these genes are transcribed later, during spermiogenesis (Figure 3B) [20–24].

To test which gene or genes might cause midpachytene toxicity, we utilized a transgenesis system in which each of the nine expressed Y genes was introduced into XY males as autosomally integrated bacterial artificial chromosomes (BACs). The location of these transgenes on fully synapsed autosomes allowed them to evade meiotic silencing and to be expressed during pachytene (Figure 3C). We reasoned that expression of one or more Y genes during midpachytene in XY males would phenocopy the stage IV block seen in XYY males. For each line, we performed transgene copy-number quantitation, RNA FISH to confirm pachytene expression, quantitative RT-PCR (qRT-PCR) to compare the expression level of the Y gene in the transgenic testis to that in XY males, and histology to look for evidence of stage IV arrest.

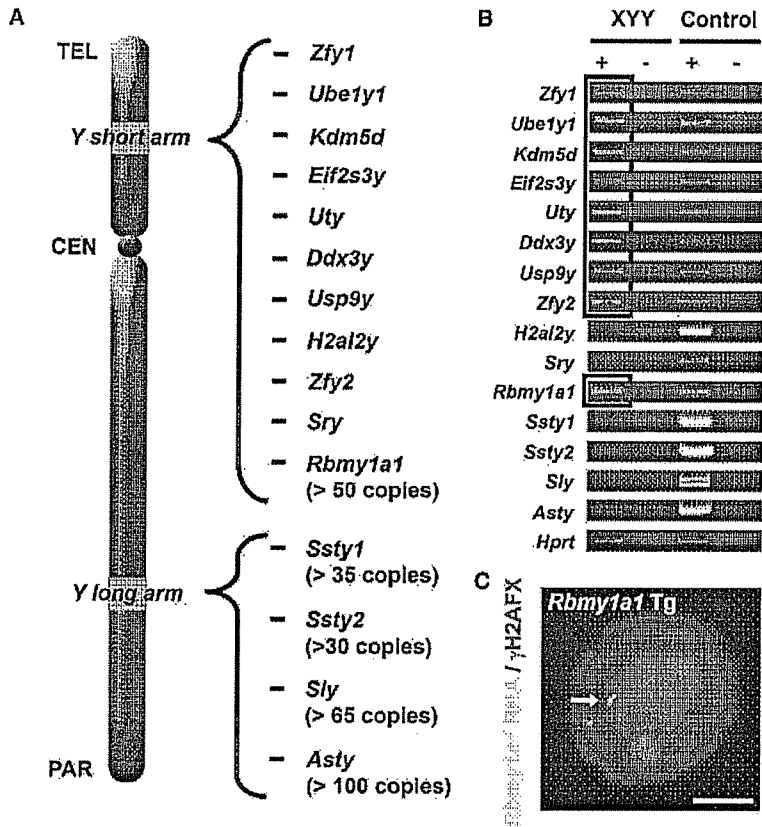


Figure 3. Search for Candidate Y-Linked XYY Pachytene Arrest Genes

(A) List of the 15 known Y-linked genes in mouse. The following abbreviations are used: TEL, telomere; CEN, centromere; PAR, pseudoautosomal region.

(B) Expression of the 15 Y-linked genes assayed by RT-PCR in 15.5 days postpartum (dpp) XYY testes. At this age, the most advanced cells of the first spermatogenic wave are in midpachytene. Adult testes from XY males act as a positive control. The nine genes expressed in XYY males are highlighted by red boxes. Note that all nine genes have been previously shown to be repressed during normal male meiosis as a result of meiotic sex chromosome inactivation [37].

(C) Representative RNA FISH image showing expression of a Y transgene, in this case *Rbmy1a1* (green), in early-pachytene cells. The transgenic copies express the transgene, whereas the endogenous Y-linked copies, incorporated within the sex body (red), are silent. RNA FISH images for the six remaining lines are shown in Figure S1A. Scale bar represents 5 μ m. See also Figures S1 and S2.

First, we analyzed transgenic lines for seven of the nine candidate Y-linked genes (copy numbers shown in parentheses), *Ube1y1* (3-4), *Kdm5d* (11-12), *Eif2s3y* (26-32), *Uty* (8), *Ddx3y* (1), *Usp9y* (20-22), and *Rbmy1a1* (4-5; see Table S1 available online). For each line, we detected pachytene expression of the transgene by RNA FISH (Figure 3C; Figure S1A), and qRT-PCR showed that the expression level for the Y gene was higher than that in noncarrier males (Figure S1B). For all seven lines, transgenic males were fertile and transmitted the transgene to their offspring, indicating that expression of the transgene was not pachytene lethal (Figure S2). Histology showed grossly normal spermatogenesis in each line (Figure S2) with no evidence of stage IV arrest. We were also able to generate a male expressing all of these transgenes except *Ddx3y*; this male also had no stage IV block (data not shown). We conclude that overexpression of *Ube1y1*, *Kdm5d*, *Eif2s3y*, *Uty*, *Ddx3y*, *Usp9y*, and *Rbmy1a1* is not grossly detrimental to male meiosis.

We then examined the effect of misexpressing the remaining two Y genes, *Zfy1* and *Zfy2*, on male meiosis. The *Zfy1/Zfy2* paralogs originated as a result of a duplication event in recent evolutionary history and as such are similar, sharing 94% amino acid homology [25]. We generated seven *Zfy1* and four *Zfy2* transgenic male founders (Figure 4A). In the former case, the BAC also contained *Ube1y1*, because *Zfy1*-only-containing BACs were not available; however, our earlier analysis had shown that *Ube1y1* overexpression was not deleterious to male meiotic cells.

In contrast to the other Y transgenics, the first four *Zfy* founder males, comprising two *Zfy1/Ube1y1* transgenics (Z1

lines 1 and 2, copy number 14 in both cases) and two *Zfy2* transgenics (Z2 lines 1 and 2, copy numbers 2 and 6, respectively), were sterile. Interestingly, the testis weights in these males were low (Figure S3D), and histology showed in all four cases a stage IV block (Figure 4B; Figure S3A). In each founder, the transgene was expressed during pachytene (Figure 4B; Figures S4A and S4E). qRT-PCR showed that for line Z2-2, the *Zfy* expression level was lower than that in XYY males (Figure 4C), arguing against the possibility that the midpachytene arrest was an artifact resulting from transgene overexpression (see Figure 4 legend for further discussion). Thus, pachytene expression of *Zfy1/2* in XY males phenocopies the stage IV arrest phenotype.

The next three founder males, all transgenic for *Zfy1/Ube1y1* (Z1 lines 3, 4, and 5, copy numbers 3, 23, and 6, respectively) were fertile (Figure 4A; Figure S3D). However, none of these males transmitted the transgene to their offspring, suggesting that they were mosaics. Histology showed pachytene defects, with stage IV apoptosis, pachytene deficiency, and agametic tubules (Figure S3B). To follow the fate of the transgene-carrying germ cells, we carried out *Zfy1* RNA FISH. In all three males, a subpopulation of early-pachytene cells carried the transgene (18%, 22%, and 8% of early-pachytene cells in males Z1-3, 4, and 5, respectively; $n = 100$; Figure 4D; Figures S4B and S4E) and the RNA FISH signals originated from outside the sex body, showing that the transgene had integrated on an autosome. In contrast, 0%, 2%, and 0% of late-pachytene cells carried the transgene in males Z1-3, 4, and 5, respectively (Figure 4D; Figures S4B and S4E). Thus, as observed in the nonmosaic *Zfy* transgenic males, transgene-carrying germ cells in these founders were eliminated during midpachytene.

In contrast to the first seven *Zfy* founders, the remaining two *Zfy1/Ube1y1* transgenics (Z1 lines 6 and 7, copy numbers 2-3 and 1, respectively) and one *Zfy2* transgenic (Z2 line 3, copy number 1) were fertile and transmitted the transgene (Figure 4A). If expression of either *Zfy1/Zfy2* is meiotic lethal, how might transmission be possible? For two lines, we identified problems with transgene expression: Z1 line 6 showed

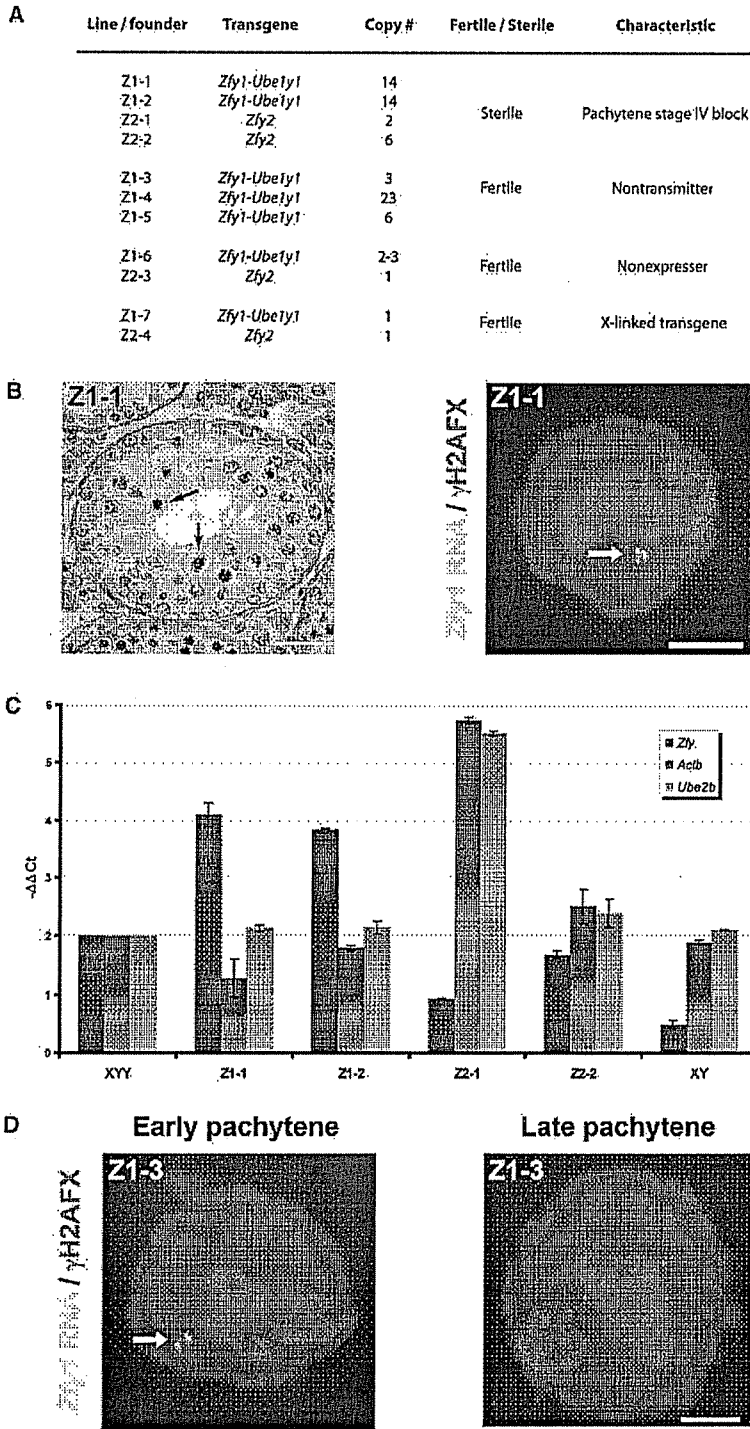


Figure 4. *Zfy* Misexpression at Pachytene Causes Midpachytene/Stage IV Germ Cell Loss

(A) Table of all *Zfy* transgenic founders and lines generated. Eleven *Zfy1/Zfy2* transgenic founders were generated, among which four were sterile. Of the remaining seven, three did not transmit the transgene, two had no pachytene transgene expression, and two had transgene integration on the X chromosome.

(B) Left: PAS-stained stage IV tubule from *Zfy1/Ube1y1* line 1, showing midpachytene arrest. Arrows point to dying pachytene cells. Scale bar represents 20 μ m. Right: RNA FISH (green) for *Zfy1* in the same founder showing expression at early pachytene. Histology and RNA FISH images for the three other sterile founders are shown in Figures S3A and S4A.

(C) Relative *Zfy* transcript levels in sterile *Zfy* transgenic founders and XY males. Transcript levels for *Zfy* and two controls (*Actb* and *Ube2b*) were quantified by qRT-PCR in testes of *Zfy* transgenic founders and directly compared to testes of XY males. *Dazl* was used for normalization. Because the *Zfy* founders arrest at midpachytene, XY testes were stage matched by harvesting at 15.5 dpp. Relative transcript levels are expressed as $-\Delta\Delta Ct$, i.e., $-(\Delta Ct_{Zfy \text{ transgenic founder}} - \Delta Ct_{XY})$. $-\Delta\Delta Ct$ for XY was arbitrarily set to 2. Error bars represent standard error of two cDNA dilutions. *Zfy* levels are higher than those in XY males in two transgenic males (Z1-1 and Z1-2) and lower than XY males in one (Z2-2). The *Actb/Ube2b* levels were consistently elevated in line Z2-1; therefore *Zfy* levels in this founder could not be reliably measured. Note that even in the Z1-1 and Z1-2 transgenics, the *Zfy* expression levels per early-pachytene cell may in reality be equivalent to or lower than that in XY males. This is because in the Z1-1 and Z1-2 transgenics, 99% and 100% of early-pachytene cells, respectively, express *Zfy* (Figure S4E), whereas in XY males, only around one-quarter of early-pachytene cells show Y-Y synapsis and as a result misexpress *Zfy* (see Results and Discussion).

(D) RNA FISH for *Zfy1* in mosaic founder Z1-3, showing expression in early but not late pachytene. See Figure S4B for analysis of other mosaics. See also Figures S3 and S4.

not cause midpachytene toxicity; this silencing was confirmed by RNA FISH (Figures S4C and S4E).

To investigate whether males carrying a *Zfy2* transgene on the X chromosome would also be fertile, we carried out a targeted integration strategy whereby a single copy of *Zfy2* was introduced into the *Hprt* locus in XY embryonic stem cells [26]. We generated an expressing line (Z2 line 4), which subsequently proved to be fertile (Figure 4A; Figures S3C and S3D) and in which the X-encoded copy of *Zfy2* was silenced during pachytene (Figures S4C and S4E). Subsequently, we crossed lines Z2 line 4 and Z1 line 7 in order to generate a male carrying both *Zfy1* and *Zfy2* on the X chromosome. This male was also fertile (Figures S3C and S3D). Together, these findings show that *Zfy1/2* expression during pachytene in XY males is sufficient to phenocopy the XYY stage IV midpachytene block.

The processes that drive midpachytene arrest in male mice with defective synapsis and/or recombination are unclear. Activation of a pachytene checkpoint and failure in MSCI are

indistinguishable *Zfy1* levels compared to noncarriers by qRT-PCR (Figure S4F, left), and Z2 line 3 showed no expression of *Zfy2* by RNA FISH (Figure S4F, right). The final transgenic (Z1 line 7; Figure 4A; Figure S3C) transmitted the transgene only to daughters, showing that the transgene had integrated on the X chromosome. Because the X chromosome undergoes MSCI, the transgene would be silenced and would therefore

both proposed to play a role [2, 3]. In this study, we show that pachytene expression of Y-linked genes elicits an arrest phenotype; XYY males exhibit a stage IV meiotic block that is dependent on Y-Y synapsis, and the same block can be created in XY males by pachytene expression of a single Y gene pair, *Zfy1/2*. These observations provide a compelling case for *Zfy1/2* misexpression causing the pachytene arrest observed in XYY mice. In XYY men, one of the two Y chromosomes is often lost during the spermatogonial divisions, and this gives rise to XY germ cells that complete spermatogenesis and support fertility [15, 27]. However, XYY men in which XY germ cell lines do not originate are almost invariably sterile [15]. Y-Y synapsis occurs at high frequency in pachytene cells from such patients [28, 29]. Although the gene content on the Y chromosome varies between humans and mice [30, 31], *ZFY/Zfy* is conserved; thus, misexpression of *ZFY* during pachytene in XYY men may contribute to their germ cell arrest phenotype. The *Zfy* genes are transcription factors thought to act principally in transcriptional activation [25]; future studies will determine which targets link pachytene *Zfy* misexpression to stage IV arrest.

How do these findings impact on our understanding of the stage IV pachytene arrest phenotype seen in other meiotic mutants? A recent report found MSCI failure in *Dmc1*, *Msh5*, *Dnmt3l*, and *Spo11* mutants [10]; this study used *Zfy2* RNA FISH to follow silencing, and in all cases it was found to be misexpressed during pachytene. Our demonstration that *Zfy1/2* expression causes stage IV apoptosis substantiates earlier suggestions that MSCI failure contributes to the increased severity of these mutations' effects on male relative to female meiosis [3, 10]. However, it should be noted that in these models, MSCI failure also affects the X chromosome. An earlier study on T(X;16)16H males noted that cells in which the X, but not the Y, chromosome escapes silencing are also selected against during pachytene [5], and we have recently found that stage IV arrest also occurs in this model (unpublished data). Thus, the X chromosome almost certainly contains pachytene-lethal genes.

Previous studies have demonstrated that any chromosome that fails to synapse with its homologous partner is silenced during pachytene [11, 12]. The role of this silencing in meiotic development is unclear, but it may serve to prevent illegitimate recombination between nonhomologs [32], to halt transcription from damaged DNA [33], or to eliminate germ cells with synaptic errors [1]. The discovery that meiotic silencing is not specific to the X and Y chromosomes counters an early hypothesis that MSCI evolved primarily to inactivate toxic sex-linked genes [34]. We favor a model in which sex-linked gene expression during pachytene was tolerated prior to the evolutionary divergence of the sex chromosomes, when the proto-X and Y chromosomes engaged in full synapsis. The subsequent rapid evolution of X and Y genes would have given rise in some instances to modified forms whose expression could no longer be tolerated during pachytene.

A challenge for the future will be to unravel the relative influences of MSCI failure versus a putative checkpoint on stage IV arrest. This requires mouse mutants with defective synapsis and/or recombination but normal MSCI. Recently, a hypomorphic mutation of *Trip13* was generated that exhibits a partial stage IV block and defective meiotic DNA double-strand break repair but normal H2AFX phosphorylation within the XY chromatin [35, 36]. Although MSCI still needs to be studied in these mice, they have the potential to enable the identification of the molecular pathways underlying such a checkpoint.

Supplemental Information

Supplemental Information includes four figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2010.11.010.

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