The expression of Y-linked Zfy2 in XY mouse oocytes leads to frequent meiosis 2 defects, a high incidence of subsequent early cleavage stage arrest and infertility

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ABSTRACT
Outbred XYΔ1Rb female mice that lack Sry due to the 11 kb deletion SryΔ1Rb have very limited fertility. However, five lines of outbred XYd females with Y chromosome deletions YΔ(Y)1Ct, YΔ(Y)5Ct that deplete the Rbmy gene cluster and repress Sry transcription were found to be of good fertility. Here we tested our expectation that the difference in fertility between XO, XYΔ1 and XYΔSry– females would be reflected in different degrees of oocyte depletion, but this was not the case. Transgenic addition of Yp genes to XO females implicated Zfy2 as being responsible for the deleterious Y chromosomal effect on fertility. Zfy2 transcript levels were reduced in ovaries of XYΔ1 compared with XYΔSry– females in keeping with their differing fertility. In seeking the biological basis of the impaired fertility we found that XYΔSry–, XYΔ1 and XO,Zfy2 females produce equivalent numbers of 2-cell embryos. However, in XYΔSry– and XO,Zfy2 females the majority of embryos arrested with 2-4 cells and almost no blastocysts were produced; by contrast, XYΔ1 females produced substantially more blastocysts but fewer than XO controls. As previously documented for C57BL/6 inbred XY females, outbred XYΔSry– and XO,Zfy2 females showed frequent failure of the second meiotic division, although this did not prevent the first cleavage. Oocyte transcriptome analysis revealed major transcriptional changes resulting from the Zfy2 transgene addition. We conclude that Zfy2-induced transcriptional changes in oocytes are sufficient to explain the more severe fertility impairment of XY as compared with XO females.

KEY WORDS: Meiosis 2 defects, First cleavage, Preimplantation failure, XY female infertility, Y gene expression in oocytes, Zfy2, Mouse

INTRODUCTION
XO mice, although fertile, have a reduced oocyte pool and a shorter reproductive lifespan (Burgoyne and Baker, 1981; Burgoyne and Baker, 1985; Lyon and Hawker, 1973). For XY female mice the majority of data point to their being sterile, or nearly so, suggesting that the Y chromosome further impairs fertility. Thus, C57BL/6 (inbred) XOs are fertile (Hunt, 1991), whereas C57BL/6 XY females are sterile (Biddle and Nishioka, 1988; Eicher et al., 1982; Taketo-Hosotani et al., 1989), including those carrying the Sry deletion SryΔ1Rb (termed XYΔSry– females; our unpublished data). When outbred, XYΔSry– females have very limited fertility (Mahadevaiah et al., 1993). However, five exceptional fertile lines of outbred XY females were generated by the Cattanach laboratory; all had deletions in the Y chromosome short arm (Yp) between Sry and the centromere that resulted in a marked reduction in gene copies within the Rbmy cluster and substantial or complete repression of Sry transcription (Capel et al., 1993; Laval et al., 1995; Mahadevaiah et al., 1998). The objective of the present study was to determine why XYΔSry– female mice are substantially less fertile than XO female mice: our working hypothesis was that this was due to a deleterious effect of Y gene expression in oocytes. Under this hypothesis, the improved fertility of XYd females is attributed to a reduction of the deleterious Y gene expression as a consequence of the proximal Yp deletions.

RESULTS
XYΔSry– females have severely impaired fertility relative to XO females, whereas XYΔ1 and XYΔSry– females are significantly less affected
The production crosses were such that the four genotypes XO, XYΔSry–, XYΔ1 and XYΔSry– all had a maternally derived X chromosome; each is generated in a separate cross using a common pool of outbred XX mothers. The Yp gene content of the variant Y chromosomes is illustrated in Fig. 1.

In Fig. 2 we present the results of two comparisons. The XO versus XYΔSry– comparison shows that addition of the YΔSry– chromosome in the context of a single maternally derived X chromosome results in a substantial reduction in fertility over their reproductive lifetime, with a 3.9-fold reduction in the number of litters and a 9.6-fold reduction in total offspring. The comparison of the XYΔSry– breeding data with the XYΔ1 and XYΔSry– breeding data reveals a 3.1-fold and 3.2-fold increase in the number of litters and a 6.5-fold and 6.4-fold increase in total offspring with the substitution of the YΔSry– chromosome with YΔ1 and YΔSry–, respectively. These data demonstrate that the addition of a YΔSry– chromosome is deleterious for female fertility and that YΔ1 and YΔSry– are less deleterious than YΔ1. Because the improved fertility of XYΔ1 and XYΔSry– females was comparable, we restricted further analysis to XYΔ1 females.

The difference in fertility between XO, XYΔSry– and XYΔ1 females is not explained by the extent of oocyte loss
XO females with a paternal X (X0O) have a reduced oocyte pool due to increased perinatal oocyte loss (Burgoyne and Baker, 1985)
that is thought to reflect asynapsis-driven transcriptional repression of the X chromosome during pachytenie (Baarends et al., 2005; Burgoyne et al., 2009; Turner, 2007; Turner et al., 2005). Furthermore, oocyte deficiency has been reported in both XYsry− and C57BL/6 XY females (Lovell-Badge and Robertson, 1990; Park and Taketo, 2003). Although oocyte count data are not available for XOs with a maternal X (XpO), in XYY~sry~ and XY~d~ females the X usually fails to synapse with the Y, and, as in XOs, may be asynapsed or self-synapsed (Mahadevaiah et al., 1998) (our unpublished observations on XY~d~). Thus, we expected perinatal oocyte loss as a consequence of X chromosome silencing, and our first objective was to determine if XY~sry− females had a more depleted oocyte pool than XO and XY~d−1 females.

We assessed oocyte numbers at 5 days postpartum (dpp) using the distributed point count method used in the study of XpO females (Burgoyne and Baker, 1985). As we expected, the number of X chromosomes has a substantial effect (Fig. 3A), with an overall 23% reduction in oocyte numbers in conjunction with a single X (oocyte size distribution data are recorded in supplementary material Fig. S1). The reduction in oocyte numbers in the single X genotypes is also apparent in the pool and growing oocyte populations at later ages (Fig. 3B-D).

There were substantial differences between the mean XX oocyte pool sizes across the three crosses and the XX and single X means appeared to covary (Fig. 3A). An ANOVA of the XX data with ‘cross’ and ‘mother’ as factors revealed that there was no significant effect of cross, but there was a substantial and significant (P=0.0062) maternal effect (supplementary material Table S1A). Since each mother provided only one litter, this ‘maternal’ effect could equally be a litter effect. This maternal/litter effect compromised the planned XO versus XYsry− and XYsry~ versus XY~d−1 comparisons. We therefore divided the individual oocyte counts for the single X genotypes by the mean oocyte counts for the XX females from their respective litters to compensate for the maternal/litter effect, and carried out ANOVA analyses on the resulting ratios. This revealed that at 5 dpp there is a significant (P=0.0078) reduction in oocyte numbers in XYsry− as compared with XO females, but no significant difference as compared with XY~d−1 (supplementary material Table S1B). Comparison of oocyte pool and growing population sizes at 28 dpp revealed no significant differences between the three single X genotypes (supplementary material Table S1C) and that between XYsry− and XY~d−1 at 35, 42 and 56 dpp revealed no differences between these genotypes (supplementary material Table S1D).

We conclude that there is a major reduction in oocyte numbers in female mice with a single maternally derived X chromosome as compared with XX littermates. However, XYsry− females do not have additional sustained oocyte deficiency that could explain their much more severely compromised fertility relative to XO and XY~d−1 females.

Zfy2 (but not Zfy1) expression in ovaries is implicated as the cause of the poor fertility of XYsry− females

Our working hypothesis for the improved fertility of XY~d−1 as compared with XYsry− females was that the Del(Y)1Ct deletion reduced Yp gene expression that is deleterious for oocytes. The deletion is located within a repeated DNA region with size estimates ranging from ~1 to 4 Mb (J. E. Alfoldi, PhD thesis, Massachusetts Institute of Technology, 2008) (Mahadevaiah et al., 1998). It resulted in a greater than 10-fold reduction in the number of copies of Rbmy that lie adjacent to the Y centromere and to the transcriptional silencing of Sry, which is postulated to be a consequence of bringing Sry close to the repressive effects of centromeric heterochromatin (Capel et al., 1993; Mahadevaiah et al., 1998). We first checked by RT-PCR for ovarian expression of Yp genes; nine Yp genes were expressed in XYsry− ovaries and, among these, Rbmy was clearly reduced in XYsry− ovaries (Fig. 4A). However, RBMY protein was not detectable even in XYsry− ovaries (Fig. 4B). We also utilised a transgenic line with an estimated 4-5 copies of Rbmy that lie adjacent to the Y centromere and to the transcriptional silencing of Sry, which is postulated to be a consequence of bringing Sry close to the repressive effects of centromeric heterochromatin (Capel et al., 1993; Mahadevaiah et al., 1998). We first checked by RT-PCR for ovarian expression of Yp genes; nine Yp genes were expressed in XYsry− ovaries and, among these, Rbmy was clearly reduced in XYsry− ovaries (Fig. 4A). However, RBMY protein was not detectable even in XYsry− ovaries (Fig. 4B). We also utilised a transgenic line with an estimated 4-5 copies of Rbmy that lie adjacent to the Y centromere and to the transcriptional silencing of Sry, which is postulated to be a consequence of bringing Sry close to the repressive effects of centromeric heterochromatin (Capel et al., 1993; Mahadevaiah et al., 1998). We first checked by RT-PCR for ovarian expression of Yp genes; nine Yp genes were expressed in XYsry− ovaries and, among these, Rbmy was clearly reduced in XYsry− ovaries (Fig. 4A). However, RBMY protein was not detectable even in XYsry− ovaries (Fig. 4B). We also utilised a transgenic line with an estimated 4-5 copies of Rbmy that lie adjacent to the Y centromere and to the transcriptional silencing of Sry, which is postulated to be a consequence of bringing Sry close to the repressive effects of centromeric heterochromatin (Capel et al., 1993; Mahadevaiah et al., 1998). We first checked by RT-PCR for ovarian expression of Yp genes; nine Yp genes were expressed in XYsry− ovaries and, among these, Rbmy was clearly reduced in XYsry− ovaries (Fig. 4A). However, RBMY protein was not detectable even in XYsry− ovaries (Fig. 4B). We also utilised a transgenic line with an estimated 4-5 copies of Rbmy that lie adjacent to the Y centromere and to the transcriptional silencing of Sry, which is postulated to be a consequence of bringing Sry close to the repressive effects of centromeric heterochromatin (Capel et al., 1993; Mahadevaiah et al., 1998). We first checked by RT-PCR for ovarian expression of Yp genes; nine Yp genes were expressed in XYsry− ovaries and, among these, Rbmy was clearly reduced in XYsry− ovaries (Fig. 4A). However, RBMY protein was not detectable even in XYsry− ovaries (Fig. 4B). We also utilised a transgenic line with an estimated 4-5 copies of Rbmy that lie adjacent to the Y centromere and to the transcriptional silencing of Sry, which is postulated to be a consequence of bringing Sry close to the repressive effects of centromeric heterochromatin (Capel et al., 1993; Mahadevaiah et al., 1998). We first checked by RT-PCR for ovarian expression of Yp genes; nine Yp genes were expressed in XYsry− ovaries and, among these, Rbmy was clearly reduced in XYsry− ovaries (Fig. 4A). However, RBMY protein was not detectable even in XYsry− ovaries (Fig. 4B). We also utilised a transgenic line with an estimated 4-5 copies of Rbmy that lie adjacent to the Y centromere and to the transcriptional silencing of Sry, which is postulated to be a consequence of bringing Sry close to the repressive effects of centromeric heterochromatin (Capel et al., 1993; Mahadevaiah et al., 1998). We first checked by RT-PCR for ovarian expression of Yp genes; nine Yp genes were expressed in XYsry− ovaries and, among these, Rbmy was clearly reduced in XYsry− ovaries (Fig. 4A). However, RBMY protein was not detectable even in XYsry− ovaries (Fig. 4B). We also utilised a transgenic line with an estimated 4-5 copies of Rbmy that lie adjacent to the Y centromere and to the transcriptional silencing of Sry, which is postulated to be a consequence of bringing Sry close to the repressive effects of centromeric heterochromatin (Capel et al., 1993; Mahadevaiah et al., 1998).
were almost completely sterile, with only one pup produced from five matings (compared with an average of 36.5 offspring per female for XO siblings); in marked contrast, XO, Zfy2

nf females produced an average of 42.8 offspring (compared with 53.0 for XO female siblings) (Fig. 5A). These comparisons not only reveal the severe effect on fertility of the Zfy2 transgene in the context of a single X chromosome, but also point to there being no major effect on fertility due to the insertion of either transgene into the Hprt locus.

Of the Yp genes expressed in the XY Sry– ovaries, Zfy2 is just distal to Sry and thus might also be subject to transcriptional repression as a consequence of the deletion (Fig. 1). qRT-PCR analysis revealed that XYd– ovaries did indeed have lower Zfy2 levels than XY Sry– ovaries, whereas the single copy X-linked Zfy2 transgene produced significantly higher transcript levels than the endogenous Zfy2 present in XY Sry– (Fig. 5B). In agreement with the presumption that Sry and Zfy2 transcription are repressed in XYd– because they are much closer to the Y centromeric heterochromatin, the next most proximal gene expressed in XY ovaries, Usp9y, is also significantly repressed in XYd–, whereas the most distal gene, Zfy1, is unaffected (Fig. 5B).
The full-length proteins encoded by Zfy1 and Zfy2 are predicted to be zinc-finger transcription factors that bind the same DNA target sequences (Grants et al., 2010; Taylor-Harris et al., 1995); this suggests that Zfy1 may contribute to the impaired fertility of XY females. We therefore introduced into XO females either a single copy of a Y genomic BAC containing Zfy1 and Uba1y that had inserted on the X chromosome (Zfy1P0) or 13-14 copies of the same BAC inserted on an autosome (Zfy1P6) (Royo et al., 2010). Surprisingly, in neither case was there any significant effect on fertility (Fig. 5C). We now know that Zfy1 is a substantially less potent transcription factor than Zfy2 for two reasons. First, the majority of Zfy1 transcripts in XX, Zfy1 transgenic ovaries lack exon 6, which removes most of the transactivation domain of the resulting protein (Fig. 5D). Second, the transactivation domain of the minority full-length Zfy1-encoded protein is 5.5-fold less active than that of the Zfy2-encoded protein (N.V., S.K.M., F.D., P.S.B. and Michael J. Mitchell, unpublished data).

Zfy2 expression impairs fertility by causing substantial preimplantation embryo arrest

In view of the results implicating Zfy2 as the cause of XY female infertility, and the data showing that oocyte depletion does not explain the poor fertility of XY<sup>Sp</sup>- females, we proceeded to check for ovulation and the potential for subsequent embryonic development. We naturally mated females (age range 6-11 weeks) from a selection of the genotypes for which we had breeding data, collected eggs/embryos from the oviducts at 1.5 days post-coitum (dpc) when fertilised eggs are expected to have progressed to the 2-cell stage, and if 2-cell embryos were present we cultured them for 4 days. The mean numbers of eggs and embryos obtained for each genotype are given in Table 1.

From the oocyte quantitation the only consistent effect of genotype was the marked reduction in oocyte numbers in genotypes with a single X (Fig. 3); this reduction affected the oocyte pool and the number of females of each genotype are shown. Rbmy transgene addition does not alter the fertility of XO or XY<sup>Sp</sup>- females.

(D) Comparison by qRT-PCR of Rbmy transcript levels (normalised to the germ cell-specific transcript Dazl) in 7-week-old XY<sup>Sp</sup>- and XO, Rbmy ovaries. XO, Rbmy ovaries have substantially higher Rbmy transcript levels than XY<sup>Sp</sup>- males, but they fall far short of the levels in XY<sup>Sp</sup>- males. *P<0.05, **P<0.001; NS, non-significant. Error bars indicate s.e.m.

Fig. 4. Rbmy expression in ovaries and the consequences for fertility. (A) RT-PCR analysis of Yp genes in testis from 8-week-old XY male (positive control) and in ovaries from 8-week-old XY<sup>Sp</sup>- and XX (negative control) females. Dazl amplification confirms that germ cells are present; β-actin is a loading control. In XY<sup>Sp</sup>- oварies, spermatid-specific transcripts (Zfy2 from Cypt promoter, H2a2y) are not expressed, whereas Zfy1, Uba1y, Kdm5d, Eif3s3y, Uty, Ddx3y, Usp9y, Zfy2 (germ cell) and Rbmy (markedly reduced in XY<sup>Sp</sup>-) are expressed. (B) Western blot detection of RBMY in 17 dpp testis (lanes 1, 2 and 4 are positive controls, with 50% loading for lane 2; lane 3 is a negative control) and in 7-week-old ovaries (lanes 5-7, Rbmy carriers; lane 8, negative control); actin is a loading control. RBMY is detected in the positive testis controls, although markedly reduced in the XY<sup>Sp</sup>- testes (8-fold-depleted Rbmy cluster). No expression could be detected in Rbmy-positive ovaries, including XY<sup>Sp</sup>- that has a complete Rbmy complement. (C) Rbmy transcript levels and fertility. The mean number of offspring per female and the number of females of each genotype are shown. Rbmy transgene addition does not alter the fertility of XO or XY<sup>Sp</sup>- females.
embryos it is important that all the genotypes expressing Y genes are compared with appropriate XX or XO controls. The reason for the fertility impairment associated with the presence of Zfy2 was already apparent after one day of culture (2.5 dpc) in that the three genotypes with the most severe fertility impairment (XX, Zfy2, XO, Zfy2 and XY Sry–) had substantially more embryos remaining at the 2-cell stage relative to appropriate controls: XX, Zfy2 46% versus XX, Zfy2 0%; XO, Zfy2 89% versus XO, Zfy2 4%; XY Sry– 57% versus XO 16% (Fig. 6A). The expected poorer development of embryos from all 1X genotypes is apparent in supplementary material Fig. S2A-E; viability tests on arrested 2-cell embryos are presented in supplementary material Fig. S2F. Strikingly, after three further days of culture (5.5 dpc), when viable embryos should have reached the blastocyst stage, the same three genotypes still had large numbers at the 2- to 4-cell stages: XX, Zfy2 25%; XO, Zfy2 55%; XY Sry– 41% (Fig. 6A). The most important statistic in relation to the underlying cause of the severe impairment of XY Sry– fertility and the improved fertility of XYd-1 is the proportion of embryos that reach the blastocyst stage by 5.5 dpc: 1.7% for XY Sry– but 43.2% for XYd-1. XO, Zfy2, which has the most severe fertility impairment, produced no blastocysts. For the 1X genotypes there is a compelling correlation between the proportion of embryos per litter that reach the blastocyst stage and their relative breeding performance as measured by the total number of offspring over their reproductive lifetime (Fig. 6B). The salient features of these major differences in preimplantation development in culture were apparent in embryos flushed from the uterus at 3.75 dpc (Fig. 6C).

### Zfy2-induced preimplantation embryo arrest is preceded by meiotic anomalies

Many of the 2-cell embryos of XY Sry– and XO, Zfy2 females, when flushed from oviducts at 1.5 dpc, lacked a polar body; the XO, Zfy2 embryos, in particular, also had morphological abnormalities (Fig. 7A; supplementary material Table S2A). For XX embryos it is known that the first polar body frequently does not survive to the 2-cell stage but the second polar body is almost invariably present. The frequent lack of a polar body in embryos from XY Sry– and XO, Zfy2 females thus points to a failure of the second meiotic division. To investigate this further we made DAPI-stained preparations of 1.5 dpc 2-cell embryos from XO, XO, Zfy2 and XY Sry– mothers. The 2-cell embryos from XO females invariably had a second polar body, occasionally together with a first polar...
body; by contrast, 69% and 46% of 2-cell embryos from XO, Zfy2 and XY Sry–, respectively, had no polar bodies (Fig. 7B,C; supplementary material Table S2B). These preparations also revealed that the blastomere nuclei were often markedly displaced from the centre, and occasionally blastomeres had two or more nuclei (Fig. 7D,E; supplementary material Table S2B).

Failure of the second meiotic division has been documented for C57BL/6 XY females using in vitro matured and activated oocytes (Villemure et al., 2007). However, the MF1 strain background is associated with poor in vitro maturation. We therefore assessed progression through the second meiotic division using ovulated eggs obtained from females that were mated to aspermic males, and were then activated in vitro with SrCl2. We collected data for the 1X genotypes XO, XO, Zfy2 and XY Sry– and the 2X genotypes XX, XX, Zfy2 and XX, Zfy2. From samples of eggs processed immediately following collection it was apparent that assembly of the metaphase II (MII) spindle was retarded in the 1X genotypes relative to the 2X genotypes, with XO, Zfy2 the most markedly

Table 1. The impact of Zfy2, Zfy1, Y\textsuperscript{Sry–} and Y\textsuperscript{6–1} on ovulation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Fertility status</th>
<th>Age (days)</th>
<th>1-cell</th>
<th>≥2-cell</th>
<th>Ovulations</th>
</tr>
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<tr>
<td>XX</td>
<td>5</td>
<td>Normal</td>
<td>68.4±4.6</td>
<td>0.4±0.2</td>
<td>11.2±0.7</td>
<td>11.6±0.7</td>
</tr>
<tr>
<td>XX, Zfy2\textsuperscript{a}</td>
<td>5</td>
<td>Like XX</td>
<td>74.6±3.1</td>
<td>0.4±0.2</td>
<td>9.4±1.0</td>
<td>9.8±0.9</td>
</tr>
<tr>
<td>XX, Zfy2</td>
<td>3</td>
<td>&lt;&lt; XX</td>
<td>76.0±0.6</td>
<td>0.3±0.3</td>
<td>9.3±1.4</td>
<td>9.7±1.8</td>
</tr>
<tr>
<td>X dose 2</td>
<td>13</td>
<td></td>
<td>73.0±2.3</td>
<td>0.4±0.0</td>
<td>10.0±0.6</td>
<td>10.4±0.6</td>
</tr>
<tr>
<td>XO</td>
<td>6</td>
<td>&lt; XX</td>
<td>57.8±5.6</td>
<td>0.8±0.5</td>
<td>9.2±1.4</td>
<td>10.0±1.5</td>
</tr>
<tr>
<td>XO, Zfy2\textsuperscript{a}</td>
<td>2</td>
<td>Like XO</td>
<td>56.5±18.5</td>
<td>1.5±0.5</td>
<td>9.5±2.5</td>
<td>11.0±2.0</td>
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<tr>
<td>XO, Zfy2</td>
<td>5</td>
<td>&lt;&lt;&lt; XX</td>
<td>65.4±3.3</td>
<td>1.2±0.5</td>
<td>12.8±1.7</td>
<td>14.0±1.4</td>
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<tr>
<td>XO, Zfy1\textsuperscript{a}</td>
<td>2</td>
<td>Like XO</td>
<td>72.5±0.5</td>
<td>0.0±0.0</td>
<td>11.0±2.0</td>
<td>11.0±2.0</td>
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<tr>
<td>XO, Zfy1</td>
<td>3</td>
<td>Like XO</td>
<td>63.3±1.2</td>
<td>0.0±0.0</td>
<td>9.0±1.2</td>
<td>9.0±1.2</td>
</tr>
<tr>
<td>XY\textsuperscript{Sry–}</td>
<td>4</td>
<td>&lt;&lt;&lt; XO</td>
<td>73.8±2.7</td>
<td>1.0±0.5</td>
<td>14.5±1.3</td>
<td>15.5±1.0</td>
</tr>
<tr>
<td>XY\textsuperscript{6–1}</td>
<td>6</td>
<td>Like XO</td>
<td>62.8±4.4</td>
<td>1.0±0.5</td>
<td>10.2±1.4</td>
<td>11.2±1.0</td>
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<tr>
<td>X dose 1</td>
<td>28</td>
<td></td>
<td>64.6±3.8</td>
<td>0.8±0.3</td>
<td>10.9±1.2</td>
<td>11.9±1.3</td>
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The numbers of 1-cell (probably unfertilised eggs) and ≥2-cell embryos obtained from oviducts of the various genotypes at 1.5 dpc; the total numbers provide an estimate of the ovulation rate. There were no significant differences between genotypes or between the 2X and 1X chromosome genotypes. Data are mean ± s.e.m.

Fig. 6. The impact of Zfy2, Zfy1, Y\textsuperscript{Sry–} and Y\textsuperscript{6–1} on preimplantation development. (A) Percentage of embryos by stage of development after 1 and 4 days of culture (embryos at 2.5 dpc and 5.5 dpc, respectively). There is a striking retarding effect of the Zfy2 transgene in XX and XO mice, and of Y\textsuperscript{Sry–} in XY Sry– mice, with many embryos exhibiting 2- to 4-cell arrest. (B) Lifetime offspring production by the various genotypes of females with a single X chromosome is strongly correlated (R\textsuperscript{2}=0.9773) with the proportion of embryos that reach the blastocyst stage in culture. (C) Percentage of embryos by stage of development at 3.75 dpc after development in vivo. Eighteen to 64 embryos were collected per genotype, originating from at least two females per genotype.
affected (supplementary material Table S2C, Fig. S3A). After activation, the 2X comparisons (which are free of the effects of X dosage deficiency) revealed a marked effect of the functional Zfy2 transgene, with 50% remaining arrested at MII and, of the 50% that did reach anaphase/telophase, 29% showed decondensation of the chromatin (Fig. 7F); this should not occur until completion of

Fig. 7. Zfy2-elicited abnormalities originating before the 2-cell stage. (A) All XO 2-cell embryos have at least one polar body (arrows indicate examples); XO, Zfy2 frequently lack polar bodies (brackets indicate examples) and have blastomeres of unequal size and abnormal shape. (B) Polar body counts from around 25 DAPI-stained 2-cell embryos per genotype. (C) Examples of polar bodies and their nuclear morphology. PB, polar body; l, live; d, dead; d/f, dead/fragmenting. (D) Increased frequency of eccentrically located nuclei, and occasional additional nuclei, in blastomeres of XO, Zfy2 and XY Sry− females. Around 50 embryos per genotype were assessed. (E) Examples of eccentric and multiple nuclei. (F) Impairment of the second meiotic division assessed after SrCl₂ activation.
Table 2. Classification of 289 Zfy2-regulated genes according to the subsequent fate/usage of their mRNAs

<table>
<thead>
<tr>
<th></th>
<th>Stable mRNA* n=212 (73%)</th>
<th>Culled mRNA* n=77 (27%)</th>
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<tbody>
<tr>
<td>Zfy2 upregulated*</td>
<td>Retained1</td>
<td>Removed1</td>
</tr>
<tr>
<td>Zfy2 downregulated*</td>
<td>40</td>
<td>34</td>
</tr>
<tr>
<td>Zfy2 downregulated§</td>
<td>58</td>
<td>39</td>
</tr>
</tbody>
</table>

*Data from Su et al. (Su et al., 2007) indicating changes in steady-state mRNA levels between GV and MII oocytes.
†Data from Chen et al. (Chen et al., 2011) indicating changes in polysomal mRNA levels between GV and MII oocytes.
‡Data from this study.
§Data from this study.

telophase. Among the 1X genotypes, all of the XO oocytes were either at MII with a clear spindle or had progressed further through the second meiotic division; they nevertheless remained retarded relative to XX, and 25% had precocious chromatid decondensation. The two 1X Zfy2-expressing genotypes were remarkable in that ~60% of embryos showed precocious chromatid decondensation (Fig. 7F; supplementary material Fig. S3B). We conclude that Zfy2-induced transcriptional changes severely disrupt the second meiotic division but do not block the first cleavage.

Zfy2-induced alterations to the oocyte transcriptome

To identify changes in the oocyte transcriptome induced specifically by Zfy2 we collected transcriptome data for fully grown germinal vesicle (GV) oocytes collected from XX, XX.Zfy2-20/d, XX.Zfy2 and XXZfy1 lo or hi females. Following a stringent filtering strategy (see Materials and Methods) to focus on Zfy2-specific transcriptional changes that are potentially linked to XY female infertility, we identified 359 Zfy2-regulated genes – 163 upregulated and 196 downregulated. We compared this list of Zfy2-regulated genes with the gene lists of Su et al. (Su et al., 2007) and Chen et al. (Chen et al., 2011), which respectively relate to genes regulated by mRNA culling during oocyte maturation and mRNAs recruited to polysomes (actively translating) during oocyte maturation. Of the 359 Zfy2-regulated genes, 289 could be matched to these other two datasets, and of these 212 are stable transcripts that therefore potentially affect the proteome of later stage oocytes and/or the zygote (Table 2; supplementary material Table S3, tab 2). We also compared our data to results from a comparison of XY and XX oocytes in a C57BL/6 model of XY female infertility (Xu et al., 2012). Eight upregulated genes and 20 downregulated genes were significantly regulated in the same direction in both experimental systems (supplementary material Table S3, tab 2).

Finally, we performed a clustering analysis using DAVID (Huang et al., 2009) to detect pathways or functional annotation groups enriched among Zfy2-regulated genes (supplementary material Table S3, tab 4). This showed no common functions among the downregulated genes, but there was a significant enrichment among upregulated genes for functional categories related to homeobox gene function and oocyte/embryo polarity. Interestingly, Hoxd8, Otx1 and Gli3 were highlighted by this analysis as well as by the comparison with the Xu et al. data (Xu et al., 2012) (supplementary material Table S3, tab 5).

DISCUSSION

Our working hypothesis was that the markedly poorer fertility of outbred XY SY-Hy as compared with XO female mice is due to Y gene expression in the oocytes, and that this Y gene expression is reduced as a consequence of the proximal Yp deletion present in the much more fertile XY4 females. Our findings confirm this hypothesis and implicate expression of the Zfy2-encoded zinc-finger transcription factor. Although the ultimate cause of the severe fertility impairment proved to be a widespread preimplantation arrest at the 2- to 4-cell stages, the embryos were already abnormal at the 2-cell stage, with a marked deficiency of polar bodies attributable to frequent failure of the second meiotic division.

Reduction of the oocyte pool in XY female mice

XY females share features with XO females that are expected to compromise their fertility: (1) the transcriptional silencing of the X chromosome during pachytene (except when self-synapsed) leading to oocyte loss (Baarends et al., 2005; Turner, 2007; Turner et al., 2005); (2) the deleterious consequences of maternal X dosage deficiency for preimplantation development (Burgoyne and Biggers, 1976); (3) the generation of embryos lacking an X chromosome (YO or YY) that die early in preimplantation development; and (4) the generation of embryos with a single paternal X (XpY and XpO) that are selected against during pregnancy, particularly when the embryos have an inbred genetic background (Burgoyne et al., 1983; Hunt, 1991; Jamieson et al., 1998). It is therefore important to compare XY females with XO females, rather than with XX females.

Based on previous observations suggesting substantial oocyte deficiency in XY SY-Hy and C57BL/6 XY females (Lovell-Badge and Robertson, 1990; Park and Taketo, 2003), our initial focus was on the possibility that XY SY-Hy females might have increased depletion of the oocyte pool in the perinatal period, but this proved not to be the case. Importantly, because the oocyte count data were obtained using the same quantitation technique used previously for XpO females (Burgoyne and Baker, 1985), these data show that a single maternal or paternal X is associated with substantial oocyte deficiency at this age. This is in agreement with the evidence that meiotic silencing of the X chromosome during pachytene leads to oocyte loss, and that in surviving oocytes the X undergoes self-synapsis (Baarends et al., 2005; Turner et al., 2005). The data on oocyte sizes (supplementary material Fig. S1) also establish that the reduced oocyte growth, previously suggested to be a consequence of the presence of a paternal X (Burgoyne and Baker, 1985), is also seen in mice with a single maternally derived X, implying that it is an X dosage effect. We also identified a substantial maternal/litter effect on oocyte numbers that needs to be taken into account in studies that seek to compare oocyte numbers between genotypes. The biological basis for this substantial maternal/litter effect is unknown and it will be important in the future to collect data from XX females that derive from a series of mothers, each of which provided more than one litter, so that the mother/litter confound is resolved.

Maternal Zfy2-induced meiotic errors and subsequent preimplantation arrest as the cause of XY female mouse infertility

From our comparison of preimplantation development of embryos from outbred XY SY-Hy and XO females, it was apparent that the greatly impaired fertility of the former is linked to substantial arrest at the 2- to 4-cell stage. However, there was also compelling

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evidence that the 2-cell embryos from XY<sup>Sty</sup>- frequently lacked second polar bodies due to a prior failure of the second meiotic division, and that the blastomeres occasionally had multiple nuclei. These phenomena and their aetiology have been documented in great detail for embryos obtained from C57BL/6 inbred XY females (Merchant-Larios et al., 1994; Obata et al., 2008; Villemure et al., 2007); substantial 2-cell arrest is also seen in outbred XY females with a Sertoli cell-specific ablution of Sox9 function (Lavery et al., 2011). The importance of the present study is that Y deletion mapping along with Zfy2 transgene addition to XO females establishes that Zfy2 expression in oocytes is sufficient to reproduce these phenomena, and thus provides an explanation for the markedly poorer fertility of XY as compared with XO female mice.

**Zfy2-induced changes to the GV oocyte transcriptome**

Our transcriptome analysis identified 359 genes as candidates for the effect of Zfy2 on female fertility, 28 of which were in common with a previous study comparing C57BL/6 XY and XX oocytes (Xu et al., 2012). Pathway analysis identified a group of homeobox/polarity-related genes upregulated by Zfy2, three of which were also found in the C56BL/6 study. Disruption of meiosis 2 by Zfy2 might thus be due to altered oocyte polarity. However, it is hard to select candidates for further investigation since in fully grown GV oocytes a substantial proportion of mRNAs are ‘stored’, with translation deferred until the proteins are required (Conti et al., 2012; Flèmr et al., 2010; Li et al., 2010), and there is also extensive culling of mRNAs once they are no longer required (Chen et al., 2011). Although our array data, being derived from GV oocytes, cannot relate directly to the question of culling, the majority of Zfy2-regulated transcripts are flagged as unculled in the Chen et al. study (Table 2; supplementary material Table S3, tab 2). Moreover, none of the key mediators of culling is regulated by Zfy2. We therefore presume that the majority of Zfy2-induced changes are likely to persist through meiosis and into the early embryo. To fully unpick the complexity of the response to Zfy2 we would require information on the proteomic changes during meiotic maturation and after fertilisation. However, very few (47/359) of the Zfy2-regulated genes featured in a recent proteomic study comparing GV oocytes, MII-arrested oocytes and fertilised zygotes (Wang et al., 2010), probably because there is a bias toward abundant proteins.

Importantly, we have shown that Zfy2 transgene addition to XX oocytes produces the same (although less penetrant) meiotic and preimplantation phenotypes as the addition to XO oocytes. Future work can therefore utilise XX transgene carriers, which are much easier to produce. Our focus for the future is to distinguish between the direct and indirect targets of the transcription factor ZFY2, but our attempts to obtain ZFY2-specific antibodies have been unsuccessful. We are therefore generating mice with a Zfy2 transgene with a flag tag to enable direct ZFY2 targets to be identified in oocytes.

**MATERIALS AND METHODS**

**Mouse crosses and breeding tests**

We chose an outbred MFI albino (NIMR colony) genetic background for this study because all the genotypes have at least some fertility on this background and we had extensive prior data relating to MFI XY<sup>Sty</sup>- females. We used MFI mothers from a common pool of females to produce XO, XY<sup>Sty</sup>- and XY<sup>o</sup> females with a maternally derived X, together with XX sibling controls. (1) XO females were produced from X<sup>Sty<sub>dl1Rlb</sub></sup>* × XX matings (Burgoyne and Evans, 2000). (2) XY<sup>Sty</sup>- females, with the 11 kb Sry<sup>Sty<sub>dl1Rlb</sub></sup> deletion (Gubbay et al., 1992; Lovell-Badge and Robertson, 1990), were produced from XY<sup>Sty</sup>-× XX matings, in which the father’s Sry<sup>Sty<sub>dl1Rlb</sub></sup> deletion is complemented by the transgene Tg(Sry)<sub>2Ei</sub> (Mahadevaiah et al., 1998). (3) XY<sup>L1</sup>- and XY<sup>L3</sup>- females carrying the large proximal Yp deletions Y<sup>Del(Y)1Ct</sup> or Y<sup>Del(Y)3Ct</sup> that are associated with fully penetrant sex reversal (Capel et al., 1993; Laval et al., 1995; Mahadevaiah et al., 1998) were produced from XY<sup>Sty</sup>,Sry × XX matings.

For breeding tests, these females, together with their XX sibling controls, were mated at 6 weeks to MF1 males and offspring recorded daily. The MF1 males were replaced if no litters were produced for 5 weeks and the matings were continued until the females had not produced any litters for at least 2 months. The same female genotypes were used to provide oocytes for oocyte counting with a single litter being obtained from each female, and also to provide preimplantation embryos.

For addition of candidate Y genes we used previously described transgenic lines (Royo et al., 2010; Vernet et al., 2011). (1) Rbmy: four to five copies of an Rbmy BAC (RP24-211C16) originating from the proximal Yp Rbmy cluster. (2) Zfy2: one copy of a Zfy2 BAC inserted by cassette-mediated exchange (CME) into the Hprt locus on the X chromosome. (3) Zfy2<sup>loc</sup>: one copy of a non-functional Zfy2 BAC inserted by CME into the Hprt locus. (4) Zfy1<sup>fi</sup>: one copy of a Zfy1-UBaly BAC (RP24-327G6) located on the X chromosome. (5) Zfy1<sup>hi</sup>: 13-14 copies of the Zfy1-UBaly BAC located on an autosome. These transgenes were used to produce XY<sup>L1</sup>- and/or XO carrier females; the transgenes varied as to the extent to which they have been backcrossed to MF1, so the breeding performance of carrier females was compared with non-transgenic siblings. The same female genotypes also provided preimplantation embryos.

**Oocyte counting**

Oocytes were counted from a single ovary per female using 6 μm sections of Bouin-fixed wax-embedded ovaries, stained with Hematoxylin and Eosin; the scorer was blind as to genotype. At 5 dpp the oocytes do not have a single nucleolus that can be used as a counting marker, a minority have commenced growth with a substantial size range, and the oocyte distribution across sections is non-random with respect to size and the numbers present. We therefore used the distributed point count method (Burgoyne and Baker, 1985) that was designed for estimating oocyte counts at this age. In essence, a squared grid is used to distribute sample points evenly over the total section area, with all sections included in the analysis. Each sample point is recorded as having ‘hit’ either a germinal or a somatic cell, which together with a measure of total ovarian volume allows germinal and somatic tissue volumes to be calculated. During the sampling process an estimate of the relative frequencies of the different oocyte sizes is obtained, which together with the germinal tissue volume allows an oocyte count to be obtained.

At 28-56 dpp, oocytes were counted in every twentieth section. At this age ‘growing oocytes’ (in stage II-VI follicles) have a single nucleolus that can be used as a counting marker; ‘pool oocytes’ (in stage I follicles) still may have multiple nucleoli and were counted when the nucleus was judged to be cut near the middle (Baker et al., 1980; Jones and Krohn, 1961).

We identified a substantial and statistically significant maternal or litter effect (each mother provided a single litter) on the size of the oocyte pool (supplementary material Table S1), which compromised comparisons of oocyte numbers between single X genotypes, which necessarily come from different mothers/litters. To compensate for the maternal effect, each oocyte count for single X genotypes was divided by the mean oocyte count for XX litters; the resulting ratio data were then utilised for the statistical comparisons of oocyte numbers.

**Transcript analysis**

**Sample collection and RNA extraction**

Ovaries or testes were frozen in liquid nitrogen. Fully grown cumulus-enclosed oocytes at GV stage were collected in M2 medium (Sigma-Aldrich) from ovaries of 4-week-old females 45-48 hours after intraperitoneal injection of PMSG (Intervet), denuded of cumulus cells using a drawn-out Pasteur pipette, frozen in liquid nitrogen with a minimal volume of M2 medium, and pooled in batches of ~100 cells coming from at least two females per genotype.

Total RNA was extracted from frozen ovaries or testes with Trizol (Invitrogen) and from frozen GV oocytes using the RNAqueous-Micro Kit (Ambion) according to the manufacturer’s protocols.
Semi-quantitative and quantitative transcript analysis

2 μg of total RNA from testis or ovary was DNase I treated (Invitrogen) using standard protocols. Reverse transcription (RT) of polyadenylated RNA was performed with Superscript II reverse transcriptase according to the manufacturer's protocols (Invitrogen) in a final volume of 20 μl; a minus RT control was included for each sample.

For semi-quantitative RT-PCR, 35 cycles were performed using Thermoprime Plus DNA polymerase (ABgene), with Dazl to monitor germ cell content and β-actin as a loading control. Adult XY testis and adult XX ovary samples served as positive and negative controls, respectively.

Quantitative RT-PCR (qRT-PCR) for Zfy2, Zfy1, Usp9y and Rhmy was performed as described previously (Cocquet et al., 2009) using ovary RNA samples, with 7-week-old XO ovary and 17 ddp XY testis as positive and negative controls, respectively. Quantitation by qRT-PCR of Zfy1 transcripts with and without exon 6 from Zfy1lo and Zfy1hi transgenic ovas was as described previously (Decarpentrie et al., 2012), with 7-week-old XX ovary and 15 ddp XY testis as positive and negative controls, respectively. All qRT-PCR reactions were carried out in triplicate per assay. The difference in PCR cycles with respect to ΔΔCt (for a given experimental sample) was calculated from the mean ΔΔCt of the 17 ddp or 15 ddp tests reference samples (ΔΔCt). The fold change in expression was calculated as the mean of the power 2(ΔΔCt) for each genotype.

Primers are listed in supplementary material Table S4.

Microarray analysis
cDNAs were amplified using the Ovation Pico WTA System 2 Kit (NuGen) and biotin labelled using Encore biotinIL (NuGen). Probes were hybridised to MouseWG-6 v2 Expression Beadchip (illumina) using the direct hybridisation protocol and the data were scanned using a bead array reader (illumina).

Two batches of GV oocyte total RNA were amplified and analysed separately. Batch 1 consisted of triplicates of ~100 GV oocytes pooled from either XX, XX/Zfy2lo, XX/Zfy1hi or XX/Zfy2 females. On completing the analysis we found that 226/309 XX/Zfy2 oocytes came from females that also carried an Eif2s3y transgene (hereafter denoted XX/Zfy2+Eif2s3y). Consequently, the dysregulation of gene expression observed in these 'contaminated' samples could be a consequence of the expression of Zfy2, Eif2s3y, or both. Batch 2 consisted of triplicates of oocytes from XX/Zfy1hi and XX/Zfy2 mice. The transcriptome data were normalised using cubic spline normalisation as implemented in Beadstudio (Illumina). The dataset was deposited at Gene Expression Omnibus under accession number GSE52218.

Expression changes in transgenics were measured relative to XX controls, and the significance determined using the Illumina custom error model with Benjamini/Hochberg false discovery rate correction. In order to focus on the genes specifically regulated by Zfy2 and consequently potentially linked to the infertility phenotype, we selected genes that were: (1) expressed in at least one of the uncontaminated samples (16,089 genes remaining); (2) significantly regulated (P<0.05) in batch 2 XX, Zfy2 oocytes (2655 remaining; supplementary material Table S3, tab 1); (3) regulated ≥1.5-fold in XX/Zfy2 relative to wild type (867 remaining); (4) not significantly regulated in a pairwise comparison of batch 1 with batch 2 samples (473 remaining); (5) regulated at least twice as strongly in XX/Zfy2 as in any of the control genotypes (XX/Zfy1hi, XX/Zfy1lo and XX/Zfy2lo), and at least twice any residual batch effect (359 remaining; supplementary material Table S3, tab 2). Note that Zfy1lo is considered a control because this transgene did not affect fertility despite substantial changes in the transcriptome.

The final set of 359 Zfy2-regulated genes comprised 163 that were upregulated and 196 that were downregulated. For these, the agreement between batch 1 and batch 2 samples is very high (r²=0.9094; supplementary material Table S3, tab 3), indicating that these are genuine transcriptional responses to the Zfy2 transgene. We tested whether any specific gene categories were enriched among the regulated genes using DAVID (Huang et al., 2009), by comparing the lists of genes that were upregulated and downregulated by Zfy2 with the background list of all oocyte-expressed genes. Indexing for DAVID used the Entrez gene identification number (id) for each gene and high classification stringency was used for the functional clustering.

For comparisons to other data sets obtained using the Affymetrix platform rather than the Illumina platform, genes were matched according to their Entrez id, or by gene symbol if no match was found for the Entrez id.

Western blotting

Western blotting was performed as described previously (Cocquet et al., 2010). Briefly, 10-15 μg of ovary or testis protein extract were run on an 8% SDS-polyacrylamide gel. After transfer and blocking, membranes were incubated overnight with anti-RBMY antibody [1/500 (Turner et al., 2002)]. Anti-actin antibody (1/50,000; Sigma, A3854) was used for normalisation. Incubation with the corresponding secondary antibody coupled to peroxidase, and detection by chemiluminescence, were carried out as described by the manufacturer (Pierce). XY testis (complete Rhmy gene cluster) and XSexO testis [8-fold depleted Rhmy cluster (Mahadeviah et al., 1998)] provided positive controls; XO,Sry,Eif2s3y transgenic tests (Mazeyrat et al., 2001) and XO ovary provided negative controls.

Embryo collection, culture and processing

Females over 2 months of age were caged with MF1 males for up to 3 days. The morning when copulation plugs were found was defined as 0.5 dpc. At 1.5 dpc, oocytes/embryos were flushed from the oviduct with M2 medium using a 30G needle and examined under a stereo microscope. Embryos that had progressed to at least the 2-cell stage were cultured in 20 μl drops of KSOm (Lawitts and Biggers, 1993) (prepared according to the Jackson Laboratory medium preparation protocol) covered with mineral oil (FertiPro) in a humidified chamber at 37°C supplemented with 5% CO2. Development of embryos was observed each day up to 5 days. As a check on our visual assessment of embryo viability, we stained 2-cell embryos flushed at 1.5 dpc and some arrested 2-cell embryos after 2 days in culture, in a 1:1 mixture of Trypan Blue:M2 medium for 40 minutes. They were washed in M2 and photographed under brightfield (Leica DMI 4000B microscope). Embryos flushed from the uterus at 1.5, 2.5 and 3.75 dpc were also photographed to provide data on development in vivo.

For visualisation of polar body and blastomere nuclei at early cleavage stages, embryos were collected in drops of M2 medium under oil. They were then processed individually as follows: (1) assessed number of polar bodies (Wild M5 dissection microscope); (2) washed in a drop of phosphate buffered saline (PBS) and transferred to a glass slide; (3) fixed for 1 minute in 4% paraformaldehyde/0.05% Triton X-100 (3.747 ml PBS, 1.25 ml 16% buffered saline (PBS) and transferred to a glass slide; (4) dried on warming pad at 35°C for at least 1 hour; (4) rinsed in distilled water for 30 seconds; (5) dried on a warming pad at 35°C for at least 1 hour. The embryos and fluids were handled throughout with fine glass pipettes while observing the embryo under the dissection microscope; if an embryo detached, the fluid was carefully withdrawn to make it reattach. Up to eight embryos were placed on each slide, located within circles drawn with black felt marker on the underside of the slide. Nuclei were stained with DAPI diluted in the mounting medium (Vectashield with DAPI; Vector Laboratories) and images captured using a DeltaVision microscope (Applied Precision).

Collection and activation of ovulated oocytes

Females were mated with aspermic males. Oocytes were collected from the oviduct on the morning the copulatory plug was found, incubated at 37°C in 300 μl hyaluronidase (Sigma Type IV) in M2 for 3-5 minutes, denuded of cumulus cells by repeated pipetting in M2, and split into two groups per female. The first group was immediately fixed with 4% parafomaldehyde in PBS for 1 hour, permeabilised with 0.5% Triton X-100 (or saponin) in PBS for 0.5-1.0 hour, and immunostained for tubulin using mouse anti-alpha tubulin (1:200; Abcam, ab 80779), followed by goat anti-mouse IgG Alexa 488 (1:500; Invitrogen, A-11001). The second group was incubated in 5 mM SrCl2 in Ca/Mg-free M16 for 1.5 or 2 hours before fixation and staining. The oocytes were then transferred to glass slides and mounted in Vectashield containing DAPI and scored for spindle (tubulin) and chromatin (DAPI) status using a Leica DMRA2 microscope.

Statistical analysis

The General Linear Models Analysis of Variance software of NCSS 8 statistical software was used for all genotype comparisons of breeding
performance and the number of oocytes, with Tukey-Kramer adjustment of probability values when there were multiple comparisons.

For qRT-PCR comparisons, a one-tailed Student’s t-test assuming unequal variances was performed on the power 2(AuAC)<100 values using eXcel (Microsoft).

For microarray analysis, cubic spline normalisation of expression data and all subsequent filtering steps were performed using BeadStudio and eXcel; P-values for all comparisons were calculated using the custom error model implemented in BeadStudio.

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Competing interests
The authors declare no competing financial interests.

Author contributions
N.V., M.S., S.K.M., F.D., O.A.O., T.T. and P.S.B. contributed to experimental design and collection of data; N.V., P.J.I.E., T.T. and P.S.B. analysed the data; O.A.O. had overall responsibility for mouse colony management; A.R. assisted with oocyte collection for transcriptome analysis and genotype analyses; N.V., P.J.I.E. and P.S.B. wrote the manuscript.

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