

# Kit/stem cell factor receptor-induced activation of phosphatidylinositol 3'-kinase is essential for male fertility

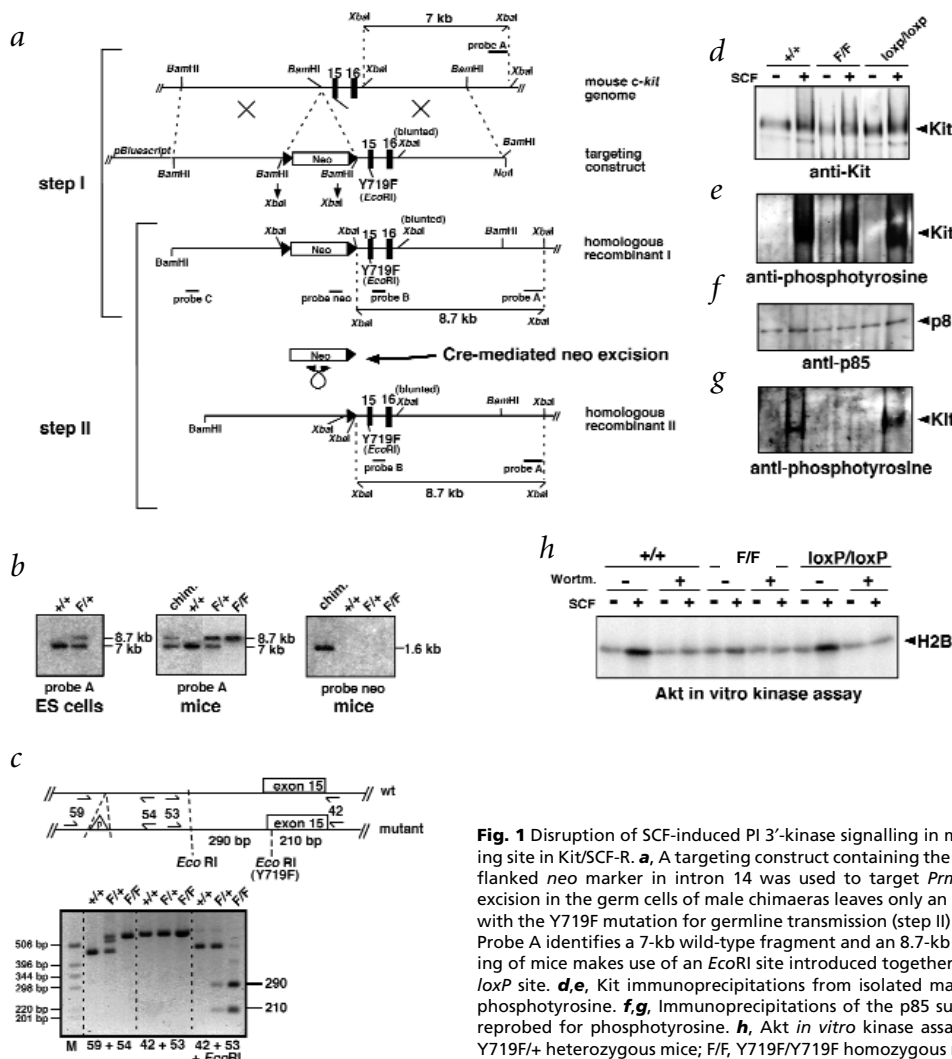
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The *c-kit*-encoded transmembrane tyrosine kinase receptor for stem cell factor (Kit/SCF-R) is required for normal haematopoiesis, melanogenesis and gametogenesis<sup>1–3</sup>. However, the roles of individual Kit/SCF-R-induced signalling pathways in the control of developmental processes in the intact animal are completely unknown. To examine the function of SCF-induced phosphatidylinositol (PI) 3'-kinase activation *in vivo*, we employed the Cre-*loxP* system<sup>4</sup> to mutate the codon for Tyr719, the PI 3'-kinase binding site in Kit/SCF-R, to Phe in the genome of mice by homologous recombination. Homozygous (Y719F/Y719F) mutant mice are viable. The mutation completely dis-

rupted PI 3'-kinase binding to Kit/SCF-R and reduced SCF-induced PI 3'-kinase-dependent activation of Akt by 90%. The mutation induced a gender- and tissue-specific defect. Although there are no haematopoietic or pigmentation defects in homozygous mutant mice, males are sterile due to a block in spermatogenesis, with initially decreased proliferation and subsequent extensive apoptosis occurring at the spermatogonial stem-cell level. In contrast, female homozygotes are fully fertile. This is the first report so far demonstrating the role of an individual signalling pathway downstream of Kit/SCF-R in the intact animal. It provides the first *in vivo* model for male

sterility caused by a discrete signalling pathway defect affecting early germ cells.

We previously reported that PI 3'-kinase is required for SCF-induced cell proliferation and survival *in vitro*<sup>5,6</sup>, and wanted to examine its role *in vivo* by specifically disrupting Kit/SCF-R-induced PI 3'-kinase activation. The mutation of the single PI 3'-kinase-binding site in Kit/SCF-R (refs 5,7,8), Y719F, was generated by gene targeting in embryonic stem (ES) cells with 13.6 kb of homologous sequence containing the desired point mutation in *Kit* exon 15 and a *loxP*-flanked ('floxed') *neo* marker in intron 14 (Fig. 1a). The introduced *loxP* sites were used for subsequent Cre-mediated excision of the gene *neo* to avoid potential transcriptional interference. To achieve this, the ES cells used contained a Cre recombinase transgene under



**Fig. 1** Disruption of SCF-induced PI 3'-kinase signalling in mice by mutation of the PI 3'-kinase-binding site in Kit/SCF-R. **a**, A targeting construct containing the Y719F mutation in *Kit* exon 15 and a *loxP*-flanked *neo* marker in intron 14 was used to target *Prm*-Cre ES cells (step I). Cre-mediated *neo* excision in the germ cells of male chimeras leaves only an extra 87 bp behind in the intron together with the Y719F mutation for germline transmission (step II). **b**, Southern-blot analysis of *Xba*I digests. Probe A identifies a 7-kb wild-type fragment and an 8.7-kb gene-targeted fragment. **c**, PCR genotyping of mice makes use of an *Eco*RI site introduced together with the Y719F mutation, and shows the *loxP* site. **d, e**, Kit immunoprecipitations from isolated mast cells probed for Kit and reprobed for phosphotyrosine. **f, g**, Immunoprecipitations of the p85 subunit of PI 3'-kinase probed for p85 and reprobed for phosphotyrosine. **h**, Akt *in vitro* kinase assays using histone H2B as a substrate. F+/+, Y719F/+ heterozygous mice; F/F, Y719F/Y719F homozygous mice.

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**Table 1 • Breeding results**

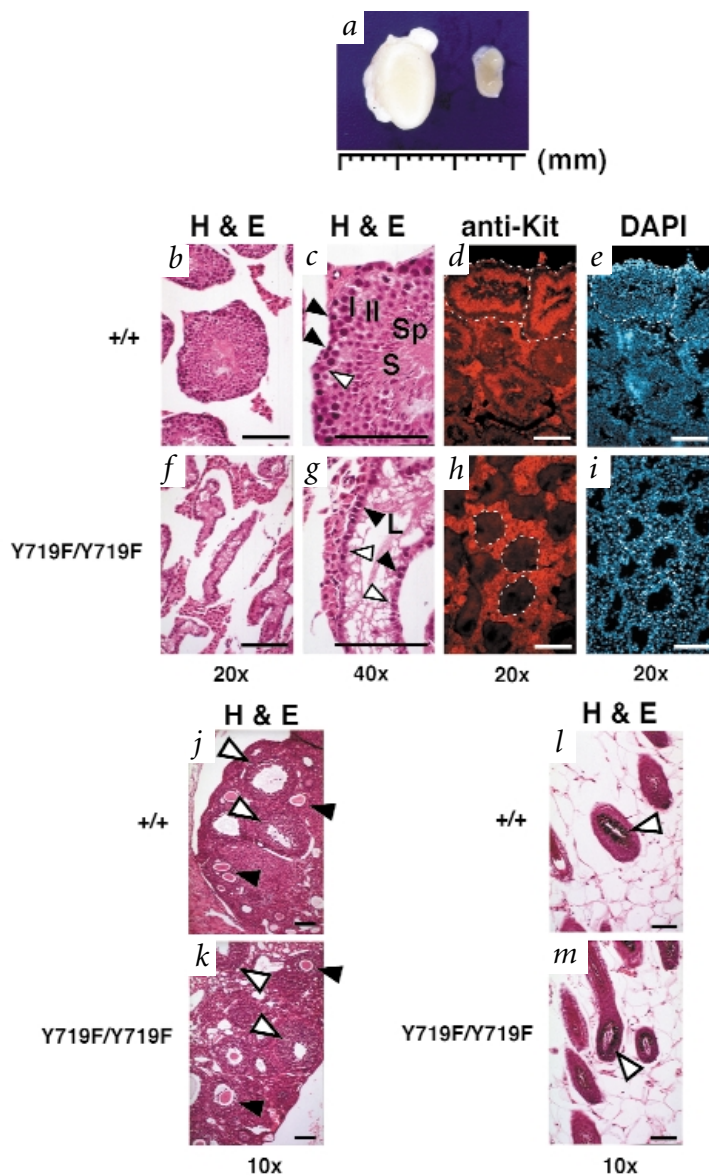
Female	+/+	Y719F/+	Y719F/Y719F
Male			
+/+	7.8±0.32 (M) 6.3±0.6 (I)	8.6±0.35 (M) 5.8±1.1 (I)	8.3±0.28 (M) 6.4±0.7 (I)
Y719F/+	7.7±0.31 (M) 5.7±0.9 (I)	7.6±0.28 (M) 6.2±1.2 (I)	8.0±0.34 (M) 6.6±0.6 (I)
Y719F/Y719F	0 (M) 0 (I)	0 (M) 0 (I)	0 (M) 0 (I)

Each number is the average litter size obtained from matings between B6D2F1/SV129 mice ('M'; mixed background) or between SV129 mice ('I'; inbred strain). Mean  $\pm$  S.E.M.,  $n > 15$  for 'M';  $n = 6$  for 'I'. Each mean represents breedings between a minimum of 7 males and 15 females for 'M', and 3 males and 6 females for 'I'.

control of the protamine-1 (*Prm1*) promoter, which is active in male haploid germ cells<sup>9</sup>. Accordingly, although male chimaeric mice derived from targeted ES cells contained the *neo* gene in somatic tissues, we found it was absent from all mutant offspring (Fig. 1*a-c*). As a consequence, only one *loxP* site and surrounding *Xba* linker sequences, totalling 87 bp, were left behind in intron 14 of *Kit* upstream of the desired point mutation. Both the *loxP* site and the point mutation were detected by PCR genotyping, as we had introduced a diagnostic *Eco*RI restriction site together with the Y719F codon (Fig. 1*c*). Homozygous Y719F/Y719F and heterozygous Y719F/+ mice were born at the expected mendelian ratios in the F<sub>3</sub> generation, had normal body weights and good general health status. To ensure that we had disrupted PI 3'-kinase signaling from the Kit/SCF-R in mutant mice, we examined primary mast cells from bone marrow of +/+ and Y719F/Y719F age-matched mice, as mutant mast cells turned out to be fully viable (Fig. 1*d-h*, and data not shown). We also included mast cells from gene-targeted control mice (*loxP/loxP*), generated in parallel with the mutants, that contained the *loxP* site but not the Y719F mutation. Kit/SCF-R from Y719F/Y719F and control mice was of the same size and was expressed at comparable levels (Fig. 1*d*). The Y719F-Kit receptor was also kinase active and autophosphorylated on tyrosine residues upon SCF stimulation (Fig. 1*e*). The regulatory p85 subunit of PI 3'-kinase was expressed at similar levels in mast cells from mutants and controls (Fig. 1*f*). Although p85 co-precipitated similar amounts of Kit/SCF-R from SCF-stimulated +/+ and *loxP/loxP* control cells, no association with Kit/SCF-R was detected in the Y719F/Y719F mutant mast cells (Fig. 1*g*). To determine whether there was any indirect activation of PI 3'-kinase by the mutant receptor, we assayed for SCF-induced Akt activation (Fig. 1*h*). Activation of the serine/threonine kinase Akt is PI 3'-kinase dependent and provides a sen-

sitive measure for PI 3'-kinase activity<sup>10</sup>. SCF activated Akt five-fold in +/+ and *loxP/loxP* mast cells in a wortmannin-sensitive manner, but at only 12% of wild-type levels in Y719F/Y719F mast cells, and this was completely inhibited by wortmannin. Given the complete disruption of PI 3'-kinase binding to the mutant receptor, and that Akt is a sensitive PI 3'-kinase-dependent target, these results suggest that approximately 90% of SCF-induced PI 3'-kinase activation is abolished in mutant cells. Most likely, the mutant Kit/SCF-R can activate indirect pathways leading to PI 3'-kinase, for example via Ras, Src and Gab1 (refs 11,12), accounting for the residual 10% activation.

Heterozygous mice and homozygous mutant females were fully fertile, but Y719F/Y719F males were sterile (Table 1). This was the case both for the 'mixed background' (B6D2F1×129/Sv) mice as well as for inbred (129/Sv) mice. Mutant males exhibited normal libido and produced vaginal plugs, but their testes were severely hypoplastic (Fig. 2*a*). Adult (4-months-old) littermate mice and age-matched *loxP/loxP* controls were perfusion-fixed and their testes dissected (Fig. 2*b-i*). Seminiferous tubules of +/+ mice contained spermatogonial type A and B stem cells mixed with Sertoli cells close to the basement lamina, then primary and secondary spermatocytes, spermatids and finally spermatozoa



**Fig. 2** Testes, ovaries and hair follicles of adult mice. **a**, Testes from +/+ (left) and littermate Y719F/Y719F (right) mouse. **b-i**, Sections of +/+ and Y719F/Y719F testes show spermatogonial stem cells (closed arrow heads) and Sertoli cells (open arrow heads). I, primary spermatocytes; II, secondary spermatocytes; S, spermatids; Sp, spermatozoa. White stippled lines (**d-e** and **h-i**) indicate the capsules of some individual seminiferous tubules. Note the outer layer of Kit-positive cells in +/+ testes (**d**) and presence of cells within the capsules of seminiferous tubules from Y719F/Y719F mutant mice (**i**). Kit staining (**d,h**) was with Cy-3-conjugated secondary antibodies, **e,i**, DAPI stainings of the same sections. **j-k**, Sections of ovaries showing oocytes (closed arrowheads) and ruptured ovarian follicles (open arrowheads). **l-m**, sections of hair follicles in lumbar skin. Arrowheads indicate melanocytes. H & E indicates haematoxylin/eosin staining. Bars, 100  $\mu$ m.

Table 2 • Blood parameters

	Genotype			
	+/+ (M) (mean ± s.e., n=8)	Y719F/Y719F (M) (mean ± s.e., n=8)	+/+ (I) (mean, n=4)	Y719F/Y719F (I) (mean, n=4)
RBC (x10 <sup>6</sup> /μl)	10.0 ± 0.5	9.13 ± 1.0	9.8	9.6
hgb (g/dl)	15.1 ± 0.8	14.5 ± 1.3	15.5	15.6
Hct (%)	48.3 ± 2.8	45.6 ± 3.8	48.7	49
MCV (fl)	48.0 ± 2.3	45.6 ± 3.8	50.5	51.3
MCHC (g/dl)	31.3 ± 1.1	31.7 ± 0.8	31.8	31.8
RDW (%)	19.8 ± 1.8	19.5 ± 2.1	19.3	20.2
Plat (x10 <sup>3</sup> /μl)	1149 ± 286	952 ± 212	830	817
MPV (fl)	6.8 ± 0.4	6.8 ± 0.3	6.1	5.7
WBC (x10 <sup>3</sup> /μl)	7.0 ± 1.5	8.4 ± 2.2	8.9	9.3
Neu (%N)	18.3 ± 4.9	24.7 ± 4.7	17	28
Lymph (%L)	78.7 ± 6.2	73.3 ± 4.5	68.5	82.5
Mono (%M)	2.5 ± 1.9	1.2 ± 1.0	4	0.5
Eos (%E)	0.5 ± 0.5	1.6 ± 1.6	0	0.5
Baso (%B)	N.D.	N.D.	N.D.	N.D.

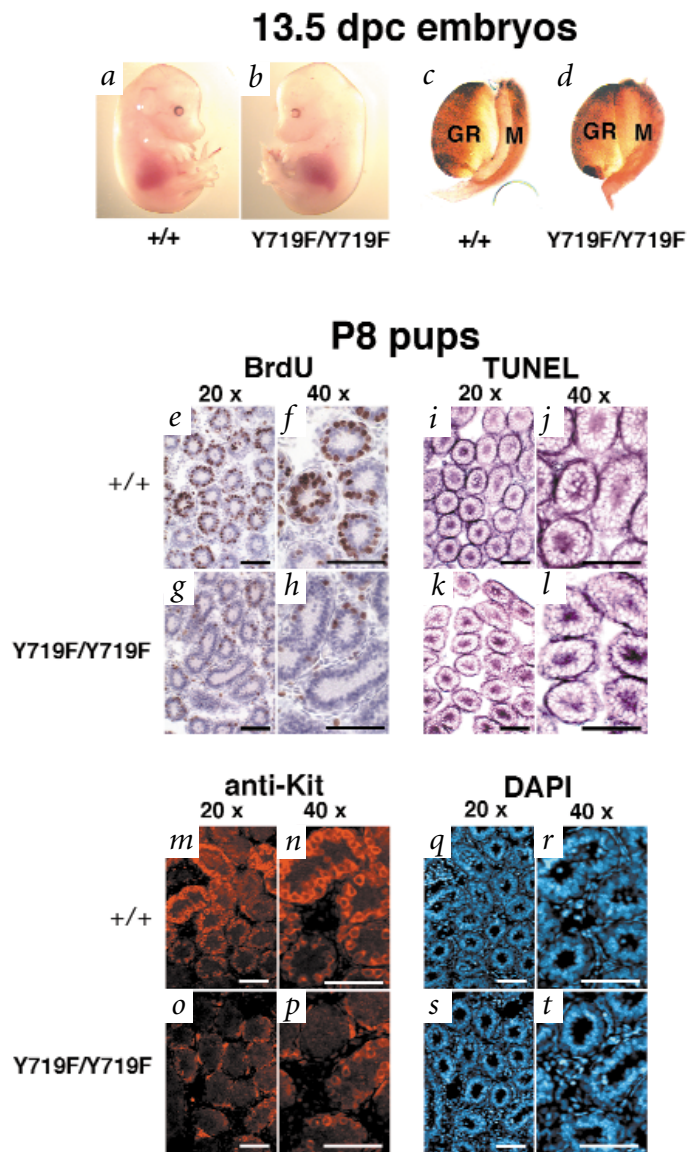
Peripheral blood obtained by retro-orbital bleedings was subjected to automated complete blood cell counts, platelet counts and differential counts as well as manual differential counts on blood smears. M, mixed background mice, B6D2F1/SV129; I, inbred strain mice, SV129; mean ± s.e. (sample standard error,  $\sigma_{n-1}$ , where n=8) for M; mean (n=4) for I; RBC, red blood cells; Hgb, haemoglobin; Hct, haematocrit; MCV, mean red cell volume; MCHC, mean cell haemoglobin concentration; RDW, red cell distribution width; plat, platelets; MPV, mean platelet volume; WBC, white blood cells; Neu, neutrophils; Lymph, lymphocytes; Mono, monocytes; Eos, eosinophils; Baso, basophils; N.D., not detected.

closest the lumen (Fig. 2b–c). Kit/SCF-R was strongly expressed in the single layer of outer spermatogonial cells, and in secondary spermatocytes, spermatids and spermatozoa, but it was absent in primary spermatocytes and Sertoli cells (Fig. 2d–e). Moreover, Kit was expressed in Leydig cells in the stromal interstitial tissue of +/+ mice, as previously reported<sup>13</sup>. In contrast, tubules of Y719F/Y719F mice contained only two distinct cell types, Sertoli cells and spermatogonial stem cells, in a single outer cell layer. Cellular debris filled the tubular lumina (Fig. 2f–g). No Kit-expressing cells were found in the seminiferous tubules of Y719F/Y719F mice, despite intense Kit staining of the testicular interstitium (Fig. 2h–i). Based on their proximity to the basement membrane, morphology, nuclear size and absence of Kit staining, it is possible that the spermatogonial cells remaining in testes of the adult mutant animals are undifferentiated stem cells, in accordance with two recent reports<sup>14,15</sup>. These results would suggest that the testicular hypoplasia and infertility of Y719F/Y719F males are caused by an early spermatogonial stem cell differentiation block<sup>13,16</sup>. Although Kit/SCF-R has been implicated in oogenesis and is expressed on oocytes<sup>17</sup>, ovaries of Y719F/Y719F females were of normal size and contained normal number and size of oocytes and ruptured follicles, consistent with the intact fertility of female mutant mice (Fig. 2j–k).

As Kit/SCF-R is also required for melanogenesis and haematopoiesis<sup>1–3</sup>, we next examined pigmentation and blood parameters in the mutant mice. Y719F/Y719F mice had normal skin and retinal pigmentation, and melanocytes were present at normal numbers in the base of hair follicles (Fig. 2l–m). All haematological parameters examined, including white cell and differential counts, were similar in littermate Y719F/Y719F and +/+ mice (Table 2) and similar to those from *loxP/loxP* control mice (data not shown). In particular, haemoglobin (Hgb), mean cell volume (MCV) and mean cell haemoglobin concentration (MCHC) were all normal in the mutant mice. This excludes macrocytic anaemia, one aspect of the mouse *W* and human piebald phenotypes, which both are due to naturally occurring loss-of-function mutations in Kit/SCF-R. Moreover, we did not detect an increased number of immature precursors or blast cells in smears from bone marrow or peripheral blood (data not shown). Finally, an extensive expression analysis by flow cytometry of the cell surface differentiation markers CD3e, CD4, CD5, CD8a, CD24 (HSA), CD25 (IL2-R $\alpha$ ), CD44, CD45 (B220), Gr-1, Mac-1 and CD117 (c-Kit) was performed on cells from thymus,

spleen and bone marrow of five-week-old wild-type and mutant animals. The number of cells expressing each marker and the expression mean of marker-positive cells were virtually identical in wild-type and mutant cells; in particular, Kit expression levels were equivalent in Y719F/Y719F mice (data not shown). These data indicate that SCF-induced PI 3'-kinase signalling is not essential for melanogenesis or to sustain normal haematopoiesis of non-challenged mice.

To exclude a major pre-natal defect(s) in development and migration of primordial germ cells, we examined isolated genital ridges from Y719F/Y719F and +/+ littermate embryos at 13.5 days post coitus (dpc). Primordial germ cells (PGC) start entering the genital ridges at 11.5 dpc and are easily detectable by alkaline phosphatase staining until approximately 14.5 dpc, when the definitive gonads start developing<sup>18</sup>. The developing gonads of Y719F/Y719F and +/+ embryos were of similar size, and alkaline phosphatase-stained whole mounts showed that PGC were present not only in wild-type but also mutant animals (Fig. 3a–d). Although more careful quantitation of PGC differentiation and migration awaits future studies, these results together with those from adult testes suggest that mutant males have a major defect in spermatogonial stem cell differentiation. To characterize this further we examined testes from post-natal day 8 (P8) and P10 pups. Spermatogonial cells start expressing Kit/SCF-R at around P7 (refs 13,14). Only spermatogonial and Sertoli cells are present in P8 pups, whereas preleptotene spermatocytes start appearing in testes at P10 (ref. 19). At P8 the organization, morphology and cell types of seminiferous tubules from mutant mice were indistinguishable from those of wild type (Fig. 3e–t, and data not shown). However, *in vivo* BrdU labelling of P8 pups showed that germ-cell proliferation was decreased in Y719F/Y719F mice compared with +/+ litters (Fig. 3e–h). No apoptotic cells were observed in either +/+ or homozygous mutant mice at this stage (Fig. 3i–l). In mutant and wild-type testes the distribution of Kit-immunopositive cells in parallel sections was similar to that of BrdU-positive cells, consistent with previous reports that proliferating spermatogonial cells are Kit-positive<sup>13,14</sup> (compare Fig. 3m–p with Fig. 3e–h). Accordingly, fewer Kit-positive cells were observed in mutant seminiferous tubules (Fig. 3o,p) despite the presence of cells in all tubules (Fig. 3q–t). In P10 pups a reduction in germ-cell proliferation was observed (Fig. 4a–d). At this stage the tubules from Y719F/Y719F mice still contained only spermatogonial and Sertoli cells organized in two outer cell layers,



**Fig. 3** Primordial germ cells in genital ridges and cell proliferation, apoptosis, and Kit positive cells in parallel sections of testes from P8 pups. **a–d**, Embryos at 13.5 dpc were genotyped and their genital ridges dissected out. PGC were visualized by alkaline phosphatase staining of whole mounts of male genital ridges. **e–h**, BrdU labelling *in vivo* shows proliferating cells (brown) and hematoxylin counter stain (blue) in testes sections. **i–l**, TUNEL *in situ* assay on parallel sections visualized with nitroblue-tetrazolium/X-phosphate reveals no apoptotic cells at this stage. **m–p**, Kit-positive cells (red) observed in the outer cell layer within the seminiferous tubules and scattered in the interstitium. Fewer Kit-positive cells are seen in the mutant testis. **q–t**, DAPI staining of the same sections as in **m–p**.

the floxed *neo* marker from the intron we tried to minimize the risk of transcriptional interference and/or hypomorphic alleles, and the results from our analysis of haematopoietic cells indicate that we achieved this goal. To our knowledge this is also the first report of a single defined point mutation in any gene resulting in a complete early male germ-cell differentiation block this early as the only defect. The low number of apoptotic germ cells preclude analysis of signalling directly in these cells, but our data clearly suggest that PI 3'-kinase is essential for SCF-induced spermatogenesis, at least in part, through regulation of proliferation and cell survival. It has been shown that blocking antibodies to Kit/SCF-R inhibit spermatogonial development<sup>16</sup> and that SCF together with LIF can support primordial germ cell survival *in vitro*<sup>22–24</sup>. These data and our findings here suggest that the spermatogenic defect is cell-autonomous, but this issue remains to be addressed directly. Kit/SCF-R is encoded by the mouse dominant white-spotting (*W*) locus, for which a number of naturally occurring mutations have been described. Haematopoiesis and melanogenesis are most often affected, whereas in mice heterozygous for the *W* mutation, decreased fertility is only rarely observed<sup>1,2</sup>. Based on this fact, and the central role of PI 3'-kinase in regulating migration, survival and proliferation of numerous cell types<sup>25–27</sup>, it is surprising that haematopoiesis and melanogenesis are intact in the Y719F/Y719F mutant mice. It is also unclear why only male, but not female fertility is affected by compromising Kit/SCF-R-induced PI 3'-kinase signalling. The loss-of-

function *W* mutations, however, are a different type of mutation than Y719F, as they negatively affect all signalling pathways from the receptor<sup>1–3</sup>. It is possible that the residual 10% SCF-induced PI 3'-kinase activation in mutant mice suffices to sustain haematopoiesis and melanogenesis, but not spermatogenesis, because a higher threshold level is required in rapidly dividing germ cells. Nevertheless, the observed tissue- and gender-specific phenotype is highly unexpected. A specific inhibitor of PI 3'-kinase binding to Kit/SCF-R in adults or adolescents might prove clinically useful, for instance as a male contraceptive, or to preserve fertility by inhibiting germ-cell proliferation during cancer chemotherapy<sup>15</sup>.

whereas the tubules from +/+ mice were organized in 3–4 cell layers containing primary spermatocytes as well (Fig. 4, and data not shown). Extensive apoptosis was observed in some tubules from Y719F/Y719F mice (Fig. 4e–h). Although the outer layer of germ cells in +/+ mice was Kit-positive (Fig. 4i–j), most tubules in P10 mutant pups did not express Kit/SCF-R (Fig. 4k–l), despite the presence of one or two outer cell layer(s) (Fig. 4o,p). The cells in these are morphologically indistinguishable from the Sertoli cells and more undifferentiated, Kit-negative spermatogonia seen in adult mutants. Our results suggest that testicular development is largely normal until P8 in mutant mice, as the testes are normally organized and contain the different cell types, including germ cells, at roughly normal numbers. However, only a few Kit-positive spermatogonial cells are generated in mutant testes at this stage. Eventually these undergo apoptosis, leaving only Kit-negative, more undifferentiated spermatogonial stem cells and Sertoli cells behind. These two cell types might persist in the mutant testes throughout life (Fig. 2f–i; refs 14,15).

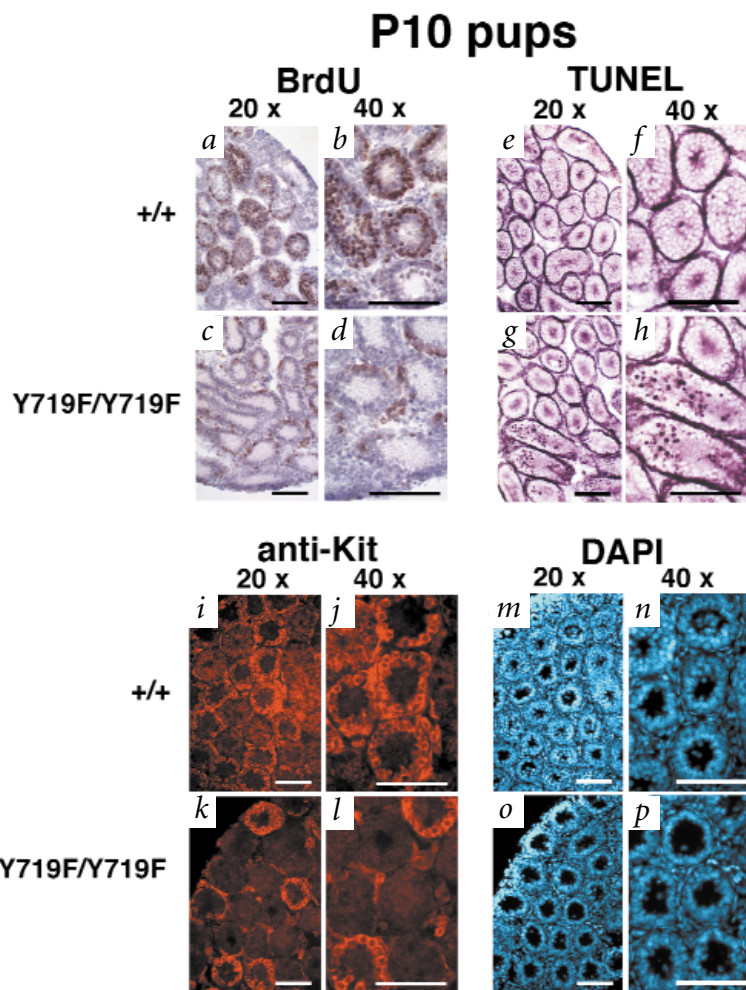
Two other reports have attempted to analyse the role of individual phosphorylation site(s) in a receptor protein tyrosine kinase (RPTK) *in vivo*<sup>20,21</sup>. In this study, we introduced a phosphorylation-site point mutation in the germ line of mice making only minimal changes to the RPTK-encoding gene. By excising

function *W* mutations, however, are a different type of mutation than Y719F, as they negatively affect all signalling pathways from the receptor<sup>1–3</sup>. It is possible that the residual 10% SCF-induced PI 3'-kinase activation in mutant mice suffices to sustain haematopoiesis and melanogenesis, but not spermatogenesis, because a higher threshold level is required in rapidly dividing germ cells. Nevertheless, the observed tissue- and gender-specific phenotype is highly unexpected. A specific inhibitor of PI 3'-kinase binding to Kit/SCF-R in adults or adolescents might prove clinically useful, for instance as a male contraceptive, or to preserve fertility by inhibiting germ-cell proliferation during cancer chemotherapy<sup>15</sup>.

## Methods

**Gene targeting, generation of *Prm*-Cre embryonic stem cells and Y719F-Kit/SCF-R homozygous mutant mice.** A13.6-kb *Bam*HI/*Bam*HI genomic *Kit* fragment covering exon 15 was cloned from a 129/Sv mouse genomic library. To create a targeting construct, a 1.2-kb *loxP*-flanked *neo* cassette from pOG277 (ref. 9) was cloned into the intron 606 bp upstream of exon 15 (Fig. 1a). A 1.5-kb *Xba*I fragment covering exons 15 and 16 was subjected to site-directed mutagenesis changing the codon for Y719 to F and simultaneously introducing a diagnostic *Eco*RI site: GAATATATG → GAATTCATG. The mutated fragment was blunted and, after complete sequence verification, used to replace the wild-type 1.5-kb fragment

**Fig. 4** Cell proliferation, apoptosis, and Kit-positive cells in parallel sections of testes from P10 pups. **a–d**, BrdU labelling *in vivo* shows proliferating cells (brown) and haematoxylin counter stain (blue). **e–h**, TUNEL *in situ* assay on parallel sections visualized with nitroblue-tetrazolium/X-phosphate shows dark apoptotic cells in the mutant testis (**g–h**) at this stage. **i–l**, Kit-positive cells (red) observed in the outer cell layer of tubules and scattered in the interstitium. Fewer Kit-positive cells are seen in the mutant testis. **m–p**, DAPI staining of the same sections as in **i–l**.



(Fig. 1a). A unique *NotI* site 3' of the homologous sequence was used to linearize the targeting construct before electroporation into 129/SvJae *Prm-Cre* ES cells<sup>9</sup>. Transfected *Prm-Cre* ES cell clones were selected in G418, and resistant colonies screened by Southern blotting. Internal and external probes verified correct targeting and absence of concatamers in several clones that contained the Y719F mutation. A cross-over event between the *neo* cassette and the Y719F mutation was identified in one clone, which consequently had wild-type sequence as well as Y719. This ES cell clone was introduced into the germ line of mice and served as a *loxP*-containing control. Three Y719F/+ and the control ES cell clones were injected into C57BL/6J blastocysts. Five chimaeras derived from a Y719F/+ ES cell clone and two from the control ES clone, respectively, transmitted through the germ line. They were crossed with B6D2F1/J mice and inbred with 129/Sv+*P+<sup>tyr-c+</sup>Mgf-SII*/J mice, and the resulting mice were intercrossed to obtain homozygous mutant mice of both mixed (B6D2F1x129/Sv) or inbred (129/Sv) background.

**Genotyping.** Southern blot and PCR on genomic DNA was performed on both ES cell and mouse DNA. To pre-screen for homologous recombination in the ES cells, genomic DNA from 192 G418-resistant ES cell clones was digested with *XbaI*. Southern blot with the 3' external probe A gave a targeting frequency of 21%. Genomic DNA samples from 9 of the positive clones were then probed with the internal probes *neo*, B and C on different combinations of *XbaI*, *XbaI-BamHI*, and *Sall* digests to exclude concatamerization, inversions and uneven cross-over. Genomic DNA from tail biopsies of resulting mice was extracted using 'Gitschier lysis buffer'<sup>28</sup>. The *neo* gene was present in all the chimaeras, but efficiently excised in all offspring, in accordance with the Cre recombinase being expressed in the haploid germ cells of male chimaeras. Excision of the *neo* gene and presence of the Y719F-Kit mutation was further verified by PCR on genomic DNA using primers 54 and 59 surrounding the *loxP* sites, and primers 42 and 53 surrounding the mutated exon 15. The size difference between +/+ and Y719F/Y719F was 87 bp, as expected for one *loxP* site with the surrounding *XbaI* linkers. The middle PCR band seen in DNA from Y719F/+ is due to formation of a heteroduplex between wild type and mutant PCR product, as shown by re-running the purified band after denaturation as well as by sequencing. For subsequent generations of mice, routine genotyping was done by PCR using primers 54 and 59.

**Isolation of primary bone marrow-derived mast cells, immunoprecipitation and Western blotting.** Bone marrow cells were collected by flushing the marrow cavity of femurs, and mast cells were derived by selective growth for 6 weeks in IL-3-containing medium (Opti-Mem I; Gibco BRL, 10% fetal bovine serum, 0.5 ng/ml recombinant murine IL-3 R & D Systems). Medium was replaced daily and cells transferred to new dishes to remove adherent cells, including macrophages and megakaryocytes. Immunoprecipitation, western blot and Akt *in vitro* kinase assays were done as described<sup>6,29</sup>, using extracts from  $9 \times 10^6$  mast cells per lane. Briefly, cells were starved for 12 h in Opti-Mem I medium without IL-3 and containing only 0.5% serum, before stimulation with 100 ng/ml murine SCF (R & D Systems, Inc.) for 8 min at 37 °C, where indicated. The mouse monoclonal antibodies U5 and U10 recognize the p85-Bcr

domain. Kit was detected using an affinity-purified rabbit anti-serum against the C-terminus of human Kit, Kit-C1-affi<sup>29,30</sup>, which also recognizes rodent Kit, or with an affinity-purified goat anti-serum against the C terminus of mouse Kit, M-14 (Santa Cruz). The rabbit anti-serum against Akt recognizes the C terminus, and the monoclonal antibody 4G10 (UBI) was used to detect phosphotyrosine.

**Cell proliferation, apoptosis, immunofluorescence and alkaline phosphatase studies.** Eight and 10 day old pups derived from Y719F/Y719F (female) × Y719F/+ (male) crosses were injected intraperitoneally with 50 µg/g bodyweight of a 5 mg/ml aqueous solution of BrdU (Zymed Laboratories) and killed after 2 h. Testicles were dissected and tail biopsies taken for genotyping. After fixation in 4% paraformaldehyde, testes were cryosectioned, and parallel sections processed for either BrdU staining, TUNEL assay or immunofluorescence staining. BrdU incorporation was detected using a primary biotinylated mouse anti-BrdU monoclonal antibody, incubation with peroxidase-conjugated streptavidin, and development using diaminobenzidine (all from Zymed Laboratories). Cells were counter-stained with haematoxylin. TUNEL staining was performed using the *In situ* cell death detection kit, AP (Boehringer), using nitroblue tetrazolium chloride together with 5-bromo-4-chloro-3-indolyl phosphate as substrate for alkaline phosphatase. Immunofluorescence staining was performed using the Kit-C1-affi ab (0.3 µg/ml) as primary antibody, and Cy-3-conjugated goat-anti rabbit Ig as secondary. Specificity was confirmed using the M-14 anti-Kit antibody (Santa Cruz). Nuclei were counter-stained with DAPI. Perfusion fixation, paraffin embedding and sectioning, and haematoxylin-eosin staining was performed on adult testes, ovaries and skin lumps from the lumbar region, according to standard procedures. Genital ridges were isolated and stained as described<sup>18</sup>.

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