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Sall1, a causative gene for Townes–Brocks syndrome, enhances the canonical Wnt signaling by localizing to heterochromatin

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Abstract

The *Spalt* (*sal*) gene family plays an important role in regulating developmental processes of many organisms. Mutations of human *SALL1* cause the autosomal dominant disorder, Townes–Brocks syndrome (TBS), and result in ear, limb, anal, renal, and heart anomalies. Targeted deletion of mouse *Sall1* results in kidney agenesis or severe dysgenesis. Molecular mechanisms of *Sall1*, however, have remained largely unknown. Here we report that Sall1 synergistically activates canonical Wnt signaling. The transcriptional activity of Sall1 is related to its nuclear localization to punctate nuclear foci (pericentromeric heterochromatin), but not to its localization or association with β-catenin, the nuclear component of Wnt signaling. In contrast, the RNA interference of Sall1 reduces reporter activities of canonical Wnt signaling. The N-terminal truncated Sall1, produced by mutations often found in TBS, disturbs localization of native Sall1 to heterochromatin, and also down-regulates the synergistic transcriptional enhancement for Wnt signal by native Sall1. Thus, we propose a new mechanism for Wnt signaling activation, that is the heterochromatin localization of Sall1.

The *Spalt* (*sal*) gene family plays important roles in regulating developmental processes of many organisms. In *Drosophila* development, *sal* is a region-specific homeotic gene, which specifies cell fate decisions of chordontonal precursors in the peripheral nervous system [1,2], regulates tracheal development [3], controls terminal differentiation of photoreceptors [4], and determines proper placement of wing veins [5,6].

Humans have four *sal* related genes (*SALL1*, *SALL2*, *SALL3*, and *SALL4*) and mice also have four (*Sall1*, *Sall2*, *Sall3*, and *Sall4*) [7–15]. Heterozygous mutations of human *SALL1* lead to Townes–Brocks syndrome, with features of dysplastic ears, preaxial polydactyly, imperforate anus, and (less commonly) kidney and heart anomalies [16]. With homozygous deletion in mice the kidney had severe defects which meant that *Sall1* has an essential role in kidney development [17]. The molecular mechanisms of Sall1 have remained obscure.

Sall1 encodes a protein that contains 10 zinc finger motifs. The most N-terminal zinc finger is a single C2HCtype and is conserved only in vertebrates (*Drosophila sal* does not have the N-terminal C2HC zinc finger) [18]. The other zinc fingers are of the C2H2-type and are arranged as doublets with a third finger associated with the second pair. Recently, it was reported that Sall1 functions as a transcriptional repressor, by being localized to pericentromeric heterochromatin and is associated with histone deacetylase (HDAC) complex [19,20]. When linked to a heterologous DNA-binding

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domain (GAL4 DNA-binding domain), Sall1 protein is capable of repressing transcription on the synthetic reporter containing tandem GAL4-binding sites upstream of a promoter. The native DNA-binding site and direct target genes regulated by Sall1 proteins have remained to be determined.

In *Drosophila* wing development, *sal* is activated downstream of *dpp* (bone morphogenetic protein 4 ortholog) and in tracheal development is activated downstream of *wingless* (Wnt ortholog) [5,21–23]. We did research to determine if mammalian Sall1 is involved in signaling pathways related to development. We report here that Sall1 synergistically enhanced reporter activities of the canonical Wnt signal, by localizing to heterochromatin. Our evidence indicates that this mechanism may be related to the cause of TBS.

Materials and methods

Plasmids. The complete coding *Sall1* cDNA was fused to the HA-tag by replacing the PCR fragment amplified using the primer containing HA-tagged sequence and the *Sal*I restriction enzyme site (f, GTCGACA CCATGTACCCATACGACGTCCCAGACTACGCTTCGCGGAG GAAGCAAGCG; r, GAGCAGAAGGTCTGATAATTC). The *Sal*I–*Not*I digested HA-tagged *Sall1* cDNA fragment was cloned into the *Xho*I–*Not*I sites of pCAGEN, a mammalian expression vector driven by the CAG promoter [24,25].

Sall1 truncated forms were generated as follows; zinc finger region 1 (Zn 1) encoding 1–288 amino acids (*Sal*I-*Sac*I fragment) was inserted into pCAGEN vector, zinc finger region 2 (Zn 2) encoding 289–598 amino acids (*Sac*I–*Apa*I fragment), zinc finger region 3 (Zn 3) encoding 599–857 amino acids (*Apa*I–*Xho*I fragment), zinc finger region 4 (Zn 4) encoding 858–1105 amino acids (*Xho*I–*Spe*I fragment), and zinc finger region 5 (Zn 5) encoding 1106–1324 amino acids (*Spe*I–*Not*I fragment) were inserted into appropriate restriction sites in pCMV-HA vector (Clontech). The N-terminal half form of HA-tagged Sall1 encoding 1–598 amino acids (Zn1-2) was inserted into the pCAGEN vector, and the C-terminal half form encoding 599–1324 amino acids (Zn 3–5) inserted into the pCMV-HA vector.

The Sall1-GFP fusion was generated by inserting the d2EGFP fragment from pd2EGFP-1 vector (Clontech) at the 3′ terminal region of Sall1 in pBluescript II KS (−) vector in-frame. *Sal*I–*Not*I fragment of the Sall1-d2EGFP fusion was excised and inserted into the pCAGEN vector. To generate GFP-fused Sall1 truncated forms, the pCMV-HA-NLS-d2GFP vector was constructed. The PCR fragment coding a single nuclear localization signal from SV40 was inserted into the pCMV-HA vector (pCMV-HA-NLS vector) and then the fragment coding d2EGFP following intact multiple cloning sites excised from pd2EGFP-1 vector was inserted and the pCMV-HA-NLS-d2EGFP vector was generated. Sall1 truncated forms fused to d2EGFP were generated as follows; zinc finger region 1 encoding 1–288 amino acids (*Sal*I–*Sac*I fragment), zinc finger region 1–2 encoding 1–598 amino acids (*Sac*I–*Apa*I fragment), and zinc finger region 3–5 encoding 599–1324 amino acids (*Spe*I–*Not*I fragment) were inserted into appropriate restriction sites in pCMV-HA-NLS-d2EGFP vector (Clontech) in-frame. The cDNA fragment of zinc finger region 1′ (Zn 1′) encoding 1–435 amino acids was generated by the combination of the *Sal*I–*Sac*I fragment encoding 1–288 amino acids and the fragment encoding 299–435 amino acids that was amplified using primers containing *Sma*I restriction enzyme site in the reverse one (f, ATTAGC ACAGAGCCTTGCTAGC; r, CCCGGGGGACATTTGG TGGCTTGCTTTTTC). The combined cDNA fragment was inserted into the pCMV-HA-NLS-d2EGFP vector in-frame. The cDNA encoding zinc finger region 1′ (1–435 amino acids) without NLS was generated by combination of the *Sal*I–*Sac*I fragment encoding 1–288 amino acids and the fragment encoding 299–435 amino acids fused to d2EGFP, which was excised from the pCMV-HA-NLS-Zn 1′-d2EGFP vector, as described above, and inserted into appropriate sites in the pCAGEN vector. To generate the Zn 1′-DsRed2 fusion, the *Age*I–*Xho*I DsRed2 fragment without NLS was excised from pDsRed2-Nuc vector (Clontech), and inserted into the pCAGEN vector with the *Sal*I–*Age*I Zn 1′ fragment excised from the pCMV-HA-NLS-Zn 1′-d2EGFP vector.

The fragment coding the complete β-catenin cDNA was excised from pBJ-myc-β-catenin by *Bam*HI [26] and inserted into appropriate sites in the pCMV-myc vector (Clontech).

Protein interaction assay. BOSC23 cells were transiently transfected with 4 μg pCAGEN-HA-Sall1 or each pCMV-HA-Sall1 truncated form and 3 μg pCMV-myc-β-catenin. After 48 h, cells were washed with phosphate-buffered saline, lysed for 10 min on ice with 600 μl buffer A (10 mM Hepes–KOH, pH 7.8, 10 mM KCl, 1.5 mM $MgCl_2$, 0.05% NP-40, 0.5 mM DTT, and 10% v/v protease inhibitor cocktail for mammalian cell extract (SIGMA)), and spun at 2300*g* for 1 min. The supernatant was discarded and then the pellet was suspended in 300 μl buffer C (20 mM Hepes–KOH, pH 7.8, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 10% v/v protease inhibitor cocktail for mammalian cell extract), rotated at 4 °C for 30 min and then spun at 20,000*g* for 30 min. The supernatant was diluted with an equal volume of buffer C containing 50% glycerol.

For the immunoprecipitation assay, the extract was diluted with an equal volume of buffer D (20 mM Hepes–KOH, pH 7.8, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 10% v/v protease inhibitor cocktail for mammalian cell extract), twice the volume of the dilution buffer (25 mM Hepes–KOH, pH7.8, 2.5 mM EDTA, 0.1% NP-40, and 10% v/v protease inhibitor cocktail for mammalian cell extract) in a 1.5 ml micro tube and then incubated on ice for 10 min. After removal of particulate cell debris by centrifugation at 20,000*g* for 5 min, the supernatant was incubated overnight with 2 μg of anti-HA high affinity (Roche) at 4 °C. HA-tagged protein and its interacting proteins were isolated by precipitation with protein G–Sepharose beads (Amersham–Pharmacia) for 3 h at 4 °C. The beads were washed three times with wash buffer (20 mM Hepes–KOH, pH 7.8, 375 mM NaCl, 1 mM $ZnCl_2$, 1 mM EDTA, 0.5 mM DTT, 1% NP-40, 10% glycerol, and 1 mM PMSF) and eluted by boiling in Laemmli sample buffer. HA-tagged protein and its interacting proteins were separated by SDS–polyacrylamide gel electrophoresis on 8% gels, transferred to Immobilon Transfer Membranes (Millipore), blocked in 3% nonfat dried milk, and incubated with anti-HA antibody (Roche) or anti-myc antibody (SC-40, Santa Cruz Biotechnology). Antibody reactivity was detected using horseradish peroxidase-labeled secondary antibodies anti-mouse (KPL) and horseradish peroxidase-labeled secondary antibodies anti-rat (KPL) and ECL detection reagents (Amersham–Pharmacia).

Monoclonal antibody for human SALL1. To generate a monoclonal antibody for human SALL1, we cloned the cDNA fragment of human SALL1 encoding 258–499 amino acids amplified using primers containing *Kpn*I restriction site at the 5′ end (f, GGTACCCGCTTCT CAGAATGCAGACTTG; r, GGTACCTTGTGTTTGAAGAATGC CTC). The PCR fragment was cloned into the *Kpn*I sites of pBACsurf-1, which is a baculovirus transfer vector designed for expression of target proteins on the virion surface. Recombinant virus was produced, purified, and then immunized [27]. Monoclonal antibody for human SALL1 was obtained after screening by immunoblotting using the extract from HEK293 cells. These selected clones were cross-reactive for both human SALL1 and mouse Sall1 proteins.

Cell culture, transfection, and RNAi. NIH-3T3 cells and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (SIGMA) containing 10% fetal bovine serum. For reporter assay, cells were plated in a 6-well plate at a density of 1×10^5 cells per well. For transfection of plasmids, FuGENE6 (Roche Molecular Biochemicals) was used according to the manufacturer's direction. For transfection

of double-stranded (ds) RNA oligos, LIPOFECTAMINE 2000 (Invitrogen) was used according to the manufacturer's direction. Five ds RNAi oligos for SALL1 were designed and synthesized by DHARMACON. Sequences of these oligos are as follows:

No. 6 (5′-AAGGUCUUUGGGAGUGACAGU-3′),
No. 7 (5′-AAGAGAAAUACCCUCAUAUCC-3′),
No. 15 (5′-AAUGAUUCAUCCUCAGUGGGU-3′),
No. 18 (5′-AAGGGUAAUUUGAAGCAGCAC-3′),
No. 21 (5′-AAGUUCCCAGAAAUGUUCCAG-3′).

The probe used for Northern blots was the *Kpn*I fragment of human SALL1 cDNA, excised from the pBACsurf-human SALL1 vector described above. For Western blotting, we used a monoclonal antibody for human SALL1 as described and a monoclonal antibody for GAPDH (Ambion).

Reporter assay. An internal control reporter pRLTKmini, which has the minimal thymidine kinase promoter, was constructed by removing the *Bgl*II–*Eco*RI fragment from the promoter region of pRLTK (Promega). TOPflash (Upstate Biotechnology) is a luciferase reporter containing three copies T cell factor (TCF) binding sites upstream of the thymidine kinase minimal promoter and FOPflash (Upstate Biotechnology) is the negative control for TOPflash containing mutant TCF binding sites. In the reporter assay using Wnt supernatant, 0.5 μg TOPflash or FOPflash reporter plasmid, 0.05 μg pRLTKmini control reporter plasmid were transiently introduced with 1.0 μg Sall1 or Sall1 truncated mutant expression plasmids in NIH3T3 cells. In the reporter assay using HEK293 cells, the same amounts of reporters were introduced with 0.1 μg pCAGEN-HA-Sall1. In the reporter assay using the N-terminal truncated Sall1 (Zn 1′) plasmid, 0.1–1.0 μg of this plasmid was transiently introduced with 0.1 μg pCAGEN-HA-Sall1 in HEK293 cells. After 24 h, the transfected cells were stimulated by threefold-diluted Wnt supernatants, produced from Wnt3a overexpressing L cells (ATCC) [28]. After 48 h, the transfected cells were lysed with 150 μl lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.1% NP-40), frozen and thawed three times, and then spun at 20,000*g* for 10 min. The supernatant was then assayed using Dual-Luciferase Reporter Assay System (Promega). Luminescent reporter activity was measured using LUMAT LB 9507 luminometer (EG&G BERTHOLD). In the reporter assay using the β-catenin expression vector, 0.3 μg pCMV-myc-β-catenin with 0.1–1.0 μg pCAGEN-HA-Sall1 was transiently introduced into NIH3T3 cells. In HEK293 cells, 0.01 μg pCMV-myc-β-catenin with 0.01–0.1 μg pCAGEN-HA-Sall1 was used. Forty-eight hours after transfections, cells were used in the same way as described above. In the reporter assay combined with RNA interference, the final concentration 20 μM of each si RNA oligo was transfected in Opti-MEM (Invitrogen) as the serum free condition using LIPOFECTAMINE 2000 (Invitrogen). After 6 h, the serum free medium was replaced with Dulbecco's modified Eagle's medium (SIGMA) containing 10% fetal bovine serum, then reporter plasmids were transfected in the same way as described above. In all reporter assays, EGFP expression plasmid, pCMV-EGFP, was used to normalize the DNA content of the transfection. All transfections were normalized to *Renilla* luciferase activity and were replicated. All reporter assays were repeated at least three times and representative data are shown.

Analysis of protein localization by confocal microscopy and immunocytochemistry. Total 1.0 μg of Sall1-GFP fusion plasmid or Sall1 mutant GFP fusion plasmids or β-catenin plasmid was transiently introduced into NIH3T3 cells plated in Lab-Tek II Chamber Slide w/ Cover RS Glass Slide (Nalge Nunc International). Twenty-four hours after transfection, cells were fixed in phosphate-buffered saline (PBS) containing 2% paraformaldehyde, 0.1% Triton X-100, and 2 μg/ml of 4,6-diamidino-2-phenylindole (DAPI) at 4 °C for 20 min, washed for 5 min in PBS three times at room temperature, and then blocked with 10% goat serum at room temperature for 30 min. Cells were then incubated for 1 h at room temperature with an anti-myc antibody diluted in PBS containing 1% goat serum (1:1000), and detected using a rhodamine-conjugated secondary antibody anti-mouse (CHEMICON). The localization of proteins was detected using confocal microscopy Radiance 2100 (Bio-Rad).

Results

Sall1 has the potential to activate the canonical Wnt signaling

As the expression of *spalt* is regulated by several signaling pathways (*dpp*/BMP, *wingless*/Wnt, etc.) in *Drosophila* development, luciferase assays were done using several reporters to examine the potential involvement of mammalian Sall1. In following experiments, we selected and used two cell lines; NIH3T3 cells and HEK293 cells. Endogenous Sall1 was detected in HEK293 cells, in RNA and protein levels, but not in NIH3T3 cells (data not shown). Among reporters tested (BMP, TGF-β, retinoic acid, LIF, and Wnt) [25,29–32], the Wnt responsive reporter consistently showed a synergistic response to Sall1 (Figs. 1A and B) in both cell lines. Sall1 expression vector was introduced with the Wnt responsive reporter (TOPflash) that contains multiple TCF binding sites or the control reporter (FOPflash), and these cells were stimulated by the supernatant from L cells stably expressing Wnt-3A. Sall1 alone only weakly activated the TOPflash reporter in both cell lines. In the presence of Wnt stimulation, however, Sall1 synergistically activated the TOPflash reporter in both cell lines, but not so the control FOPflash reporter. Therefore, Sall1 synergistically activates the canonical Wnt signal. In these settings, activation status of β-catenin was not altered as determined by Western blotting, ruling out the possibility of a secondary production of Wnt ligands by Sall1 or Wnt stimulation upstream of β-catenin (data not shown).

When the canonical Wnt pathway is activated, β-catenin avoids the ubiquitin–proteosome pathway following the phosphorylation by GSK3-β and accumulates in the cytoplasm to move into the nucleus and function as the transcriptional coactivator of the TCF/LEF transcription factor [33]. In NIH3T3 cells, expression of β-catenin alone activated gene expression on the TOPflash reporter only slightly, but co-expression of Sall1 and β-catenin synergistically increased its reporter activity in dependent manner regarding the amount of Sall1 (Fig. 1C). In HEK293 cells, Sall1 also enhanced the luciferase reporter by β-catenin (Fig. 1D). Sall1 by itself could not bind to TCF binding sites in the TOPflash reporter, as determined by an electromobility shift assay using nuclear extracts from Sall1-introduced HEK293 cells (data not shown). Therefore, Sall1 may possibly function as a coactivator for β-catenin in the canonical Wnt signaling.

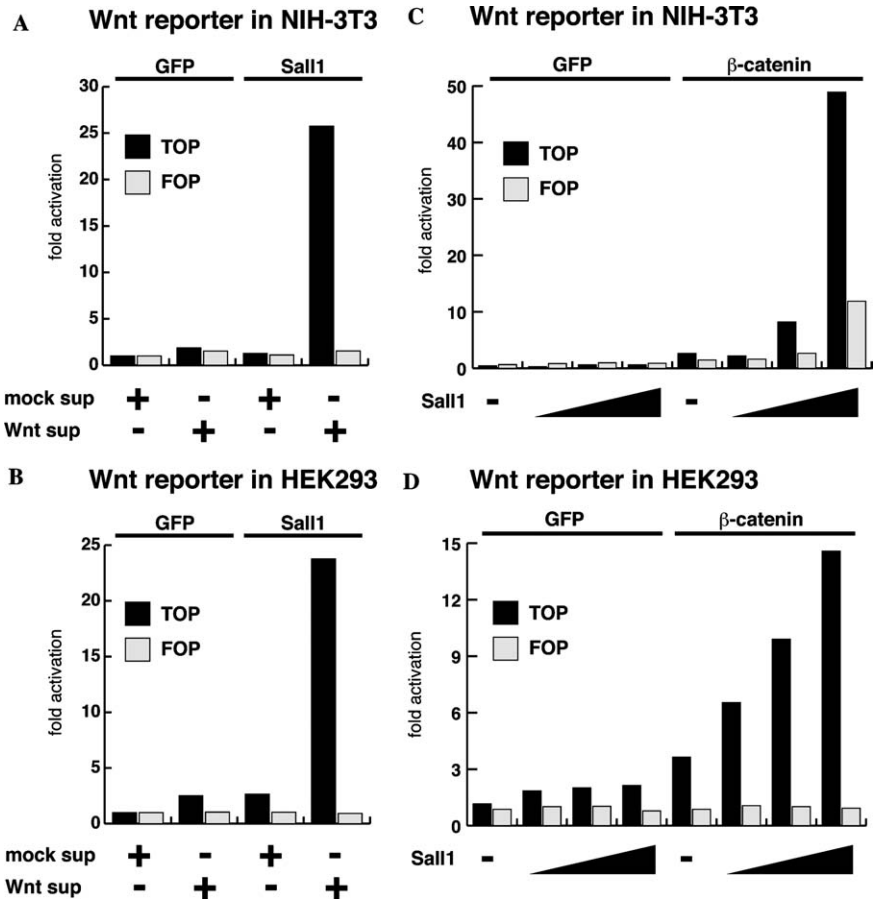


Fig. 1. Sall1 synergistically enhanced canonical Wnt signaling pathway. (A,B) The reporter assay responsive to Wnt signaling. TOPflash as the reporter plasmid responsive to Wnt signaling, FOPflash as negative control were introduced into NIH-3T3 cells (A), HEK293 cells (B). These cells were stimulated by the Wnt supernatant (Wnt sup) from L cell stably expressing Wnt-3A or the mock supernatant (mock sup) from normal L cells. (C,D) TOPflash reporter assay by expression of β-catenin, with or without Sall1. pCMV-myc-β-catenin (0.3 μg) (the β-catenin expression vector) and 0.1–1.0 μg of pCAGEN-HASall1 were introduced in NIH-3T3 cells (C). pCMV-myc-β-catenin (0.01 μg) (the β-catenin expression vector) and 0.01–0.1 μg of pCAGEN-HASall1 were introduced in HEK293 cells (D). In all reporter assays, pCMV-EGFP (the control vector) was used to normalize the DNA content of the transfection, and pRLTKmini was used as the internal control reporter plasmid.

Endogenous Sall1 participates in Wnt signaling in HEK293 cells

To determine if Sall1 endogenously participates in canonical Wnt signaling, we depleted the endogenous human SALL1 protein in HEK293 cells via double-strand RNA (siRNA)-mediated interference and did reporter assays. We designed five kinds of ds RNAi oligos for human SALL1 (Nos. 6, 7, 15, 18, and 21), and assessed their potential to deplete endogenous SALL1 mRNA, using Northern blot analysis (Fig. 2A). Oligo No. 18 most effectively, and Nos. 6 and 7 weakly, reduced endogenous SALL1 mRNA in HEK293 cells, but No. 21 had no effect (Fig. 2A). Therefore, we selected No. 21 as a negative control in following experiments, as it did not reduce the amount of either SALL1 or GAPDH (Figs. 2B and C). When used in reporter assays, oligo No. 18 most effectively, oligo No. 6 less effectively, down-regulated the activity on the TOPflash reporter, in proportion to their efficiency to reduce endogenous SALL1 mRNA, while a negative control, No. 21, did not do so (Fig. 2D). Therefore, endogenous SALL1 also participates in canonical Wnt signaling, at least in HEK293 cells.

Sall1 interacts with β-catenin

We next did immunoprecipitation studies to examine the interaction between Sall1 and β-catenin. Nuclear extracts were prepared from BOSC23 cells, transiently introduced with both HA-tagged Sall1 and myc-tagged β-catenin expression vectors. In these extracts, Sall1 effectively interacted with β-catenin (Fig. 3B). To further determine the domains of Sall1 required for interactions with β-catenin, we also did immunoprecipitation studies using deletion mutants of Sall1 with β-catenin. Sall1 has a total of 10 zinc fingers including multiple double-zinc finger motifs and we constructed five truncated mutants

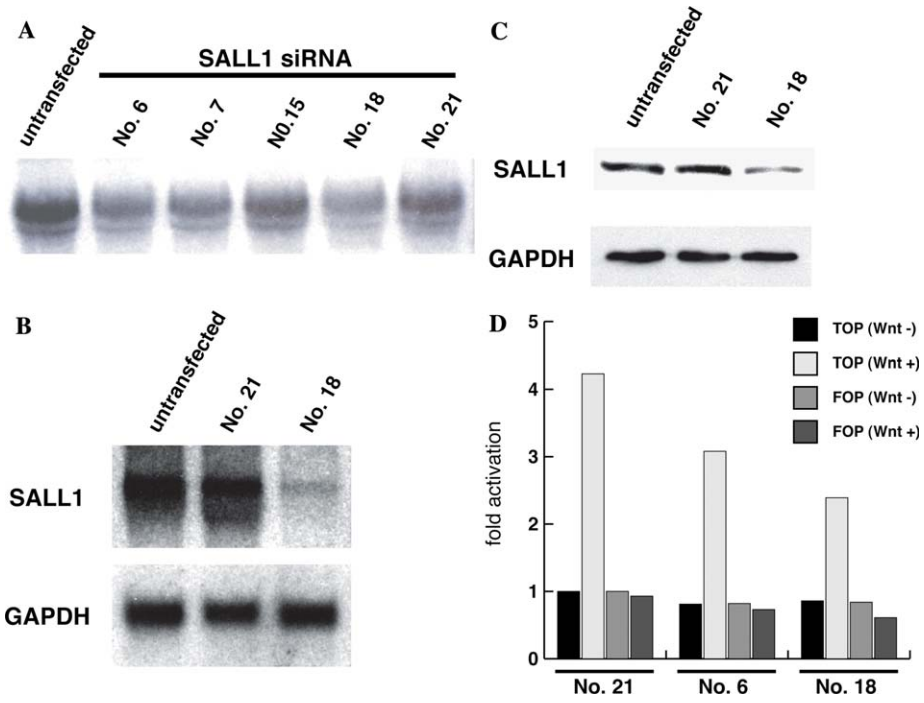


Fig. 2. RNA interference for endogenous Sall1 specifically down-regulates the TOPflash activity in HEK293 cells. (A,B) Northern analysis for assessing the efficiency of five designed siRNA oligos for human SALL1 to reduce the amount of endogenous SALL1 mRNA. (C) Western analysis for assessing the efficiency of SALL1 siRNA oligos to reduce the amount of endogenous SALL1 protein. (D) The reporter assay using SALL1 siRNA oligos in HEK293 cells. siRNA oligo, No. 18 most efficiently, and No. 6 less efficiently, down-regulated the reporter activity on TOPflash reporter in proportion to its efficiency to reduce endogenous SALL1 mRNA, while No. 21 had no effect.

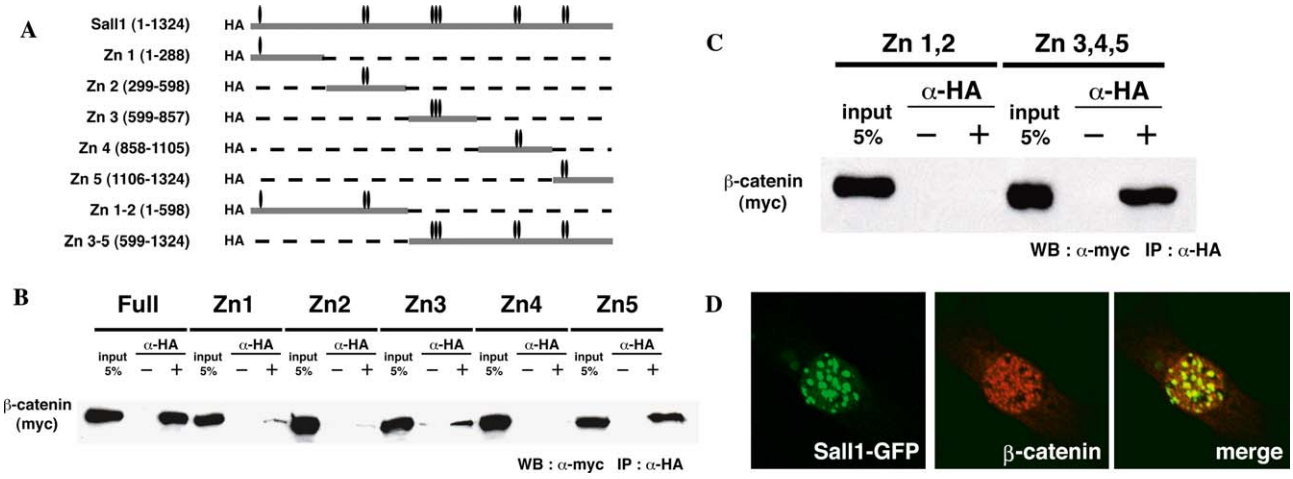
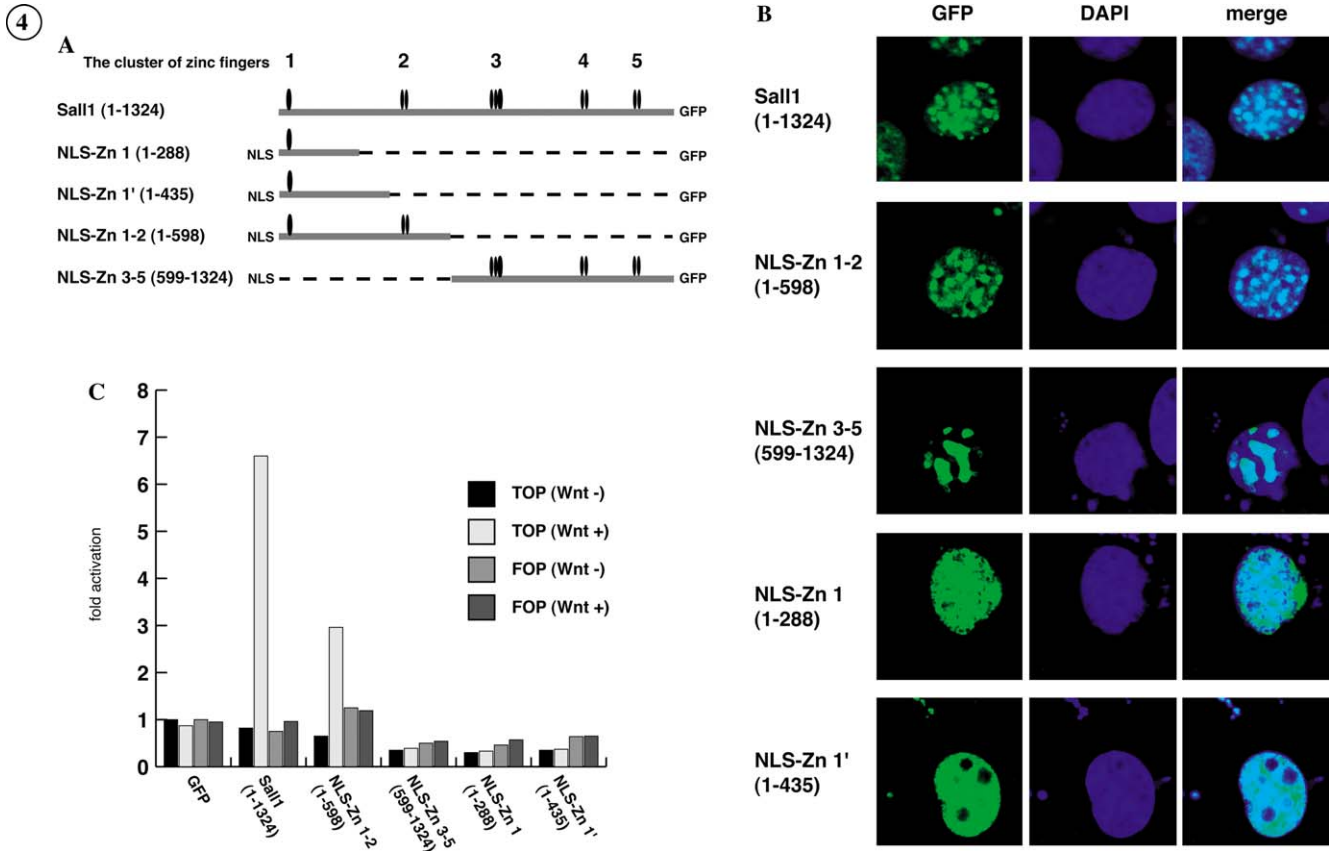


Fig. 3. Localization of Sall1, which has the potential to associate with β-catenin, does not overlap with those of β-catenin in the nucleus. (A) Diagram of HA-tagged full-length Sall1 and its deletion mutants used in protein interaction assays. Positions of the zinc fingers are depicted as ovals. The numbers in parentheses indicate the number of coding amino acids. (B,C) Protein interaction assay by immunoprecipitation using nuclear extracts from BOSC23 cells, transiently introduced with myc-β-catenin and HA-Sall1 or its mutants. Immunoprecipitation was done using an anti-HA antibody and detected using an anti-myc antibody. (D) The localization of Sall1 in the nucleus did not overlap with that of β-catenin. NIH3T3 cells on glass coverslips were transfected with full-length Sall1-GFP and myc-tagged β-catenin, stained with anti-myc. Cells were viewed using confocal microscopy.

encoding clusters of each zinc finger motif (Zn 1, 2, 3, 4, and 5) (Fig. 3A). The immunoprecipitation was done using lysates from BOSC23 cells introduced with these truncated mutants and β-catenin. The truncated form, encoding the most C-terminal double-zinc finger (Zn 5), had the highest affinity with β-catenin, and the form encoding the triple Zn finger (Zn 3) had a lesser affinity for β-catenin (Fig. 3B). To confirm these results, we


4
A
The cluster of zinc fingers 1 2 3 4 5
Sall1 (1-1324)
NLS-Zn 1 (1-288)
NLS-Zn 1' (1-435)
NLS-Zn 1-2 (1-598)
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NLS
GFP
B
GFP
DAPI
merge
Sall1 (1-1324)
NLS-Zn 1-2 (1-598)
NLS-Zn 3-5 (599-1324)
NLS-Zn 1 (1-288)
NLS-Zn 1' (1-435)
C
fold activation
TOP (Wnt -)
TOP (Wnt +)
FOP (Wnt -)
FOP (Wnt +)
GFP
Sall1 (1-1324)
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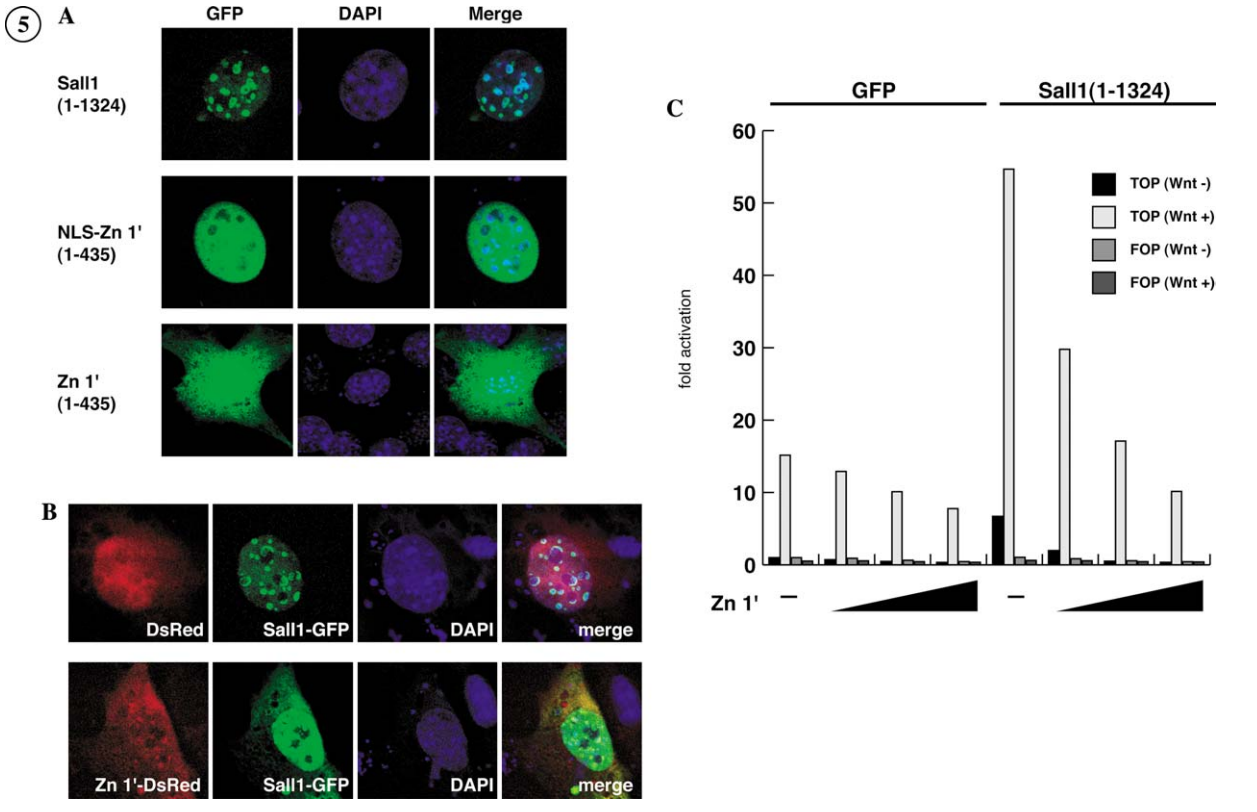
NLS-Zn 3-5 (599-1324)
NLS-Zn 1 (1-288)
NLS-Zn 1' (1-435)


5
A
GFP
DAPI
Merge
Sall1 (1-1324)
NLS-Zn 1' (1-435)
Zn 1' (1-435)
B
DsRed
Sall1-GFP
DAPI
merge
Zn 1'-DsRed
Sall1-GFP
DAPI
merge
C
GFP
Sall1(1-1324)
fold activation
TOP (Wnt -)
TOP (Wnt +)
FOP (Wnt -)
FOP (Wnt +)
Zn 1'

divided Sall1 into halves and constructed two other mutant forms; Zn 1–2, the N-terminal half of Sall1, encoded first and second Zn finger regions, and Zn 3–5, the C-terminal half, encoded third, fourth, and fifth Zn finger regions (Fig. 3A). β-Catenin strongly interacted with the C-terminal half domain encoding Zn 3–5, but not with the N-terminal half domain encoding Zn 1–2 (Fig. 3C).

We next assessed whether the localization of Sall1 in the nucleus correlated with those of β-catenin in NIH3T3 cells, because NIH3T3 cells were useful to distinguish between euchromatic and heterochromatic regions than in HEK293 cells by confocal microscopy. Sall1 was localized to punctate nuclear foci (pericentromeric heterochromatin) as reported [19,20]. β-Catenin in the nucleus was also localized to the punctate nuclear foci, but its localization pattern only partially overlapped with Sall1 (Fig. 3D). This suggests that not all Sall1 in nucleus associates with β-catenin. We also assessed whether, upon Wnt stimulation, the localization pattern of Sall1 changes to overlap with that of β-catenin. Sall1 localization, however, did not change with or without Wnt stimulation (data not shown). Thus, Sall1 is not a simple coactivator for β-catenin on the TOPflash reporter.

The transcriptional activity of Sall1 correlates with its localization in the nucleus

Recently, Sall1 was reported to localize to punctate nuclear foci (pericentromeric heterochromatin) and its nuclear localization to correlate with its transcriptional repression [19,20]. We categorized 10 zinc fingers of Sall1 to five clusters of zinc fingers (Zn 1, 2, 3, 4, and 5) (Fig. 4A). To assess whether the localization of Sall1 in the nucleus also correlates with its transcriptional activation in Wnt signaling, we constructed Sall1-GFP or Sall1 mutants-GFP (Fig. 4A) and examined localization of these GFP-fusion proteins in NIH-3T3 cells using confocal microscopy (Fig. 4B). To focus localization of those mutants only in nucleus, the nuclear localization signal from SV40 was fused to the N-terminal of each GFP-fusion mutant. The full-length Sall1-GFP was localized as a small speckled pattern in the nucleus and co-localized with 4,6-diamidino-2-phenylindole (DAPI) and heterochromatin protein 1 (HP1), as reported [19,20] (Fig. 4B and data not shown). The N-terminal half of Sall1, Zn 1–2, was also localized in a similar fashion to the full-length Sall1, but localization of the C-terminal half, Zn 3–5, looked different; larger speckles or aggregates than those of the full-length and N-terminal half of Sall1-GFP (Fig. 4B). It was reported that the most N-terminal zinc finger domain that binds to HDAC complex is essential and sufficient for its repressor activity [19]. Therefore, we constructed Zn 1′ encoding 1–435 amino acids, which was reported to be the minimal truncated form as a transcriptional repressor (Fig. 4A). Unexpectedly, Zn 1′, which was reported to localize to heterochromatin in COS-1 cells, showed a uniform localization in the nucleus (Fig. 4B). The most N-terminal single zinc finger region, Zn 1, also showed uniform localization in the nucleus (Fig. 4B). Therefore, its localization to heterochromatin requires both the N-terminal single zinc finger (Zn 1) and the following double zinc fingers (Zn 2).

We next did luciferase reporter assays using full-length Sall1 protein and these Sall1 truncated forms. NIH-3T3 cells, introduced with these truncated Sall1 expression plasmids and TOPflash reporter, were stimulated by Wnt supernatants (Fig. 4C). Interestingly, Zn 3–5 (C-terminal half), that has the potential to bind β-catenin, had no activity in the luciferase assay. In contrast, Zn 1–2 (N-terminal half), that has the capacity to localize to heterochromatin, activated the TOPflash reporter, regardless of no interaction with β-catenin (Figs. 3B and C). This indicates Sall1 localization to heterochromatin, but not its association with β-catenin, correlates with its transcriptional activation in Wnt signaling. As Zn 1 and Zn 1′, which have only the first zinc finger, did not show synergistic enhancement of the reporter activity nor localization to heterochromatin, both zinc finger regions 1 and 2 are required for localization to heterochromatin and its synergistic transcriptional enhancement in Wnt signaling.

Fig. 4. The N-terminal half region of Sall1 has the potential to be localized to heterochromatin and to enhance synergistically Wnt reporter activity. (A) Diagram of GFP fused full-length Sall1 and its deletion mutants used in the confocal microscopy. Mutants are fused to NLS in N-terminus and to EGFP in C-terminus. (B) NIH3T3 cells grown on glass coverslips were transfected with corresponding deletion mutants of Sall1-GFP (green) or Sall1-truncated mutants-GFP, counterstained with DAPI (blue) to identify heterochromatin in nucleus, and viewed using confocal microscopy. (C) The reporter assay responsive to Wnt signaling using Sall1-GFP or Sall1 mutants-GFP plasmids. NIH3T3 cells were transfected with 1.0 μg of each Sall1-GFP or Sall1 mutants-GFP, and after 24 h, stimulated with Wnt sup or the mock sup.

Fig. 5. Zn 1′, which is produced by mutations often observed in human TBS, disrupts the localization of native Sall1 protein and its transcriptional activity in Wnt signaling in a dominant-negative fashion. (A) NIH3T3 cells were transfected with corresponding deletion mutants of Sall1-GFP (green), NLS-Zn 1′-GFP, or Zn 1′-GFP without NLS, and stained with DAPI (blue), and viewed using confocal microscope. (B) Full-length Sall1-GFP was introduced into NIH3T3 cells, together with DsRed (upper panels) or Zn 1′-DsRed (lower panels). DAPI staining (blue) shows heterochromatin. (C) The reporter assay responsive to Wnt signaling using the N-terminal truncated mutant Zn 1′. HEK293 cells were transfected with 0.1–1.0 μg Zn 1′ plasmid with or without 0.1 μg HA-Sall1 plasmid in addition to reporter plasmids, and after 24 h, stimulated by the Wnt sup or the mock sup.

Zn 1′, which is produced by mutations often observed in human TBS, disrupts the localization of native Sall1 protein and its transcriptional activity in Wnt signaling in a dominant-negative fashion

It was recently reported that mice carrying a mutation which caused the production of the truncated Sall1 protein, Zn 1′, recapitulated the abnormalities found in human TBS [34,35]. We confirmed that Zn 1′-GFP protein without NLS was localized uniformly in the cytoplasm and in the nucleus, as reported (Fig. 5A). As Zn 1′ was also reported to associate with all Sall family members, probably through the conserved N-terminal glutamine-rich domain [34,36], we hypothesized that Zn 1′ without NLS may affect the localization of native Sall1 protein and its synergistic enhancement of Wnt signaling. We constructed Zn 1′ protein fused to DsRed, introduced it with full-length Sall1-GFP fusion in NIH-3T3 cells, and examined the localization of these proteins using confocal microscopy. When co-expressed with the control DsRed protein, full-length Sall1-GFP remained to be localized to heterochromatin (Fig. 5B). Zn 1′ fused to DsRed, however, affected the localization of Sall1-GFP; Sall1-GFP was localized in both the cytoplasm and the nucleus. Sall1-GFP in the nucleus was no longer localized to heterochromatin, being uniformly localized together with Zn 1′-DsRed (Fig. 5B). This indicates that Zn 1′ functions as a dominant-negative form for native Sall1 proteins, by inhibiting the native Sall1 from localizing to heterochromatin. To assess whether Zn 1′ also functions as the dominant-negative form in Wnt signaling, we next did luciferase reporter assays of HEK293 cells using the Zn 1′ truncated forms without NLS. As HEK293 cells express endogenous Sall1, Zn 1′ introduction down-regulated the activity of endogenous Sall1 on the TOPflash reporter in a dose-dependent manner (Fig. 5C). When introduced with exogenous native Sall1, Zn 1′ also efficiently inhibited synergistic activation of the Wnt responsive reporter by native Sall1 (Fig. 5C). This indicates that the N-terminal truncated Sall1, Zn 1′, which is caused by high-frequent mutations in TBS, also functions as a dominant-negative form in canonical Wnt signaling. We propose that this newly defined mechanism of Wnt signaling activation by heterochromatin localization of Sall1 may explain one of the causative mechanisms of TBS.

Discussion

Sall plays important roles in a variety of organs, but its molecular mechanisms have remained largely unknown.

Here we show that Sall1 functions as a transcriptional activator specifically in the canonical Wnt signaling pathway. The luciferase activity on the TOPflash reporter stimulated by Wnt-3a was synergistically activated by the introduction of Sall1 (Fig. 1). We also tested other unrelated zinc finger proteins on the TOPflash reporter, and found that the effect on Wnt canonical pathway was specific to Sall1 (data not shown). The synergistic activity on TOPflash reporter by Sall1 was also observed by transfection of β-catenin and Sall1. The introduction of ds RNAi oligo for human SALL1 to HEK293 cells not only reduced the amount of endogenous SALL1 protein, but also led to down-regulation of the TOPflash reporter activity (Fig. 2). Activation of Wnt signaling by Sall1 did not correlate with its localization with β-catenin, but rather with its localization of heterochromatin (Figs. 3 and 4). Further, the N-terminal truncated form of Sall1 (Zn 1′), which was reported to lead human TBS abnormalities in mice [34], disturbed localization of the native Sall1 and also down-regulated the synergistic activity on TOPflash reporter by native Sall1 (Fig. 5).

In two previous reports, Sall1 was seen to function as a transcriptional repressor on the artificial promoter containing tandem GAL4 binding sites, when linked to the heterologous GAL4 DNA-binding domain, and also that Sall1 associated with HDAC and several components of the chromatin remodeling complex (MTA1, MTA2, and RbAp46/48) [20,34]. Therefore, Sall1 could repress gene expression by recruiting the HDAC complex. It was not, however, reported that native Sall1 functions as a transcriptional repressor. We found that the native form of Sall1 could function as a transcriptional activator in Wnt signaling essential for many developmental processes and that its activity correlated with its localization to heterochromatin [37]. The increase of Sall1 proteins may squelch some transcriptional repressor complex, including HDAC, or be associated with chromatin remodeling factors to alter the chromatin structure near the promoter region of Wnt target genes.

Another C2H2 type zinc finger protein Ikaros functions as both transcriptional repressor and activator, and is localized to pericentromeric heterochromatin [38–40]. Ikaros associates with DNA-dependent ATPase Mi-2 included in the NuRD chromatin remodeling complex [41]. Ikaros has six C2H2 type zinc fingers; the N-terminal zinc finger cluster consisting of four zinc fingers functions as the DNA-binding domain and C-terminal two zinc fingers as a dimerization domain [42]. When linked to a heterologous GAL4 DNA-binding domain, Ikaros functions as a transcriptional repressor on the reporter containing tandem GAL4 binding sites. On the other hand, the native form of Ikaros enhances activity of the reporter that contains tandem Ikaros binding sites upstream of the thymidine kinase promoter, and also enhances activity of the reporter containing no Ikaros binding sites but only Sp1

transcription factor binding sites. When Ikaros is not localized to heterochromatin caused by point mutations in its DNA-binding domain or dimerization domain, Ikaros does not enhance activities of these reporters. Therefore, localization of Ikaros to heterochromatin correlates with its transcriptional activation [38]. It was also reported that Sall proteins interact with all family members through its conserved glutamine-rich domain [36]. Therefore, a similar mechanism may function both for Sall1- and Ikaros-dependent activation. As the Zn 1–2 region of Sall1 was required and is sufficient for its heterochromatin localization (Fig. 4), these zinc fingers may bind to target sequences in heterochromatin, directly or indirectly. The identification of DNA sequences or molecules in heterochromatin compartments, which are required for heterochromatin localization of Sall1, would elucidate the mechanism of heterochromatin localization of Sall1 and eventually the mechanism of the activation of Wnt signaling by Sall1.

It is to be noted that Sall1-dependent activation is not general but rather it is specific to Wnt, at least among several pathways tested (BMP, TGF-β, retinoic acid, and LIF). The Wnt signal is regulated by multiple steps and large numbers of agonists and antagonists bind to β-catenin and TCF [26,43–48]. In addition, there is emerging evidence that more complicated mechanisms function in Wnt activation, including the transcriptional regulation by remodeling chromatin structure and by sumoylation [49,50]. In the former mechanism, it was reported that Brahma (Brm)/Brahma-related gene-1 (Brg-1), a component of mammalian SWI/SNF or RSC chromatin remodeling complex, binds to β-catenin, changes the chromatin structure by its ATPase activity, and then enhances Wnt-dependent transcription [49]. On the other hand, when an ARID domain protein Osa is contained in the Brm/Brg-chromatin remodeling complex, it tightens the chromatin structure and represses Wnt-dependent gene expression [50]. In the latter mechanism, the sumoylation of Lef-1 by PIASy, a member of E3 SUMO ligase, transfers Lef-1 to the nuclear body, a specific subcompartment in the nucleus, and then suppresses its transcriptional activity [51]. Tcf-4 is also sumoylated by PIASy, transferred to the PML nuclear body, and activates Wnt-dependent transcription [52]. It is to be noted that Sall1 also interacts with SUMO-1 and ubiquitin-conjugation enzyme UBE2I (human homolog of yeast UBC9), and is sumoylated [53], though its physiological relevance to Wnt signal remains to be determined. In this study, we propose another mechanism of Wnt signal activation by heterochromatin localization of Sall1. Further elucidation of this mechanism will lead to better understanding of not only the Wnt signal but also phenotypes observed in various species ranging from *Drosophila* to humans lacking SALL/spalt functions.

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