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ERM is required for transcriptional control of the spermatogonial stem cell niche

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Abstract

Division of spermatogonial stem cells[1] produces daughter cells that either maintain their stem cell identity or undergo differentiation to form mature sperm. The Sertoli cell, the only somatic cell within seminiferous tubules, provides the stem cell niche through physical support and expression of surface proteins and soluble factors[2,3]. Here we show that the Ets related molecule[4] (ERM) is expressed exclusively within Sertoli cells in the testis and is required for spermatogonial stem cell self-renewal. Mice with targeted disruption of *ERM* have a loss of maintenance of spermatogonial stem cell self-renewal without a block in normal spermatogenic differentiation and thus have progressive germ-cell depletion and a Sertoli-cell-only syndrome. Microarray analysis of primary Sertoli cells from ERM-deficient mice showed alterations in secreted factors known to regulate the haematopoietic stem cell niche. These results identify a new function for the Ets family transcription factors in spermatogenesis and provide an example of transcriptional control of a vertebrate stem cell niche.

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Author Information Full Affymetrix data sets have been deposited with the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) as accession series GSE2205. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions.

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Ets family transcription factors share a unique Ets DNA-binding domain and participate in a variety of developmental processes[5]. ERM[4,6] belongs to a subfamily of Ets factors that also includes Pea3 and ER81 ([ref. 5]). Pea3 and ER81 are important for normal neuronal development[7,8]. ERM is expressed in several tissues including brain, lung and testis[6]. To study the function of ERM *in vivo*, we generated mice with an inactivated *ERM* allele (*ERM*$^{-}$) (Supplementary Fig. 1). Heterozygous *ERM*$^{+/-}$ mice are normal, and interbreeding heterozygous *ERM*$^{+/-}$ mice yielded a mendelian (1:2:1) distribution of *ERM*$^{-/-}$, *ERM*$^{+/-}$ and *ERM*$^{+/+}$ in viable offspring (60:147:77, respectively), indicating that ERM is not critical for embryonic development or viability. The most marked phenotype in *ERM*$^{-/-}$ mice is the disruption of spermatogenesis, whereas other organs did not reveal obvious anatomical defects (Supplementary Fig. 2).

Spermatogenesis is a cyclic process involving the differentiation of spermatogonial stem cells, meiotic cell division and the formation of haploid spermatids. Sertoli cells are the only somatic cells within seminiferous tubules and provide the immediate environment for developing germ cells[2]. A balance between spermatogonial stem cell self-renewal and differentiation in the adult testis is essential for the maintenance of cyclic waves of spermatogenesis and fertility. Although *ERM*$^{+/-}$ males were fertile, adult *ERM*$^{-/-}$ males were sterile (n = 12). ERM$^{-/-}$ males had a significantly decreased testicular size (Fig. 1a). At 4 weeks of age, seminiferous tubules of wild-type and *ERM*$^{-/-}$ mice seemed similar, with multiple layers of germ cells indicating the normal initiation of spermatogenesis (Fig. 1b, c). By 6 weeks many *ERM*$^{-/-}$ tubules underwent progressive germ-cell maturation and depletion (Fig. 1d) and by 10 weeks most tubules were devoid of any germ cells (Fig. 1e), containing only morphologically normal Sertoli cells at the basement membrane, which expressed GATA-1 (Fig. 1f), a marker of mature Sertoli cells.

Histological analysis indicates that the primary defect in *ERM*$^{-/-}$ testis is the depletion of spermatogonia rather than a developmental block in spermatogenic differentiation. First, at 4 weeks or earlier, *ERM*$^{-/-}$ seminiferous tubules showed normal spermatogenic differentiation (Fig. 1c), indicating that *ERM*$^{-/-}$ primordial germ cells and embryonic gonocytes could give rise to spermatogonial stem cells and that a normal wave of spermatogenesis had occurred. However, by 6 weeks (Fig. 1g–k), an array of tubules showed either a selective loss of spermatogonia only or a combined loss of preleptotene and pachytene spermatocytes (Fig. 1h, i). Many tubules contained only elongated spermatids and Sertoli cells, with depletion of all precursor germ cells (Fig. 1j), whereas others were devoid of germ cells altogether (Fig. 1k). These data indicate that ERM deficiency is permissive of normal spermatogenic differentiation but causes germ-cell depletion through an initial loss of spermatogonial stem cells.

To assess the depletion of spermatogonia at the molecular level, we used microarray analysis to compare gene expression between wild-type and *ERM*$^{-/-}$ testis at 4 weeks of age, when the testes of the two phenotypes have nearly identical histological appearances. Of 50 genes whose expression was reduced to less than one-third in *ERM*$^{-/-}$ testis compared with the wild type, the greatest reduction was observed for spermatogonia-specific genes (Fig. 2a and Supplementary Table 1). For example, the spermatogonia marker Stra8 ([ref. 9]) was reduced 19-fold, and other spermatogonia-selective transcripts, including RNA-binding-motif protein (Rbm), Dazl, lymphoid-specific helicase (Lsh) and cellular retinoic-acid-binding protein (CRABP) were reduced 7-fold, 3.5-fold, 9-fold and 14-fold, respectively, in *ERM*$^{-/-}$ testis. By contrast, several markers of more mature germ cells were unchanged in expression, including the spermatid markers[10] protamine 1 (Prm1) and Prm2, and transition proteins 1 and 2. Microarray results were confirmed by reverse-transcriptase-mediated polymerase chain reaction (RT–PCR) analysis in three distinct pairs of 4-week-old wild-type and *ERM*$^{-/-}$ littermates (Fig. 2a). Thus, at 4 weeks, when *ERM*$^{-/-}$ and wild-type testes are

very similar in histological appearance, spermatogonia-specific genes were already greatly underrepresented in *ERM*$^{-/-}$ testes, again indicating that the defect is the loss of maintenance of spermatogonia. Finally, the loss of spermatogonial stems cells in *ERM*$^{-/-}$ mice was indicated by the absence of Plzf[11] expression in *ERM*$^{-/-}$ testis in comparison with wild-type testis (Fig. 2b, c). The rare cells with morphological similarity to spermatogonia that remained in *ERM*$^{-/-}$ testis at 6 weeks of age were negative for Plzf expression, indicating that these are committed spermatogonia rather than undifferentiated spermatogonia.

Failure to maintain spermatogonia in *ERM*$^{-/-}$ testis could result from either an intrinsic requirement for ERM in germ cells or cell-extrinsic requirement for ERM in Sertoli or other cells. We first analysed ERM expression in c-*kit*$^{W/W\text{-}v}$ testes (Supplementary Fig. 3a), which have a Sertoli-cell-only phenotype[12]. ERM expression was increased in germ-cell-free c-*kit*$^{W/W\text{-}v}$ testis relative to wild-type testis, indicating that it is expressed by somatic cells. In addition, ERM was expressed in isolated Sertoli cells, but not in isolated spermatogonia, pachytene spermatocytes or round spermatids (Supplementary Fig. 3b), whereas Stra8 was expressed exclusively in spermatogonia, as expected. Further, we used several additional methods to show that ERM is exclusively expressed within Sertoli cell in the testis. First, by *in situ* hybridization, ERM messenger RNA was localized to the periphery of seminiferous tubules in wild-type testis but was absent centrally (Fig. 3a). The non-functional *ERM*$^{-}$ mRNA transcript was detected in the Sertoli-cell-only *ERM*$^{-/-}$ testis at 10 weeks (Fig. 3b), indicating direct expression by Sertoli cells. Second, we examined ERM expression histologically with an IRES-LacZ reporter cassette targeted to the ERM locus (N. Kurpios, S. Arber, J.A. Hassell, unpublished observations). ERM expression in *ERM*$^{+/IRES\text{-}LacZ}$ testis was found exclusively in Sertoli cells, was first detectable between 3 and 4 weeks of age and persisted throughout adulthood (Fig. 3c and Supplementary Fig. 4). This onset of ERM expression precedes the timing of spermatogonial loss, which is consistent with a requirement for ERM in the adult stem cell niche in the testis. Third, a fusion protein of ERM and green fluorescent protein (GFP) was localized to the nucleus of TM4 Sertoli cells (Fig. 3d). Last, we generated an ERM-specific monoclonal antibody, 3H7 (Supplementary Fig. 5), which identified ERM protein expression to be present exclusively within Sertoli cell nuclei of wild-type testis (Fig. 3e) and to be undetectable in *ERM*$^{-/-}$ testis (Fig. 3f). Thus, ERM expression in testis is specific to Sertoli cells, indicating that the loss of spermatogonia in *ERM*$^{-/-}$ testes might not be due to a cell-intrinsic defect of spermatogonia but rather to alterations in the microenvironment provided by Sertoli cells.

Signalling by fibroblast growth factor (FGF) has been reported to regulate the expression of ERM in Zebrafish[13], and FGF9 deficiency in mice causes a possible defect in Sertoli cell differentiation[14], prompting us to examine the effect of FGF on ERM expression in Sertoli cells (Fig. 3g). ERM mRNA was induced by FGF1, FGF2 and FGF9, but not by FGF7 and FGF10, in the murine Sertoli cell line TM4 (Fig. 3g). These results indicate a potential activity of FGF receptor signalling in regulating ERM expression and Sertoli cell function *in vivo*.

Spermatogonial depletion could result from alterations in spermatogonial proliferation or apoptosis. We therefore examined cell proliferation in *ERM*$^{-/-}$ testes at 3 and 4 weeks by labelling *in vivo* with bromodeoxyuridine (BrdU) (Fig. 4a–d). At 3 weeks, before the loss of germ cells, BrdU incorporation by spermatogonia was normal in *ERM*$^{-/-}$ testis (Fig. 4a, b). However, at 4 weeks, BrdU incorporation was almost absent in *ERM*$^{-/-}$ testis, compared with labelling in wild-type (Fig. 4c, d). To determine whether this loss was due to increased apoptosis, we used TdT-mediated dUTP nick end labelling (TUNEL), which showed no alteration in TUNEL staining in *ERM*$^{-/-}$ testes at 4 or 6 weeks (Fig. 4e, f, and Supplementary Table 2). Further, expression of proapoptotic and antiapoptotic genes in the

Bcl-2 family also showed no difference between *ERM*$^{-/-}$ and wild-type testes (Fig. 4g). These results indicate that the defect was caused by decreased self-renewal of spermatogonial stem cells. Finally, serum hormone levels were measured to determine whether the pituitary–testis axis had contributed to the phenotype. We found no significant difference in serum levels of testosterone or follicle-stimulating hormone between wild-type and *ERM*$^{-/-}$ mice (Supplementary Table 3). Together, these results indicate that ERM expression by Sertoli cells might be required for spermatogonial stem cell self-renewal, and that loss of spermatogonial stem cells in *ERM*$^{-/-}$ testis is due to a defect in the stem cell niche provided by ERM-expressing Sertoli cells rather than an endocrine disorder.

Several somatic signalling pathways regulating spermatogonial stem cell self-renewal have recently been discovered[3,15,16]. Mutations in two genes, promyelocytic zinc-finger (Plzf)[11,17] and glial-cell-derived neurotrophic factor (GDNF)[3] result in a defect in mammalian spermatogonial stem cell self-renewal. By comparison, the self-renewal defect in *ERM*$^{-/-}$ testes is more severe and differs mechanistically from that caused by mutations in Plzf or GDNF. First, Plzf is a transcription factor expressed by spermatogonia[11,17], not by Sertoli cells, and the loss of Plzf causes a spermatogonia-intrinsic defect. Loss of spermatogonia in Plzf-deficient mice is not complete even at 8 months[11,17], indicating that spermatogonial self-renewal can occur for extended periods in the absence of Plzf, in contrast to more rapid and complete loss of spermatogonia in *ERM*$^{-/-}$ testes. Second, GDNF[3], which is secreted by Sertoli cells, was implicated in spermatogonial self renewal because GDNF$^{+/-}$ mice show gradual spermatogonial depletion[3]. GDNF$^{-/-}$ testes have not been analysed because of embryonic lethality.

To test whether ERM regulates GDNF, we performed a microarray analysis of purified wild-type and *ERM*$^{-/-}$ primary Sertoli cells (Supplementary Fig. 6 and Supplementary Table 4). GDNF expression was unchanged in *ERM*$^{-/-}$ testis in primary Sertoli cells from 4-week-old wild-type and *ERM*$^{-/-}$ testes, indicating that it is not a target of ERM. In contrast, several other genes were greatly reduced in *ERM*$^{-/-}$ Sertoli cells. Genes with the greatest reduction included the chemokines, CXCL-12 (SDF-1), CXCL5 (LIX) and CCL7 (MCP-3), reduced 9-fold, 10-fold and 25-fold, respectively (Supplementary Fig. 6 and Supplementary Table 4). SDF-1 and CXCL5 have been implicated in regulating the stem cell niche in other systems[18,19]. SDF-1 is involved in haematopoietic stem cell (HSC) migration, retention and self-renewal[18] and is required for the migration of primordial germ cells towards the genital ridge[20]. CXCL5 was recently implicated in HSC maintenance[19]. Conceivably these chemokines are niche signalling molecules regulating spermatogonial stem cell self-renewal, but lethality caused by deficiencies of SDF-1 and its receptor CXCR4 prevent an immediate analysis of their role in adult spermatogenesis[21,22]. Matrix metalloproteinase-12 (MMP-12) showed a tenfold reduction in *ERM*$^{-/-}$ Sertoli cells. Interestingly, MMP-9, a related MMP family member, is involved in the recruitment of HSCs to the bone marrow niche[23].

Interactions with niche cells are crucial for maintaining stem cell character, and several molecules produced by niche cells can regulate the capacity of the niche to support stem cell self-renewal[24]. As an example of the transcriptional control of a stem cell niche, ERM provides the opportunity to explore how the capacity of the stem cell niche to maintain spermatogonial self-renewal is transcriptionally coordinated.

Methods

Generation of ERM mutant mice

ERM exons 2–5 encoding the initiation codon and transcriptional activation domain were deleted (Supplementary Fig. 1a). The targeting vector was constructed in pLNTK by using a 1.6-kilobase (kb) genomic fragment (left arm) upstream of the mouse ERM exon 2, and a 4-

kb genomic fragment (right arm) downstream of exon 5. The left arm was generated by PCR from genomic DNA with the use of the oligonucleotides left arm forward (f), 5′-TTTT*GTCGAC*GCGGCCGCTTTTGGAATCTCTTAGGG AAGTTT-3′ (*Sal*I tailed), and left arm reverse (r), 5′-CCC *CTCGAG* TTTCCCTCTTGCCTGTGTAGCCA-3′ (*Xho*I tailed). The 1.6-kb PCR fragment was digested with *Xho*I and *Sal*I and ligated into the *Xho*I site of pLNTK vector. The right arm was generated by PCR with the use of the oligonucleotides right arm forward (f), 5′-AAAA*CTCGAG*ATACAAAGGATTGCAAAGGCT-3′ (*Xho*I tailed), and right arm reverse (r), 5′-GGGA*CTCGAG*TTCTGAAATTG TTTGGCCTTGGA-3′ (*Xho*I tailed), digested with *Xho*I and ligated into the *Sal*I site of targeting vector. The targeting vector DNA was electroporated into MC50 embryonic stem cells (a gift from R. Schreiber). Positive clones were identified by Southern blot analysis with 5′ and 3′ probes (Supplementary Fig. 1a). *In vitro* Cre-mediated neo excision was performed on two distinct recombinant clones, 1CD3 and 1CC5, generating neo-deleted clones E7 and A7, respectively. Blastocyst injection was performed for all four clones and each generated germline transmission of the targeted ERM allele. Male chimaeras were crossed with 129SvEv females to establish ERM mutants on the 129SvEv genetic background. Homozygous mice were obtained by intercrossing heterozygous siblings. The phenotypes for all four lines were indistinguishable grossly and microscopically. For the results shown in this study, the E7 neo-deleted strain was used.

In situ hybridization

A 345-base-pair fragment of the ERM cDNA was obtained by RT–PCR with the use of the oligonucleotides ERM-345(f), 5′-CCGAGTT GTCGTCCTGTAG-3′, and ERM-345(r), 5′-ACTGGCTTTCAGGCATCATC-3′, and cloned into pGEM-Teasy vector used for the synthesis of anti-sense and sense probes. Cryostat sections were hybridized with ^{35}S-labelled antisense RNA (cRNA) probe.

Generation of ERM-specific monoclonal antibody, and histology

ERM region encoded by exons 7 and 8, lacking homology to Pea3 and ER81, was amplified by RT–PCR with the use of the primers 5′-GGAATTCCATATGTGTGCCTA CGATAGGAAGCCTCCC-3′ and CGGGATCCTTATCTCTGTTCTGATGGA TACTGG-3′ and cloned into *Nde*I/*Bam*HI sites of pET28a (Novagen). His-tagged ERM recombinant protein (12 kDa) was induced with 1 mM isopropyl *β*-D-thiogalactoside in *Escherichia coli* BL21 (Invitrogen) and purified by Ni^{2+}-nitrilotriacetate and size-exclusion chromatography. Hybridomas were generated from immunized hamsters and screened by ELISA against purified ERM protein. The hybridoma 3H7 monoclonal antibody (mAb) was used as supernatant for immunohistochemistry.

Immunohistochemistry was performed on sections fixed in 10% formalin. mAb 3H7 was used with goat anti-hamster biotinylated secondary antibody at 1:1000 dilution. Anti-GATA-1 rat mAb (Santa Cruz) was used at 1:100 dilution. Anti-Plzf antibody (Calbiochem) was used at 1:1000 dilution. Vectastain ABC kit and DAB substrate kit (Vector Laboratories) were used for immunohistochemistry. Sections were counterstained with haematoxylin. Analysis of the ERM–LacZ cassette used frozen sections stained overnight with 5-bromo-4-chloro-3-indolyl-*β*-Dgalactoside staining buffer at 37 °C, counterstained with nuclear Fast red.

An ERM-GFP fusion protein was created by deletion of the IRES from the plasmid ERM-RV[25] by Quick change mutagenesis (Strategene) with the oligonucleotides ERM-GFP top (5′-CTTCGCTTACGTGAGCAAGGGCGAGGAGC-3′ and ERM-GFP bot (5′-CCTTGCTCACGTAAGCGAAGCCTTCGGTGTA-3′), to produce ERM-GFP-RV. TM4

cells were infected with ERM-GFP-RV or GFP-RV and purified by cell sorting. Cellular localization of ERM-GFP fusion protein was captured by confocal microscopy.

TM4 cells were maintained in serum-free medium for 24 h and treated with medium alone or 1 nM FGF1, FGF2, FGF7, FGF9 and FGF10 (PeproTech) or 10% fetal calf serum. RNA was harvested after 3 h and real-time RT–PCR was performed for ERM and glyceraldehyde3phosphate dehydrogenase using an ABI Prism 7700 Sequence Detector (Applied Biosystems). ERM-specific primers used for this RT–PCR were 5′-CAGGAGCCCCGAGATTACTG-3′ and 5′-CCGCCTCTCATGTAGGATGAC-3′. Data are represented as fold expression of normalized ERM expression over the medium control.

Cell proliferation and apoptosis assays

Mice were injected intraperitoneally with BrdU (Sigma) at a concentration of 50 mg kg^{-1}. After 4 h, testes and small intestine were isolated and fixed in 10% buffered formalin. Paraffin-embedded sections were processed with the BrdU *in-situ* detection kit (BD Biosciences). Germ-cell apoptosis was analysed by TUNEL labelling with Apoptag *in situ* apoptosis detection kit (Intergen) in accordance with the manufacturer's protocol. Expression of proapoptotic and antiapoptotic genes in 4-week-old wild-type and $ERM^{-/-}$ testes was quantified by the mAPO-2 Multi-probe RNase protection assay kit (BD Biosciences) in accordance with the manufacturer's protocol.

Microarray analysis of wild-type and $ERM^{-/-}$ testes and primary Sertoli cells

Wild-type and $ERM^{-/-}$ testes ($n = 4$) were isolated at 4 weeks of age, and total RNA was extracted separately with RNeasy kit (Qiagen). RNA (10 μg) was pooled, and biotinylated cRNA target was independently generated from each pool. Each cRNA was hybridized to an Affymetrix U74Av2 Murine Genome Array. Microarray analysis of primary Sertoli cells isolated[26] from wild-type or $ERM^{-/-}$ mice was carried out similarly.

RT–PCR analysis

We used semiquantitative PCR for confirmation of Affymetrix gene array results in Fig. 2a and Supplementary Fig. 5. RT–PCR amplifications were titrated within the linear range for each primer pair. Primers used are listed in Supplementary Table 5.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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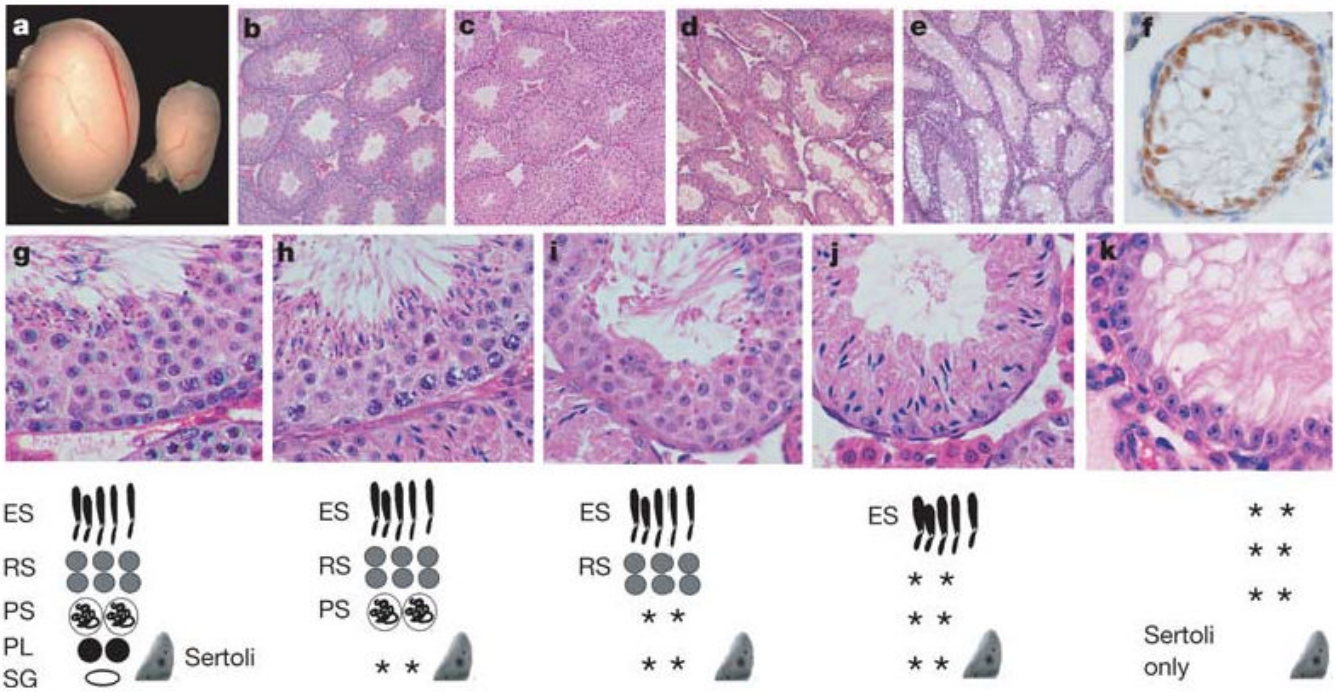

Figure 1. Spermatogonial depletion and Sertoli-cell-only syndrome in *ERM*$^{-/-}$ mice

a, Ten-week wild-type (left) and *ERM*$^{-/-}$ (right) testes. **b**, Wild-type seminiferous tubules. **c**–**e**, Progressive germ-cell loss in *ERM*$^{-/-}$ testis at 4 weeks (**c**), 6 weeks (**d**) and 10 weeks (**e**). **f**, Persistent Sertoli cells in *ERM*$^{-/-}$ tubules indicated by GATA-1 expression at 10 weeks. **g**–**k**, Normal spermatogenesis with spermatogonial depletion. Histology of wild-type (**g**) or *ERM*$^{-/-}$ (**h**–**k**) testes at 6 weeks of age. Diagrams of seminiferous epithelium are shown at the bottom: ES, elongated spermatid; RS, round spermatid; PS, pachytene spermatocyte; PL, preleptotene spermatocyte; Sg, spermatogonium; Sertoli, Sertoli cell; asterisks, missing cell populations.

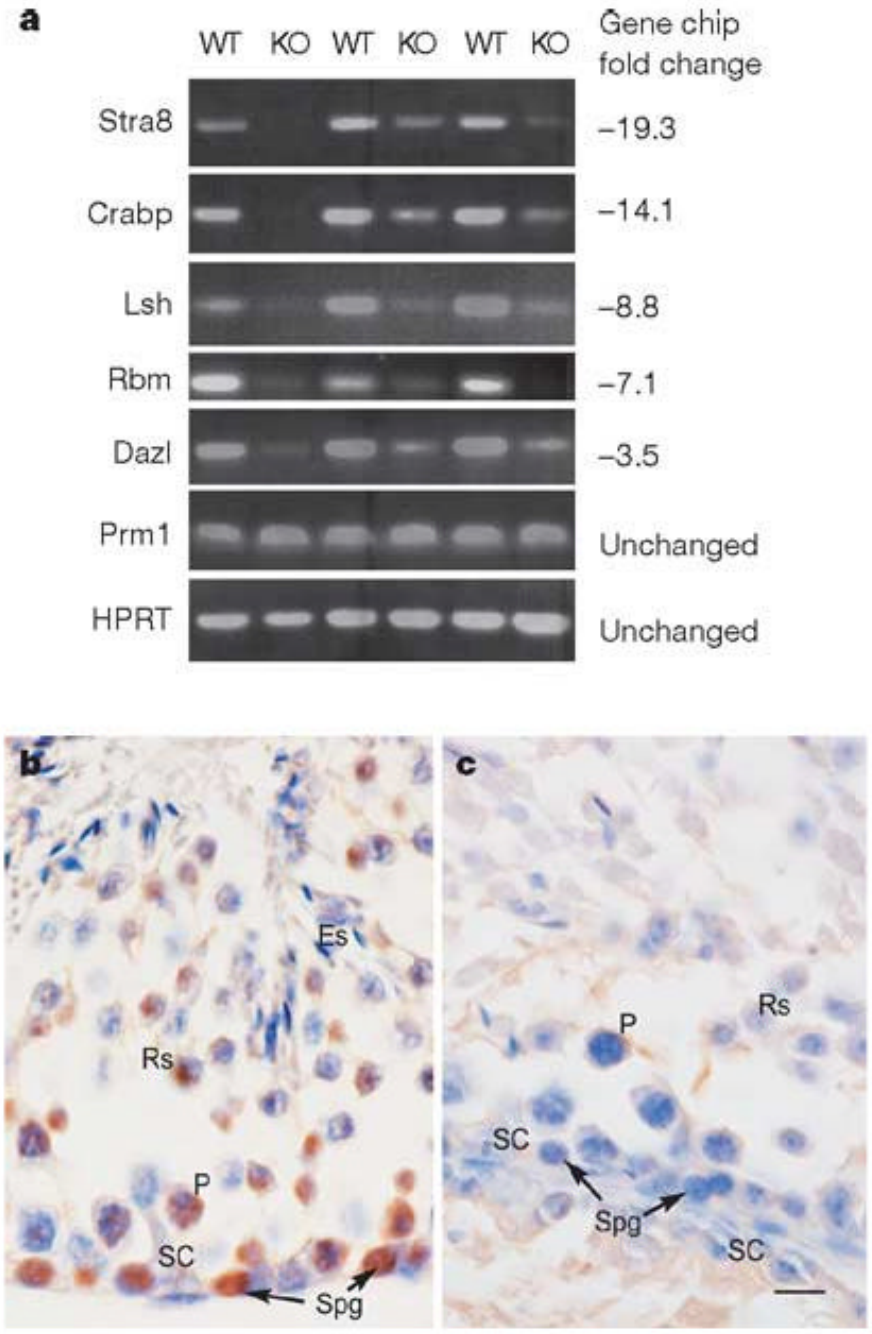

Figure 2. Selective reduction of spermatogonia-specific genes in *ERM*$^{-/-}$ testes

a, RT–PCR analyses of three independent pairs of wild-type (WT) and *ERM*$^{-/-}$ (KO) male littermates are shown for the indicated genes. Numbers at the right represent the relative fold reduction measured by microarray analysis (Supplementary Table 1). HPRT, hypoxanthine–guanine phosphoribosyltransferase. **b**, **c**, Expression of Plzf in 6-week-old wild-type (**b**) and *ERM*$^{-/-}$ (**c**) testes by immunohistochemistry. Cells indicated are as follows: ES, elongated spermatid; RS, round spermatid; PS, pachytene spermatocyte; Spg, spermatogonium; Sc, Sertoli cell. Scale bar, 10 μm.

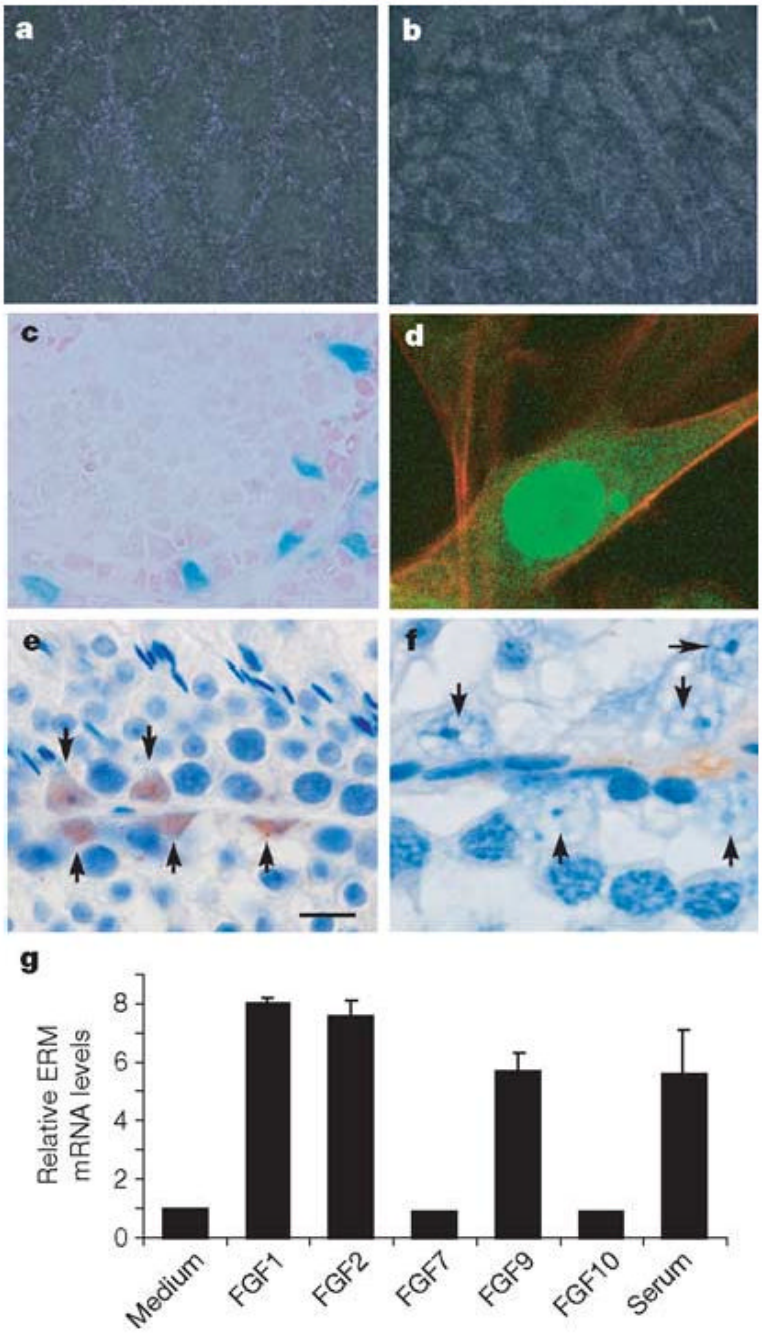

Figure 3. ERM expression in testis is restricted to Sertoli cells

a, **b**, In situ hybridization for ERM mRNA in 10-week wild-type (**a**) and ERM$^{-/-}$ (**b**) testes. **c**, Testes from 6-week *ERM*$^{IRES\text{-}LacZ}$ heterozygous males were stained for LacZ expression. **d**, TM4 Sertoli cells were infected with ERM-GFP-RV retrovirus and analysed by confocal microscopy. **e**, **f**, Immunolocalization of ERM protein in adult wild-type (**e**) and ERM$^{-/-}$ (**f**) testis with anti-ERM monoclonal antibody. Arrows indicate nuclei of Sertoli cells in wild-type (**e**) and ERM$^{-/-}$ (**f**) testes. **g**, ERM mRNA expression in TM4 cells after treatment with various FGFs. Data are fold induction (means + s.d. for two independent measurements) compared with ERM expression in medium.

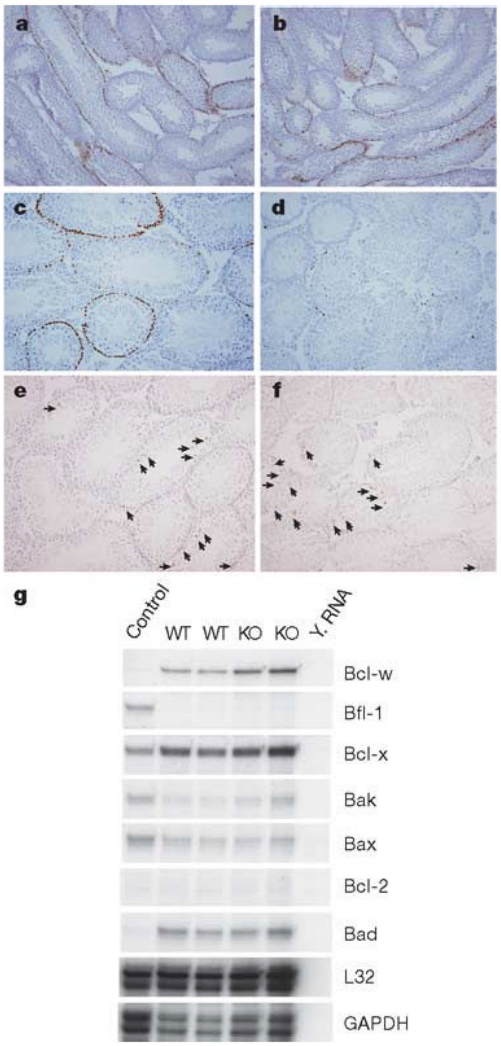

Figure 4. Failure of stem cell self-renewal causes spermatogonial depletion

a–**d**, Loss of proliferating spermatogonia in *ERM*$^{-/-}$ testis, demonstrated by BrdU labelling in vivo of wild-type (**a**, **c**) and *ERM*$^{-/-}$ (**b**, **d**) testes at 3 weeks (**a**, **b**) and 4 weeks (**c**, **d**). **e**, **f**, Comparable levels of spermatogonial apoptosis, determined by TUNEL staining, in wild-type (**e**) and *ERM*$^{-/-}$ (**f**) testes at 4 weeks of age. Arrows indicate apoptotic cells. **g**, Expression of apoptosis-related genes was unchanged in *ERM*$^{-/-}$ testes as indicated by RNase protection assay of total RNA from 4-week-old wild-type (WT) and *ERM*$^{-/-}$ (KO) testes. GAPDH, glyceraldehyde3phosphate dehydrogenase. Y. RNA, Yeast tRNA.