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The murine homolog of *SALL4*, a causative gene in Okihiro syndrome, is essential for embryonic stem cell proliferation, and cooperates with *Sall1* in anorectal, heart, brain and kidney development

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Mutations in *SALL4*, the human homolog of the *Drosophila* homeotic gene *spalt* (*sal*), cause the autosomal dominant disorder known as Okihiro syndrome. In this study, we show that a targeted null mutation in the mouse *Sall4* gene leads to lethality during peri-implantation. Growth of the inner cell mass from the knockout blastocysts was reduced, and *Sall4*-null embryonic stem (ES) cells proliferated poorly with no aberrant differentiation. Furthermore, we demonstrated that anorectal and heart anomalies in Okihiro syndrome are caused by *Sall4* haploinsufficiency and that *Sall4*/*Sall1* heterozygotes exhibited an increased incidence of anorectal and heart anomalies, exencephaly and kidney agenesis. Sall4 and Sall1 formed heterodimers, and a truncated Sall1 caused mislocalization of Sall4 in the heterochromatin; thus, some symptoms of Townes-Brocks syndrome caused by SALL1 truncations could result from SALL4 inhibition.

KEY WORDS: *Sall4*, *spalt*, Embryonic stem cells, Okihiro syndrome, Townes-Brocks syndrome, Organogenesis, Mouse

INTRODUCTION

Early differentiation of the mammalian embryo leads to the development of two distinct lineages – the inner cell mass (ICM) and the trophectoderm. Cells of the ICM are pluripotent and give rise to the epiblast and eventually to all the fetal tissues, while trophectoderm cells have restricted potential and give rise to the trophoblast cell layers of the placenta. Embryonic stem (ES) cells are established from the ICM and have the potential to generate chimeric mice when introduced into a blastocyst. When cultured in vitro, ES cells differentiate into a variety of cell lineages; thus, human ES cells are possible candidates for cell therapies, although there are some ethical issues to overcome. Maintenance of mouse ES cell pluripotency requires constant suppression of their differentiation by both extrinsic and intrinsic factors. Leukemia inhibitory factor (LIF) and bone morphogenetic protein 4 (BMP4) are the two major extrinsic signals (Niwa et al., 1998; Matsuda et al., 1999; Ying et al., 2003). LIF induces phosphorylation and nuclear localization of STAT3, a transcription factor essential for the LIF-dependent pathway for self-renewal. BMP4 induces phosphorylation and nuclear localization of Smad1, and the subsequent upregulation of Id helix-loop-helix proteins that block neural differentiation and maintain the pluripotency of ES cells in the presence of the LIF signal. Intrinsic factors include *Oct3/4*, *Nanog* and *Eras*. *Oct3/4* and *Nanog* are expressed at high levels in ES cells and have been shown to be essential for maintaining pluripotency in ES cells (Chambers et al., 2003; Mitsui et al., 2003; Nichols et al., 1998). *Eras* and *Utf1* are also expressed abundantly in ES cells, and they play crucial roles in the proliferation of stem cells (Takahashi et al., 2003; Nishimoto et al., 2005).

The *spalt* (*sal*) gene was first isolated from *Drosophila* and it encodes a protein characterized by multiple double zinc-finger motifs of the C2H2 type. *sal* acts as a region-specific homeotic gene, and is required for the specification of the head and tail regions during early development (Jurgens, 1988; Kuhnlein et al., 1994). During the later stages of development, *sal* regulates pattern formation and cell fate decisions in the wing disc (de Celis et al., 1996; Nellen et al., 1996), trachea (Kuhnlein and Schuh, 1996) and sensory organ development (de Celis et al., 1999). *sal* is expressed at the anteroposterior boundary of the wing imaginal discs, and its expression is controlled by the *dpp* (decapentaplegic) gene (de Celis et al., 1996; Nellen et al., 1996).

Humans and mice each have four known Sal-related genes (known as *SALL1-SALL4* in humans and *Sall1-Sall4* in mice). Mutations in *SALL1* on chromosome 16q12.1 have been associated with Townes-Brocks syndrome, an autosomal dominant disease characterized by dysplastic ears, a preaxial polydactyly, imperforate anus and, less commonly, kidney and heart anomalies (Kohlhase et al., 1998). Mice deficient in *Sall1* show kidney agenesis or severe dysgenesis, but other phenotypes observed in the human disease are not apparent (Nishinakamura et al., 2001). This discrepancy could be explained by the formation of truncated SALL1 proteins as a result of mutations in *SALL1*, as comparison with *Sall1*-null mice showed that mutant mice producing a truncated Sall1 protein exhibited more severe defects, including renal agenesis, exencephaly, as well as limb and anal deformities (Kiefer et al., 2003). It has been reported that Sall1 also functions as a

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transcriptional repressor by localizing in the heterochromatin and interacting with components of chromatin remodeling complexes such as histone deacetylase (HDAC)1, HDAC2, retinoblastoma-associated protein 46/48 (RbAp46/48), metastasis-associated protein (MTA)1 and MTA2 (Kiefer et al., 2002).

It is still unclear if *SALL2* is associated with human disease; however, it has been reported that Sall2 functions as a tumor suppressor (Li et al., 2001; Li et al., 2004). *Sall2*-deficient mice show no apparent phenotype, and mice lacking both *Sall1* and *Sall2* show kidney phenotypes comparable with those of *Sall1* knockout mice (Sato et al., 2003). Although no diseases are thus far directly linked to *SALL3*, this gene is located in a region that is commonly deleted in cases of 18q deletion syndrome (Kohlhase et al., 1999). Individuals with this deletion exhibit hearing loss, cardiac problems, mental retardation, midfacial hypoplasia, delayed growth and limb abnormalities (Strathdee et al., 1997). *Sall3*-null mice die on the first postnatal day and deficiencies in the cranial nerves and abnormalities in the oral structures are present (Parrish et al., 2004). Mutations in *SALL4* cause an autosomal dominant disorder known as Okihiro syndrome, which is characterized by limb deformity, eye movement deficits and, less commonly, anorectal, ear, heart and kidney anomalies (Al-Baradie et al., 2002; Kohlhase et al., 2002).

To investigate the roles of Sall family genes and their functional redundancy in organogenesis, we generated *Sall4*-deficient mice. In this study, we report that the mouse *Sall4* gene was found to be unexpectedly essential for early embryogenesis, and for the proliferation of ES cells. We further reveal the importance of the heterodimerization of Sall4 and Sall1 in vivo, which could explain the underlying genetic mechanisms involved in Townes-Brocks syndrome that is caused by truncations of SALL1.

MATERIALS AND METHODS

Generation of *Sall4*-deficient mice (*Sall4-del*)

A *Sall4*-targeting vector was constructed by incorporating the 5′ *Aor51*HI-*Eco*RI 8.2-kb *Sall4* fragment and the 3′ *Bam*HI-*Eco*RV 2.3 kb fragment into a vector that contained the β-galactosidase gene (*lacZ*), the neomycin resistance (*Neo*^{r}) gene (pGK-Neo) and the diphtheria toxin A subunit (pMC1DTA) in tandem. This construct deletes all the eight zinc-finger domains of *Sall4* and results in the fusion of 39 amino acids at the N terminal of Sall4 and β-galactosidase (Fig. 1A). This strategy is identical to the one we used to delete *Sall1* (Nishinakamura et al., 2001). Five out of 114 G418-resistant E14.1 ES clones were correctly targeted and two were used to generate germline chimeras that were bred with C57BL/6J females. E14.1 ES cells were maintained on mitomycin C-treated primary embryonic fibroblasts in the presence of LIF (10^3 U/ml) and serum in all the procedures and experiments described in this paper. The primer sequences used for genotyping were as follows: 5′-GAGGACTCCATACCGGTGAA-3′, 5′-GTGCCCAGCTTCTTCAAGTC-3′ and 5′-CCTCTTCGCTATTACG-CCAG-3′ (the length of the amplified segment was 272 bp in the wild-type allele and 379 bp in the mutated allele). As the *Sall4*-deficient mice (*Sall4-del*) lacked proper *lacZ* expression, we also generated mice using the *Sall4-IRES-β-geo* vector described below.

Targeted disruption of both alleles of *Sall4*

The *Sall4-IRES β-geo* vector was constructed by incorporating the 5′ *Pac*I-*Eco*RI 4.2 kb fragment, the *Eco*RI-*Sal*I 6.0 kb *IRES-β-geo* fragment and the 3′ *Apa*LI-*Eco*RV 6.0 kb fragment into a vector that contained pMC1DTA (see Fig. S1 in the supplementary material). The *Sall4-IRES-Hyg* vector was generated by a similar method. These vectors confer drug resistance only when they are incorporated into the promoter regions of genes that are expressed in ES cells, including *Sall4* (thus facilitating the isolation of homologous recombinants). Indeed, when tested in wild-type ES cells, homologous recombinants were obtained at a frequency of 68.8% (11/16) by using the *Sall4-IRES-β-geo* vector and at 66.7% (8/12) with the *Sall4-IRES-Hyg* vector. The *Sall4-IRES-β-geo* clones were used to generate another strain of mice (*Sall4-βgeo*) for monitoring *Sall4* expression, as shown in Fig. 4D,E. This strain showed phenotypes identical to the original *Sall4*-deficient mice (*Sall4-del*).

Generation of a *Sall4* floxed allele

The *Sall4*-flox vector was constructed by incorporating the 5′ *Hin*dIII-*Hin*dIII 3.5 kb fragment, the *Hin*dIII-*Apa*LI 4.7 kb fragment and the 3′ *Apa*LI-*Eco*RV 6.0 kb fragment into a vector that contained pGK-Neo flanked by Frt and loxP sequences and pMC1DTA (see Fig. S1 in the supplementary material). LoxP sequences were placed so that exon 2 and 3 were excised upon Cre treatment, resulting in disruption of all zinc-finger motifs. Five out of 170 clones were correctly targeted (flox/+) and one of them was further transfected with the *Sall4-IRES-Hyg* vector. Both resulting

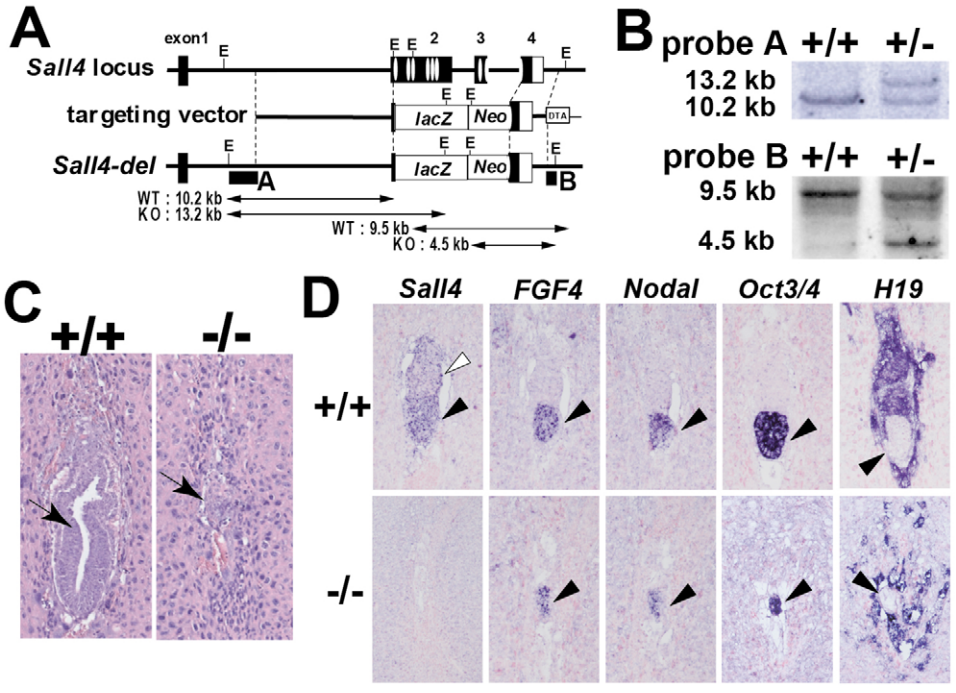

Fig. 1. Embryonic lethality of *Sall4*-deficient mice. (**A**) Targeting strategy of *Sall4*. Ovals represent the zinc-finger domains. E, *Eco*RI. (**B**) Southern blot analysis using the probes described in Fig. 1A. (**C**) Hematoxylin and Eosin staining of wild-type (+/+) and *Sall4*-deficient (–/–) embryos at E6.5. Arrow indicates epiblast. (**D**) In situ hybridization of *Sall4*, epiblast markers (*Fgf4*, *Nodal* and *Oct3/4*), and a marker for trophectoderm and extra-embryonic ectoderm (*H19*) in wild-type (+/+) and *Sall4*-deficient (–/–) embryos at E5.8. H19 signal is absent in the epiblast of both +/+ and –/– embryos. Black arrowhead, epiblast; white arrowhead, extra-embryonic ectoderm. Serial sections from two wild-type and two *Sall4*-deficient embryos are shown. Left three columns and right two comuln are from different embryos.

clones, flox/– and +/–, proliferated normally. Neo^r placed in intron 2 did not affect Sall4 expression as shown in Fig. 3C. These ES cells were infected with adenovirus expressing Cre under the CAG promoter (AxCANCre provided by RIKEN Bioresource Center) (Niwa et al., 1991; Kim et al., 2002) at a m.o.i (multiplicity of infection) of 50. After incubation for 1 hour, cells were diluted and plated onto 6-well plates coated with mitomycin C-treated embryonic fibroblasts.

Histology and blastocyst culture

Histological examination was performed as described earlier (Nishinakamura et al., 2001). In situ hybridization was performed using the AmpMap Kit and an automated Discovery System (Ventana) according to the manufacturer's protocols. Blastocyst culture and immunosurgery were also performed as described earlier (Nichols et al., 1998). cDNA was synthesized using SuperScript III CellsDirect cDNA Synthesis System (Invitrogen). Primer sequences used for RT-PCR are available upon request. 5-Bromo-2′-deoxyuridine (BrdU) labeling and detection kit I (Roche) was used to examine proliferation of the blastocysts upon 30 minutes of BrdU incorporation.

Chimera formation

Two independent *Sall4*-deficient clones were transfected with *pCAG-GFP-IRES-puro* and selected on puromycin-resistant embryonic fibroblasts (Tucker et al., 1997). Cells retaining ubiquitous green fluorescent protein (GFP) expression from each *Sall4*-deficient clone were injected into blastocysts, and consistent results were obtained. Frozen sections of the chimeras were stained by an anti-GFP antibody (Molecular Probes) and detected using ImmunoPure metal enhanced DAB substrate kit (Pierce).

Proliferation and rescue analysis of ES cells

For the proliferation assay, 1×10^4 cells were plated per well in 24-well plates in triplicate on mitomycin C-treated primary embryonic fibroblasts in media containing LIF and serum, and passaged at the same density every 4 days for 16 days to determine the cumulative cell number. A BrdU Flow Kit (BD Biosciences) was used for cell cycle analysis of ES cells after 2 hour incorporation of BrdU. For rescue analysis, the *Sall4* expression vector (*Sall4* in *pCAG-IRES-puro*) or *GFP* expression vector (negative control) was introduced into *Sall4*-deficient cells by electroporation. Multiple clones were selected on puromycin-resistant embryonic fibroblasts and expanded.

siRNA transfection

The siRNA duplexes were designed to target the coding region of mouse *Sall4* cDNA at nucleotide 2761-2785 and synthesized by Invitrogen. Sall4-siRNA or control siRNA containing the same GC content was transfected into D3 embryonic stem cells using Lipofectamine2000 (Invitrogen) according to the manufacturer's instruction, except for the maintenance of D3 cells, which were cultured on gelatin-coated plates in the presence of LIF. D3 cells at a density of 1×10^4 cells per well in 24-well plates were transfected in triplicate with 25 pmol siRNA, and counted every day. The negative control siRNA showed no growth or morphological impairment compared with the mock-transfected or the non-transfected cells.

Immunocytochemistry and confocal microscopy

The anti-Sall1 monoclonal antibody (Sato et al., 2004) and an anti-Sall4 polyclonal antibody raised against polypeptide (MAKHQFPHFLEENKI) corresponding to amino acids 1050-1064 were used. A monoclonal anti-Sall4 antibody was generated using *Sall4* cDNA encoding amino acids 95-216. The following additional antibodies were used for staining blastocysts and ES cells: monoclonal anti-Oct3/4 (Santa Cruz), rabbit antisera against Oct3/4 (Niwa et al., 2005) and monoclonal anti-Cdx2 (BioGenex). GFP-fused *Sall4* and *Sall1* zinc-finger mutants in which the cysteine residues were replaced by glycine were produced using PCR. NIH 3T3 cells were plated onto six-well plates at a density of 1×10^5 cells per well 1 day prior to transfection. The cells were transfected with 3 μg of the plasmids using FuGENE 6 (Roche) and cultured for 48 hours prior to analysis by confocal microscopy. Construction of *Sall1-GFP* and *Sall1*$^{1\text{-}435}$*-DsRed*, immunoprecipitation, immunocytochemistry and confocal microscopy were performed as described previously (Sato et al., 2004).

RESULTS

Sall4-deficient mice die shortly after implantation

Fig. 1A,B depict the strategy used to delete all the eight zinc-finger domains of *Sall4*. As *Sall4*-null mice did not survive beyond embryonic day (E) 6.5 (Table 1), the embryos were examined at E5.5-6.5 (Fig. 1C,D). Although wild-type embryos developed an egg cylinder with a central proaminiotic cavity, seven out of the 26 embryos (26.9%) derived from the heterozygous intercross barely retained a structure that resembled the epiblast. These embryos were identified as *Sall4*-null by in situ hybridization, while *Sall4* expression was observed in the epiblast, and less abundantly in visceral endoderm and extra-embryonic ectoderm in the wild-type embryos (Fig. 1D). Though the areas occupied by the epiblast markers (*Oct3/4*, *Fgf4* and *Nodal*), the trophectoderm and extra-embryonic ectoderm marker (*H19*), and the extra-embryonic ectoderm marker (*Bmp4*) were significantly reduced, they were still present in *Sall4*-null embryos (Fig. 1D and data not shown). These data demonstrate that *Sall4* is essential for embryonic development during the peri-implantation period, but not for commitment to the epiblast or extra-embryonic lineages.

Sall4 is required for inner cell mass proliferation in blastocysts in vitro

As the drastic impairment of *Sall4*$^{-/-}$ embryos hindered further examination, blastocysts from *Sall4*$^{+/-}$ intercrosses (E3.5) were examined. At this stage, the Sall4 protein was expressed both in the inner cell mass (ICM) and trophectoderm in the wild-type (Fig. 2A). Homozygotes were, however, indistinguishable from wild-type or heterozygotes, and expression of *Oct3/4* (ICM marker) and *Cdx2* (trophectoderm marker) was not altered (Fig. 2A), indicating that lineage commitment between these two lineages occurs normally. Next, cultured blastocysts were investigated for phenotypic changes. The embryos hatched from their zonae and attached to the plates, and the trophectoderm grew in an identical manner. By day 5 in culture, all homozygotes (n=14) showed significantly reduced outgrowth of ICM compared with wild-type and heterozygote (n=43) (Fig. 2B). At day 3 of culture, when the phenotype was becoming apparent, *Sall4*-deficinet ICM was positive for Oct3/4, as determined by immunostaining (Fig. 2B), and expression of the lineage markers examined by RT-PCR was not impaired: *Oct3/4* (ICM), *Gata6* and *Gata4* (primitive endoderm), eomesodermin and *H19* (trophectoderm) (Fig. 2C). These results suggest that lineage commitment does occur in the absence of Sall4. By contrast, at day 3 of culture, BrdU incorporation was significantly reduced in *Sall4*-deficient ICM when compared with wild type or heterozygote, while an apparent increase in apoptosis, as determined by TUNEL assay, was not detectable (Fig. 2D; data not shown), indicating that *Sall4* is required for ICM proliferation. Next, the trophectoderm was removed by immunosurgery to rule out secondary effects of trophectoderm abnormality. Of 54 ICMs, 41 (76%) grew into a spheroid structure with a primitive endoderm surrounding it, and all

Table 1. Genotyping of heterozygous crosses

Stage	+/+	+/–	–/–	n.d.	Total
E3.5	10	16	8	2	36
E6.5-7.5	7	16	0	5	28
E8.5-10.5	5	10	0	9	24
E11.5-13.5	10	14	0	3	27
E14.5-15.5	10	12	0	4	26
P0	80	86	0	0	166

n.d., not determined. Only implantation sites were detected.

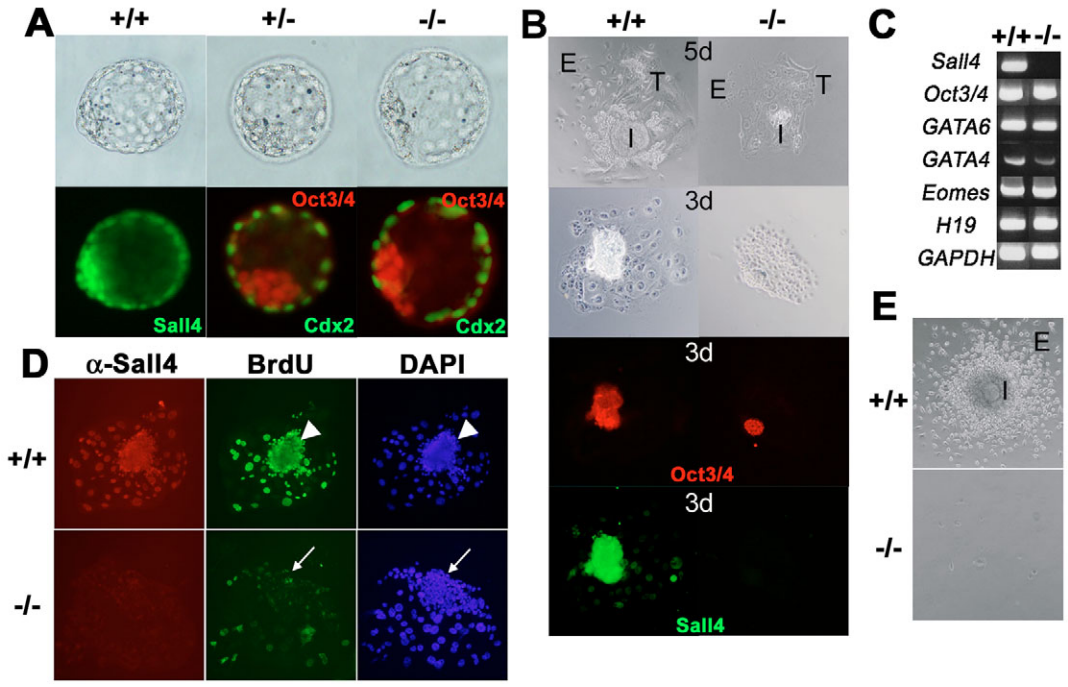

Fig. 2. Requirement of *Sall4* for inner cell mass proliferation in blastocysts in vitro. (**A**) Normal development of wild-type (+/+), heterozygous (+/–) and *Sall4*-null (–/–) blastocysts (E 3.5). Sall4 is expressed in the inner cell mass and trophectoderm (left column). Oct3/4 (red) and Cdx2 (green) staining shows that commitment to the inner cell mass and trophectoderm occurs normally in the *Sall4* homozygotes (compare middle and right columns). (**B**) Reduction of inner cell mass in *Sall4*-null blastocysts cultured in vitro. Uppermost row shows phase-contrast photo at 5 day of culture. Second row shows phase contrast at 3 day of culture. Lower two rows show immunostaining of Oct3/4 and Sall4. E, primitive endoderm; I, inner cell mass; T, trophectoderm. (**C**) RT-PCR analysis of markers in blastocysts cultured for 3 days. All the lineage markers are expressed. (**D**) Reduced proliferation of the inner cell mass of *Sall4*-null blastocysts cultured for 3 days. BrdU incorporation of the inner cell mass is reduced in *Sall4*-null blastocysts (arrow), compared with wild type (arrowhead). (**E**) Failure of growth of *Sall4*-null inner cell mass free from the trophectoderm.

the ICMs were genotyped as wild-type or heterozygote. By contrast, 13 out of 54 (24%) ICMs showed very few signs of growth (Fig. 2C rightmost column), suggesting that, independent of trophectoderm, *Sall4* is essential for ICM outgrowth.

Sall4-null ES cells show reduced proliferation

The above data prompted us to investigate Sall4 functions in ES cells, as these cells are derived from the ICM. To obtain *Sall4*-null ES cells, a vector containing the hygromycin resistance gene (*Hyg*) was introduced into heterozygous cells containing the *Neo*^{r} allele (see Fig. S1 in the supplementary material). We also attempted the reverse order, and both experiments resulted in a relatively frequent isolation of heterozygous cells in which the initial targeted allele was retargeted by the second vector. However, *Sall4*-null ES cells were isolated at a very low frequency (Table 2). To confirm these results, we constructed other versions of the vectors that contained promoterless *β-geo* or *Hyg*, thus facilitating the isolation of homologous recombinants (see Fig. S1 in the supplementary material). However, when introduced into heterozygous cells, most homologous recombinants showed retargeting of the mutated allele, and *Sall4*-null cells were obtained at a low frequency (Table 2). Therefore, the absence of *Sall4* is disadvantageous to ES cells.

We next tried inducible knockdown of *Sall4* by introducing siRNA oligonucleotides into ES cells. siRNA against *Sall4* efficiently reduced *Sall4* expression by day 2 after transfection, but *Sall4* expression recovered by day 4 (Fig. 3A), which was also confirmed by Sall4 immunostaining on ES colonies (data not shown). *Sall4*-siRNA-treated cells showed transient reduced growth with similar kinetics to the *Sall4* expression, while negative control siRNA had no effect (Fig. 3B). By contrast, colony morphology and Oct3/4 staining were not altered in the absence of Sall4 (data not shown). These data suggest that *Sall4* may be required for proliferation of ES cells.

Next, heterozygous cells were generated containing a floxed allele of *Sall4* by homologous recombination (see Fig. S1 in the supplementary material). When the *Sall4-IRES-Hyg* vector was introduced into this clone, both alleles were targeted with a similar frequency (wild-type allele, 10/23; floxed allele, 11/23), resulting in two types of cells: flox/– and +/– (Fig. 3C). As shown by western blot, *Neo*^{r} in intron 2 of the floxed allele did not affect Sall4 expression, and there was no difference in proliferation between the two types of cells. Upon infection with adenovirus expressing Cre, flox/– cells became almost *Sall4*-null by day 3, while +/– cells served as a negative control, as determined by western blot (Fig. 3C). Indeed, when cells were subsequently replated and single clones

Table 2. Generation of *Sall4*-null ES cells

Experiment	Vectors		Selection at second round	Number of colonies	Integration event		
	First round	Second round			Random	Retargeting	Second allele
1	*pGK Neo*	*pGK Hyg*	Hyg	134	116	16	2
2	*pGK Hyg*	*pGK Neo*	Neo	115	68	44	3*
3	*IRES Hyg*	*IRES β-geo*	Neo	20	12	8	0
	IRES Hyg	*IRES β-geo*	Neo+Hyg	13	12	0	1*

Two rounds of targeting were carried out to disrupt both the alleles of *Sall4* in ES cells.
*Two out of three clones in Experiment 2 and one clone in Experiment 3 were finally dominated by contaminating heterozygous cells.

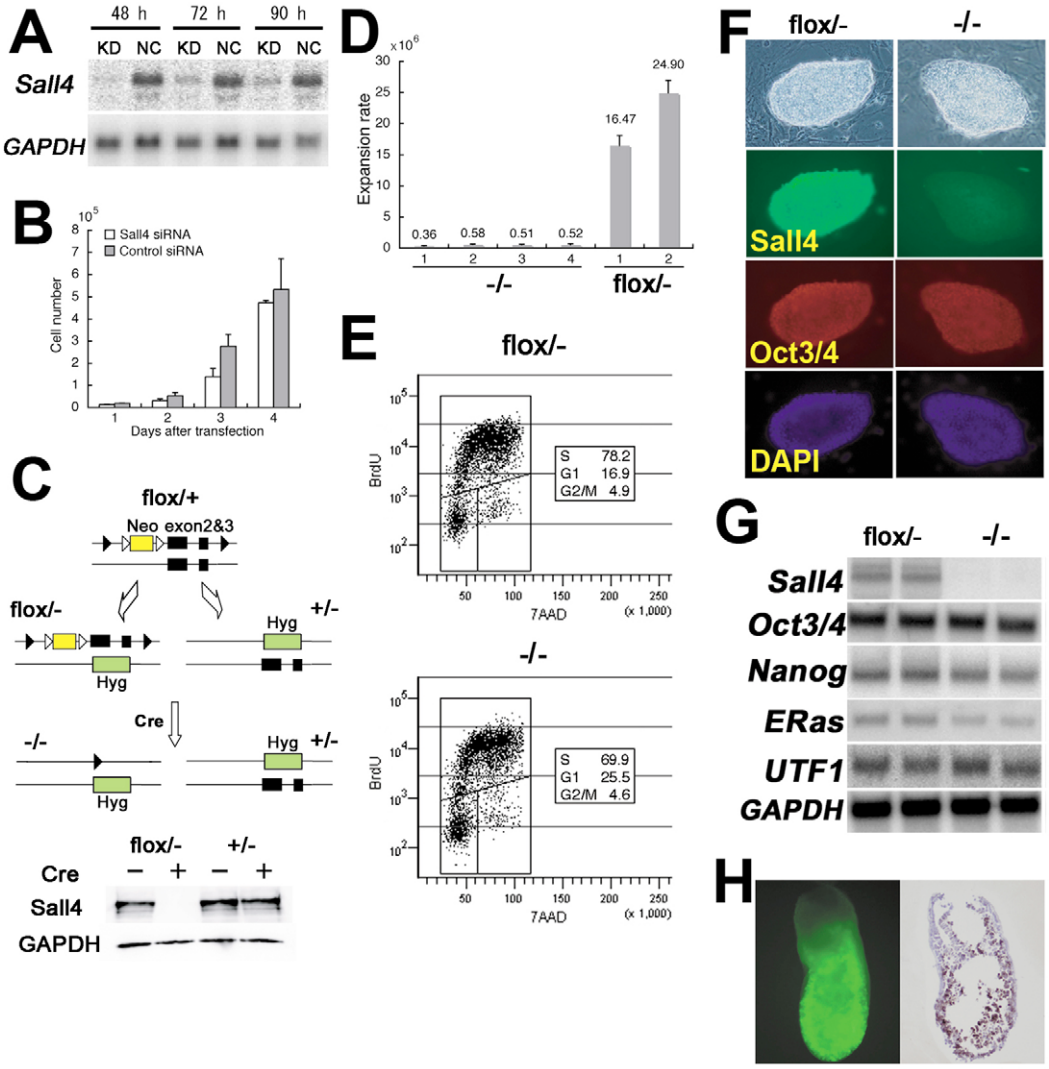

Fig. 3. Reduced proliferation of *Sall4*-null ES cells. (**A**) Northern blot analysis showing the reduction of *Sall4* upon Sall4-siRNA treatment. KD (knockdown), treated with Sall4-siRNA; NC (negative control), treated with control siRNA. (**B**) Transiently reduced proliferation of ES cells upon Sall4-siRNA treatment. Cells were counted in triplicate. (**C**) Conditional disruption of *Sall4* in ES cells. When a *Sall4-IRES-Hyg* vector was introduced into cells heterozygous for a floxed allele of *Sall4* (flox/+), both alleles were targeted with a similar frequency, resulting in two types of cells: flox/– and +/–. Upon infection with adenovirus expressing Cre, flox/– cells became almost *Sall4*-null by day 3, determined by western blot (lowest panels). White triangle, Frt; black triangle, loxP. (**D**) Reduced proliferation of *Sall4*-null ES cells. Cell expansion rate over 16 days is shown, using *Sall4*-null (–/–) versus flox/– cells obtained upon the same Cre treatment. Analysis was carried out in triplicate. (**E**) Reduced S phase and increased G1 phase in the *Sall4*-null ES cells. Consistent data were obtained from two independent experiments using three *Sall4*-null cells, and the representative data is shown. (**F**) Normal morphology and positive staining of Oct3/4 of a *Sall4*-null ES colony. (**G**) Northern blot analysis of *Sall4*-deficient ES cells. Two heterozygous (flox/–), and two *Sall4*-null ES (–/–) clones are shown. (**H**) Chimeric embryo formation from *Sall4*-null ES cells transfected with GFP. (left) High contribution of GFP-expressing cells in the E7.5 embryo. (Right) A section of the chimera was stained by an anti-GFP antibody and detected by DAB.

were picked, 44 out of 47 clones were *Sall4* null, and all these *Sall4*-null clones grew significantly slower than the clones retaining *Sall4* despite being treated identically (Fig. 3D), but could be cultured for a prolonged period of time (more than 1 month). None of the clones from the other groups (flox/– without Cre, +/– with or without Cre) showed impaired proliferation, and re-expression of *Sall4* cDNA under a ubiquitous promoter in *Sall4*-null cells restored proliferation (data not shown), confirming that this phenotype was *Sall4* dependent. Cell cycle analysis revealed that *Sall4*-null cells showed a decreased S-phase and increased G1-phase compared with heterozygous cells (Fig. 3E). These data suggest that *Sall4* absence in ES cells leads to inefficient G1/S transition, which may possibly explain the phenotypes observed in blastocyst culture and embryos in vivo.

These *Sall4*-null ES cells formed compact colonies and were morphologically indistinguishable from heterozygous or wild-type cells (Fig. 3F). No significant differences were observed in LIF-induced STAT3 phosphorylation or BMP4-induced Id family upregulation between wild-type and *Sall4*-null ES cells, suggesting that the absence of *Sall4* does not affect the response to the two major extrinsic signals involved in maintaining the pluripotency of ES cells (data not shown). In the *Sall4*-null ES cells, *Oct3/4* and *Nanog*, which are essential for pluripotency, and *Eras* and *Utf1*, which are important for the growth properties of the ES cells, continued to be expressed, which was confirmed by northern blot and immunostaining (Fig. 3F,G). Although expression of *Oct3/4* and *Utf1* was unaltered in the absence of *Sall4*, the expression of *Nanog* and *Eras* was slightly decreased, and this was detailed in the discussion. Expression of markers for primitive endoderm (*Gata6*), mesoderm (*T*), and trophectoderm (*Cdx2*) was not detected (data not shown), which suggests that the proliferation defect observed in the absence of Sall4 is not secondary to aberrant differentiation. Embryoid bodies were formed, though were smaller, from *Sall4*-null ES cells, and *Sall4*-deficient cells produced markedly smaller tumors than did heterozygous cells when transplanted into nude mice (data not shown), again suggesting that *Sall4* absence does not affect pluripotency of ES cells but proliferation. To further confirm that *Sall4*-null cells are undifferentiated, we injected these cells into

Table 3. Increased death rate of *Sall4* heterozygous mice after birth

Age	+/+	+/–
0-3 weeks	1	13
3-6 weeks	0	8
Total: dead/born (%)	1/80 (1.3)	21/86 (24.4)

Dead pups from the heterozygous crosses (described in Table 1) were scored for 6 weeks after birth.

blastocysts to generate chimeras. At E7.5, *Sall4*-null cells tagged with GFP contributed highly to the embryos (Fig. 3H). Taken together, our data indicate that Sall4 is essential for proliferation but not pluripotency of ES cells.

Sall4 haploinsufficiency results in anorectal and heart anomalies and exencephaly

Genotyping from heterozygous crosses showed that nearly half the *Sall4* heterozygous mice died in utero (Table 1). Out of 86 born heterozygous mice, 13 were runt and died or were eaten by their mothers within 3 weeks (Table 3). Eight more died within the next 3 weeks and six of these mice had significantly dilated bowels and apparent anal stenosis (Fig. 4A, open arrowhead). The number of enteric ganglia in these heterozygotes was not affected (data not shown); hence, gastrointestinal dilation is likely to be a secondary effect of anal dysplasia. When examined at E17.5-18.5, ventricular septum defects were also observed in some of the heterozygotes (Fig. 4B and Table 4). These phenotypes partially mimic the Okihiro syndrome caused by *SALL4* mutations in humans, and, thus, anorectal and heart anomalies could be caused by *SALL4* haploinsufficiency. Other phenotypes observed in Okihiro syndrome were not detected in *Sall4*-heterozygous mice. All the heterozygotes that survived beyond 3 weeks (n=73) had normal appearing extremities and closer examination of newborns did not show any anomalies in digit, metacarpus or metatarsus formation (n=6) (Fig. 4C). Though abducens nerves and nuclei are reported to be responsible for the abnormal eye movements in Okihiro syndrome (Al-Baradie et al., 2002; Kohlhase et al., 2002), the abducens nuclei in adult heterozygous mice (n=6) were properly formed, and oculomotor and trochlear nuclei, eye and ocular muscles were also unaffected (Fig. 4D and data not shown). Inner ear structures at E17.5 (n=9) and in adults (n=6) were not impaired (Fig. 4E; data not shown). Thus, in mice, not all Okihiro phenotypes were caused by Sall4 haploinsufficiency.

We also found other phenotypes not reported in humans. Some heterozygotes (three out of 26 examined at E11.5-15.5) exhibited exencephaly (Fig. 4F), and four out of 73 *Sall4* heterozygous mice that survived up to 3 weeks exhibited tail flexion anomalies (data not shown). As exencephaly and a kinked tail are caused by failure of the neural tube to close, *Sall4* may also play an important role in this event.

Sall4 genetically interacts with *Sall1*

Compound heterozygotes were generated to investigate functional redundancy among members of the *Sall* family. Surprisingly, no *Sall1/4* compound heterozygotes survived after birth, whereas mice having other genetic combinations (*Sall1/2*, *Sall1/3*, *Sall2/3*, *Sall2/4* and *Sall3/4*) survived. The *Sall1/4* heterozygotes exhibited uni- or bilateral renal agenesis (Fig. 5A), exencephaly (data not shown), anorectal malformations (Fig. 5B) and ventricular septum defects (data not shown); the incidence of these phenotypes was significantly increased in comparison with *Sall4* heterozygotes (Table 4), suggesting a genetic interaction of *Sall4* and *Sall1* in vivo. Next, we determined if the expression of *Sall4* and *Sall1* overlapped in the affected organs. At E8.5, a stage at which the neural tubes close, both *Sall4* and *Sall1* were expressed in the mesenchyme of the anterior portion (Fig. 5C, filled arrowhead) and in all tissues of the tail region (Fig. 5C, open arrowhead). At E11.5, *Sall4* and *Sall1* were expressed in the anorectal region (Fig. 5D, arrowhead). In the heart at E11.5, *Sall4* was detected in the myocardium, including the developing interventricular septum, while *Sall1* was expressed not only in the myocardium but also in the endocardium, thus overlapping with *Sall4* in the myocardium (Fig. 5E). We further checked to determine if Sall1 and Sall4 were colocalized in ES cells. Endogenous Sall4 was localized in the punctate nuclear foci that colocalized with 4,6-diamidino-2-phenylindole (DAPI) (Fig. 5F), indicating that Sall4 is localized in the constitutive heterochromatin. Endogenous Sall1 was also localized in the heterochromatin and overlapped with Sall4. Immunoprecipitation using lysates from ES cells showed that endogenous Sall4 binds to Sall1 (Fig. 5G). Therefore, these two genes probably form heterodimers in the developing brain, heart and anorectal regions.

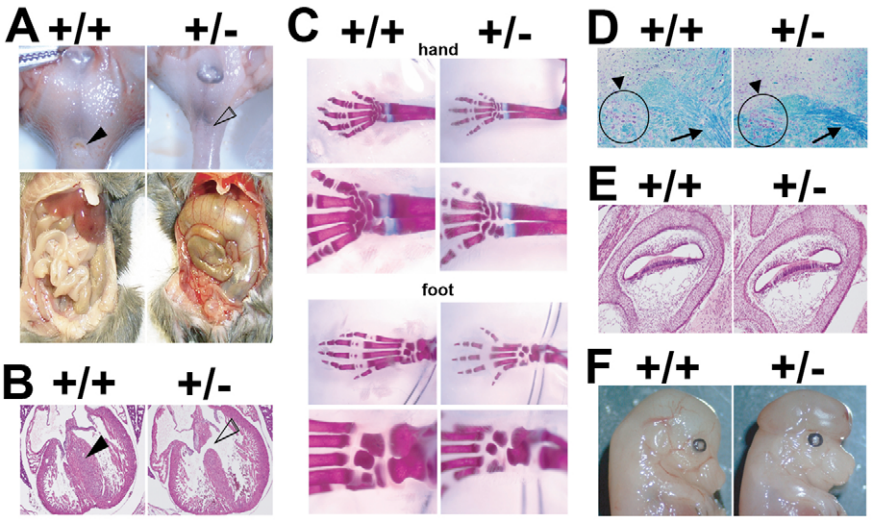

Fig. 4. Phenotypes caused by *Sall4* haploinsufficiency. (**A**) Anal stenosis (above) and megacolon (below) of 5-week-old *Sall4*-heterozygous mice. Black arrowhead, anus in wild type; open arrowhead, imperforate anus in the heterozygotes. (**B**) Ventricular septum defect in *Sall4* heterozygotes at E18.5. Black arrowhead, ventricular septum in wild-type; open arrowhead, ventricular septum defect in the heterozygotes. Hematoxylin and Eosin staining. (**C**) Normal formation of digits, metacarpus and metatarsus in new born *Sall4* heterozygotes. Stained with Alcian Blue and Alizarin Red S. (**D**) Normal formation of abducens nuclei (arrowhead) and facial nerve (arrow) in 8-week-old *Sall4* heterozygotes. Serial sections were examined by Klüver-Barrera staining. (**E**) Normal development of inner ear structure in *Sall4* heterozygotes at E17.5. Hematoxylin and Eosin staining. (**F**) Exencephaly of *Sall4*-heterozygous mice at E14.5.

Table 4. Phenotypic exacerbation in *Sall4/Sall1* double heterozygous mice

	Sall4+/–	*Sall1+/–*	*Sall4+/– Sall1+/–*
Renal agenesis*	0/43 (0%)	2/61 (3.3%)‡	16/38 (42.1%)§
Exencephaly*	2/43 (4.6%)	0/61 (0%)	17/38 (44.7%)
Anorectal malformations†	4/14 (28.6%)	0/14 (0%)	11/16 (68.8%)
Ventricular septum defects†	2/10 (20.0%)	0/10 (0%)	7/10 (70.0%)

*Examined at E13.5-P0.
†Examined at E17.5-18.5.
‡Two *Sall1* heterozygotes had unilateral kidney agenesis.
§Six out of 16 double heterozygotes had no kidneys bilaterally, and ten had unilateral kidney agenesis.

However, in the developing kidney, the two genes did not overlap (data not shown). Thus, heterodimer-independent mechanisms may exist in kidney development.

Truncated Sall1 disturbs heterochromatin localization of Sall4, and functions in a dominant-negative manner

When various *Sall4* and *Sall1* constructs with mutations in the zinc-finger domains were generated, we observed that the most C-terminal double zinc-finger domain (Zn4) of Sall4 was essential and sufficient for localization to the heterochromatin (Fig. 6A). In the case of Sall1, two double zinc fingers (Zn 4 and 5) were required, and were sufficient for heterochromatin localization (Fig. 6B). Thus, these C-terminal double zinc fingers constitute heterochromatin localization domains. *SALL1* mutations in Townes-Brocks syndrome are likely to produce C-terminally truncated proteins. Truncated *Sall1* fused to *DsRed* (*Sall1*$^{1\text{-}435}$*-DsRed*) was ubiquitously located throughout the cytoplasm and euchromatin (Fig. 6C), as this mutant lacked the C-terminal heterochromatin localization domain. Co-transfection of *Sall1*$^{1\text{-}435}$*-DsRed* and *Sall4-GFP* showed disturbance in Sall4 localization in the heterochromatin. Thus, the C-terminally truncated SALL1 proteins in Townes-Brocks syndrome probably dimerize with SALL4 in a dominant-negative manner, resulting in mislocalization of SALL4 in the heterochromatin. Considering the anal and heart anomalies and exencephaly in *Sall1/4* double heterozygotes, the phenotypes observed in Sall1 truncations could be explained by the functional reduction of Sall4.

DISCUSSION

In this study, we showed that mouse *Sall4* is essential for early embryogenesis and for proliferation of ES cells. We also provided both in vivo and in vitro evidence for the dimerization of Sall4 and Sall1 and revealed that the C-terminally truncated Sall1 in Townes-Brocks syndrome caused mislocalization of Sall4 in a dominant-negative manner. As human *SALL4* is a causative gene for the Okihiro syndrome, characterized by limb deformity and eye movement abnormalities, the indispensable role of mouse *Sall4* in early embryogenesis and proliferation of ES cells was entirely unexpected. Blastocyst culture analysis combined with the trophectoderm removal technique identified the importance of *Sall4* in the inner cell mass that

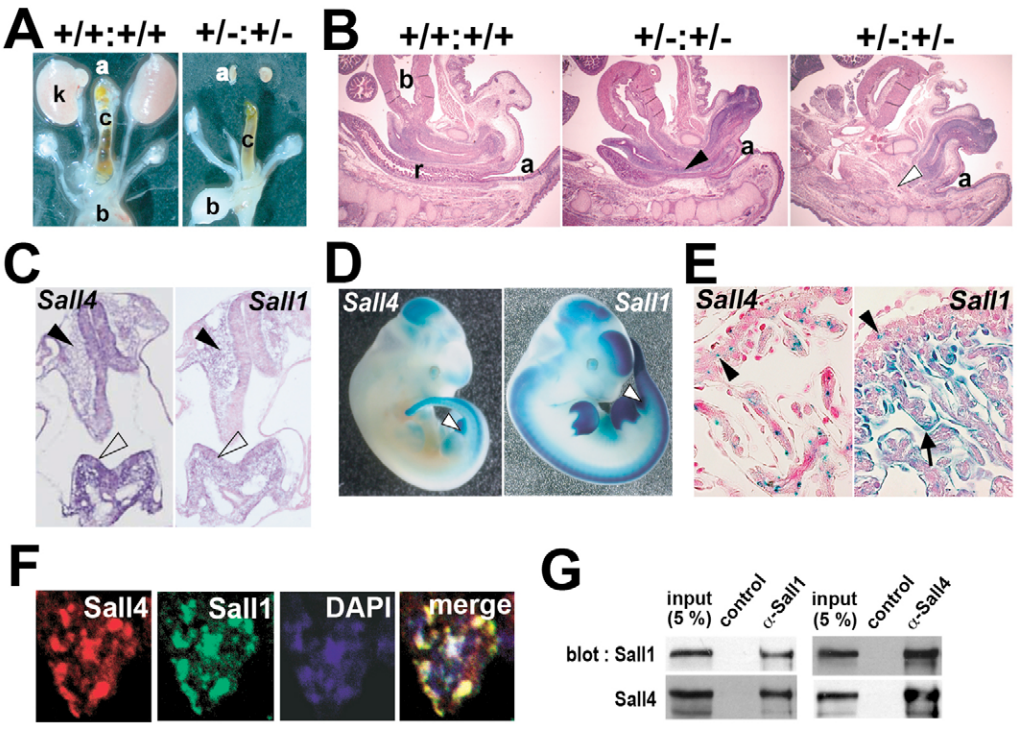

Fig. 5. Genetic interactions of *Sall4* and *Sall1*. (**A**) Bilateral renal agenesis in *Sall1/4* heterozygotes. Six out of the 38 compound heterozygotes analyzed had this phenotype, while 10 had unilateral agenesis. a, adrenal glands; b, urinary bladder; c, colon; k, kidney. (**B**) Anal stenosis in *Sall1/4* heterozygotes at E17.5 (right two panels). Black arrowhead shows complete stenosis of the rectoanal junction; white arrowhead shows absence of the rectum. a, anus; b, urinary bladder; r, rectum. (**C**) In situ hybridization of *Sall4* and *Sall1* at E8.5. The upper side is the anterior region of the embryo (transverse section). Black and white arrowheads indicate the mesenchyme and neuroepithelium, respectively. (**D**) *Sall4* and *Sall1* expression in the anorectal region at E11.5 (arrowheads). Heterozygotes of *Sall4-βgeo* and *Sall1-lacZ* (Nishinakamura et al., 2001) were stained using X-gal. (**E**) Overlap of *Sall4* and *Sall1* in the developing heart at E11.5. *Sall4* is expressed in myocardium (arrowhead), while *Sall1* is expressed in myocardium (arrowhead) and endocardium (arrow). *Sall4-βgeo* and *Sall1-lacZ* mice were stained using X-gal. (**F**) Immunocytochemistry of Sall4 and Sall1, and counterstaining with DAPI in ES cells. (**G**) Binding of Sall4 and Sall1 shown by immunoprecipitation using ES cell lysates.

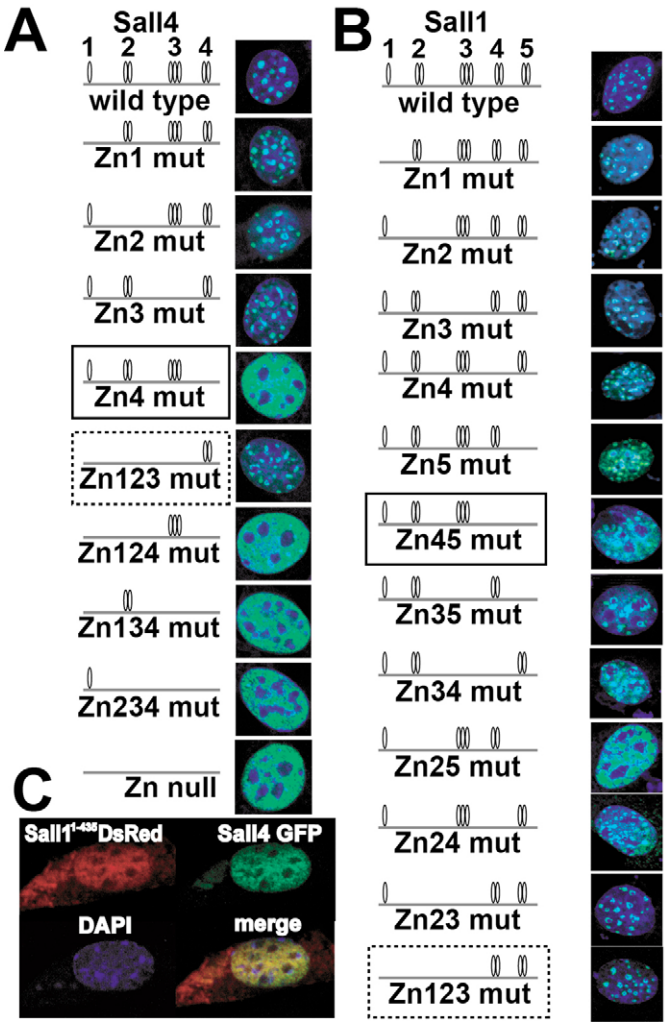

Fig. 6. Mislocalization of Sall4 from the heterochromatin by a truncated Sall1 that functions in a dominant-negative manner. (**A**) Requirement of the C-terminal zinc finger (Zn4) of Sall4 for heterochromatin localization (rectangle). Zn4 is also sufficient for heterochromatin localization (broken outline). Mutants of Sall4-GFP fusion were expressed in NIH 3T3 cells. Zinc-finger clusters are categorized as Zn1, Zn2, Zn3 and Zn4, as shown. (**B**) Requirement of the C-terminal zinc fingers (Zn4 and Zn5) of Sall1 for heterochromatin localization (rectangle). Zn4 and Zn5 are also sufficient for heterochromatin localization (broken outline). Mutations in Zn2 and Zn5 also show a defect in heterochromatin localization, though these two clusters are not sufficient for proper localization in the heterochromatin. (**C**) Co-transfection of truncated *Sall1* (*Sall1*$^{1\text{-}435}$-*DsRed*) and *Sall4-GFP* into NIH 3T3 cells. Heterochromatin localization of Sall4 is disrupted by C-terminally truncated Sall1.

is the origin of the embryo proper. As ES cells are derived from the inner cell mass, it is understandable that *Sall4*-null ES cells showed significantly reduced proliferation, which may possibly explain the phenotypes observed in blastocyst culture and embryos in vivo.

How can Sall4 regulate the proliferation of ES cells? As Sall1 is localized in the heterochromatin and binds to the components of chromatin remodeling complexes [namely HDAC1, HDAC2, RbAp46/48, MTA1 and MTA2 (Kiefer et al., 2002)], Sall4 could also bind HDAC complexes and deacetylate the histone lysine residues, followed by recruitment of histone-methylating and DNA-methylating complexes. Centromeric and pericentromeric regions, which comprise constitutive heterochromatin, are required for spindle formation in mitosis, and it is possible that the absence of Sall4 affects these structures. Alternatively, Sall4 could suppress downstream genes, including cell cycle inhibitors, by inducing heterochromatin formation in the euchromatic promoters (known as facultative heterochromatin formation). More detailed examination in the heterochromatin structure in *Sall4* mutant ES cells, as well as identification of downstream target genes of Sall4, is required for further elucidation of Sall4 functions. It is interesting that *Nanog* and *Eras* were slightly decreased in *Sall4*-deficient cells. *Nanog* heterozygous cells show no reduction in proliferation, and *Nanog*-deficient cells differentiate into primitive endoderm (Mitsui et al., 2003). Though *Eras* could be partly responsible for impaired proliferation of *Sall4*-null ES cells, *Eras*-null mice had no abnormalities in vivo (Takahashi et al., 2003). Thus, it is unlikely that a reduction of these genes could explain the phenotypes of *Sall4* deficiency. As ES cells are suitable for quantitative biochemical approaches, we expect to apply the detailed analysis of Sall4 function in ES cells to the study of organ formation during later stages of development.

We demonstrated that Sall4 and Sall1 form heterodimers and that truncated Sall1 proteins altered Sall4 localization in a dominant-negative manner. Townes-Brocks syndrome caused by *SALL1* mutations exhibits limb, anal, ear, kidney and heart anomalies, and mice retaining truncated Sall1 proteins show similar phenotypes as well as exencephaly. As human families with severe phenotypes are unlikely to survive, exencephaly could be one of the phenotypes of SALL1 truncations. Considering the anal and heart anomalies and exencephaly in *Sall1/4* double heterozygotes, at least these three phenotypes observed in Sall1 truncations can be explained by the functional reduction of Sall4. Thus, we propose that some symptoms of Townes-Brocks syndrome caused by SALL1 truncations result from the inhibition of SALL4 functions that is due to heterodimer formation.

SALL4 mutations in humans cause the autosomal dominant disorder Okihiro syndrome, which is characterized by limb deformity, eye movement (abducens nerve) abnormalities, and anorectal, ear, heart, and kidney anomalies. Some *Sall4* heterozygous mice had anal and heart anomalies, suggesting that these phenotypes are caused by *Sall4* haploinsufficiency. This is important because most phenotypes in Townes-Brocks syndrome could be caused by the dominant-negative effect of the truncated Sall1, but not by haploinsufficiency of Sall1. However, other anomalies to limbs, abducens nuclei, inner ears and kidneys were not detected in *Sall4* heterozygous mice; thus, these phenotypes are not explained by *Sall4* haploinsufficiency in mice. In humans, C-terminally truncated SALL4 proteins could function in a dominant-negative manner, as is the case for SALL1 truncations. Recently, a gene trap allele retaining truncated *Sall4* was reported and this strain exhibited digit and heart anomalies (Koshiba-Takeuchi et al., 2006). If this allele serves as a dominant-negative form, it is possible that this trap allele has more severe phenotypes than ours, although other phenotypes are not described. Direct comparison of the two mouse strains on the same genetic background would test this possibility.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/15/3005/DC1

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