Targeted disruption of the synovial sarcoma-associated SS18 gene causes early embryonic lethality and affects PPARBP expression

D.R.H. de Bruijn1, W.J.M. Peters1, S.M. Chuva de Sousa Lopes3, A.H.A. van Dijk1, M.P. Willems1, R. Pfundt1, P. de Boer2 and A. Geurts van Kessel1,*

1Department of Human Genetics and 2Department of Obstetrics and Gynaecology, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands and 3Wellcome Trust/Cancer Research UK Gurdon Institute of Cancer and Developmental Biology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, UK

Received June 16, 2006; Revised and Accepted August 13, 2006

The synovial sarcoma-associated protein SS18 (also known as SYT or SSXT) is thought to act as a transcriptional co-activator. This activity appears to be mediated through the SWI/SNF proteins BRG1 and INI1 and the histone acetyl transferase p300. Here, we report that disruption of the mouse Ss18 gene results in a recessive embryonic lethal phenotype, due to placental failure caused by impairment of placental vascularization and/or chorio-allantoic fusion. This phenotype resembles the p300 knockout phenotype, but is distinct from the Brg1 and Ini1 knockout phenotypes. Through expression profiling of knockout embryos, we observed altered expression of genes known to affect placental development, including the peroxisome proliferator-activated receptor-binding protein (Pparbp). Since Pparbp null mutant embryos display a similar, lethal phenotype with placental failure, we suggest that the functional and phenotypic co-linearities between Ss18 and p300 may also include the transcriptional co-activator Pparbp. Additional interbreeding of Ss18 and Ss18l1 (Crest) mutant mice indicates that these two functionally and structurally related genes may act synergistically during critical stages of embryonic development.

INTRODUCTION

Synovial sarcoma is an aggressive soft tissue tumor predominantly affecting children and young adults. Cytogenetically, these tumors are characterized by a recurring chromosomal translocation, t(X;18)(p11; q11), which is found in over 95% of all cases (1,2). As a result of this translocation, the SS18 gene on chromosome 18 is fused to either one of three closely related SSX genes on the X chromosome, SSX1, SSX2 or SSX4 (3–6).

The SS18 gene is well conserved during evolution (7,8) and displays a high degree of homology to the SS18-like 1 gene (SS18L1, also known as Crest) (8,9). Using RNA in situ hybridization, we previously observed ubiquitous SS18 expression in the embryo proper at early stages of mouse embryonic development (embryonic day 12.5, E12.5), whereas in later stages (E14.5; E18.5), Ss18 expression was confined to mainly cartilagineous, neuronal and epithelial derived tissues (7). Additional expressed sequence tag (EST) analysis (using the unigene EST profile viewer) revealed Ss18 expression at appreciable levels in pre-implantation (E0–E6.5), gastrulation (E6.5–E7.9) and mid-gestation (E8–E12) embryos. A similar EST analysis, as well as our previous RNA in situ hybridization analysis (7), revealed ubiquitous Ss18 expression in adult mouse tissues. The nearest Ss18 homolog, Ss18l1, displays a more restricted expression pattern, most markedly in brain (9) (our unpublished data).

The SS18 protein is thought to act as a transcriptional co-activator and is located in nuclear speckles, distinct from known nuclear domains such as coiled bodies, splicing factor speckles or PML oncogenic domains (10–12). The SS18 and SS18L1 proteins contain two functional domains, an SS18 N-terminal homology (SNH) domain and a glutamine, proline, glycine and tyrosine rich QPGY domain (12,13). The SNH domain interacts directly with the acute leukemia-associated transcription factor AF10 (13), the
SWI/SNF ATPases BRM and BRG1 (12,14), the histone acetyl-transferase p300 (15) and the co-repressor mSin3A (16). Mediated by the AF10 protein, Ss18 associates with yet another SWI/SNF protein, IN11 (17,18) and the histone methyltransferase hDOT1L (19). Given the high degree of homology between the SNH domains of Ss18 and Ss18L1 (8), similar protein–protein interactions may also relate to Ss18L1. Support for this latter notion comes from the observation that Ss18L1 associates with p300 and the p300 homolog CBP (9). The Ss18 QPGY domain, which encompasses the C-terminal 205 amino acids, can act as a transcriptional co-activation domain and is able to initiate multimerization of the Ss18 protein (11,12,14). Structurally, the Ss18 QPGY domain is related to two other SWI/SNF proteins, i.e. ARID1A (BAF250a/SMARCF1) and ARID1B (ELD/OSA1/BAF250b) (18). Since the Ss18 protein lacks obvious DNA-binding domains, the QPGY-mediated transcriptional co-activator functions may act through chromatin modification. As such, the above described SNH domain-mediated protein–protein interactions may serve to tether Ss18 to its cognate DNA (chromatin) targets.

On the basis of Ss18 expression patterns observed and the multitude of Ss18-mediated protein–protein interactions found, we raised the question what the in vivo phenotypic consequences of anomalous Ss18 expression might be. Such an analysis may shed light on the Ss18-related genetic networks that govern the processes of normal and abnormal (cancer) development. Previously, severe phenotypes were observed in Ini1 and Brg1 mutant mice, including perinatal growth retardation (E3.5–E5.5) lethality of null mutant embryos, enhanced tumor formation in heterozygous knockout animals and growth arrest of fibroblasts derived from homozygous knockout embryos (20,21). A less severe phenotype was observed in p300 knockout mice, including early gestational lethality (E9.5–E10.5) lethality of 50% of the p300 heterozygous and all of the p300 homozygous knockout mice (22). Interestingly, a similar early gestational lethality was observed in compound p300/Cbp heterozygous embryos, indicating that, during embryonic development, the mouse is sensitive to p300/Cbp gene dosage effects (22). No predisposition to tumor formation was observed in p300 heterozygous knockout animals (22). Ss18I1 null mutant mice displayed an even less severe phenotype, with postnatal lethality after day 12 resulting from central nervous system defects (9). This latter observation is in full agreement with the brain-specific Ss18I1 expression.

Here, we report that a functional knockout of the mouse Ss18 gene results in a recessive embryonic lethal phenotype, which resembles the p300 knockout phenotype and is attributed to placental failure. Through expression profiling, we identified a set of genes, including the Pparbp gene, whose expression was significantly altered in the Ss18 mutant embryos. Since Pparbp null mutants also display an embryonic lethal phenotype, including placental failure (23), we suggest that the observed functional and phenotypic co-linearities between Ss18 and p300 may also include the transcriptional co-activator Pparbp. Additional interbreeding of Ss18 and Ss18I1 mutant mice indicated that these two highly related genes may act synergistically during critical stages of embryonic development.

RESULTS AND DISCUSSION

Ss18 null mutation results in early gestational lethality

In order to elucidate the role of the Ss18 gene in normal and abnormal (tumor) development, we have used a classical replacement strategy to generate a null mutation in the mouse Ss18 gene (Fig. 1). Ss18 heterozygous F1 mice were born in Mendelian ratios, with a mean litter size of 6.5. These mice were phenotypically normal and did not display enhanced tumor formation (data not shown). After intercrossing the Ss18 heterozygous mice, a cohort of 219 live-born progeny (62 litters) was obtained, which was genotyped at weaning. This cohort included Ss18 wild-type (Ss18 wt; 42.9%) and Ss18 heterozygous (Ss18 hs; 57.1%) mice, but no Ss18 homozygous knockout (Ss18 ko; 0.0%) mice (Table 1), suggesting that the Ss18 knockout mutation leads to a recessive embryonic lethal phenotype. The Ss18 heterozygous offspring was significantly under-represented in this cohort ($P = 0.02$). A similar under-representation has been reported for p300 heterozygous mice (22). In addition, we observed that the mean litter size in this cohort (3.5) (Table 1) deviates considerably from the mean litter size observed in the F1 generation (6.5) (discussed earlier). Since this difference cannot be attributed entirely to the loss of Ss18 knockout embryos in utero, we set out to assess litter sizes prenatally. In a cohort of 83 embryos (E9.5–E11.5), derived from Ss18 heterozygous intercrosses, we observed a mean litter size of 8.3 (Table 1), indicating that the overall embryonic loss is 58%. Combined with the significant under-representation of Ss18 heterozygous mice at weaning (discussed earlier), we conclude that this number includes all Ss18 knockout mice and approximately half of the Ss18 heterozygous mice.

After genotyping the 83 embryos mentioned above, we found all three Ss18 genotypes to be present in Mendelian ratios (Table 1). A macroscopic analysis of the E9.5 embryos revealed that 90% of the Ss18 knockout embryos exhibited growth retardation. The extent of this growth retardation varied from rudimentary embryos within the, apparently intact, embryonic membranes (33%) to embryos that were lagging behind and, by somite count, were estimated to be at stage E8.5 (67%). A similar growth retardation was observed in 43% of the Ss18 heterozygous embryos, thereby providing an explanation for the observed under-representation of these mice at weaning (discussed earlier). A morphological survey of stage E7.5 ($n = 15$), E8.5 ($n = 10$), E11.5 ($n = 12$) and E13.5 ($n = 16$) knockout embryos revealed no overt growth retardation at early stages (E7.5 and E8.5), whereas at later stages, some (E11.5) or all (E13.5) of the growth-retarded embryos displayed features of active resorption, indicating that the Ss18 knockout-induced embryonic lethality is initiated after stage E8.5, but before E9.5.

In order to assess the potential influence of genetic background on the observed phenotype, the Ss18 targeted mice with a mixed genetic background were crossed back for six generations into a C57BL/6 genetic background. Subsequently, heterozygous offspring was again intercrossed and the phenotypes of E9.5 embryos were evaluated. At this stage, we found that a majority of the knockout embryos
already displayed signs of resorption (data not shown), indicating that the onset of embryonic lethality was earlier when compared with embryos with a mixed genetic background (discussed earlier). Again, growth retardation was also observed in a subset of the heterozygous embryos. Together, these results indicate that the genetic background does indeed affect the level of penetration of the Ss18 knockout phenotype, but not the phenotype itself.

Ss18 knockout embryos show placental failure

Given the ubiquitous nature of Ss18 gene expression during early embryonic development and the Ss18 knockout-induced embryonic lethality before E9.5, we set out to perform a detailed histological analysis of conceptuses at E5.5 (n = 9), E7.5 (n = 15) and E9.5 (n = 16). Completely in line with the above macroscopic analysis, this microscopic analysis failed to reveal any significant differences between the Ss18 wild-type, heterozygous and homozygous knockout embryos at E5.5 and E7.5 (data not shown). At E9.5, all wild-type embryos displayed normal histologies without any signs of growth retardation (Fig. 2A). Heterozygous embryos were less well developed and were in the process of embryonic turning and neural tube closure (Fig. 2B, arrows). All knockout embryos were heavily retarded, displaying kinked partially closed neural tubes and little or no signs of embryonic turning, indicating that a developmental arrest had occurred just after gastrulation (Fig. 2C and D, arrows). This phenotype is reminiscent of that observed by others in p300 knockout embryos (22). Approximately 50% of the Ss18 knockout embryos were hemorrhagic with dilated blood vessels in the head region and with a swollen heart, indicative of oxygen shortage. At this stage of development (E9.5), a tight association of fetal and maternal blood vessels in the newly forming labyrinth layer of the developing placenta is essential for an efficient exchange of gas and nutrients between the embryonic and maternal circulations.

Normal placental development is initiated by the fusion of the chorionic and allantoic membranes (chorio-allantoic fusion) at E8.0–E8.5 (24), thereby forming the chorionic plate, which is composed of trophoblast cells. Through a subsequent process of vascular branching, fetal and maternal blood vessels enter this layer at E9.0, inducing the development of the placental labyrinth layer, which is made up of syncytiotrophoblasts. Upon closer examination of the E9.5 Ss18 knockout placentas, defects became apparent. The chorionic plate of all knockout placentas were composed of densely packed trophoblasts and did not exhibit vascularization (Fig. 2G and H). In contrast, the chorionic trophoblasts in

---

**Table 1. Overview of Ss18 heterozygous intercrosses, their litter sizes and genotypes**

<table>
<thead>
<tr>
<th>Age</th>
<th>Number of progeny</th>
<th>Litter size</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ss18 wt</td>
</tr>
<tr>
<td>Ss18 hz ♀ × Ss18 hz ♂</td>
<td>weaning</td>
<td>219</td>
<td>3.5</td>
</tr>
<tr>
<td>Ss18 hz ♀ × Ss18 hz ♂</td>
<td>E9.5–E11.5</td>
<td>83</td>
<td>8.3</td>
</tr>
</tbody>
</table>

**Figure 1.** Targeted disruption of the Ss18 gene in mice. Top: schematic representation of the Ss18 wild-type locus (WT), with the first three Ss18 exons (shown as open blocks) and all BglII (Bg) and SstI (S) sites. The targeting vector is shown with the restriction sites used to clone the 5' and 3' homology arms, the Neomycin resistance cassette (Neo') and the HSV-TK gene. Middle: the targeted Ss18 allele with the incorporated Neomycin cassette. The location of the probes (solid gray boxes) and the diagnostic restriction fragments before and after homologous recombination are shown above the wild-type and knockout alleles. Bottom: Southern blot analysis of DNA from ES cells digested with SstI (left) and BglII (right), hybridized with the 5' and 3' probe, respectively. The bands corresponding to the wild-type allele (wt) and the targeted allele (ko) are indicated. The ES clones used to generate Ss18 mutant mice are indicated (5E9 and 2F12).
the E9.5 placentas of wild-type and heterozygous littermates had already started to participate in the formation of the labyrinth layer, together with the allantoic mesenchyme (Fig. 2E and F). Overall, the number of fetal blood vessels (identified by the nucleated embryonic blood cells) in the prospective labyrinth was strongly reduced in SS18 knockout placentas when compared with normal placentas (Fig. 2I–L). Fetal blood vessels in the knockouts were either located outside the placenta or exclusively near the base where the surrounding trophoblast tissue was very compact, and never in close proximity to the maternal sinuses (Fig. 2K and L, arrows). In contrast, the close association of maternal and fetal blood vessels could readily be observed in normally developing placentas (Fig. 2I and J, arrows). On the basis of these observations, we conclude that the process of vascular branching is impaired in the SS18 knockout embryos. This SS18 knockout phenotype closely resembles that of Gcm1 and Dlx3 mutant mice (25,26). In addition, we observed the formation of a so-called ‘allantoic ball’ in a subset of SS18 knockout embryos (data not shown). This structure is indicative of impaired chorio-allantoic fusion and is similar to what has previously been observed in embryos carrying null mutations of the Wnt7b or Integrin-α4 genes (27,28).

Taken together, our data indicate that placental development is impaired in all SS18 knockout embryos, either through incomplete chorio-allantoic fusion or through a defect in the subsequent process of vascular branching morphogenesis. These developmental anomalies result in a lack of oxygen and/or nutrients in the SS18 knockout embryos, followed by developmental arrest and, ultimately, embryonic death (between E8.5 and E9.5). Subsequently, at E11.5, the embryonic membranes collapse, thereby initiating the process of resorption.

**SS18 knockout embryos show disturbed expression of placental lineage-specific genes**

On the basis of our observation that the embryonic lethal SS18 knockout phenotype is due to placental failure, we set out to analyze the expression of pre-selected embryonic and placental lineage-specific genes. To this end, we employed nine E9.5 embryos (including the embryonic membranes), three of each SS18 genotype, from two different litters and with different degrees of developmental delay. Using semi-quantitative RT–PCR, we analyzed the expression of the trophoblast giant cell specific placental lactogen 1 gene (PL1, Csh1) and the distal-less homeobox 3 gene (Dlx3), which is expressed in the chorionic plate and labyrinth (29). By doing so, we observed a clear correlation between elevated Csh1 expression and more extensive growth retardation, both in the knockout

---

**Figure 2.** Histological analysis of E9.5 wild-type (A, E and I), heterozygous (B, F and J) and knockout (C, D, G, H, K and L) embryos. The wild-type embryo (A) and placenta (E and I) are developed normally. The heterozygous embryo is in the process of embryonic turning and neural tube closure (B, arrows) and displays normal placental development (F and J) with a well-vascularized chorionic plate (J, doubleheaded arrow) and maternal and embryonic blood vessels in close proximity (J, arrows). Both knockout embryos are smaller in size (C and D) with partially closed neural tubes and little or no signs of embryonic turning (C and D, arrows). The chorionic plate of the knockout embryos (K and L, doubleheaded arrow) has formed but displays a lack of vascularization, and the fetal blood vessels are not found in close proximity to maternal blood vessels (K and L, arrows).
and in the heterozygous embryos (Fig. 3). An entirely opposite expression pattern was observed for the Dlx3 gene, which was found to be expressed at the lowest levels in the most extremely retarded embryos (Fig. 3). The latter is in full agreement with our observation that labyrinth development is disturbed in Ss18 knockout placentas. Together, these results demonstrate that a high Csh1/Dlx3 expression ratio is indicative for a more retarded phenotype in the Ss18 mutant embryos (Fig. 3). Furthermore, we observed a decreased expression of the early brain and limb bud-specific Wnt7a gene (30,31) in all knockout embryos tested (Fig. 3), which is in full agreement with the impaired neural tube development and the extensive growth delay observed in the previous morphological and histological analyses.

Microarray-mediated identification of Ss18 downstream target genes

Since the Ss18 protein is thought to act as a transcriptional co-activator, we set out to identify additional genes displaying transcriptional deregulation in the knockout embryos. Therefore, we performed expression profiling on 8K oligonucleotide microarrays, using linear amplified RNA (aRNA) from E9.5 embryos, including the embryonic membranes. For these experiments, three embryos were selected: one Ss18 null mutant and two wild-type littermates (Fig. 3, arrowheads). In accordance with its relatively mild phenotype, the knockout embryo exhibited a low Csh1/Dlx3 ratio but a clear Wnt7a expression (Fig. 3). The wild-type embryos were not growth retarded. In a total of three hybridizations, each aRNA was labeled with Cy3 and Cy5 and hybridized to the arrays. Since the mouse 8K oligonucleotide set was printed in duplicate on the microarray, 3093 met the inclusion criteria (see Materials and Methods). Normalized 2log expression ratios of the genes corresponding to these probes were used for a two-class significance analysis of microarrays (SAM) method (ko/wt versus wt/wt), and 283 genes were identified that displayed SAM derived q-values of less than 5%. From this list, all genes were selected that displayed mean ko/wt ratios outside the pre-set threshold levels (see Materials and Methods) and that differed significantly from the mean wt/wt ratios. By doing so, a list of 22 genes was generated which included the Ss18 and Csh1 genes (Table 2). As expected, the Ss18 gene exhibited significant (over 8-fold) down-regulation in the Ss18 knockout embryo, whereas the Csh1 gene exhibited a slight up-regulation in the Ss18 knockout embryo, which is in support of our previous RT–PCR data (Fig. 3). Interestingly, we found that four additional genes (Fzd2, Ywhaz, Slc1a7 and Pparbp) exhibited significant down-regulation in the knockout embryo. In order to confirm and extend these latter observations, we performed realtime quantitative RT–PCR expression analyses of these putative Ss18-target genes in an expanded set of 15 E9.5 embryos. (not shown). By doing so, we confirmed the microarray results for all four genes and found that the Pparbp and Ywhaz genes displayed down-regulation in all knockout embryos tested (Fig. 3), indicating that these genes may actually represent Ss18 downstream target genes. The peroxisome proliferator-activated receptor-binding protein gene (Pparbp, also known as PBP or TRAP220) is of particular interest. The Pparbp protein contains two LXXLL motifs, which are necessary for its binding to nuclear receptors, like the thyroid hormone receptor (32), the peroxisome proliferator-activated receptor-gamma (33) and others (34). In addition, several independent knockout models for the Pparbp gene exhibited embryonic lethality with heart failure, impaired neuronal development, extensive apoptosis, angiogenic defects, impaired PPAR-gamma mediated lipogenesis and, interestingly, placental failure (23,36–38). We conclude that several placental lineage-specific genes are aberrantly expressed in the Ss18 knockout embryos and that concomitant down-regulation of Pparbp expression may contribute to the embryonic lethal phenotype observed.

Ss18–Ss18l1 compound mutants display embryonic and perinatal lethality

Previously, we reported that the Ss18l1 gene exhibits a high degree of homology to the Ss18 gene (8). In addition, it was found that, like Ss18, the Ss18l1 gene can fuse to SSX in human synovial sarcoma (39). On the basis of these observations, we questioned whether the Ss18 knockout phenotype that we observed can be modulated by Ss18l1, or vice versa. We obtained Ss18l1 (Crest) heterozygous mice (9) in a mixed C57Bl6/129 background from Professor A. Ghosh (University of California at San Diego, USA) and intercrossed these with Ss18 heterozygous mice (also of mixed background). The offspring generated by these crosses included compound Ss18 heterozygous/Ss18l1 heterozygous mice in the normal Mendelian ratios (data not shown). Subsequently,
these compound Ss18 heterozygous/Ss18I1 heterozygous mice were bred with Ss18 wild-type/Ss18I1 heterozygous mice. At birth, the offspring of these crosses should contain all three Ss18I1 genotypes (Ss18I1-wt: 25%, Ss18I1-hz: 50%, Ss18I1-ko: 25%), combined with either a wild-type or a Ss18I1 heterozygous genotype. In a cohort of 14 litters (comprising 112 pups), we observed a significantly reduced number of Ss18I1 knockout mice (5% versus the expected 25%). Postnatally, these mice displayed the expected phenotype, i.e. growth retardation and subsequent death after day 12 (9). To our surprise, all these Ss18I1 knockout mice contained two wild-type Ss18I1 alleles. Since we were able to identify Ss18I1 heterozygous/Ss18I1 knockout embryos in E9.5 and E18.5 litters of similar crosses, we assume that these latter compound mice die perinatally. Taken together, we conclude that haplo-insufficiency of the Ss18I1 gene aggravates the Ss18I1 knockout phenotype and, thus, that the Ss18I1 and Ss18I1 genes may act synergistically during critical stages of early embryonic development.

In conclusion, we show that the Ss18I1 protein is essential for early placental development. The observed Ss18 knockout phenotype is reminiscent of the p300 knockout phenotype, but appears to be less severe than the Inil and Brg1I knockout phenotypes. Notably, the early gestational lethality of all homozygous knockout mice, combined with the partial lethality among heterozygous embryos, the strain-induced differences in penetration of the phenotypes and the absence of tumor predisposition in heterozygous animals, highlights the co-linearity between the p300 and Ss18 knockout phenotypes.

We propose that this co-linearity may be extended to the Pparbp transcriptional co-activator which, like Ss18 and p300, exerts a knockout phenotype which includes embryonic lethality and placental failure. During critical stages of embryonic development, Pparbp may act as an Ss18I1 downstream target.

**MATERIALS AND METHODS**

**Cloning and sequencing procedures**

All cloning procedures were essentially as described before (7,8,13,40). Sequence analyses were performed at the DNA Sequencing Facility of the Radboud University Nijmegen Medical Centre. DNA and protein databases were searched using BLAST and/or BLAT search algorithms at the NCBI or UCSC, respectively (www.ncbi.nlm.nih.gov; genome.ucsc.edu).

**Construction of the targeting vector and homologous recombination**

The genomic Ss18 sequence of mouse strain 129/Sv was obtained previously (13). In the Ss18 targeting vector (Fig. 1) a 13 kb Ss18 genomic fragment, including exons 1–3, was replaced by a neomycin resistance cassette (40) embedded in 5' and 3' homology arms of 4.7 and 4.6 kb, respectively (Fig. 1). For negative selection, the herpes simplex virus thymidine kinase (HSV-TK) gene was added to the 5'-arm. Tissue culture of ES cells and conditions for electroporation of the targeting construct were as described previously (40).
Transfected ES cells were selected in G418 (300 μg/ml) and FIAU (1 μM), and picked after 10 days of growth. Identification of correct homologous recombination in the selected clones was performed by Southern blot analysis and hybridization with the 5‘ probe, which detected a 9.7 kb wild-type band and a 6.9 kb knockout band, respectively (Fig. 1). All clones displaying the correct 5‘ knockout band were expanded and subsequently screened with the 3‘ probe, which detected a 6.0 kb wild-type band and a 7.6 kb knockout band, respectively (Fig. 1). Next, all clones displaying correct 5‘ and 3‘ homologous recombination events were screened with a Neo probe to ensure that all contained a single integration of the targeting construct and, finally, karyotyped using standard procedures (data not shown).

**Generation and breeding of Ss18 knockout mice**

Blastocyst injections with two independently targeted ES cell lines (Fig. 1) were performed as described previously (40). Both ES cell lines (2F12 and 5E9) gave rise to overt chimeric males which transmitted the targeted allele to their offspring. F1 mice were generated by mating the chimeric males with C57Bl/6 females. No differences were observed between embryos derived from the two different ES cell lines. Homozygous mutant Ss18 embryos were generated by intercrossing Ss18 heterozygous mice of the F1 or F2 generation. The Ss18 genotype was determined by PCR performed on genomic DNA, extracted from toe or tail biopsies or, in the case of embryos, yolk sacs or homogenized embryos. Lack of Ss18 expression in the knockout embryos was confirmed by RT–PCR on E9.5 embryos (Fig. 3). Information about the primers used to detect the knockout and wild-type alleles is available upon request. Genotyping of the Ss18II knockout and wild-type alleles was performed as described (9).

**Embryo isolation, histology and RNA isolation**

Mouse conceptuses were dissected at different gestational stages (day 0.5 defined as noon of the day in which a vaginal plug was detected) and the actual embryo ages were estimated according to somite count. For histological examination, embryos were fixed in paraformaldehyde, embedded in paraffin, sectioned (7 μm) and stained with hematoxylin and eosin using routine procedures. For genotyping and RT–PCR, E9.5 embryos were homogenized and split into two portions. From one portion, total RNA was extracted using the RNAeasy micro-kit (Qiagen) according to the instructions of the manufacturer. From the other portion, DNA was isolated using the QIAmp micro kit (Qiagen) to be used for genotyping purposes.

**RNA amplification and microarray hybridization**

Approximately 500 ng of total RNA from selected E9.5 mouse embryos was amplified using the Amino Allyl MessageAmp kit (Ambion) according to the instructions of the manufacturer. Typically, this yielded 50 μg of amplified RNA, of which 2 μg was used per hybridization. Subsequently, the amino-allyl labeled aRNA samples were labeled with monoreactive Cy5 and Cy3 dyes (Amersham Biosciences), and unincorporated dyes were removed using Microcon YM-30 microcolumns (Millipore). Next, Cy-3 and Cy-5 labeled aRNAs were mixed and supplemented with 1.5 μl 10 mg/ml poly(dA) (Amersham) and 10 μg COT1 DNA (Life Technologies), after which the mixture was concentrated to 60 μl using Microcon microcolumns. Subsequently, this mixture was denatured (2 min, 98°C), supplemented with 60 μl 2× hybridization buffer (50% formamide, 5× SSC, 0.1% SDS) and incubated at 42°C before loading onto oligo-based microarrays and hybridization in a GeneTAC hybridization station (Genomic Solutions). On these microarrays, an 8K mouse oligonucleotide set (Compugen) was printed in duplicate at the Microarray Core Facility of the Radboud University Nijmegen Medical Centre. All hybridizations were performed at 42°C for 16–20 h. After hybridization, the slides were washed at 42°C in buffer 1 (1× SSC, 0.2% SDS) for 5 min, in buffer 2 (0.1× SSC, 0.2% SDS) for 4 min and in buffer 3 (0.1× SSC) for 5 min at room temperature. Finally, the slides were rinsed briefly with 0.01× SSC and dried by centrifugation for 10 min at 300g. The slides were scanned on an Affymetrix 428 scanner (Affymetrix) using software package version 1.0 (Affymetrix).

**Microarray analysis and normalization**

The acquired microarray images were analyzed with Genepix Pro 4.0 software (Axon instruments). DNA spots were automatically segmented, local backgrounds (B635 and B532) were subtracted and total Cy5 and Cy3 intensities (F635 and F532, respectively), as well as the median of pixel-by-pixel intensity ratios of the two dyes, were calculated for each spot. Spots were discarded if the signal intensities were below a priori set thresholds (F635median ≤ 1.4× B635 or F532median ≤ 1.4× B532), if they exhibited poor hybridization signals or if they were saturated (F635median = 65534 or F532 = 65534). The, 2log transformed, fluorescence ratios were normalized using LOESS curve fitting (41) and analyzed using a multiple t-testing SAM method (42). The threshold levels, indicating down- or up-regulation of a gene, were set at −0.6 and 0.6, respectively.

**ACKNOWLEDGEMENTS**

The authors thank Professor Dr Anirvan Ghosh of the Division of Biology, University of California, San Diego, La Jolla, CA 92039, USA, for providing the Ss18II (Crest) mutant mice, and Professor Dr Christine Mummery of the Hubrecht Laboratory, Utrecht, The Netherlands, for advice and support. We thank Walter van der Vliet for the mouse oligonucleotide microarrays and the Central Microarray Facilities of the Radboud University Nijmegen Medical Centre and the Dutch Cancer Institute for advice and support. We thank the staff of the Nijmegen animal facilities for expert technical assistance. This work was supported by grants from the Dutch Cancer Society (KWF) and the Fundacao para a Ciencia e Tecnologia (FCT, SFRH/BD/827/2000).

*Conflict of Interest statement*. None declared.
REFERENCES


39. Storlazzi, C.T., Mertens, F., Mandahl, N., Gisselsson, D., Isaksson, M.,
fusion gene, SS18L1/SSX1, in synovial sarcoma. *Genes Chromosomes

40. de Bruijn, D.R., Oerlemans, F., Hendriks, W., Baats, E., Ploemacher, R.,
Wieringa, B. and Geurts van Kessel, A. (1994) Normal development,
growth and reproduction in cellular retinoic acid binding protein-I

41. Workman, C., Jensen, L.J., Jarmer, H., Berka, R., Gautier, L.,
variability in DNA microarray experiments. *Genome Biol.*, 3,
research0048.

Sci. USA*, 98, 5116–5121.