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A *Sall4* Mutant Mouse Model Useful for Studying the Role of *Sall4* in Early Embryonic Development and Organogenesis

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Summary

SALL4 is a homologue of the *Drosophila* homeotic gene *spalt*, a zinc finger transcription factor, required for inner cell mass proliferation in early embryonic development. It also interacts with other transcription factors to control the development of the anorectal region, kidney, heart, limbs, and brain. Truncating mutations in *SALL4* cause Okihiro syndrome, manifest as Duane anomaly, radial ray defects and sensorineural and conductive deafness. We report the characterization of a novel murine *Sall4* null allele created by bacterial recombineering in ES cells. Homozygous mutant mice exhibit early embryonic lethality. Heterozygous mutant mice recapitulate phenotypic features of Okihiro syndrome including deafness, lower anogenital tract abnormalities, renal hypoplasia, anencephaly, Hirschprung's disease, and skeletal defects. This phenotype shows important differences in cardiac and ear manifestations to previously characterized *Sall4* mutant alleles and should prove useful for the investigation of the influence of modifier alleles and protein interactions on the transcriptional regulatory function of *Sall4*.

Keywords

bacterial recombineering; *Sall4*; Okihiro syndrome; mouse; organogenesis; embryonic stem cells

SALL4 is a member of the *SALL* gene family of zinc finger transcription factors (Kohlhase *et al.*, 1996), containing multiple C2H2 double zinc finger domains, homologous to the *Drosophila spalt (sal)* homeotic gene important in pattern formation and cell specification (de Celis *et al.*, 1996, 1999; Jurgens, 1988; Kuhnlein *et al.*, 1994). Murine *Sall4* (Kohlhase *et al.*, 2002a) is required for inner cell mass proliferation in early embryonic development (Sakaki-Yumoto *et al.*, 2006). It also interacts with other transcription factors, such as *Sall1* and the *Tbx* family of transcription factors to control the development of the anorectal region, kidney, heart, limbs and brain, by acting on downstream targets including *Fgf10* (Kiefer *et al.*, 2003; Koshiba-Takeuchi *et al.*, 2006; Sakaki-Yumoto *et al.*, 2006). Mutations in *SALL1* are associated with Townes-Brock syndrome (OMIM 107480) (Kohlhase, 2000; Powell and Michaelis, 1999) and mutations in *SALL4* cause Okihiro syndrome (OMIM 607323), an autosomal dominant human familial disorder, characterized by a triad of Duane

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anomaly, radial ray limb defects, and deafness (Okiihiro *et al.*, 1977). Less penetrant developmental anomalies includes atrial septal defect (ASD), renal hypoplasia, anal stenosis or imperforate anus, and Hirschprung's disease (Kohlhase *et al.*, 2005). Nonsense, truncating and missense mutations, plus chromosomal deletions, resulting in loss of one or all of the zinc finger domains of *SALL4* have been described in families with Okiihiro syndrome (Al-Baradie *et al.*, 2002; Borozdin *et al.*, 2004; Kohlhase *et al.*, 2002b; Miertus *et al.*, 2006). Although the syndrome is thought to arise from haploinsufficiency of *SALL4* it is still unclear why some truncations result in more severe phenotypes than others (Kohlhase *et al.*, 2005).

Using bacterial recombineering technology in ES cells (Liu *et al.*, 2003), we have generated mice with a targeted disruption of the gene. Homozygous mutant mice are embryonic lethal, while heterozygous mice display a range of developmental defects that recapitulate many of the features of Okiihiro syndrome. The phenotype in heterozygous mice is associated with a reduction of *Sall4* mRNA transcript, suggesting that it is due to haploinsufficiency of the *Sall4* gene. This phenotype shows important differences in cardiac and ear phenotypes to previously characterized *Sall4* mutant alleles (Koshiba-Takeuchi *et al.*, 2006; Sakaki-Yumoto *et al.*, 2006) and should prove useful for the investigation of the influence of modifier alleles and protein interactions on the transcriptional regulatory function of *Sall4*.

RESULTS

To inactivate *Sall4* we generated a targeting vector (*Sall4*-TV1) by bacterial recombineering which was designed to delete exons 2-4 (Fig. 1a). This deletion removes all zinc finger domains of the protein and is expected to result in a null allele. The *Sall4*-TV1 was used to target the locus in AB2.2 ES cells. Targeted clones were obtained at a rate of 15% (Fig. 1b). Two of these targeted clones were used to establish the *Sall4^{Brdm1}* allele in mice (Fig. 1c). PCR analysis of E3.5 blastocysts identified all three genotypes to be present (Fig. 1d). RT-PCR was performed on E8.5 embryos (Fig. 1e). A reduction of mRNA transcript was evident in *Sall4^{m1/+}* embryos compared with *Sall4^{+/+}* embryos. Whole mount analysis of *Sall4^{m1/+}* embryos at E10.5 revealed that *Sall4* mRNA was expressed with the expected tissue distribution, namely midbrain, branchial arch, and limb bud mesenchyme (Kohlhase *et al.*, 2002a; Sakaki-Yumoto *et al.*, 2006), however, the levels of *Sall4* mRNA were very low compared with wild type embryos (Fig. 1e,f).

Sall4^{Brdm1/+} (*Sall4^{m1/+}*) mice were intercrossed and the resultant litters were genotyped (Table 1). Homozygous mutant (*Sall4^{m1/m1}*) mice were not identified, thus the *Sall4^{Brdm1}* allele was homozygous lethal. The litter sizes from the *Sall4^{m1/+}* intercrosses were significantly lower than expected, because of the decreased numbers of heterozygous as well as homozygous mice (Table 1). To determine the time of death of the *Sall4^{m1/m1}* mice, timed matings were performed and litters were genotyped ($n = 11$, E8.5; $n = 4$, E9.5; $n = 4$, E10.5; $n = 9$, E12.5; $n = 14$, E14.5; $n = 54$, E15.5; $n = 8$, E17.5). *Sall4^{m1/m1}* embryos were not detected at any of the stages examined beyond E8.5, indicating that *Sall4^{m1/m1}* embryos die between E3.5 and E8.5. As well as *Sall4^{m1/m1}* embryonic lethality, a large number of heterozygous *Sall4^{m1/+}* pups did not survive to weaning (Table 2). The estimated percentage *Sall4^{m1/+}* pups that died before weaning varied from 16 to 55% depending on the type of cross and backcross generation (Table 2).

Phenotypic abnormalities were identified in surviving heterozygous embryos and adult mice. Exencephaly was observed in 5/121 (4%) of conceptuses between E14.5 and E18.5 (Fig. 2a). All of the exencephalic embryos were determined to be *Sall4^{m1/+}* by genotyping.

Histological analysis of 40 embryos and neonates (20 *Sall4*^{m1/+}; 20 *Sall4*^{+/+} littermate controls) between E15.5 and P4 revealed 7/20 (35%) heterozygous mice with unilateral renal agenesis; and 5/20 (25%) heterozygous mice with renal hypoplasia (Fig. 2b-e). No wild type mice were identified either with unilateral agenesis or hypoplasia of a kidney.

Nine percent (5/56) adult *Sall4*^{m1/+} mice exhibited defects of the lower anogenital tract, ranging from imperforate anus, anovaginal fistula formation, and persistent common cloaca (Fig. 2f), to Hirschsprung's disease due to absence of enteric neurons in the distal colon (Fig. 2g-l).

Adult *Sall4*^{m1/+} and *Sall4*^{+/+} littermate control mice were tested for impaired hearing by the Preyer reflex at weekly intervals upto 12 weeks of age (Table 3). Fifty percent (9/18) *Sall4*^{m1/+} mice showed an absent or weak Preyer reflex by 12 weeks of age, with a mean age of onset of 58 days. Significantly, only 5% (2/43) of the wild type control mice lost their Preyer reflex by 12 weeks of age ($P < 0.05$, Student's *t*-test). Middle ear inflammation was significantly increased in the *Sall4*^{m1/+} mutants (67%) compared with the control group (22%) ($P < 0.01$, Student's *t*-test) both macroscopically (Fig. 3a-d) and microscopically (Fig. 3e-h), suggesting that the progressive hearing loss in mutants may be, at least in part, conductive. The tympanic membrane ossicles, cochlea, and vestibular system all appeared normal (15 *Sall4*^{m1/+} and 18 *Sall4*^{+/+}). Scanning electron microscopy revealed no significant differences in the appearance of cochlear sensory hair cells of *Sall4*^{m1/+} compared with wild type mice (6 *Sall4*^{m1/+} and 4 *Sall4*^{+/+}) (Fig. 3i,j). However, the possibility of a functional defect resulting in an additional sensorineural component to the hearing loss cannot be excluded. This could be investigated in the future by ABR testing. It would also be pertinent to investigate the expression of *Sall4* in the middle and inner ear.

Although auditory renal and anogenital tract defect were readily observable in *Sall4*^{+/-} mice in our model, other features of the human syndrome were mild or non-apparent. Occasionally, *Sall4*^{m1/+} mice displayed skeletal defects consisting of shortening of the cranial bones and lateral deviation of the nasal bones, plus absence of the triquetrum, one of the small carpal bones (data not shown). Unlike other described *Sall4* mutant alleles cardiac defects were not apparent, as analyzed by MRI (data not shown).

Many of the developmental defects described here recapitulate features of Okihiro syndrome in humans, caused by loss of function mutations in *SALL4* (Kohlhase *et al.*, 2005). Humans have been not been reported with homozygous mutations within the *SALL4* gene, probably because this would result in early embryonic lethality. Similarly exencephaly is not described in the human syndrome. Like Okihiro syndrome in humans, the nonlethal defects in the surviving heterozygous mice show variable penetrance. This may be due to the influence of modifier alleles in the mixed genetic background.

It is pertinent to compare our model with other recently described *Sall4* mutant alleles, which have similar though not identical phenotypes (Koshiba-Takeuchi *et al.*, 2006; Sakaki-Yumoto *et al.*, 2006). A *Sall4* gene trap allele (Koshiba-Takeuchi *et al.*, 2006) with an insertion in exon 2 of the gene exhibits a variable embryonic phenotype ranging from VSD to mild limb abnormalities. The heart defects increased in severity when *Sall4*^{+/-} mice were crossed to *Tbx5*^{+/-} mice or backcrossed to Black Swiss mice. Compound *Sall1*^{+/-}/*Sall4*^{+/-} mice also displayed a more severe cardiac phenotype. In our model, VSDs were not observed—this may be due to the difference in genetic background, namely a predominantly C57BL/6 background compared with a Black Swiss background. Alternatively, the genetrap *Sall4*^{+/-} allele may act through a dominant negative function rather than just haploinsufficiency, resulting in a more severe phenotype.

A targeted *Sall4* allele has also been described (Sakaki-Yumoto *et al.*, 2006). This also deletes all zinc finger domains of the gene but retains the distal portion of exon 4. The phenotype described mentions similar preimplantation embryonic lethality of homozygotes. Heterozygotes show increased postnatal lethality, plus embryonic exencephaly, renal agenesis, anorectal malformations, and heart defects. However, limb defects are not described, and there is no structural evidence for any eye defect. The reason for the differences in observed phenotype between structurally similar alleles, and between the animal model and the human syndrome is unclear. This may be due to different effects on Sall4 protein-protein interactions and subsequent nuclear localization or transcriptional activation. Sall4 has been shown to interact with other proteins including Sall1 (Kiefer *et al.*, 2003; Koshiba-Takeuchi *et al.*, 2006; Sakaki-Yumoto *et al.*, 2006). In fact, the phenotype of mice with the truncating *Sall1* mutation is partly due to dominant negative action on *Sall4* (Kiefer *et al.*, 2003). This appears to be via a direct effect on nuclear localization of Sall4 in heterochromatin domains (Sakaki-Yumoto *et al.*, 2006). A recently described missense mutation (H888R) in the most C terminal zinc finger motif of *SALL4* in humans results in a mild form of Okihiro syndrome and cranial midline defects (Miertus *et al.*, 2006). This mutant is suggested to have increased DNA binding affinity, which is hypothesized to affect the binding of adjacent zinc fingers, and the transcriptional regulation function of *Sall4* by making the dissociation of mutant Sall4 from DNA more difficult (Miertus *et al.*, 2006).

In summary, we describe a novel mutant *Sall4* mouse model. This provides an animal model for studying the function of *Sall4*, which has subtle phenotypic differences to previously described models. Further investigations of Sall4 interactions in these different models may help to elucidate the nature of the genetic control of *Sall4* transcriptional regulation.

METHODS

Sall4 Targeting Vector

A replacement vector was constructed using bacterial recombineering (Liu *et al.*, 2003). The retrieval vector consisted of a 16,772 bp portion of *Sall4* (NT 4943 to NT 21715) containing exons 2, 3, and 4 from the RPCI21 PAC library clone [RP21-431J12] (Osoegawa *et al.*, 2000), and was constructed from PCR amplified left and right homology arms of 248 and 244 bp, respectively. These were cut with *NotI/HindIII* and *HindIII/SpeI* respectively, and ligated into a vector backbone, including the MC1-HSV *tk* negative selection cassette. This retrieved fragment was used to generate a targeting vector which replaces 8,332 bp of *Sall4* from NN 9502 [mouse chromosome 2: 168256993] at the 3' end of intron 1 to NN 17824 [mouse chromosome 2: 168265315] at the 3' end of exon 4. A *loxP-PGK-EM7-NeobpA-loxP* cassette (PL452) was used as the positive selection marker. A mini-targeting vector was constructed using the amplified homology arms of 252 bp (left) and 226 bp (right). After recombineering, the *Sall4* targeting vector (Sall4-TV) contained 8.45 kb total homology to the *Sall4* locus (4559 bp, left arm of homology; 3891 bp, right arm of homology). Primer details available on request.

Gene Targeting and Genotyping

Gene targeting in AB2.2 ES cells (129S7/SvEvBrd-*Hprt*^{b-m2}; 129S7) were carried out as previously described (Abuin and Bradley, 1996). Targeted clones were identified by Southern blot hybridization as previously described (Ramirez-Solis *et al.*, 1993). A 609 bp fragment (mouse chromosome 2: 168,251,675-168,252,284) was used as the 5' external probe. This probe detects a 9.0 and 6.3 kb *SpeI* fragment from the wild type and targeted alleles, respectively. A 428 bp 3' external probe (mouse chromosome 2: 168,269,837-168,270,265) was used to confirm the structure of the targeted allele at the 3' end. ES cell clones were used to establish transmission within the germ line of mice by standard

procedures. Mice were genotyped by PCR using genomic DNA extracted from the tail. The targeted allele was maintained and analyzed in a mixed genetic background consisting of ~75% C57BL/6J^{Tyr^C-Brd} and 25% 129/SvEvBrd contribution. Primer details are available on request.

RT-PCR

Total RNA was extracted from E8.5 embryos using the RNeasy Mini-Kit (Qiagen GmbH, Germany). Five micrograms of total RNA was reverse transcribed using SuperscriptTM II RT kit (Invitrogen) and 2 pmol of oligo-dT primers according to manufacturers' instructions. Control RT-PCR of β -actin was performed simultaneously. The RT-PCR products were visualized by running on an agarose gel. PCR primers are available on request.

In Situ and Whole Mount Hybridizations

Mouse embryos and brain tissue (C57BL/6J^{Tyr^C-Brd}) were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Eight micrometer sections were hybridized with a digoxigenin-labeled antisense RNA probe to the mouse *Salil4* gene (RefSeq ENSMUSG00000051739). Transcription was performed using an Ambion Maxi-Script kit with the addition of digoxigenin UTP (Roche). Fifty nanograms of probe was used for hybridization at 60°C for 6 h, using a Ventana Discovery platform with BlueMap and RiboMap kits, according to manufacturers' guidelines. The final stringency of washing was 0.1× SSC at 75°C. Sections were counterstained with Nuclear Fast Red. For whole mounts, the probe was hybridized at 62°C for 18 h on an Intavis hybridization platform, according to manufacturers' protocols.

Histopathology

Adult tissues were fixed in 10% neutral buffered formalin for 48 h, embedded in paraffin wax, and sectioned at 4 μ m intervals. Embryos for histopathology were fixed and processed similarly, but bisected and serially sectioned at 4 μ m intervals in the sagittal plane. Sections were stained with haematoxylin and eosin. For acetyl cholinesterase staining, segments of distal bowel in OCT were snap frozen in liquid nitrogen and cryostat sections cut for enzyme histochemistry (Bancroft, 2002).

Hearing, Middle Ear Dissection, and Inner Ear Clearing

Hearing was assessed blind to genotype. Hearing ability was monitored at weekly intervals by elicitation of the Preyer reflex (ear flick) or startle response, using a click box to deliver a 20 kHz, 90 dB SPL stimulus. After culling, the middle ear was photographed through the tympanic membrane, before the middle ear was dissected open and examined. Ossicles were examined in situ, before being removed for more detailed study. Half heads were fixed in Bodian's fixative and cleared with glycerol as described previously (Steel and Smith, 1992). The inner ear was examined for signs of malformation.

Scanning Electron Microscopy

Inner ears from P40 mice were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, the organ of Corti exposed, and samples processed using the osmium tetroxide-thiocarbohydrazide (OTOTO) method (Hunter-Duvar and Mount, 1978). After critical point drying and sputter coating, samples were analyzed using an Hitachi S4800 FE scanning electron microscope at 5 kV (Hitachi Instruments, San Jose, CA).

Skeletal Analysis

Adult mice were X rayed using a Faxitron 43855A (Faxitron Corporation, IL). Skeletons were fixed in 70% ethanol, stained with Alcian Blue/Alizarin Red, and cleared in potassium hydroxide/glycerol according to standard procedures (Inouye and Murakami, 1976).

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FIG. 1.

Targeted mutation of *Sall4*. (a) Structure of the *Sall4* locus, the *Sall4* targeting vector (Sall4-TV), and targeted allele *Sall4^{tm1Brd}*. S, *SpeI*, triangle, *loxP* site; MC1*tK*, thymidine kinase negative selection cassette. (b) Southern blot analysis of *SpeI* digested genomic DNA hybridized with the 5' probe, +/+, wild type AB2.2 ES cells; m1/+, *Sall4^{tm1Brd}* targeted ES cells. (c) PCR analysis using allele specific primers on genomic DNA of P21 pups. (d) PCR analysis of E3.5 embryos. All three genotypes are present. (e) RT-PCR analysis on reverse-transcribed RNA from E8.5 *Sall4^{+/+}* and *Sall4^{tm1Brd}* embryos. M, DNA marker ladder. (f, g) Whole mount in situ hybridization in E10.5 embryos. (f) *Sall4* is expressed in the midbrain,

branchial arches, limb buds, and tail bud in *Sall4*^{+/+} embryos. (g) In *Sall4*^{m1/+} embryos, greatly reduced expression is seen in these areas. mb, midbrain; mx, first branchial arch; b, second branchial arch; fl, fore limb; hl, hind limb; tb, tail bud.

FIG. 2.

Developmental defects in *Sall4*^{m1/+} mice. (a) Exencephaly in E16.5 *Sall4*^{m1/+} embryo. (b, c) Normal right kidney (b) and hypoplastic left kidney (c) of E16.5 *Sall4*^{m1/+} embryo. The hypoplastic kidney is smaller, without well defined zonation, scale bar, 1 mm. C, renal cortex; M, renal medulla. (d) Normal kidney from E16.5 *Sall4*^{m1/+} embryo exhibiting well formed glomeruli and tubules, scale bar, 500 μ m. (e) Hypoplastic kidney contains primitive nephrogenic vesicles and mesenchyme, scale bar, 500 μ m. G, glomerulus; CT, proximal and distal convoluted tubules; NV, nephrogenic vesicle; M, mesenchyme. (f) Lower anogenital tract abnormalities in adult *Sall4*^{m1/+} mice. The lower rectum, vagina, and urethra all open into a persistent cloaca. UT, uterus; B, bladder; V, vagina; U, urethra; C, persistent cloaca; R, rectum; S, spinal column, scale bar, 1 mm. (g-l) Hirschprung's disease in *Sall4*^{m1/+} mouse with bowel obstruction. (g, h) Enteric ganglion cells (arrow) in the intramural and submucosal plexuses of the large bowel in *Sall4*^{+/+} mouse, (g) H&E stained section, (h) acetyl cholinesterase staining, scale bar, 100 μ m. (i-l) *Sall4*^{m1/+} mice with bowel obstruction. (i, j) Proximal dilated bowel with sparse enteric neurons (arrows), scale bar, 50 μ m. (k, l) Distal segment of bowel, enteric ganglion cells absent; scale bar, 50 μ m.

FIG. 3.

Conductive deafness in *Sall4*^{m1/+} mice. (a-d) Tympanic membrane and middle ear cavity viewed from the external auditory meatus. (a, c) Normal middle ear cavity, *Sall4*^{+/+} mice. (b, d) *Sall4*^{m1/+} mice with middle ear inflammation. TR, tympanic ring; TM, tympanic membrane; M, malleus; I, incus, scale bar, 1 mm. (e-h) Histological appearance of the middle ear cavity. (e) Normal middle ear cavity of *Sall4*^{+/+} mouse, scale bar, 500 μm. (f-h) *Sall4*^{m1/+} with suppurative otitis media, middle ear effusion and squamous metaplasia of epithelium, scale bar 100/500 μm. PD, purulent debris; C, normal ciliated epithelium; SM, metaplastic squamous epithelium. (i, j) Scanning electron micrographs show normal inner ear epithelium of *Sall4*^{+/+} and *Sall4*^{m1/+} mice, scale bar, 5 μm.

Table 1

Summary of Gestational Mortality of $Sall4^{m1/+}$ and $Sall4^{m1/ml}$ Mice in Weaned Offspring of $Sall4^{m1/+}$ Intercrosses and Backcrosses

Male × Female	Number of litters	Average litter size	No. of $Sall4^{m1/+}$ pups	No. of $Sall4^{+/+}$ pups	Ratio of $Sall4^{m1/+}$: $Sall4^{+/+}$ pups
$Sall4^{m1/+} \times Sall4^{m1/+}$	26	4.7 ± 1.1	44	77	0.57:1
$Sall4^{m1/+} \times Sall4^{+/+}$	71	5.5 ± 1.0	116	273	0.42:1
$Sall4^{+/+} \times Sall4^{m1/+}$					

121 weaned offspring of $Sall4^{m1/+}$ intercrosses and backcrosses to C57BL/6 (C57BL/6TyrC-Brd_y) were genotyped. No homozygous $Sall4^{m1/ml}$ offspring were identified amongst the 121 weaned offspring of 26 $Sall4^{m1/+}$ intercrosses ($P < 0.007$, Student's *t*-test). The average litter size for $Sall4^{m1/+}$ intercrosses (4.7 ± 1.1) and $Sall4^{m1/+}$ backcrosses to C57BL/6TyrC-Brd_y mice (5.5 ± 1.0) was significantly reduced ($P < 0.01$ and $P < 0.001$, respectively). The ratio of heterozygous:wild type pups differed in all crosses (0.57:1-0.42:1) from the expected Mendelian ratio of 1:1 for backcrosses or 2:1 for intercrosses.

Table 2
Summary of Neonatal Lethality in Offspring from $Sall4^{m1/+}$ Backcrosses and Intercrosses

Cross	Total pups born	Total pups alive at day 21	Total pups dead before day 21	Total recorded $Sall4^{+/+}$ pups at day 21	Estimated $Sall4^{m1/+}$ pups at birth	Percentage of $Sall4^{m1/+}$ pups that died before weaning
Backcrosses						
F1 $Sall4^{m1/+}$	110	95	15	57	46-53	16-28
F2 $Sall4^{m1/+}$	440	361	79	270	93-128	31-44
Intercrosses						
F1 $Sall4^{m1/+}$	75	55	20	34	34-43	38-49
F2 $Sall4^{m1/+}$	46	35	11	26	16-20	45-55

671 pups born from 113 litters from 16 different mating pairs ($Sall4^{m1/+}$ intercrosses and backcrosses to C57BL/6 (C57BL/6^{TyrC-Brd}) were analyzed. Of these, 125 pups died before P21 at various ages from P1 onwards. Of the remaining 546 pups genotyped at weaning, 387 were wild type. The estimated number of $Sall4^{m1/+}$ pups at birth and the percentage of $Sall4^{m1/+}$ pups that died before P21 was calculated assuming either a Mendelian ratio of $Sall4^{+/+}$ and $Sall4^{m1/+}$ pups in the deaths of each litter, or that all dead pups were $Sall4^{m1/+}$, to obtain an estimated percentage range of deaths in $Sall4^{m1/+}$ pups.

Table 3
Summary of Hearing Analysis and Morphological Characteristics of $Sall4^{ml/+}$ and $Sall4^{+/+}$ Mice

Genotype	Total number of mice tested for Preyer reflex	Number of animals with no Preyer reflex	Mean age (range) of loss of Preyer reflex (days)	Number of mice with macroscopic evidence of middle ear discharge	Number of mice with histological otitis media	Number of mice with SEM abnormalities
$Sall4^{+/+}$	43	2 (5%)	50 (31-83)	4/18 (22%)	0 ($n=5$)	0 ($n=4$)
$Sall4^{ml/+}$	18	9 (50%)*	58 (46-76)	10/15 (67%)**	2 ($n=3$)	0 ($n=6$)

$Sall4^{ml/+}$ mice show significantly increased progressive hearing loss in the first 3 months of life compared to wild type controls. The Preyer reflex was recorded from a total of 18 $Sall4^{ml/+}$ and 43 $Sall4^{+/+}$ mice at 12 weeks. Mice were repeat tested where possible. Significance of hearing loss was determined by comparing the number of $Sall4^{ml/+}$ mice with an absent reflex to the number of $Sall4^{+/+}$ tested that had an absent Preyer reflex at each time point using Student's t -test.

* P value < 0.05;

** P value < 0.01, Student's t -test.