Spermatid development in XO male mice with varying Y chromosome short-arm gene content: evidence for a Y gene controlling the initiation of sperm morphogenesis

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Abstract

We recently used three XO male mouse models with varying Y short-arm (Yp) gene complements, analysed at 30 days post partum, to demonstrate a Yp gene requirement for the apoptotic elimination of spermatocytes with a univalent X chromosome at the first meiotic metaphase. The three mouse models were i) XSYrO in which the Yp-derived Tp(Y)1CtSxr-a sex reversal factor provides an almost complete Yp gene complement, ii) XSxr²⁰,Eif2s3y males in which Tp(Y)1CtSxr-b has a deletion completely or partially removing eight Yp genes – the Yp gene Eif2s3y has been added as a transgene to support spermatogonial proliferation, and iii) XOSry,Eif2s3y males in which the Sry transgene directs gonad development along the male pathway. In this study, we have used the same mouse models analysed at 6 weeks of age to investigate potential Yp gene involvement in spermiogenesis. We found that all three mouse models produce haploid and diploid spermatids and that the diploid spermatids showed frequent duplication of the developing acrosomal cap during the early stages. However, only in XSxrO males did spermiogenesis continue to completion. Most strikingly, in XOSry,Eif2s3y males, spermatid development arrested at round spermatid step 7 so that no sperm head restructuring or tail development was observed. In contrast, in XSxr²⁰,Eif2s3y males, spermatids with substantial sperm head and tail morphogenesis could be easily found, although this was delayed compared with XSxrO. We conclude that Sxr²⁰ (and therefore Yp) includes genetic information essential for sperm morphogenesis and that this is partially retained in Sxr²⁰.

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Introduction

Spermatogenesis consists of three distinct phases: the proliferative phase, in which spermatogonia undergo successive mitotic divisions to assure both germ cell production and maintenance of the tissue by stem cell renewal, the meiotic phase in which spermatocytes undergo two consecutive divisions to produce haploid spermatids and the spermiogenesis phase in which haploid round spermatids differentiate into sperm. During the spermiogenesis phase, there is extensive nuclear restructuring involving the replacement of histones by protamines in conjunction with elongation and condensation of the nucleus to form the sperm head. Meanwhile, the spermatids develop an acrosome that is a cap-like structure containing the enzymes necessary to break down the outer membrane of the egg during fertilization, and a tail (flagellum) is formed.

It has long been known that spermiogenesis can occur in the absence of the second meiotic division, thus generating spermatids with a 2C DNA content ‘diploid spermatids’ (Hannappel & Drews 1979, Hannappel et al. 1980, Levy & Burgoyne 1986, Kot & Handel 1990, Mori et al. 1999). For instance, in XSxrO male mice where the X chromosome carries the Y chromosome short-arm (Yp)-derived Tp(Y)1CtSxr-a ‘sex reversal’ factor (Fig. 1), a massive apoptotic elimination occurs at the metaphase of the first meiotic division (MI) in response to the sex chromosome univalence (Levy & Burgoyne 1986, Kot & Handel 1990, Sutcliffe et al. 1991). However, a very small proportion of MI spermatocytes do not apoptose; the majority of these do not undergo the second meiotic division and remain diploid, while a minority do complete the second meiotic division to become haploid (Levy & Burgoyne 1986, Sutcliffe et al. 1991). Subsequently, the diploid and haploid cells initiate the
condensation phase of spermiogenesis and there is substantial head morphogenesis – albeit abnormal due to the absence of the Y long-arm (Burgoyne et al. 1992, Cocquet et al. 2009). The occurrence of spermiogenesis in the absence of the second meiotic division is not unique to mice with an abnormal sex chromosome complement, as it is also seen in male T(6;12)32H carriers where a reciprocal translocation between autosomes 6 and 12 results in incomplete chromosome pairing and diploid spermatids are generated (de Boer et al. 1986).

Recently, we investigated the DNA content of the meiotic products in two XO male mouse models with a deficient Yp gene complement (Vernet et al. 2011). In the first model, the Y gene complement is provided by transgenic copies of Sry (on an autosome) and Eif2s3y (on the X chromosome) and in the second Sry is replaced by Tp(Y)1CtSxr-b, a derivative of Sxr with a deletion removing most Yp genes but retaining Sry. These models are here denoted as X£SryO and X£SxrO respectively (Fig. 1). At 30 dpp in both models, meiosis was found to be blocked in the interphase between the first and the second meiotic division so that no haploid spermatids were produced. In control males at this age, both meiotic divisions are completed and elongating spermatids are already seen (Vernet et al. 2011). In the X£SryO males, we further showed that during epithelial stages II–III, the interphasic secondary spermatocytes with 2C DNA lost the SYCP3 staining typical of interphasic secondary spermatocytes; in wild-type mice, SYCP3 is lost at the same epithelial stage following the secondary spermatocyte to haploid round spermatid transition (Vernet et al. 2011). This observation therefore suggested that diploid interphasic secondary spermatocytes also enter spermiogenesis to become diploid round spermatids. However, no progression to elongating spermatid stages was seen at 30 dpp. In a separate study, Decarpentrie et al. (2012) reported that in X£SxrO testis from 30 dpp onwards, expression from the Zfy2 spermatid-specific Cyt-derived promoter was detected, which again suggests entry into spermiogenesis. Finally, Mazeyrat et al. (2001) claimed that there were some elongating spermatid stages in these two Yp gene-deficient models when analysed at substantially older ages.

These previous studies suggested that spermiogenesis was initiated in these Yp-deficient models, so we therefore sought to determine the extent of spermiogenic progression in X£OSry and X£SxrO males in comparison to that seen in X6SxrO mice, which have an almost complete Yp gene complement.

**Results**

**Completion of the second meiotic division in X£OSry and X£SxrO mice at 6 weeks of age**

In our published study (Vernet et al. 2011), the DNA content of post-meiotic cells was assessed at 30 dpp.
This age was chosen because the DNA analysis was linked to a study of MI spermatocyte apoptosis, and at later ages, apoptotic interphasic secondary spermatocytes from the previous cycle interfered with the quantitation of MI spermatocyte apoptosis. For the current study of spermiogenic progression, we wished to analyse 6-week-old males when spermatogenesis is fully established, but when there is none of the secondary damage resulting in disturbed spermatogenesis and spermatogonial and spermatocyte loss that is seen in older males (Mazeyrat et al. 2001).

The appropriate XO control males for this study are X Sxr O, which have an almost complete Yp gene complement. For comparison with previous data from 30 dpp X O Sry and X Sxr O males (Vernet et al. 2011), we first quantified the DNA content in spread spermatogenic cells from three 30 dpp X Sxr O males. In contrast to the lack of haploid spermatids in the Yp-deficient mouse models, haploid round spermatids were identified in two of the three X Sxr O males analysed with an average of 8.7% of spermatids being haploid (Table 1). At 6 weeks, all four X Sxr O males analysed had a greater proportion of haploid spermatids with an average of 44.1%. These results indicate that in X Sxr O males the second meiotic division occurs more often after the first spermiogenic wave.

Analysis of the DNA content of post-meiotic cells in four 6-week-old X O Sry and X Sxr O mice showed a low frequency of haploid spermatids in both genotypes varying from 1.5 to 18.4% (Table 1). This indicates that the block at the interphase stage of secondary spermatocytes observed at 30 dpp has become leaky by 6 weeks of age. However, the proportion of haploid spermatids obtained is significantly less than in age-matched X Sxr O males.

Acrosomal development is seen in spermatids of X O Sry and X Sxr O males but substantial head elongation is restricted to X Sxr O males

The development of the acrosome in mouse spermatids is divided into four phases, Golgi (steps 1–3), cap (steps 4–6), acrosomal (steps 7–9) and maturation phases (steps 10–16), based on light microscopic analysis of periodic acid-Schiff (PAS)-stained testis sections and transmission electron microscopy (TEM; Oakberg 1956b, Russell et al. 1990). During the Golgi phase, numerous pro-acrosomic granules are formed within Golgi vesicles followed by a clustering into a vesicle containing a single acrosomic granule that associates with the nuclear envelope. During the subsequent cap and acrosomal phases, the acrosome increases in size by fusion of additional Golgi-derived vesicles and spreads over the anterior nuclear pole. The acrosome covers two thirds of the nucleus at the end of the acrosomal phase and achieves its definitive hook shape during the maturation phase.

Although X Sxr O mice produce very few, predominantly diploid, round spermatids, these nevertheless proceed through spermiogenesis; however, the sperm formed are grossly abnormal (Levy & Burgoyne 1986, Kot & Handel 1990, Sutcliffe et al. 1991). This spermiogenic progression is reflected in acrosomal changes that become apparent in PAS-stained (Supplementary Figure 1, see section on supplementary data given at the end of this article) and peanut agglutinin lectin (Lectin–PNA)-stained sections (Fig. 2) of 6-week-old males. As in XY controls, the few round spermatids of X Sxr O males progressively developed an acrosomal structure. However, while a single acrosomal structure was found at the nucleus of wild-type spermatids, in X Sxr O round spermatids, double and sometime multiple acrosomal structures were often detected (see stages IV–VI from Fig. 2). In order to assess whether this aberrant acrosome development was a consequence of diploidy, we recorded the incidence of aberrant acrosomes in haploid and diploid spermatids in sections stained with Lectin–PNA and 4',6-diamidino-2-phenylindole (DAPI), using the contralateral testes from the two X Sxr O males with the higher percentage of haploid spermatids from the testis cell spread analysis. Diploid round spermatids can be confidently identified in the DAPI-stained sections because multiple nucleolus-like structures are observed and the nuclei are a third larger in diameter than those of haploid round spermatids. In stage IV–V tubules, we found a very significant (P<0.00001) preponderance of multiple acrosome structures (chromocentra) in diploid spermatids, although some were present in haploid round spermatids (Table 2). We therefore assessed the frequency of multiple acrosome structures in stages IV–V tubules in XY males, and this revealed a similar frequency to that obtained for the haploid round spermatids from the X Sxr O males. Elongation of the sperm head becomes apparent from stage IX onwards, and by this stage, the acrosome anomalies were no longer apparent for X Sxr O males (see stages IX–I from Fig. 2). The lack of acrosome anomalies during this acrosomic phase suggests either a fusion of the multiple acrosomal structures or selective loss of the spermatids with aberrant acrosomes.

Table 1 Haploid spermatid production in 30 dpp and 6-week-old XY and XO male mice. Data collected after DNA quantitation using DAPI fluorescence intensity measurement on SYCP3-labelled testis cell spreads. Values are mean ± S.E.M.

| Genotype | Age  | Haploid (%) | Values range |
|----------|------|-------------|--------------|
| XY (n=3) | 30   | 98.2 ± 0.9* | 96.8–100     |
| X Sxr O (n=3) | 30 | 8.7 ± 4.4 | 0–15.2       |
| XY (n=3) | 40–45 | 99.1 ± 0.8* | 97.2–100     |
| X Sxr O (n=4) | 42–45 | 44.1 ± 7.3 | 21.3–52.6    |
| X O Sry (n=4) | 41–45 | 11.4 ± 3.1* | 5.4–18.4     |
| X Sxr O (n=4) | 42–45 | 5.2 ± 1.2* | 1.5–6.9      |

*Very significantly different from age-matched X Sxr O (P<0.001; ANOVA).
As in XsraO males, acrosome development in XESry and XEsxrbO males appeared synchronous with controls, but once again, there were frequent double or multiple acrosomal structures that were no longer apparent by the acrosomal phase (Fig. 2; Supplementary Figure 1). As in XsraO males, diploid round spermatids from XESry and XEsxrbO testis show multiple DAPI bright chromocentra while haploid round spermatids contain one chromocentrum. The defects in acrosome formation in XESry males were confirmed by TEM analysis (Fig. 3). Thus, in XY control mice, the acrosome of step 3–8 round spermatids develops as a single structure at one side of the nucleus, while in XESry males two or more acrosomal structures were often found at the spermatid nucleus at the beginning of the cap phases (Fig. 3A, B, C and D). We occasionally found a discontinuous acrosomal structure at the nucleus of step 6 spermatids, but by the end of the cap phase, no obvious acrosome anomalies were observed (Fig. 3F, G and H).

Despite the comparable acrosome development, it was apparent from the PAS- and Lectin–PNA-stained sections that sperm head morphogenesis in stage IX tubules and beyond was not comparable to that in the XsraO controls. This was most obvious in XESry testes where we could see no clear evidence of the elongation or condensation of the spermatid nuclei that marks the development of the sperm head (Fig. 2, stages XII–I through IV). As it has previously been suggested that sperm head elongation can be seen to some extent in XsraO testes (Mazeyrat et al. 2001), we did further analysis of haematoxylin and eosin (H&E)- and PAS-stained sections but again we did not find any clear evidence of elongation. Indeed, close examination of the PAS-stained material suggested that XESry spermatids almost never re-orientate to bring the acrosome to face the basal membrane, which in XsraO and XY controls marks the progression from steps 7 to 8 of round spermatid development, just before elongation (Fig. 4A). As the acrosome was fully formed around the spermatid nucleus, it is clear that step 7 round spermatids are typically the most advanced germ cells in XESry testes. Interestingly, the round spermatids arrested in step 7 do not enter apoptosis directly as these cells were observed until stage V of the next cycle. However, in stages XI–IV, remnants of dying round spermatids were found in XY and XsraO seminiferous tubules from stage IX onwards and in XsraO from stage XII onwards. Scale bar, 15 μm.

Figure 2 An acrosome is formed in all three genotypes of XO male mice. Acrosomes were detected with Lectin–PNA (red) on testis sections from 6-week-old XY, XsraO, XESry and XEsxrbO mice. DAPI (blue) was used as a nuclear stain. Stages of seminiferous tubules are indicated and schematic diagrams of the spermatids appearing at each stage are represented for the XY control. Acrosome development in post-MI germ cells of XsraO, XESry and XEsxrbO mice is synchronous with the acrosome development in XY round spermatids. However, double acrosomal structures (arrowheads) are observed in spermatids from XsraO, XESry and XEsxrbO males from stage IV to stage VI. Elongated spermatids are found in XY and XsraO seminiferous tubules from stage IX onwards and in XsraO from stage XII onwards. Scale bar, 15 μm.
spermatids and some rare possibly elongating spermatids with a darkly stained nucleus (classically associated with apoptosis/necrosis) were present that could have been confused previously with elongated spermatid nuclei (Mazeyrat et al. 2001), indicating that the arrested step 7 spermatids slowly disappeared by apoptosis/necrosis (Fig. 4 B; Supplementary Figure 1). However, we have also seen apparently healthy round spermatids in the epididymis (data not shown), indicating that the arrested cells can also be shed from the epithelium.

In contrast to XEO Sry, in 6-week-old XESxrbO males, nuclear elongation was evident (Fig. 4) as previously reported for older males (Mazeyrat et al. 2001). This difference in the stage of development reached is consistent with the fact that at 6 weeks of age, XESxrbO testes were significantly larger than those from XEO Sry (57.8±0.28 mg XESxrbO and 49.8±0.33 mg XEO Sry; P=0.0218). However, relative to XSxrO and XY controls, sperm head elongation was delayed from stage IX to stage XII-I and nuclear condensation was delayed from stage XII until stages II–III (Figs 2 and 4).

**Spermatid elongation in XESxrbO in relation to ploidy**
The ‘leak’ in the sperm head elongation arrest at 61 dpp in XESxrbO mice is more extensive at 98 dpp with multiple clumps of elongating spermatids observed in the seminiferous tubules (Mazeyrat et al. 2001). In this study, in XESxrbO mice, we have also easily found tubules with elongating spermatids in 6-week-old testes, whereas at 30 dpp, elongating spermatids are absent; in XSxrO testes, elongating spermatids are frequent at 30 dpp (Supplementary Figure 2, see section on supplementary data given at the end of this article). These age and genotype effects on the frequency of elongating spermatids mirrors the age and genotype effects on the frequency of haploid spermatids, suggesting that haploidy may promote sperm head elongation. To test this, we analysed the ploidy of round and elongating spermatids by quantification of DNA FISH signals in 6-week-old males (Fig. 5). This analysis not only confirmed the low frequency of haploid round spermatids in XESxrbO males (5% haploid and 95% diploid) but also revealed that haploid and diploid spermatids can elongate with equal efficiency (6% haploid and 94% diploid) (Table 3).

### Table 2: The frequency of double/multiple acrosomal structures in haploid or diploid spermatids of stages IV–V tubules for 6-week-old XSxrO and XY male mice.

| Mouse ID | Percentage of haploid spermatids (n= number of cells analysed) | Percentage of cells with double or multiple acrosomal structures | \( \chi^2 \), P value |
|----------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------|
| XSxrO #1 | 38.7 (n=111)                                                 | 9.3 52.9                                                      | 21.8, P=0.000003    |
| XSxrO #2 | 46.6 (n=131)                                                 | 16.4 62.9                                                      | 29.0, P=0.00000007  |
| XY #1    | 100 (n=253)                                                  | 10.2 –                                                       | –                   |
| XY #2    | 100 (n=249)                                                  | 13.1 –                                                       | –                   |

\*See Table 1 for the percentages of haploid spermatids estimated from cell spreads from the contralateral testes. \( \chi^2 \) analysis comparing the frequency of double/multiple acrosomal structures in diploid vs haploid spermatids in XSxrO males.

**Figure 3** Aberrant acrosome development in XO male mice. Electron micrographs of round spermatids from XY control (A, C, E and G) and XEO Sry (B, D, F and H) males. Different steps of maturation are represented. In the Golgi phase, step 3 spermatids show a normal acrosomal structure in XY (A) and a double acrosomal vesicle (each vesicle contains an acrosomal granule; stars) in XEO Sry (B). At the beginning of the cap phase (C and D), the acrosome of step 4–5 spermatids flattens out on the nucleus and the acrosomal granule contacts the inner acrosomal membrane. While XY spermatids have a single acrosomal vesicle (C), double and here multiple acrosomal vesicles (D) are found in XEO Sry spermatids. At the end of the cap phase (E and F), the acrosome has extended over one-third of the nuclear circumference in the XY (E). Double or here discontinuous acrosomes are found in step 6 spermatids of XEO Sry male (F); arrowhead points to the acrosomal discontinuity. (G and H) In the acrosomal phase, acrosomes of XEO Sry spermatids (H) were now indistinguishable from those of XY spermatids (G). Scale bar (in H): 1 μm (A, B, C and D), 1.25 μm (E, F, G and H).
Sperm tail elongation is seen in spermatids of XESxrbO males but not in XEOSry males

The development of the sperm tail is not easy to assess in testis sections. However, it occurs in conjunction with sperm head elongation in normal and XESxraO males (Mahadevaiah et al. 1988), so it seemed likely that sperm tails would be formed in XESxrbO. To confirm this, we prepared silver-stained smears of testicular cell suspensions for all three XO male models (Fig. 6). Sperm with elongated tails were easily identified in the smears from XESxraO males and, as might be expected, none were identified in smears from XEOSry males. In XESxrbO males, sperm with some tail development were also easily found, but the sperm tail elongation was generally less extensive than in XESxraO males. Intriguingly, the sperm heads in XESxrbO males appeared less abnormal than those in XESxraO males.

Discussion

In this study, we compared spermatid development in XO males with varying Yp gene complements: XEOSry (Sry and Eif2s3y), XESxrbO (Rbmy1a1, H2al2y (Gm16501 and Gm6026), Sry, Eif2s3y and Zfy2/1 fusion gene) and XESxraO (Rbmy1a1, H2al2y, Sry, Zfy2, Usp9y, Ddx3y, Uty, Eif2s3y, Kdm5d, Ube1y1 and Zfy1). As

![Figure 4](image_url)

**Figure 4** In XEOSry male mice, spermatids arrest and accumulate at round spermatid step 7 of their development whereas those of XESxrbO mice progress to elongating spermatid stages. Histological sections of testes from 6-week-old XY, XEOry, XESxraO and XESxrbO mice.

(A) PAS/haematoxylin-stained stage IX–X testis tubule sections. At this stage, elongation is clearly seen in XY and XESxraO tubules but barely starts in XESxrbO tubules and is not seen in XEOSry (see corresponding inset). The round nuclear shape and the fact that the acrosome (purple staining) of XEOSry spermatids is often not facing towards the basal membrane (arrowheads) show that the spermatids are arrested at step 7 (they should be step 9–10). (B) H&E-stained stage XII-I testis tubule sections. We show stage XII-I seminiferous tubules because by that stage elongated spermatids should be properly formed. While in XY males, elongated spermatids form a hooked tip, this is lacking in XESxraO and XESxrbO. By this stage, many of the arrested step 7 spermatids in XEOSry have been eliminated by shedding and apoptosis/necrosis. The few that remain have darkly stained nuclei (stars and corresponding insets), which very rarely may have an elongated shape (arrows and corresponding inset). However, similar elongated ‘nuclei’ are seen in large vesicular structures classically associated with apoptosis (bottom right panel). Scale bar, 40 μm. Insets represent a 3 X magnification of some characteristic spermatids found in the corresponding pictures.

![Figure 5](image_url)

**Figure 5** Both haploid and diploid spermatids from XESxraO male mice are elongating. Spread cells from XY and XESxraO testes stained by DNA FISH with autosomal probes for either Kenq1 or Aim. In XY males, for both probes, haploid elongated spermatids display a single DNA FISH spot whereas spermatogonia show two spots characteristic of diploid cells. In XESxraO testis, haploid (one spot) and diploid (two spots) spermatids are elongating.
previously reported for XsraO males, all three XO male genotypes produced diploid and haploid spermatids, as evidenced by ploidy analysis and acrosomal development, but the proportion of haploid spermatids was substantially lower in the two severely Yp-deficient genotypes. Acrosomal anomalies were observed in all three mouse models and were more frequent in diploid spermatids. Ploidy, per se, did not appear to affect the extent of spermiogenic progression, but Yp gene deficiency had clear effects with delayed sperm head and tail morphogenesis in XsraO males and a lack of sperm head elongation and extension of the axoneme to form the sperm tail in XESxrbO males. The results are summarised in Fig. 7.

**Achievement of the second meiotic division in XO males**

Our data show that haploid and diploid spermatids are present in all three XO male genotypes with the proportion of haploid increasing between 30 days and 6 weeks of age, but the proportion of haploid spermatids is markedly higher in XsraO males than in the two Yp gene-deficient genotypes. Drawing any conclusion as to the cause of this latter difference is complicated by the fact that XsraO males have a robust, Zfy2-dependent, apoptotic elimination of spermatocytes at MI, which reduces spermatid counts to ~3% of XY controls, while both types of Yp-deficient XO males have a severely attenuated response because they lack the Y-encoded gene Zfy2 (Sutcliffe et al. 1991, Vernet et al. 2011). The Zfy2-dependent apoptotic response is considered to be a consequence of the univalent X chromosome triggering an MI spindle assembly checkpoint (MI SAC; Burgoyne et al. 1992, Vernet et al. 2011). We must therefore first consider how this difference in apoptotic response may impact on haploid vs diploid spermatid frequencies.

The existence of an MI SAC in mammals has been well documented from studies of mouse oocytes (reviewed by Sun & Kim (2012)). In the present context, the XO female mouse is most relevant for comparison with the Yp gene-deficient mouse models because in female meiosis triggering the MI SAC does not elicit an apoptotic response. XO oocytes complete both meiotic divisions, despite the fact that the X has no pairing partner and thus is predisposed to trigger the MI SAC (LeMaire-Adkins et al. 1997). The failure to trigger MI arrest is explained by recent data showing that the first meiotic division in oocytes can proceed once a ‘critical mass’ of kinetochores have achieved correct attachment to the spindle (Hoffmann et al. 2011, Nagaoka et al. 2011) – a univalent X with 1 or 2 unattached kinetochores is therefore insufficient to maintain MI arrest. In the light of the findings in XO females, it might therefore be

![Figure 6](https://www.reproduction-online.org)
expected that XO spermatocytes in the Yp-deficient models (which lack an apoptotic response) would complete both meiotic divisions to become haploid spermatids, but in fact, we have found the majority of spermatids are diploid (at 6 weeks $X^E O Sry$ had 88.6% and $X^E Sxrb$O 94.8% diploid spermatids). As we have shown, $X Sxra$O males have a substantially lower proportion of diploid spermatids (55.9% at 6 weeks); this could be explained if MI spermatocyte apoptosis preferentially removes those that are destined to form diploid spermatids, but the more exciting possibility is that a Yp gene or genes retained in $Sxra$O promotes the second meiotic division.

In XO females, it has been shown that in a proportion of oocytes, the X univalent achieves bipolar attachment to the spindle followed by segregation of the two sister chromatids; however, this is not a prerequisite for completion of the first meiotic division (Hunt et al. 1995, Hodges et al. 2001). This phenomenon has not been documented in males, but this is probably because the apoptotic response in $Sxra$O males is so efficient that there are almost no anaphase plates available for study. We have therefore started to look for evidence of bipolar attachment and segregation in $X^O Sry$ males. Our preliminary data suggest that bipolar attachment and condensation do take place, but it seems the more common fate is that the X univalent fails to attach to the spindle and is left stranded in a micronucleus (see Supplementary Table 1 and Supplementary Materials and Methods, see section on supplementary data given at the end of this article).

What is the explanation for the dramatic difference between XO females and the Yp gene-deficient XO males, with respect to completion of the second meiotic division?
division? One striking difference between the meiotic divisions of females and males is that in females the chromosomes remain condensed throughout both meiotic divisions, whereas in males there is a transcriptionally active ‘interphase secondary spermatocyte’ stage between the two meiotic divisions (Monesi 1964, Kudo et al. 2009, Vernet et al. 2011). This begs the question as to why meiosis in males requires this brief interphase between the two meiotic divisions, while meiosis in females does not. Our hypothesis is that this is to allow expression of X and/or Y genes with important functions during the divisions, but which in males (but not females) are silenced throughout pachytene and diplotene as a consequence of meiotic sex chromosome inactivation (reviewed by Turner (2007)). This model would allow for there being some Yp gene activity during this interphase that facilitates the re-condensation of the chromosomes and progression through the second division. In order to investigate this possibility, we need to compare males with the three different Yp gene complements used here but without the major difference in apoptotic selection. The apoptotic elimination in XSexrO males can be prevented by adding a minute PAR-bearing chromosome that enables the formation of a minimal sex bivalent; this restores round spermatid PAR-bearing chromosome that enables the formation of the cyte’ stage between the two meiotic divisions (Monesi 1964, Kudo et al. 2009, Vernet et al. 2011). This shows that a Yp gene while not being some Yp gene activity during this interphase that facilitates the re-condensation of the chromosomes and progression through the second division, whereas in males there is a transition from XY males. Descriptions of acrosome development in the mouse (Oakberg 1956a, Russell et al. 1990) describe the clustering of Golgi vesicles containing proacrosomic granules to form a large vesicle containing a single acrosomic granule; this associates with the nuclear envelope to mark the beginning of the ‘cap phase’, which covers spermatid stages 4–5 (in tubule stages IV–V). Nevertheless, the acrosomal cap continues to enlarge by the fusion of further Golgi vesicles and the fact that we are seeing some ‘multiple acrosome structures’ in Lectin–PNA-stained sections of stage IV–V tubules from XY control males (and in haploid spermatids from XO males) may be due to classification of some of the ongoing fusions as multiple acrosomal structures.

**Evidence of Y gene functions in sperm head remodelling and sperm tail elongation**

Our current findings show that in XSexrO males, there is an almost total block at step 7 of round spermatid development, with the result that elongation and restructuring of the nucleus to form the sperm head, and extension of the axoneme to form the sperm tail, does not take place. Intriguingly, a block at the start of spermatid elongation is also seen in mice carrying a Brca1 gene from which the E3 ligase activity was deleted (Shakya et al. 2011) and in Dicer1 knockout mice (Korhonen et al. 2011). Because sperm head remodelling and sperm tail elongation are achieved in both XSexrO (Mahadevaiah et al. 1988, Kot & Handel 2000) and XSexrO (albeit delayed), we conclude that these processes require genetic information present in SxrO that is at least partially retained in SxrP. The protein coding genes that SxrO and SxrP have in common are Sry, an estimated seven copies of Rbmy1a1 (Mahadevaiah et al. 1998) and two copies of H2al2y (Gm16501 and Gm6026); SxrP also includes a transcribed Zfy2/1 fusion gene while SxrO has retained the single copies of Zfy1 and Zfy2 from which the fusion gene was derived by an ectopic recombination event (Simpson & Page 1991). Rbmy1a1 is unlikely to be involved in spermiogenesis as it is transcribed only in spermatogonia and early spermatocytes, and no RBMY protein has been detected in spermatids (Szot et al. 2003, Lee et al. 2004). H2al2y, which encodes a variant H2A histone, is a strong candidate for sperm head restructuring as it is not detected at 21 dpp but starts to be transcribed from 23 dpp onwards; an age at which the transition from round to elongated spermatids takes place (Ferguson et al. 2009). To test this possibility, we have reinstated H2al2y expression in XSexrO males by adding a

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**Acrosome anomalies in XO males**

Our present studies indicate that in all three XO mouse models, the haploid and diploid products of meiosis enter spermiogenesis and that the initiation of acrosome development is not delayed. This shows that a Yp gene complement of just Sry and Eif2s3y is sufficient to support this initial phase of spermiogenesis. However, in all three XO male models, at the onset of the cap phase of acrosome development, we observed a high incidence of spermatids that had two or more acrosomal structures, instead of the normal single acrosomal structure. Morphologically abnormal spermatids with a deposition of ‘multiple pro-acrosomic granules’ were previously described in XSexrO mice (Kot & Handel 1990). In our more detailed analysis of all three XO male mouse models, we found frequent abnormal acrosome development in diploid spermatids resulting in multiple acrosomal structures during the cap phase. This fragmented acrosome was no longer apparent by the end of the cap phase. Our TEM analysis of XSexrO males shows intact acrosomal granules and suggests that there is fusion of the multiple acrosomal vesicles and granules after they become closely apposed to the nucleus. Although it is clear that diploid spermatids have an elevated incidence of multiple acrosome structures during the cap phase in XO males (53–63% in XSexrO males), we nevertheless found 9–16% in haploid spermatids from XSexrO males and a similar frequency in (haploid) spermatids from XY males. The incidence of spermatids that had two or more acrosomal structures, instead of the normal single acrosomal structure, is elevated in (haploid) spermatids from XO males (53–63% in XSexrO males and 9–16% in haploid spermatids from XY males) and is similar to that in Brcal knockout mice (Korhonen et al. 2011).
transgenic copy of H2a12y driven by the spermatid-specific mouse protamine-1 promoter. Disappointingly, this failed to enable sperm head elongation (or sperm tail formation) (Supplementary Figure 3 and Supplementary Materials and Methods, see section on supplementary data given at the end of this article).

The Zfy2/1 fusion gene is also an attractive candidate for the Yp spermiogenic function that is at least partially retained in Sxrd. Zfy1 and Zfy2 encode putative transcription factors and Zfy2 in particular is likely to be important for spermiogenesis because it has acquired an additional Cypt-derived promoter that drives strong expression in spermatids (Hansen et al. 2006). The Zfy2/1 fusion gene is also strongly expressed in spermatids because the promoter elements are derived from Zfy2; however, the transcripts produced are spliced like those of Zfy1 so that a substantial proportion of the transcripts will lack the exon 6 encoding the transcriptional activation domain required for transcription factor function (Decarpentrie et al. 2012). Thus, it is reasonable to suppose that both could promote sperm morphogenesis but the fusion gene would be less effective. In principle, this might be tested by comparing the separate addition of Zfy2 and Zfy2/1 transgenes to X;Osry males. However, we already know that adding Zfy2 reinstates the apoptotic elimination of MI spermatocytes with a univalent X chromosome whereas the addition of Sxr (that includes the Zfy2/1 fusion gene) does not (Vernet et al. 2011). We are therefore carrying out these transgene additions in conjunction with the introduction of the minute PAR-bearing chromosome as mentioned earlier, in order to avoid triggering this apoptotic response (Burgoyne et al. 1992).

One surprising observation was that the developing sperm head seen in cell spreads from X;SxrdO testes looked healthier and better hooked than those from X;SxrdO testes. The same observation was independently made by collaborators using testicular sperm (Monika Ward and Yasuhiro Yamauchi, personal communication). In considering why this might be, it is important to take account of the fact that in XY males there is substantial repression of X-linked genes due to the X;Y chromosome pairing that is present in XY males. MF1 XYIII males were used as normal controls; YIII is the strain of Y chromosome from which Sxr and Sxr' derive. All animal procedures were in accordance with the United Kingdom Animal Scientific Procedures Act 1986 and were subject to local ethical review.

Ploidy analysis on testis cell spreads

Nuclear DNA content was measured on surface-spread spermatogenic cells from 30 dpp and 6-week-old testes as described previously (Vernet et al. 2011) using synaptonemal complex SYCP3 staining and DAPI fluorescence intensity measurements. The cells quantified were pachytene spermatocytes (4C), spermatogonia (2C) and spermatids (2C or 1C) distinguished from secondary spermatocytes based on the SYCP3 staining pattern (Kudo et al. 2009, Vernet et al. 2011). While secondary spermatocyte interphase nuclei and early diploid spermatids contain around ten chromocenters, later diploid spermatids and haploid spermatids show three to five DAPI bright chromocenters and one chromocentrum respectively. After integrated intensity measurement of DAPI fluorescence, the values were converted to ploidy. The values were adjusted to give an arbitrary value of 4 for pachytene spermatocyte nuclei and the measurement obtained for the nuclei of the other cell types was normalised to the value of the nearest pachytene spermatocyte found on the slide. Spermatids were considered as diploid if the value obtained was above the lowest value obtained for the spermatogonia. The other spermatids
were considered to be haploid. Percentage of haploid spermatids was calculated after measurement of 50–100 spermatids for each of the three to four males analysed per genotype at each age. ANOVA analysis (General linear models, NCSS, Kaysville, UT, USA) was carried out after angular transformation of percentages.

**Histological analysis**

For standard histological analysis, testes were fixed in Bouin, rinsed with 70% ethanol, embedded in paraffin wax, sectioned at 5 μm on glass slides, and stained with H&E. Stages of seminiferous tubules were identified by the composition of germ cells near the basal membrane, as described in Ahmed & de Rooij (2009).

Acrosome formation was monitored on testis sections by i) PAS staining (which highlights the developing acrosome) of paraffin wax sections from Bouin-fixed testes followed by haematoxylin counterstaining and ii) fluorescence-tagged lectin–PNA staining of the outer acrosomal membrane using paraformaldehyde (PFA)-fixed wax sections. For the latter, testes were fixed in 4% PFA diluted in PBS overnight at 4°C, rinsed with 70% ethanol before embedding in paraffin wax. Sections (5 μm) on a glass slide were de-waxed, processed for antigen retrieval, washed in PBS and blocked for 30 min at room temperature in PBST–BSA (PBS containing 0.1% Tween 20 and 0.15% BSA). Immunolabelling for the synaptonemal complex protein SYCP3 (used as a stage marker) was performed with rabbit polyclonal anti-SYCP3 (1:100; Abcam, Cambridge, UK) diluted in PBST–BSA incubated overnight at 4°C and rinsed with 70% ethanol before embedding in paraffin wax. Sections (5 μm) on a glass slide were de-waxed, processed for antigen retrieval, washed in PBS and blocked for 30 min at room temperature in PBST–BSA (PBS containing 0.1% Tween 20 and 0.15% BSA). Immunolabelling for the synaptonemal complex protein SYCP3 (used as a stage marker) was performed with rabbit polyclonal anti-SYCP3 (1:100; Abcam, Cambridge, UK) diluted in PBST–BSA incubated overnight at 37°C. Slides were then washed in PBST, incubated at 37°C for 1 h with a mix of chicken anti-rabbit Alexa 488 (1:500; Molecular Probes, Paisley, UK) and Alexa Fluor 594-conjugated Lectin–PNA (1:700; Invitrogen Life Technologies) diluted in PBS and then washed in PBST before mounting in a medium containing DAPI (Vectashield with DAPI; Vector Laboratories, Burlingame, CA, USA). Seminiferous epithelium stages were judged from the DAPI staining and the expression pattern of SYCP3 that remains at the sister chromatids until diploid and haploid spermatids reach epithelial stages II–III (Vernet et al. 2011).

For the quantitation of acrosomal defects, we analysed spermatids from stage IV–V seminiferous tubules from two 6-week-old XSexO males. Haploid and diploid spermatids were discriminated based on nuclear size, DAPI morphology and the SYCP3 staining pattern.

**Transmission electron microscopy**

Mice were perfused, through the left ventricle (Vernet et al. 2006), with ice-cold 2.5% glutaraldehyde fixative diluted in PBS. The testes were dissected, left for 1 h in the fixative, and cut into small blocks that were kept at 4°C in the same fixative until embedding. Testes were post-fixed for 1 h in 1% osmium tetroxide, stained ‘en bloc’ for 1 h with 1% aqueous uranyl acetate, dehydrated with graded alcohol series and embedded in Epon. Ultrathin sections (60 nm) were contrasted 7 min with uranyl acetate and lead citrate and then examined using JEOL 1200EX electron microscope.

**DNA fluorescence in situ hybridization**

Fluorescence in situ hybridization (FISH) was performed on surface-spread spermatogenic cells from adult testes, as described previously (Turner et al. 2006). Digoxigenin-labelled probes were prepared using the Digoxigenin Nick Translation Kit (Roche Diagnostics), and hybridisations were carried out. The probes used against autosomal loci were chromosome 7 BAC RP23-101N20 containing one copy of the Kcnq1 locus, the chromosome 17 BAC RP23-432O2 containing one copy of the Airn locus, the chromosome 13 BAC RP23-423B15 containing several members of Hist1h family and Olfr1359 locus and the chromosome 5 BAC RP23-212A20 containing one copy of the Speer locus (BACPAC Resources Center, Children’s Hospital Oakland Research Institute, Oakland, CA, UK). After stringency washes, DNA FISH signals were developed using anti-DIG-FITC (Chemicon, Temecula, CA, USA) diluted 1:10 for 1 h at 37°C. Staging of spermatogenic cells was based on DAPI fluorescence morphology, together with immunolabelling for SYCP3 (1/100; Abcam) and the phosphorylated histone H2AX detected by anti-DIG-FITC (Chemicon, Temecula, CA, USA). After stringency washes, DNA FISH signals were developed using anti-DIG-FITC (Chemicon, Temecula, CA, USA) diluted 1:10 for 1 h at 37°C. Staging of spermatogenic cells was based on DAPI fluorescence morphology, together with immunolabelling for SYCP3 (1/100; Abcam) and the phosphorylated histone H2AX detected by a mouse MAB (1/300; Upstate, Hampshire, UK), as described previously (Turner et al. 2005).

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-12-0158.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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