Complex phenotype of mice homozygous for a null mutation in the *Sp4* transcription factor gene

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Abstract

Background: **Sp4 is a zinc finger transcription factor which is closely related to Sp1 and Sp3. All three proteins recognize the same DNA elements and can act as transcriptional activators through glutamine-rich activation domains. Unlike Sp1 and Sp3, which are ubiquitous proteins, Sp4 is highly abundant in the central nervous system, but also detectable in many other tissues.**

Results: **We have disrupted the mouse *Sp4* gene by a targeted deletion of the exons encoding the N-terminal activation domains. *Sp4* knockout mice show a complete absence of *Sp4* expression. They develop until birth without obvious abnormalities. After birth, two-thirds die within 4 weeks. Surviving mice are growth retarded. Male $Sp4^{null}$ mice do not breed. The cause for the breeding defect remains obscure since they show complete spermatogenesis. In addition, pheromone receptor genes in the vomeronasal organ appear unaffected. Female $Sp4^{null}$ mice have a smaller thymus, spleen and uterus. In addition, they exhibit a pronounced delay in sexual maturation.**

Conclusions: **The phenotype of the $Sp4^{null}$ mice differs significantly from those described for $Sp1^{-/-}$ and $Sp3^{-/-}$ mice. Thus, the structural similarities, the common recognition motif and the overlapping expression pattern of these three transcription factors do not reflect similar physiological functions.**

Introduction

The Sp family of transcription factors is composed of five proteins (Sp1, Sp2, Sp3, Sp4 and Sp5) characterized by a highly conserved DNA-binding domain at the C-terminus (Harrison *et al.* 2000; Philipsen & Suske 1999; Suske 1999; Treichel *et al.* 2001). In addition, Sp1, Sp2, Sp3 and Sp4 show similarities in their glutamine-rich N-terminal region (Suske 1999). The linkage of these four *Sp* genes to the four human *Hox* gene clusters also documents their close evolutionary relationship (Kalff-Suske *et al.* 1995, 1996; Scohy *et al.* 1998). Sequence alignments revealed that Sp1, Sp3 and Sp4 are more closely related to each other than to Sp2 (Philipsen & Suske 1999; Suske 1999). Consistently, Sp1, Sp3 and Sp4 recognize the classical GC-box and the related GT/CACC-box with identical affinity (Hagen *et al.* 1992, 1994). Sp1 and Sp3 are ubiquitously expressed, contrary to Sp4, which shows a complex expression pattern but is most abundant in neuronal tissues (Hagen *et al.* 1992; Supp *et al.* 1996).

A large variety of biological functions have been assigned to *Sp* factor-binding sites. This raises the question of which of these functions are performed by which Sp protein *in vivo*. Gene ablation studies in mice have provided important clues to the answer to this question.

Sp1 targeted embryos are severely retarded in growth, and die after day 10 of embryonic development (E10). They display a wide range of abnormalities, but all characteristic hallmarks of this developmental stage are

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present. Blastocyst injections of Sp1-deficient embryonic stem cells showed that these cells contribute efficiently to early chimeric embryos, but after E11, this declines very rapidly with no detectable contribution to any tissue of newborn animals. Thus, Sp1 deficiency causes a cell-autonomous defect, and it appears that Sp1 function is generally required for cellular survival after E10 (Marin *et al.* 1997). Sp3-deficient embryos are growth retarded and invariably die at birth of respiratory failure. The cause for the observed breathing defect is not clear. Only minor morphological alterations were observed in the lung, and surfactant protein expression is indistinguishable from wild-type mice. In addition, $Sp3^{-/-}$ mice show a pronounced defect in late tooth formation. The development of the dentin/enamel layer is impaired due to a strongly reduced expression of ameloblast-specific gene products (Bouwman *et al.* 2000). A mutation of the *Sp4* gene has been reported previously (Supp *et al.* 1996). However, the mice in this study still expressed a truncated *Sp4* mRNA fragment encoding the two strong activation domains at a high level. Thus, the question remains whether the phenotype of these *Sp4* mutant mice reflects the physiological consequences of a complete *Sp4* knockout. $Sp5^{-/-}$ mice show no overt phenotype (Harrison *et al.* 2000).

Here we describe the targeted disruption of the mouse *Sp4* gene by deleting the exons that encode for the N-terminal activation domains. We found that the complete absence of Sp4 has severe consequences for postnatal mouse development. $Sp4^{null}$ mice develop until birth without obvious abnormalities. After birth, approximately two-thirds of the knockout mice die within 4 weeks. Those that survive are size retarded. Male $Sp4^{null}$ mice do not breed and have a slightly reduced testis size. However, they show complete spermatogenesis. In addition, the pheromone receptor genes in the vomeronasal organ that are essential for mating behaviour appear to be unaffected. Female $Sp4^{-/-}$ mice have a small thymus, spleen and uterus, and they reach puberty with a pronounced delay.

Results

Targeted disruption of the mouse *Sp4* gene

A targeting vector was designed to replace sequences that encode the N-terminal activation domains of Sp4 protein (amino acids 4–557). This was obtained by replacing exons 2 and 3 of the mouse *Sp4* gene (Song *et al.* 2001) by IRES-*LacZ*-polyA/PGK-*neo* sequences in the targeting vector (Fig. 1). The *lacZ* gene is expressed under control of the endogenous Sp4 promoter. The *phosphoglycerate kinase* (PGK) promoter controls the *neomycin resistance* (*neo*) gene and the *herpes simplex virus thymidine kinase* (*hsvtk*) gene to ensure expression in embryonic stem cells.

The targeting plasmid pPNT*Sp4*i/e/IRES-*LacZ* (Fig. 1B) was linearized at a unique *Not*I site present in the vector for transfection into E14 ES cells. Cells were subsequently maintained under G418 and gancyclovir selection. A total of 200 G418 resistant colonies were analysed by Southern blotting for the homologous recombination event. Hybridization of *Eco*RV restricted DNA from individual clones with a *Bgl*II-*Bam*HI intron fragment was predicted to show a > 10 kb fragment from the wild-type locus and a 6 kb fragment from a correctly targeted locus (Fig. 1B). One clone showed the predicted mutant fragment. In addition, PCR analysis with a set of primers specific for the wild-type and the targeted *Sp4* gene confirmed the expected targeted disruption of the mouse *Sp4* gene. Hybridization with a Bluescript vector probe showed no evidence for additional unwanted random integration events in this clone. The integrity of this clone was further confirmed by karyotyping. The targeted ES clone was injected into C57BL/6 blastocysts. Breeding of male chimeras resulted in germ-line transmission of the targeted *Sp4* allele.

Complete absence of Sp4

Matings of heterozygous $Sp4^{+/-}$ animals were set up to obtain embryos deficient in both wild-type *Sp4* alleles (Fig. 1C,D). To test whether this resulted in the complete loss of *Sp4* gene expression, we performed Northern blot analyses of RNA from brain and heart of both control and $Sp4^{-/-}$ embryos (Fig. 2A). Consistent with previous results, a probe coding for an N-terminal activation region of Sp4 detected two transcripts larger than 28S RNA in wild-type and $Sp4^{+/-}$ mice. These transcripts were undetectable in $Sp4^{-/-}$ mice (Fig. 2A, lanes 1–3). In addition, a probe encoding for the zinc finger region also shows no signal in $Sp4^{-/-}$ mice (Fig. 2A, lanes 10 and 11). The absence of the Sp4 protein in homozygous $Sp4^{-/-}$ mice was confirmed by Western blot analyses with an antiserum directed against the C-terminal domain of Sp4 (Fig. 2B). From these data we conclude that the *Sp4* knockout mice lack any residual *Sp4*-specific transcripts and protein.

Expression of Sp1 and Sp3 in Sp4 null mice

To analyse whether the expression of other members of the Sp-family of transcription factors is altered in

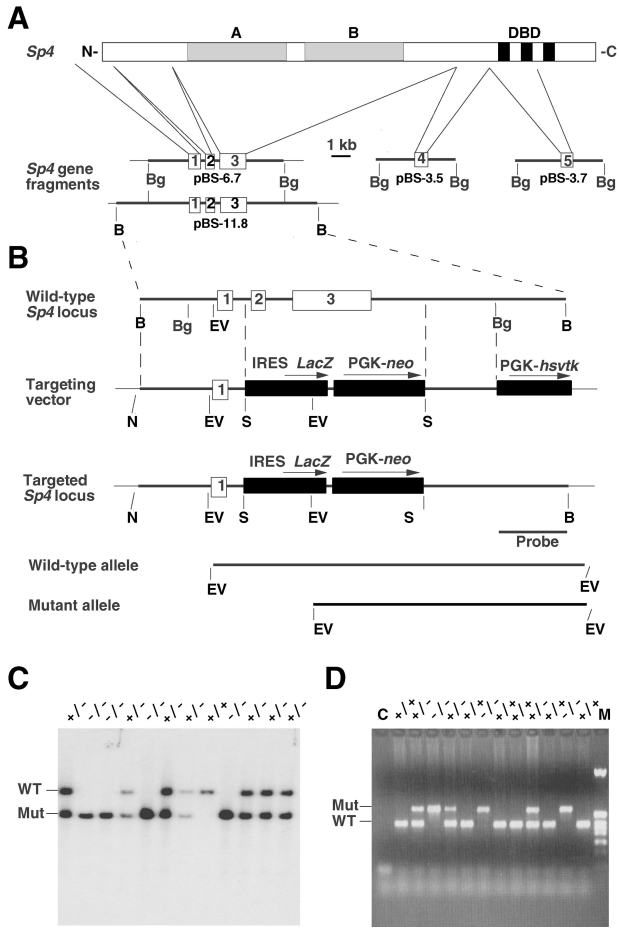

Figure 1 Targeted disruption of the mouse *Sp4* gene. (A) Schematic representation of the Sp4 protein structure and *Sp4* gene fragments. The glutamine-rich activation domains A and B and the zinc fingers (black bars) of the DNA binding domain (DBD) are indicated. Connecting lines with corresponding murine *Sp4* gene fragments indicate the derivation of individual Sp4 domains from different exons. Exons 1, 2 and 3 are present on a 6.7 kb *Bgl*II and an 11.8 kb *Bam*HI fragment. Exon 4 coding for 76 amino acids preceding the DNA-binding domain, and exon 5 coding for the first two zinc fingers are present on a 3.5-kb *Bgl*II and a 3.7-kb *Bgl*II fragment, respectively. (B) Schematic presentation of the knockout strategy. In the targeted *Sp4* locus, a cassette containing IRES-*lacZ* sequences and the *neomycin resistance* gene driven by the PGK promoter (PGK-*neo*) replaces exons 2 and 3. The targeting vector also contains a positive selection marker (PGK-*hsvtk*). Expected fragments of the wild-type and the mutant allele after restriction with *Eco*RV, and the probe used for Southern blotting are indicated. B, *Bam*HI; Bg, *Bgl*II; EV, *Eco*RV; S, *Sal*I and N, *Not*I. (C) Southern blot analysis of mouse embryos. Restriction of genomic DNA with *Eco*RV and hybridization with the probe indicated in Fig. 1B detected a $>$ 10 kb fragment of the wild-type allele (WT) and a 6 kb fragment of the mutated allele (Mut). (D) PCR analysis of mouse embryos. The primers described in Experimental procedures produced a 390 bp DNA fragment from the wild-type allele (WT) and an approximately 600 bp fragment from the targeted allele. +/+, wild-type; +/−, $Sp4^{+/-}$; −/−, $Sp4^{-/-}$; C, negative control; M, size marker.

$Sp4^{-/-}$ mice, we performed Northern analyses with *Sp1*- and *Sp3*-specific probes. These experiments revealed that the abundance of *Sp1* transcripts was unchanged (Fig. 2A, lanes 4–6 and 12–13) whereas *Sp3* transcripts were slightly (approximately twofold) enhanced in $Sp4^{-/-}$ mice (Fig. 2A, lanes 7–9). Thus, the absence of Sp4 might be partially compensated by enhanced transcription of the *Sp3* gene.

Embryonic expression of the $Sp4^{LacZ}$ allele in heterozygous $Sp4^{+/-}$ mice

Expression of *Sp4* mRNA during embryonic development was previously monitored by *in situ* hybridization (Supp *et al.* 1996). Using the $Sp4^{IRES-lacZ}$ allele driven by the *Sp4* promoter, we were able to recapitulate the endogenous expression pattern of the

Sp4 gene. In whole mounts of heterozygous E12.5 embryos, β-galactosidase activity was highest in the central nervous system but also detectable in many other tissues (Fig. 3).

Survival rates and growth of $Sp4^{-/-}$ mice

Heterozygous $Sp4^{+/-}$ mice exhibited no discernible phenotype and were able to breed. Genotyping of embryos obtained by Caesarean section shortly before the parturition date (E18.5) showed no loss of $Sp4^{-/-}$ mice up to birth (Table 1). $Sp4^{-/-}$ newborn mice showed no obvious abnormalities at birth. However, approximately half of them died within 10 days of birth (Table 1). An additional 30% died within the following 3 weeks. Their cause of death remains undetermined. A comparison of growth rates (Fig. 4) revealed that within the first 4 weeks the gain of weight of the $Sp4^{-/-}$ mice was almost arrested. After this period their growth appeared relatively normal. However, the body weight of the surviving $Sp4^{-/-}$ mice never reached that of their wild-type littermates (Fig. 4).

Table 1 Genotype distribution of *Sp4* heterozygous crossings

	Total	+/+	+ −	−/−
E18.5	22	4 (18.2%)	11 (50.0%)	7 (31.8%)
Day 10	149	50 (33.6%)	79 (53.0%)	20 (13.4%)

The genotype was determined by PCR analysis as described in Experimental procedures.

We determined standard blood parameters (red cells (haematocrit, cell volume, haemoglobin per cell), white cells, platelets) of the $Sp4^{-/-}$ mice. These fell within normal ranges. Furthermore, we determined alkaline phosphatase, aspartate aminotransferase, albumin, creatinin, lactate dehydrogenase 1, bilirubin, and urea levels in the circulation. Of these, aspartate aminotransferase and lactate dehydrogenease 1 were slightly elevated (20% increase) in the knockout mice, but this difference was not significant. Thus, the circulation is apparently normal and these data provide no clues to the physiological problems underlying the growth retardation of $Sp4^{null}$ mice.

Male $Sp4^{-/-}$ mice do not breed

Breeding of $Sp4^{-/-}$ females with wild-type male mice

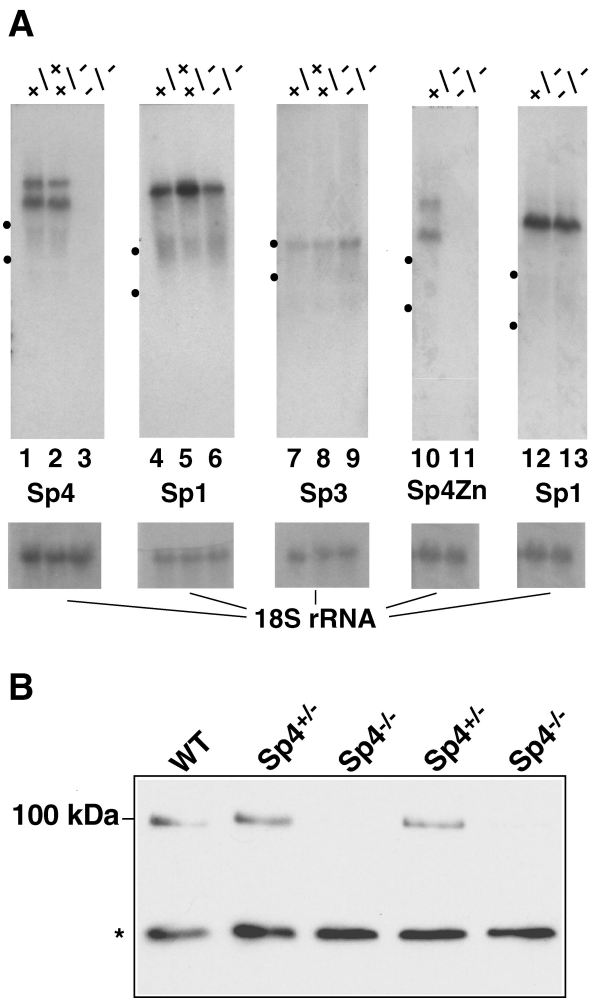

Figure 2 Complete absence of Sp4 in $Sp4^{null}$ mice. (A) Northern analyses of *Sp1*, *Sp3* and *Sp4* transcripts. RNA was extracted from heart (lanes 1–9) and brain (lanes 10–13) of wild-type (+/+), $Sp4^{+/-}$ (+/−) and $Sp4^{-/-}$ (−/−) E18.5 embryos, subjected to electrophoresis through 0.8% formaldehyde/agarose gels, and transferred to nylon membranes. The filters were hybridized with DNA-fragments encoding *Sp4* (lanes 1–3 and 10–11), *Sp1* (lanes 4–6, and 12–13) and *Sp3* (lanes 7–9). Two different *Sp4*-specific probes encoding the glutamine-rich activation domain (lanes 1–3), and the DNA-binding domain (lanes 10–11) were used to detect *Sp4* mRNA. The dots indicate the migration of 28S and 18S ribosomal RNA. As a control, the filters were probed with an 18S ribosomal RNA specific oligonucleotide. (B) Western blot analysis. Nuclear extracts (50 μg of protein) from brains of adult female wild-type (WT), heterozygous ($Sp4^{+/-}$) and *Sp4*-deficient ($Sp4^{-/-}$) mice were fractionated through 7.5% SDS–polyacrylamide gels, blotted on nitrocellulose filter and incubated with an Sp4-specific antiserum. Sp4 migrates as a 100 kDa protein. The asterisks depict an unspecific cross-reacting protein.

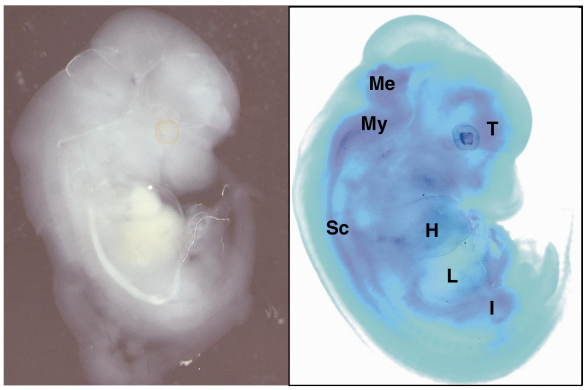

Figure 3 *LacZ* expression in heterozygous $Sp4^{+/-}$ embryos. Lateral view of E12.5 WT (left) and $Sp4^{+/-}$ (right) embryos stained for β-galactosidase activity. H, heart; L, liver; I, intestinal tract; Me, metencephalon; My, myelencephalon; T, telencephalon; Sc, spinal cord.

was sporadically successful. However, all attempts to breed adult $Sp4^{-/-}$ males with female wild-type mice were unsuccessful. This prompted us to examine whether a failure of spermatogenesis might be responsible for the observed infertility of $Sp4^{-/-}$ males. Histological analyses of testicular and epididymal cross-sections revealed complete spermatogenesis in the seminiferous epithelium (Fig. 5). In addition, luminal spermatozoa in the epididymis of $Sp4^{-/-}$ males were indistinguishable in numbers and shape from those in wild-type litter mates.

The highest expression of *Sp4* is found in the central nervous system (Hagen *et al.* 1992). Thus, Sp4 might exert its essential functions primarily in the brain. Wild-type females that were mated with $Sp4^{-/-}$ males did not contain copulation plugs, indicating an altered

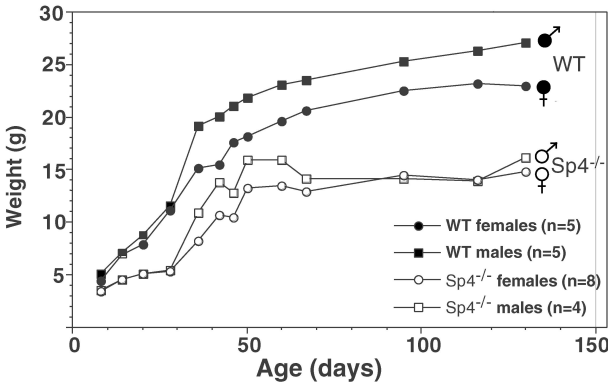

Figure 4 Growth curve (body weight vs. age) of male and female wild-type (WT) and $Sp4^{-/-}$ mice.

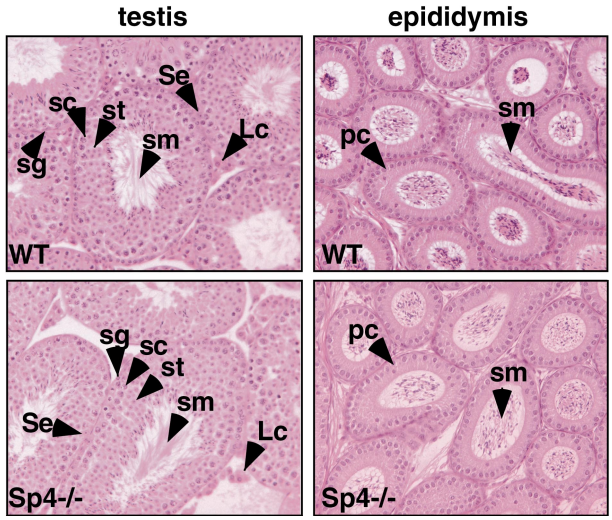

Figure 5 Histological analyses of testis (left) and epididymis (right). Haematoxylin staining revealed complete spermatogenesis in $Sp4^{-/-}$ mice. Abbreviations are: Se, Sertoli cells; Lc, Leydig cells; sg; spermatogonia; sc, primary spermatocytes; st, spermatides; sm; spermatozoa; pc, principle cell. Original magnification: × 200.

reproductive behaviour of $Sp4^{-/-}$ males. Since the hypothalamus and the vomeronasal organ (VNO) play important roles in reproductive behaviour (Buck 1995), we examined the VNO and the hypothalamus in sections, but no gross abnormalities were found in these tissues (data not shown).

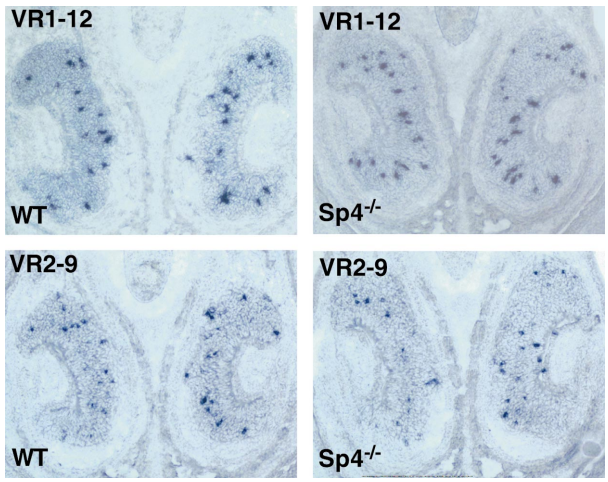

Figure 6 Analysis of VR expression by *in situ* hybridization. Cross-sections of vomero nasal organs dissected from neonatal WT and $Sp4^{-/-}$ mice were hybridized with Digoxigenin-labelled anti-sense RNA probes for the VNO receptor genes VR-12 and VR2-9.

Sexual behavioural alterations may reflect an altered gene expression pattern in specific neuronal cells of the VNO. VNO neurones express pheromone receptors of different classes (Belluscio *et al.* 1999; Herrada & Dulac 1997; Matsunami & Buck 1997). Therefore, we asked whether the expression of a subset of pheromone receptors might be altered in $Sp4^{-/-}$ mice. We analysed the expression of two different receptor genes in the VNO of $Sp4^{-/-}$ neonatal mice by *in situ* hybridization (VR1-12 from the apical zone and VR2-9 from the basal zone). The expression of both genes was unaffected in $Sp4^{-/-}$ mice (Fig. 6). Thus, we consider it unlikely that the function of the VNO is impaired in $Sp4^{-/-}$ mice.

The onset of puberty is impaired in female $Sp4^{-/-}$ mice

We determined the organ/body weight ratio of individual organs and found a significantly smaller thymus, spleen, uterus and testis in $Sp4^{-/-}$ mice compared to control mice ($P < 0.05$) (Fig. 7A). Other organs like the heart or kidney had a normal organ/body weight ratio. A reduced uterus size was also observed in heterozygous $Sp4^{+/-}$ females (Fig. 7B). The very small uterus in $Sp4^{-/-}$ females prompted us to examine the onset of puberty by determining vaginal opening time (VOT). We found that $Sp4^{-/-}$ females have a pronounced delay in vaginal opening time (VOT $>$ 200 days) compared with wild-type and heterozygous $Sp4^{+/-}$ mice (VOT $<$ 40 days). The delayed puberty is in agreement with the small uteri of $Sp4^{-/-}$ females, which is characteristic for prepubertal immaturity.

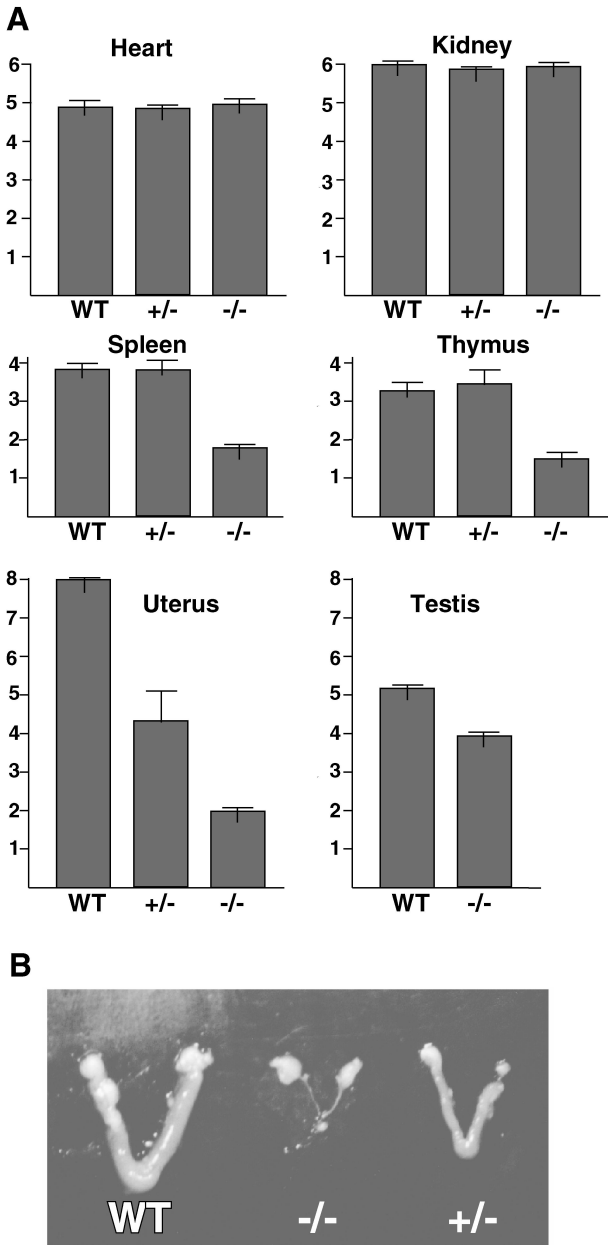

Figure 7 Organ sizes in $Sp4^{null}$ mice. (A) Relative weight of various organs. The values represent the ratio of organ mass (in mg) per body mass (in grams). (B) Uteri of 62-day-old female wild-type (WT), $Sp4^{+/-}$ (+/–) and $Sp4^{-/-}$ (–/–) littermates.

Discussion

Phenotype of $Sp4^{null}$ mice

Our results demonstrate that the transcription factor Sp4 is important for the early postnatal survival of mice, since two-thirds of the newborn die within 4 weeks post-partum. The molecular cause for the observed death, however, remains unclear.

Sp4-deficient mice that survive are impaired in their reproduction. Adult $Sp4^{-/-}$ did not breed at all and the mating of $Sp4^{-/-}$ females was only sporadically successful. The reduced testis and uterus weights of *Sp4-/–* mice indicated that Sp4 plays an important role for the function of these organs. A direct action of Sp4 in these organs seems plausible, since *Sp4* mRNA is expressed in testis and uterus (unpublished data). However, all attempts to detect a morphological alteration of the testis failed.

One could nevertheless speculate that the smaller reproductive organs are linked to the maturation of sexual functions, as reflected by the delay in the onset of puberty of $Sp4^{-/-}$ females. It is noteworthy that these animals also have a smaller thymus. The reduced

thymus size and the delay in the onset of puberty may be physiologically linked. It is known that the thymus is involved in female sexual maturation, since neonatal thymectomized mice also show a significant delay in the onset of puberty (Besedovsky & Sorkin 1974).

Sp4^{null} mice vs. *Sp4* mutant mice

A targeted mutation of the *Sp4* gene has been reported previously (Supp *et al.* 1996). Two exons coding for the three zinc fingers of the Sp4 DNA-binding domain were replaced by a targeting vector resulting in a 19 kb deletion of mouse genomic DNA. Such a large deletion, however, raises the possibility that the expression of an additional gene was disturbed. In addition, the *Sp4* gene was not completely inactivated. A truncated *Sp4* mRNA fragment encoding the two strong activation domains was still expressed at a high level (Supp *et al.* 1996). The expression of the activation domains of Sp4, however, might gain new functions or might interfere with other Sp-family members. It might act as a superactivator by interacting with glutamine-rich activation domains of other transcription factors. Indeed, such a mechanism has been demonstrated for the N-termini of Sp1 and Sp4. Both truncated proteins strongly enhance the transcriptional activity of Sp1 and Sp4 (Pascal & Tjian 1991; Hagen *et al.* 1995, and unpublished data). Therefore, it seems likely that the activation domains of Sp4 expressed *in vivo* also interfere with Sp1 or other transcription factors.

The *Sp4* knockout mice described here do not express domains of Sp4 that might interfere with other transcription factors. Neither the activation domains nor the DNA-binding domain were expressed. Nevertheless, the *Sp4*^{null} mice exhibit at least partial similarities with *Sp4* mutant mice lacking only the DNA-binding domain. Both the *Sp4*^{null} and the *Sp4* mutant strains have a high mortality rate after birth and males do not breed. Body weight/organ ratios, or the onset of the puberty were not reported for the *Sp4* mutant mice lacking only the DNA-binding domain. However, the extremely late onset of puberty is a phenotypic manifestation that would hardly have been missed by propagating this mouse strain. Thus, it seems likely that not all characteristic hallmarks of a *Sp4*^{null} mutation are detectable in mice lacking only the Sp4 DNA-binding domain.

Function of Sp-family members *in vivo*

Sp4 is closely related to the two ubiquitously expressed transcription factors Sp1 and Sp3. All three proteins recognize the same DNA elements and can act as transcriptional activators through glutamine-rich activation domains (Dennig *et al.* 1996; Hagen *et al.* 1995, 1994). The essential physiological functions, however, appear to be significantly different. In contrast to *Sp4*^{null} mice, *Sp1* mutant embryos are already severely retarded at early embryonic stages and die around day 10 of gestation (Marin *et al.* 1997). *Sp3*$^{-/-}$ mice develop until birth but die a few minutes post-partum due to respiratory failure (Bouwman *et al.* 2000). Thus, the obvious structural similarity, the common DNA recognition motif and the overlapping expression pattern do not reflect similar physiological functions. Nevertheless, there might be many overlapping functions *in vivo*. Since *Sp3* mRNA is up-regulated in *Sp4*^{null} mice, one could suggest that it might compensate at least partially for the loss of *Sp4* in *Sp4*^{null} mice. To unravel precisely the degree of redundancy between the individual Sp transcription factors, it might be necessary to generate mice deficient for two or all three GC-box binding Sp factors.

Experimental procedures

Cloning and mapping of the mouse *Sp4* gene

The cloning of the mouse *Sp4* gene has recently been described (Song *et al.* 2001). A 11.8 kb *Bam*HI fragment and three *Bgl*II fragments of 6.7 kb, 3.7 kb and 3.5 kb in length (Fig. 1A) that hybridized with the human *Sp4* cDNA were subcloned into the *Bam*HI site of the pBluescript KS vector leading to the plasmids pBS-11.5 (11.5 kb *Bam*HI fragment), pBS-6.7 (6.7 kb *Bgl*II fragment), pBS-3.7 (3.7 kb *Bgl*II fragment) and pBS-3.5 (3.5 kb *Bgl*II fragment). Subsequently the plasmids clones were mapped by Southern blotting and partially sequenced.

Generation of the *Sp4* homologous recombination construct

As a starting plasmid we chose the 7.2 kb pPNT vector (Tybulewicz *et al.* 1991) that contains PGK*neo* and PGK*hsvtk* cassettes separated and flanked by a number of unique cloning sites. We first introduced a part of the third intron of the *Sp4* gene into the multiple cloning site of the pPNT vector that separates the PGK*neo* and the PGK*hsvtk* cassettes. The 1.8 kb *Sp4* intron fragment was obtained from plasmid pBS-6.7 (Fig. 1A) as a [*Ecl*136II]/*Xba*I-*Eco*RI fragment and cloned into *Xba*I-*Eco*RI restricted pPNT plasmid leading to pPNT*Sp4*i. 5′-flanking sequences and the first exon of the *Sp4* gene were obtained from the plasmid pBS-11.5 (Fig. 1A) as a 4 kb *Not*I-[*Alw*441]/*Sal*I-*Xho*I fragment and cloned into the *Not*I and *Xho*I (adjacent sites flanking the PGK*neo* cassette) restricted pPNT*Sp4*i plasmid. The resulting 13 kb plasmid was named

pPNT*Sp4*i/e. In a final step we introduced an IRES-*LacZ* cassette obtained as a 5.7 kb *Sal*I fragment from the plasmid pGT1.8IRESβ*gal* into the *Sal*I site of pPNT*Sp4*i/e leading to the knockout construct pPNT*Sp4*i/e/IRES-*LacZ*. The plasmid pGT1.8IRESβ*gal* is a derivative of pGT1.8IRESβ*geo* (Mountford *et al.* 1994) lacking the *neomycin resistance* gene. It was constructed by cloning a 3.3-kb [*Bam*HI]/*Xba*I-*Xba*I fragment of pGT1.8IRESβ*geo* back into the backbone of *Xba*I restricted pGT1.8IRESβ*geo*.

Transfection and selection of ES cells

E14 ES cells were electroporated with 15 μg of *Not*I-linearized targeting vector pPNT*Sp4*i/e/IRES-*LacZ*. Clones were selected with G418 (200 μg/mL) and gancyclovir. Homologous recombination was analysed by Southern blotting of *Eco*RV-restricted genomic DNA with the probe indicated in Fig. 1. Unwanted random integrations were detected by hybridizing the blots with a Bluescript vector-specific probe.

Generation of chimeric and Sp4-deficient mice

The $Sp4^{+/-}$ ES cell clone was karyotyped and microinjected in C57BL/6 host blastocysts. Chimeric males were mated to C57BL/6 females, and germ-line transmission was obtained. The F1 offspring were interbred to expand the stocks and to obtain $Sp4^{null}$ mice.

Genotyping of mice by Southern blotting and PCR

DNA was prepared from tail snips and analysed for the presence of wild-type and targeted *Sp4* alleles by Southern blotting or PCR. Southern blots were performed under standard conditions using the 1.8 kb *Bgl*II-*Bam*HI fragment of the third *Sp4* intron as a probe. For PCR analyses, three primers were used, a sense primer in the *Sp4* gene amplifying the wild-type allele (5′-CCAGTAACAATCACTAGTGTTGCA-3′), a sense primer in the *neomycin resistance* gene amplifying the targeted allele (5′-CATCGCCTTCTATCGCCTTCTTGA-3′) and an anti-sense primer in the *Sp4* gene (5′-CTCACAACCATATACCAATGC AAG-3′). PCR conditions were 94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min for 30 cycles.

Northern blot analyses

Total RNA of mouse organs was extracted by the guadinium/isothiocyanate procedure using the Qiagen kit. RNA was separated through 0.8% agarose gels containing 2.2 M formaldehyde and blotted on to nylon membranes. Prehybridization and hybridization was carried out as described (Braun & Suske 1998). Gene-specific probes were obtained from appropriate plasmids or primer sets as follows. *Sp4*: 420 bp PCR-fragment amplified with A8-3 and A8-6 primers on plasmid pBS-6.7 (see above). *Sp4*Zn encoding the *Sp4* zinc finger region: 340 bp PCR fragment (nucleotides 2074–2414 of the murine Sp4 cDNA) obtained by RT-PCR using mouse RNA as template. *Sp1*: 311-bp fragment containing nucleotides 1371–1682 of the rat Sp1 cDNA (Imataka *et al.* 1992) obtained by RT-PCR using mouse RNA as template. *Sp3*: 800 bp *Pst*I fragment obtained from plasmid pBS4.6*Hin*dIII (Bouwman *et al.* 2000). The hybridization reaction with the Sp4 zinc finger encoding region as a probe contained 1 μg each of plasmids encoding *Sp1 and Sp3* to avoid cross-hybridization of the *Sp4* zinc finger probe with *Sp1 and Sp3* transcripts.

Western blotting

Nuclear extracts (50 μg of protein) were prepared from adult female brains according to Gorski *et al.* (1986), separated on 7.5% SDS-polyacrylamide gels, blotted on nylon membranes, and probed with a rabbit anti-*Sp4* serum (Santa Cruz sc-645). Primary antibodies were visualized using the Amersham ECL kit.

Whole mount LacZ staining

E12.5 embryos were dissected from uteri and fixed in 4% paraformaldehyde for 30–45 min and subsequently rinsed three times with LacZ rinse-buffer (0.1 M phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 0.01% sodium desoxycholate, 0.02% Nonidet P-40) for 30 min. LacZ staining was performed with 1 mg/mL of X-gal solution in LacZ rinse-buffer supplemented with 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide over night at 37 °C. Embryos were post-fixed in 95% ethanol over night and stored in ethanol/glycerol (1 : 1).

Organ weights

Animals were sacrificed by cervical dislocation. Organs were collected and immediately frozen on dry ice prior to weight determination.

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