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Overexpression of *Sall1 in vivo* leads to reduced body weight without affecting kidney development

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Human *SALL1* is a homologue of the *Drosophila* region-specific homeotic gene *sal*, and is also known as a causative gene for Townes–Brocks syndrome, which is characterized by multi-organ malformations. We previously demonstrated that mouse *Sall1* plays a crucial role in ureteric bud invasion during kidney development, and possibly in nephron progenitor cells in the metanephric mesenchyme. To gain insights into the *Sall1* functions in the kidney and other tissues, we generated *R26Sall1* mice, in which *Rosa26* locus stop sequences flanked by two *loxP* sites were located upstream of the *Sall1* cDNA. This allele allowed exogenous Sall1 expression in a Cre recombinase-dependent manner. *R26Sall1* mice were first crossed with *CAGCre* mice, which expressed Cre recombinase ubiquitously during embryogenesis. Mice expressing Sall1 ubiquitously were smaller in size compared with mice of other genotypes. We then crossed *R26Sall1* mice with *Six2Cre* mice expressing Cre recombinase in the metanephric mesenchyme during kidney development. However, no kidney defects were observed. Taken together, overexpression of Sall1 does not affect kidney development, but does lead to a reduced body weight, suggesting that the optimal dosage of Sall1 is required for normal mouse development.

Keywords: body size/Cre recombinase/kidney development/Rosa26/Sall1.

Abbreviations: EDTA, Ethylenediaminetetraacetic acid; ES cell, Embryonic stem cell; HA, Hemagglutinin epitope; PGK promoter, Phosphoglycerate kinase promoter; SDS, Sodium Dodecyl Sulfate; SV40, Simian virus 40.

The mammalian kidney, the metanephros, is derived from two precursor tissues, the metanephric mesenchyme and the ureteric bud. The metanephric mesenchyme induces sprouting and branching of the ureteric bud, thus forming the collecting ducts and the ureters. Reciprocally, the ureteric bud invades the mesenchyme and induces epithelialization and differentiation of the mesenchyme into the glomeruli, loop of Henle and proximal and distal tubules.

The *spalt (sal)* gene family is conserved from *Drosophila* to humans, and its members encode proteins characterized by multiple double zinc finger motifs of the C2H2 type (*1*). Humans and mice each have four known *Sal*-like genes (*SALL1–4* in humans and *Sall1–4* in mice). Mouse *Sall1* is expressed in the undifferentiated mesenchyme, as well as in more differentiated structures including renal vesicles and comma-shaped bodies. We previously reported that *Sall1*-deficient mice die within 24 h after birth owing to kidney agenesis or severe dysgenesis, and that loss of *Sall1* leads to a failure of ureteric bud invasion into the mesenchyme, the initial key step for metanephros development (*2*). We also showed that *Sall1*-high mesenchyme contains multipotent nephron progenitors that can differentiate into epithelia or glomeruli, loop of Henle and proximal and distal tubules (*3*, *4*).

Sall1 is also expressed in a variety of extra-renal tissues, including the limb buds, anus, heart and subventricular regions of the central nervous system. Consistent with this expression pattern, compound mutants of *Sall1*/*Sall4* exhibit increased incidences of exencephaly and anorectal and heart anomalies (*5*), while *Sall1*/*Sall3* mutants show limb defects (*6*). Therefore, Sall family proteins could function in heterodimeric forms during the development of certain organs. Furthermore, *SALL1* mutations in humans are associated with Townes–Brocks syndrome, an autosomal dominant disease characterized by dysplastic ears, preaxial polydactyly, imperforate anus and, less commonly, kidney and heart anomalies (*7*). Several lines of evidence suggest that mutant truncated SALL1 proteins function in a dominant-negative manner and that some symptoms of Townes–Brocks syndrome are likely to be caused by functional inhibition of other SALL family members (*8*, *9*). Indeed, mice expressing a truncated Sall1 protein show similar phenotypes to those observed in human patients with Townes–Brocks syndrome (*7*). Therefore, *Sall1* expression in the extra-renal tissues appears to have functional relevance.

In the present study, we generated the *R26Sall1* mouse strain, which allowed exogenous Sall1 expression in a Cre recombinase-dependent manner. We implemented exogenous Sall1 expression by using *CAGCre* mice that express Cre ubiquitously (*10*) as well as *Six2Cre* mice that express Cre specifically in the renal mesenchyme (*11*). We found that exogenous Sall1 is unlikely to affect kidney development. We also

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found that overexpression of Sall1 leads to a reduced body weight in mice.

Materials and methods

Gene targeting and generation of mutant mice

An HA-tagged *Sall1* fragment digested with *Sal*I and *Not*I (*12*) was inserted into the *Sal*I–*Not*I sites of *pBigT* (*13*), a vector containing a splice acceptor sequence followed by a *PGK-neo* cassette and a *tpA* stop sequence flanked by two *loxP* sites. We digested the resulting plasmid with *Pac*I and *Asc*I to release the entire floxed *neo-tpA* and *Sall1* assembly, and inserted it into the *Pac*I and *Asc*I sites of a modified version of the *pRosa26-1* vector (*14*), which contains two homologous sequences to the *Rosa26* locus flanking the inserted sequence. This plasmid was subsequently linearized with *Mlu*I and used for electroporation. Cells (2×10^7) were electroporated with 50 mg of the targeting vector, and allowed to grow on neomycin-resistant mouse embryonic fibroblasts in the presence of G418 (300 µg/ml). Among the 144 clones generated, 11 were successfully targeted as confirmed by Southern blot analysis of the genomic DNA digested with *Eco*RI. Primers (5′-AGG CGC CCG ATA GAA TAA AT-3′ and 5′-ACT CTT CCC CTC CCC CTA CT-3′) were used to generate a 607-bp 5′ probe A (Fig. 1A), which detected a 15.6-kb band for the wild-type *Rosa26* locus and a 4.3-kb band for the targeted sequence. Finally, the targeted embryonic stem (ES) cell clones were used to generate chimeric mice at the Center for Animal Resources and Development, Kumamoto University. *R26Sall1* mice were hybrids of 129/Ola and C57BL/6 mice and backcrossed to C57BL/6 mice for two generations. *CAGCre* and *Six2Cre* mice were described previously (*10*, *11*). *Six2Cre* mice were hybrids of CD1 and C57BL/6 mice and backcrossed to C57BL/6 mice for three generations. *CAGCre* mice were on the C57BL/6 background. We compared the body weights within the same litter and consistently observed reduced body weights in the *CAGCre*/*R26Sall1* mice. All animal experiments were performed in accordance with institutional guidelines and review committees.

Genotyping

Mice carrying the *R26Sall1* allele were genotyped with the following primers: Neo—forward primer (Neo F), 5′-AAG GGA CTG GCT GCT ATT GG-3′, reverse primer (Neo R), 5′-ATA TCA CGG GTA GCC AAC GC-3′; Rosa26—forward primer (Rosa26 F), 5′-GAG TTC TCT GCT GCC TCC TG-3′, reverse primer (Rosa26 R), 5′-CCG ACA AAA CCG AAA ATC TG-3′. Mice carrying the Cre allele were genotyped with the forward primer Cre 1 (5′-AGG TTC GTT CAC TCA TGG A-3′) and reverse primer Cre 2 (5′-TCG ACC AGT TTA GTT ACC C-3′). The Cre-mediated loxP excision was detected using the forward primer p1 (5′-AAG GGA GCT GCA GTG GAG TA-3′; located on the Rosa26 flanking sequence) and reverse primer p2 (5′-GGC ATC CTT GCT CTT AGT GG-3′; located on the *Sall1* exon 2). Polymerase chain reaction (PCR) amplifications were performed under identical conditions using GoTaq Flexi DNA polymerase (Promega), with a denaturation process at 95°C for 2.5 min, followed by 35 cycles of 95°C for 30 s, 58°C for 60 s and 72°C for 30 s and a final extension step at 72°C for 7 min. The PCR products were analyzed by DNA electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining.

Western blot analysis

The kidneys, brains, livers and lungs of 9-week-old *CAGCre*/*R26Sall1* mice and the kidneys of newborn *Six2Cre*/*R26Sall1* mice were subjected to western blotting analyses. The tissues were immediately dissociated in protein lysis buffer (400 mM Tris–HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 1% protease inhibitor cocktail and 1% phenylmethylsulfonyl fluoride) on ice using a sonicator (amplitude = 40, 10 times for 10 s each), followed by centrifugation at 17400 g for 5 min. The protein concentrations were determined using a protein assay kit (Bio-Rad) according to the manufacturer's instructions. Alternatively, the tissues were

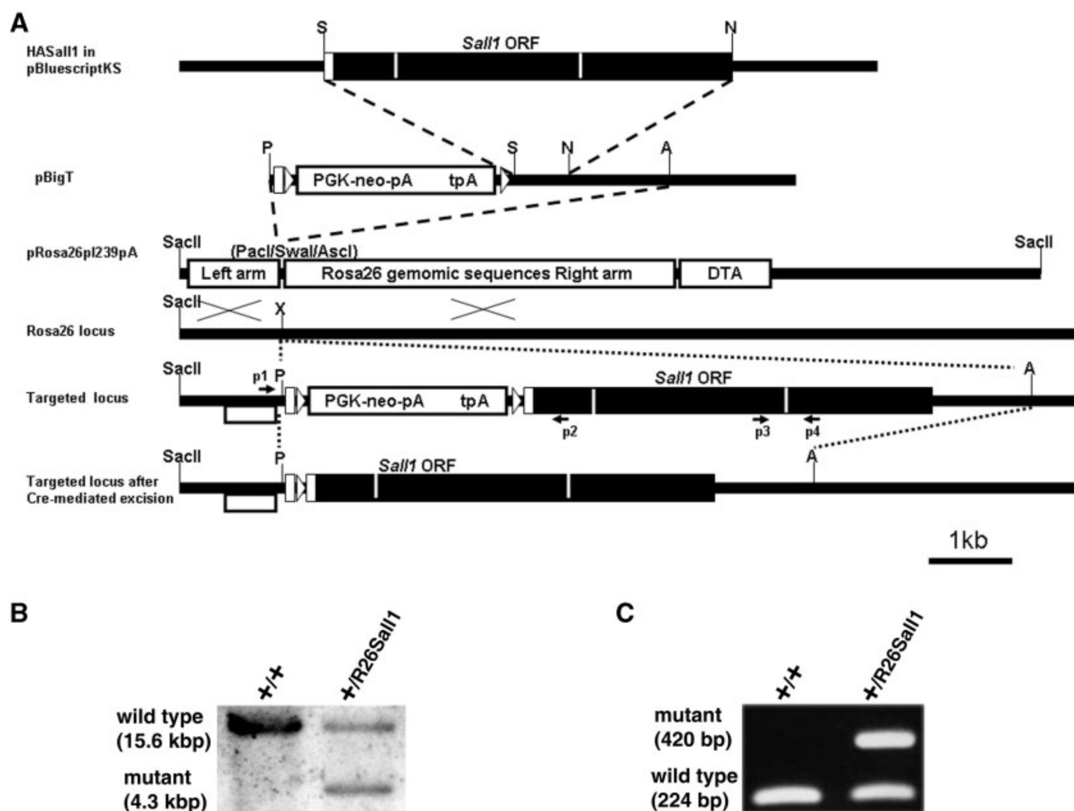

Fig. 1 Generation of *R26Sall1* mice. (A) Targeting strategy for *Sall1* in the *Rosa26* locus. The HA-tagged *Sall1* fragment was inserted into the *pBigT* vector and then into *pRosa26pl239pA*, followed by integration into the *Rosa26* locus in ES cells by homologous recombination. S, *Sal*I; N, *Not*I; RI, EcoRI; P, *Pac*I; A, *Asc*I. (B) Targeted ES clones selected by Southern blot analysis. Genomic DNA was digested with EcoRI and hybridized with probe A. (C) PCR amplification of mouse tail DNA.

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directly dissociated in SDS buffer (250 mM Tris–HCl, pH 6.8, 10% SDS, 50% glycerol, 0.02% bromophenol blue). The western blotting analyses were carried out using a standard protocol. The membranes were hybridized with anti-Sall1 (*12*; PPMX Perseus Proteomics) or anti-HA (Cell Signaling) monoclonal antibodies. Signals were detected using an ECL plus western blotting detection system (GE Healthcare) and exposure to a LAS-3000 system (FujiFilm).

Histological examination

Mice were fixed in 10% formalin, embedded in paraffin and cut into 6-μm sections. Haematoxylin and eosin staining was performed according to a standard protocol. Immunostaining was carried out using an anti-Pax2 antibody (Covance) and an automated Discovery System (Ventana) according to the manufacturer's protocols.

Results

Generation of R26Sall1 transgenic mice

To examine gain-of-function of Sall1 in various tissues *in vivo*, we generated R26Sall1 transgenic mice. HA-tagged Sall1 (*12*) was introduced into the *pBigT* vector (*13*), which contained *PGK-neo* and three SV40 terminator sequences flanked by two *loxP* sites (Fig. 1A). The *PGK-neo* sequence was used for G418 antibiotic-positive selection of murine ES cells, and the SV40 terminator sequences were used for blocking the transcription and translation of the downstream genes. The *pBigT* vector containing *HASall1* was further inserted into a modified *pRosa26-1* vector (*14*), which contained *Rosa26* genomic sequences used for homologous recombination. Finally, the entire fragment was introduced into murine ES cells by electroporation. Southern blotting and genomic PCR analyses confirmed the correct homologous recombination, and the resultant clones were used to generate germ-line chimeras and heterozygous mice (Fig. 1B and C).

Exogenous Sall1 is expressed in CAGCre/R26Sall1 mice

To gain insights into the roles of Sall1 in the kidney and other tissues, we crossed *R26Sall1* mice with *CAGCre* mice, which express Cre recombinase ubiquitously throughout their development (*10*). *CAGCre/R26Sall1* mice were born at a Mendelian frequency. We examined Cre-mediated deletion using tail DNA templates. Primers designed for the *Rosa26* genomic locus adjacent to the splicing acceptor and for *Sall1* (p1 and p2 in Fig. 1A) detected successful recombination, which produced an ~0.5-kb band (Fig. 2A). We did not detect any bands corresponding to the *Neo* sequences in *CAGCre/R26Sall1* mice, suggesting that the genomic excision of the *Neo* cassette was complete. To confirm the exogenous Sall1 expression, we performed western blotting analyses for several adult organs, including the kidney, brain and liver, all of which express endogenous Sall1, and the lung, which does not normally express Sall1 (*15*, *16*). Immunoblotting analyses using anti-Sall1 and anti-HA antibodies detected the exogenous Sall1 protein in *CAGCre/R26Sall1* mice, but not in other genotypes including *R26Sall1* mice (Fig. 2B). These data suggest that R26Sall1 mice can express exogenous Sall1 under the control of Cre recombinase.

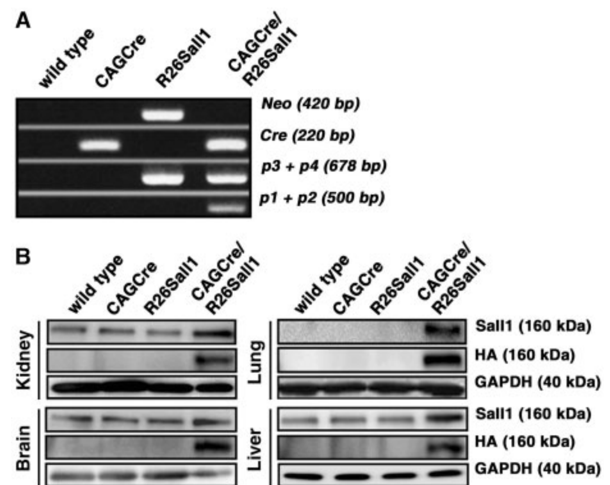

Fig. 2 Cre-mediated deletion and exogenous Sall1 expression in *CAGCre/R26Sall1* mice. (A) Cre-mediated deletion in *CAGCre/R26Sall1* mice. Tail genomic DNAs extracted from 4-week-old normal and mutant mice were analyzed by PCR. The primers used are shown in Fig. 1A. (B) Exogenous Sall1 expression in *CAGCre/R26Sall1* mice. Extracts from adult organs were subjected to western blotting using anti-HA and anti-Sall1 antibodies. HA expressions are exclusively detected in *CAGCre/R26Sall1* mice.

Ubiquitous Sall1 overexpression leads to a smaller body weight

The *CAGCre/R26Sall1* mice exhibited a reduced body size (Fig. 3A). The body weights of *CAGCre/R26Sall1* mice from 4 to 7 weeks after birth were significantly lower than those of wild-type or *R26Sall1* mice. *CAGCre* mice showed a similar tendency, but their body weights were not reduced to the same extent as those of *CAGCre/R26Sall1* mice (Fig. 3B). To exclude any adverse effects of the ubiquitous Cre expression on the body weights, the offspring of *CAGCre/R26Sall1* mice were further examined. Since the germ cells of *CAGCre/R26Sall1* mice already possessed the recombined allele, some of the offspring were expected to overexpress Sall1 in the absence of the *Cre* allele. Indeed, these mice also showed reduced body weights at 4 weeks after birth compared with those of wild-type mice (data not shown). Therefore, overexpression of Sall1 appears to impair normal growth, suggesting that the optimal dosage of Sall1 is required for the proper development of mice. Although combined reductions of Sall family members lead to defects in the limbs, kidney, heart, anus and brain (*5*, *6*), *CAGCre/R26Sall1* mice did not exhibit any abnormalities in these organs.

Overexpression of Sall1 in the kidney does not lead to apparent phenotypes

Since the reduced body size of the *CAGCre/R26Sall1* mice prevented us from examining the precise effects of Sall1 overexpression on their kidney development, we crossed *R26Sall1* mice with *Six2Cre* mice that expressed Cre recombinase specifically in the metanephric mesenchyme. Although *Sall1*-deficient mice showed kidney defects, the kidneys of the *Six2Cre/R26Sall1* mice were apparently normal (Fig. 4). Haematoxylin–eosin staining (Fig. 4A–D) and

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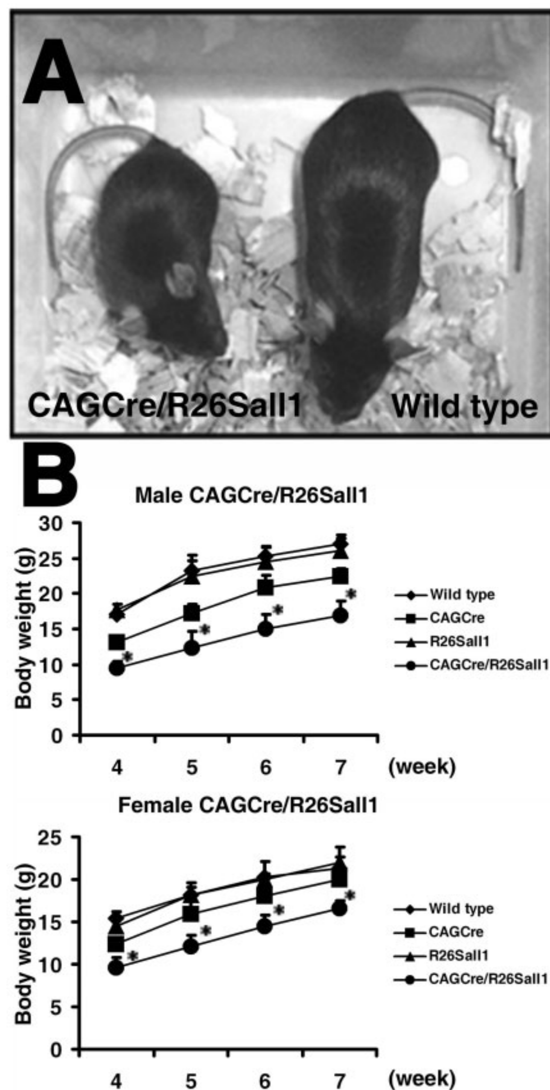

Fig. 3 Ubiquitous Sall1 overexpression leads to a smaller body weight. (A) *CAGCre/R26Sall1* mice are apparently smaller than other genotypes. (B) Growth curve of *CAGCre/R26Sall1* mice. The body weights from 4 to 8 weeks after birth were measured. Analyses by a *t*-test proved that *CAGCre/R26Sall1* mice are significantly smaller than others ($P < 0.01$, $n = 4$, 3, 5 and 4 for male and $n = 4$, 3, 4 and 5 for female wild-type, *CAGCre*, *R26Sall1* and *CAGCre/R26Sall1* mice, respectively).

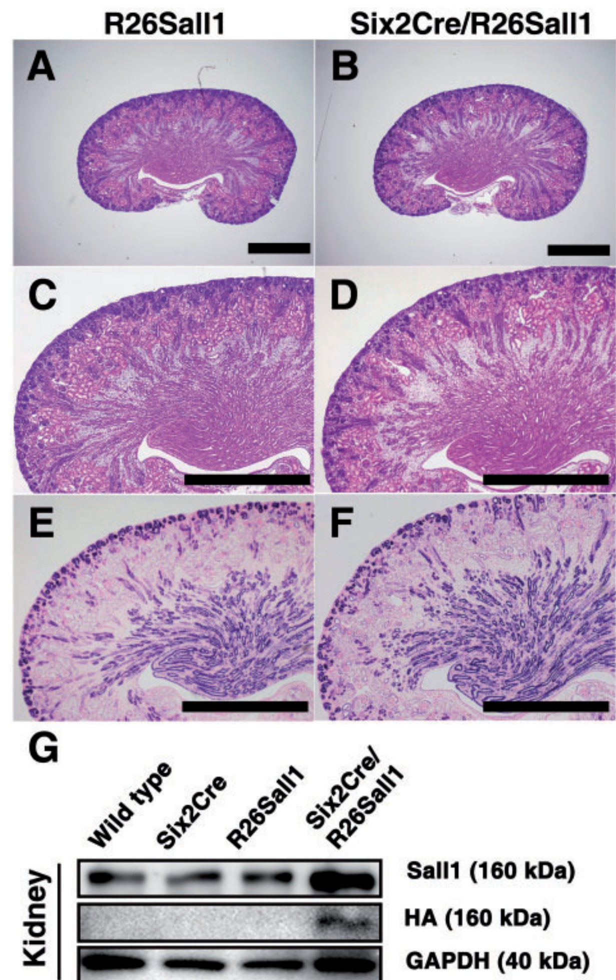

Fig. 4 Overexpression of Sall1 in the kidney does not lead to apparent phenotypes. (A and B) Haematoxylin–eosin staining of kidneys in *Six2Cre/R26Sall1* newborn mice. (C and D) Higher magnification images of (A) and (B), respectively. (E and F) Immunostaining for Pax2. Scale bar = 1 mm. (G) Western blot analysis of kidneys from newborn *Six2Cre/R26Sall1* mice.

immunostaining for Pax2 (Fig. 4E and F), which is expressed in the metanephric mesenchyme and the ureteric bud, failed to show any significant differences between *Six2Cre/R26Sall1* mice and control mice, despite the detection of exogenous Sall1 expression in the kidney of *Six2Cre/R26Sall1* mice by western blot analysis (Fig. 4G). Therefore, overexpression of Sall1 does not appear to affect kidney development, although loss of Sall1 leads to kidney agenesis or dysgenesis.

Discussion

We generated *R26Sall1* mice, in which *Sall1* was overexpressed in a Cre recombinase-dependent manner. *Six2Cre/R26Sall1* mice that expressed exogenous *Sall1* in the embryonic kidney did not show any kidney defects, although Sall1 is essential for kidney development. Absence of *Sall1* in the metanephric mesenchyme leads to a reduced size of the colony formation, suggesting that Sall1 is possibly important for cell proliferation in the mouse kidney (*17*). However, we did not detect any apparent abnormalities in kidney development or any proteinuria when we examined the mice at 6 months after birth (data not shown). Although a certain level of Sall1 is required for kidney development, an excess amount of Sall1 may not affect this process. Nonetheless, it would be worth examining the kidney functions in *Six2Cre/R26Sall1* mice under a variety of disease states.

We also found that the overexpression of *Sall1* in *CAGCre/R26Sall1* mice led to a reduced body weight of the mice. An overdose of Sall1 could possibly decrease the cell proliferation rates at the embryonic and perinatal stages. Certain transcription factors need to be maintained at appropriate levels to exert their normal functions. For example, the Oct3/4 level

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should be 50–150% of the normal level to maintain ES cells (*18*). We previously showed that Sall4 is essential for the proliferation of ES cells (*5*, *19*), although its overexpression does not lead to increased proliferation of ES cells. Therefore, it will be necessary to culture cells such as embryonic fibroblasts isolated from *CAGCre/R26Sall1* mice to assess their proliferation properties. Alternatively, it is possible that ubiquitously expressed exogenous Sall1 depresses pituitary growth hormone secretion and subsequently causes a disturbance in mouse development, since endogenous SALL1 is expressed in human pituitaries that secrete growth hormone for physiological growth and development (*20*).

The murine *Sall* family is composed of four members: Sall1, Sall2, Sall3 and Sall4 (*4*). Sall1 and Sall4 colocalize in the heterochromatin and bind to each other in ES cells, suggesting that these two proteins can form heterodimers and functionally overlap in some cases (*5*). *SALL4* is increased in human leukemia and overexpression of Sall4 in mice can cause acute myeloid leukemia (*21*). However, tumorigenesis was not observed in our *CAGCre/R26Sall1* mice at 6 months after birth (data not shown), suggesting that Sall4 may play distinct roles in tumorigenesis that are not shared by Sall1. Alternatively, expression of Sall1 may be sufficient to induce leukaemia. Although the *Rosa26* locus is extensively used for gene targeting in many organs and tissues, other loci or promoters should be examined for more abundant expression of Sall1.

In summary, we have generated a mouse line that expresses exogenous *Sall1* under the control of Cre recombinase, and demonstrated that overexpression of Sall1 leads to a reduced body weight without affecting kidney development. This mouse strain will also be useful for detecting Sall1-containing protein complexes in the kidney and other organs, which could lead to the identification of the molecular mechanisms underlying Townes–Brocks syndrome.

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Conflict of interest

None declared.

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