Murine inner cell mass-derived lineages depend on Sall4 function

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Sall4 is a mammalian Spalt transcription factor expressed by cells of the early embryo and germ cells, an expression pattern similar to that of both Oct4 and Sox2, which play essential roles during early murine development. We show that the activity of Sall4 is cell-autonomously required for the development of the epiblast and primitive endoderm from the inner cell mass. Furthermore, no embryonic or extraembryonic endoderm stem cell lines could be established from *Sall4*-deficient blastocysts. In contrast, neither the development of the trophoblast lineage nor the ability to generate trophoblast cell lines from murine blastocysts was impaired in the absence of Sall4. These data establish Sall4 as an essential transcription factor required for the early development of inner cell mass-derived cell lineages.

blastocyst | spalt | stem cells | transcription factor

Mammalian development starts with the formation of the blastocyst, which subsequently implants to give rise to a newborn embryo and all its specialized organs (1, 2). The blastocyst already harbors three distinct tissue lineages; the epiblast, the extraembryonic endoderm, and the trophectoderm (3, 4). ES cells and extraembryonic endoderm stem cells have been established from the inner cell mass (ICM), and trophoblast stem (TS) cells from the trophectoderm (5, 6). Recently, major progress has been made in understanding the transcriptional regulatory circuitry that governs these early lineage decisions in the early mouse embryo and ES cells (7, 8). Genetic studies in mice demonstrated that the transcription factors Oct4/Pou5F1, Nanog, and Sox2 are crucial regulators of epiblast and ES cell identity (9–12). In contrast, Gata6 is a transcription factor required within the primitive endoderm at the epiblast stage (13), whereas, in the absence of Cdx2, trophectoderm fate is not maintained (14). Recently, a reciprocal interaction between Oct4 and Cdx2 has been shown to be essential for the establishment of the trophectoderm lineage (15). The Spalt/Sall transcription factor family was initially discovered in *Drosophila* and later found to be conserved in *Caenorhabditis elegans*, *Xenopus*, fish, mouse, and humans (16). In *Drosophila*, Spalt proteins possess homeotic function during development and are required in photoreceptor differentiation and planar cell polarity (17). Genetic studies in *C. elegans* demonstrated that the spalt-like gene *sem-4* represses Hox and LIM domain containing transcription factors (18). In humans, mutations at the *SALL4* locus result in a range of clinically overlapping phenotypes, including Okihiro syndrome, Holt–Oram syndrome, acro-renal-ocular syndrome, and thalidomide embryopathy (19). In this study we demonstrate that murine Sall4, like Oct4, Sox2, and Nanog, is important for ICM-derived cell lineages, the epiblast, and primitive endoderm, adding to our understanding of the transcriptional components underlying early mammalian development.

Results and Discussion

Sall4 protein is already seen at the two-cell stage because of maternal contribution and then appears again in some cells of the 8- to 16-cell-stage embryo after zygotic transcription has initiated. In late blastocysts, the *Sall4* RNA and Sall4 protein become enriched in the ICM. Within the epiblast expression is maintained uniformly until the mid–late streak stages and is also evident in the derivatives of the primitive endoderm, including Reichert's membrane. (Fig. 1 *A–C* and Fig. 6 *A–C*, which is published as supporting information on the PNAS web site). By 10.5 days postcoitum (dpc), *Sall4* RNA is seen within progenitor populations of the brain, neural tube, pituitary gland, heart, liver, somites, limbs, and also in the female germ line (Fig. 6 *D–F*).

To define the roles of Sall4 in early mammalian development we generated two different *Sall4* mutant alleles via homologous recombination in ES cells. In the first mutant allele, hereafter called $Sall4^{H2bEGFP}$, exon 2, which encodes 80% of the Sall4 protein, was replaced by an internal ribosomal entry site (IRES) and a *Histone2BEGFP* coding sequence. In the second allele, $Sall4^{floxed}$, we have flanked exon 2 of *Sall4* with *loxP* sites which results in the allele, $Sall4^{\Delta exon2}$ upon Cre expression, which has lost most of the *Sall4* coding sequence including the first six zinc fingers of the Sall4 protein (Fig. 7, which is published as supporting information on the PNAS web site). Live-born homozygous *Sall4* mutant pups from heterozygous intercross matings of our two mutant alleles were never observed. Even among prestreak embryos genotyped by PCR, none of the $Sall4^{H2bEGFP}/Sall4^{H2bEGFP}$, $Sall4^{H2bEGFP}/Sall4^{\Delta exon2}$ or $Sall4^{\Delta exon2}/Sall4^{\Delta exon2}$ genetic combinations were detected. However empty implantation sites were observed regularly. Thus, *Sall4* deficiency results in periimplantation lethality before egg cylinder formation.

Blastocysts from $Sall4^{H2bEGFP}$ heterozygous intercrosses displayed in roughly 1:2:1 ratio strong : intermediate : no GFP fluorescence. Embryos sorted by fluorescence and analyzed by RT-PCR, confirmed that embryos with bright fluorescence were $Sall4^{H2bEGFP}$ homozygous embryos and lacked any Sall4 immunoreactivity (Fig. 1 *D–H*). Thus, introduction of the *H2bEGFP* coding sequence into the *Sall4* locus allows genotyping of 3.5-dpc *Sall4* mutant blastocysts by fluorescence intensity without killing them. All future experiments were done with the $Sall4^{H2bEGFP}$ allele. Immunofluorescence staining for Oct4, Nanog, Sox2, Cdx2, and Troma1 as well as RT-PCR analysis for *FGF4*, *Gata6*, and *PEM* did not reveal significant differences between WT and homozygous $Sall4^{H2bEGFP}$ mutant embryos (Fig. 1 *I–O*). Thus, development of the presumptive trophectoderm and ICM compartments, as well as proliferation and cell survival apparently proceeds normal in the absence of Sall4 protein until the blastocyst stage.

3.5-dpc embryos from $Sall4^{H2bEGFP}$ heterozygous intercrosses were sorted according to their fluorescence intensity and reimplanted separately in pseudopregnant females. In all cases indistinguishable decidual swellings at 5.5 dpc were observed. Whereas implantation sites resulting from control blastocysts contained embryos of the expected age, no ICM-derived em-

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Abbreviations: ICM, inner cell mass; TS, trophoblast stem; dpc, days postcoitum.

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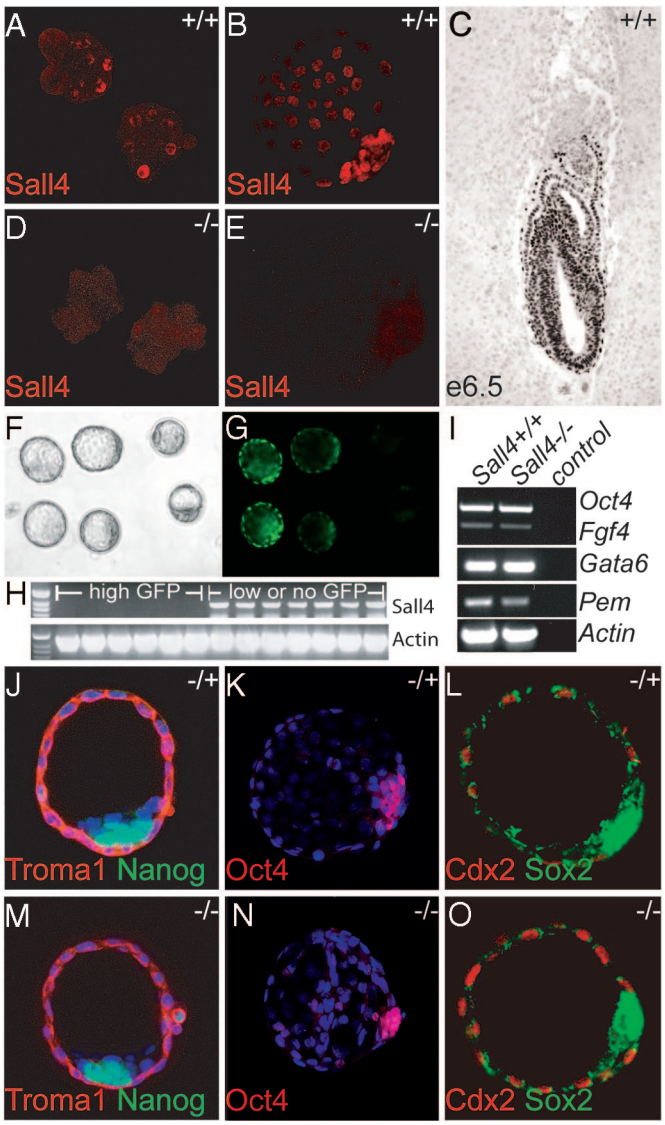

Fig. 1. Early Sall4 protein expression and marker analysis on *Sall4* mutant blastocysts Sall4 antibody staining. Sall4 protein is visible in cells at the 8- to 16-cell stage (*A*), to become enriched within cells of the ICM (*B*). At 6.5 dpc Sall4 protein is detected in ICM derivatives including epiblast, visceral, and parietal endoderm (*C*). Blastocysts from *Sall4*^{H2bEGFP} intercrosses (*F*) display different GFP fluorescence intensity and can be sorted into strong (*G Left*), medium (*G Center*), and no GFP signal (*G Right*). Bright GFP fluorescent blastocysts do not express *Sall4* RNA as shown by RT-PCR using *actin* as a control and do not show Sall4 immunostaining (*D* and *E*). Indirect immunofluorescence and RT-PCR marker analysis on WT and *Sall4* mutant expanded blastocysts does not reveal any obvious differences (*I–O*). Nanog (epiblast marker) (*J* and *M*), Troma1 (trophectoderm/epithelial marker) (*J* and *M*), Oct4 (ICM marker) (*K* and *N*), Cdx2 (trophectoderm marker) (*L* and *O*), and Sox2 (*L* and *O*) are shown. DAPI counterstaining is blue.

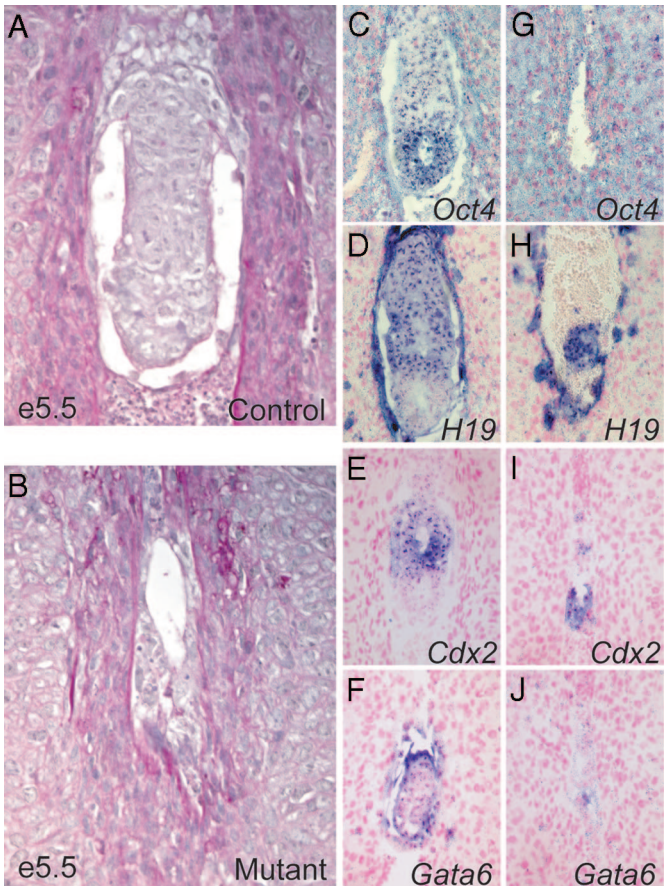

Fig. 2. *Sall4*^{H2bEGFP} mutant embryos lack ICM derivatives. Shown are paraffin sections of PAS-stained, presorted, and reimplanted control (*A*) and mutant (*B*) embryos at 5.5 dpc. Marker RNA *in situ* analysis was performed on reimplanted, pregenotyped embryos at 5.5 dpc (WT, *C–F*; mutant, *G–J*). *Sall4* mutant embryos do not form epiblast or primitive endoderm derivatives. Shown are *Oct4* (epiblast maker) (*C* and *G*) and *Gata6* (primitive endoderm marker) (*F* and *J*). Disorganized extraembryonic tissue is present in *Sall4* mutant implantation sites as shown by positive staining for *Cdx2* (*E* and *I*). Giant cells are clearly visible in *H19* staining (*D* and *H*).

bryonic structures were recognizable in *Sall4*-null blastocyst implantations (Fig. 2 *A* and *B*). This indicates that the empty implantation sites observed in heterozygous *Sall4*^{H2bEGFP} intercrosses likely result from *Sall4*^{H2bEGFP} homozygous blastocysts. RNA *in situ* hybridization was used to further characterize the *Sall4* mutant phenotype. *Oct4* showed a clear signal in the epiblast of sections from control implantation sites but was completely absent from mutant *Sall4*^{H2bEGFP} implantation sites (Fig. 2 *C* and *G*). In contrast, *H19*, which stains all extraembryonic cell types in the postimplantation embryo in a reciprocal pattern to *Oct4*, was expressed within the area corresponding to *Sall4*^{H2bEGFP} mutant implantation site, showing that extraembryonic cell types were present. Many positive cells invaded the uterine stroma, typical of trophoblast giant cells (Fig. 2 *D* and *H*). Although few *Cdx2* and *Gata6*-positive cells at 5.5 dpc were present in mutant implantation sites (Fig. 2 *I* and *J*), they did not give rise to recognizable structures at later embryonic stages (Fig. 8, which is published as supporting information on the PNAS web site). These results further demonstrate that no ICM-derived structures can be detected after implantation of *Sall4* mutant embryos.

The data presented so far suggested a specific defect in the developmental potential or viability of the ICM in *Sall4*^{H2bEGFP} mutant embryos. Therefore, *in vitro* culture experiments were performed to assess the developmental potential of *Sall4*^{H2bEGFP} mutant blastocysts. Blastocysts from *Sall4*^{H2bEGFP} intercrosses were sorted by fluorescence intensity and placed in ES cell medium. All embryos readily outgrew a layer of trophoblast giant cells independent of their genotype (Fig. 3 *A* and *D*). In WT and heterozygous embryos distinctive ICM-derived cell masses appeared and continuously grew during the culture period (Fig. 3 *A–C*). In contrast, the ICM of homozygous mutant *Sall4*^{H2bEGFP} embryos did not expand significantly (Fig. 3 *D–F*). Consistent with this observation, few mitotic cells were detected

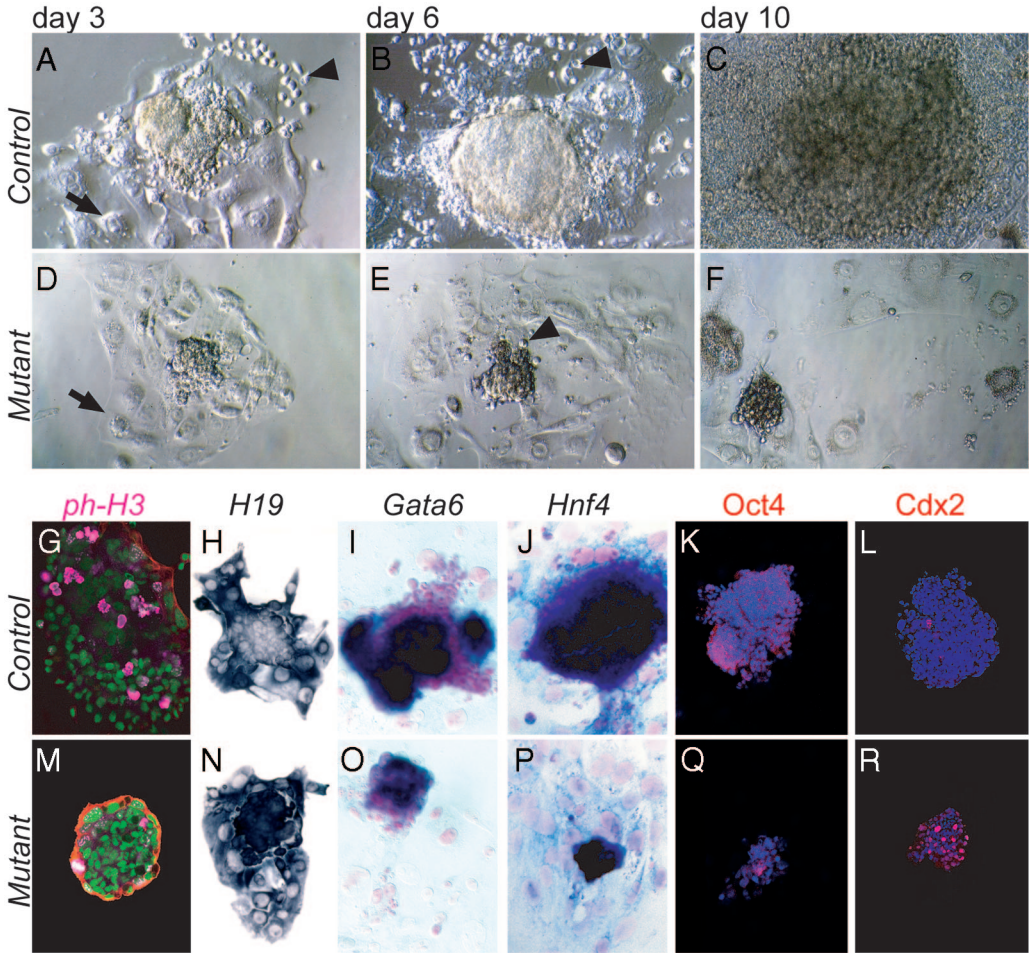

Fig. 3. *Sall4* mutant blastocysts show defective ICM development in culture. Phase contrast of WT (*A–C*) and *Sall4* mutant blastocyst (*D–F*) outgrowths cultured in standard ES cell medium plus LIF. *Sall4* mutant ICMs initially showed a small outgrowth, which did not expand further under prolonged culture conditions. Only few endoderm cells were seen compared with WT controls (arrowhead marks endoderm cells, and arrow marks giant cells). Proliferation in mutant *Sall4* outgrowths was nearly not detectable as shown by phospho-HistoneH3 immunostaining after 5 days of *in vitro* culture (*G* and *M*). Marker RNA *in situ* hybridization (*H–J* and *N–P*) and immunostaining (*K*, *L*, *Q*, and *R*) analysis on day-5 blastocyst outgrowths are shown. Note increased *H19* staining in *Sall4* mutant blastocyst outgrowths (*N*) compared with WT controls (*H*). Also, increased numbers of Cdx2-immunopositive cells are seen in mutant blastocyst outgrowths compared with controls (*L* and *R*). Few cells still express *Gata6* (*I* and *O*) and *Hnf4* (*J* and *P*). Rarely, Oct4-immunopositive cells can be detected (*K* and *Q*).

in mutant explants by phospho-HistoneH3 staining after 5 days of culture, suggesting that proliferation of the ICM-derived cell masses had ceased (Fig. 3 *G* and *M*). Importantly, mutant cells did not show an increased rate of apoptosis, as determined by TUNEL staining (data not shown). Surprisingly, RNA *in situ* hybridization performed on the ICM-derived cell mass of *Sall4*^{H2bEGFP} homozygous mutant blastocyst outgrowths showed a strong positive signal for *H19*, an extra embryonic cell marker, after 5 days of culture (Fig. 3 *H* and *N*). Likewise, a significant proportion of cells were positive for Cdx2 which was not seen in WT outgrowths (Fig. 3 *L* and *R*). In contrast, RNA *in situ* analysis for *Gata6* and *Hnf4* detected few primitive endoderm cells (Fig. 3 *I*, *J*, *O*, and *P*). Rarely, Oct4-positive cells were found after 5 days of culturing (Fig. 3 *K* and *Q*). In contrast, trophoblast cell cultures were readily obtained from WT and mutant blastocyst outgrowths cultured in medium supplemented with FGF4 and heparin (6), which differentiated into trophoblast giant cells after FGF4 withdrawal (Fig. 9, which is published as supporting information on the PNAS web site). These results demonstrate that *Sall4* deficient blastocyst cells do not in general have a proliferation block.

To further characterize the homozygous mutant *Sall4* blastocysts we used immunosurgery to remove the trophectoderm layer and to isolate the ICM from 3.75-dpc blastocysts (20). When ICMs of *Sall4*^{H2bEGFP}-null blastocysts were cultured in ES cell medium, trophoblast giant cells could be identified by their morphology whereas under the same conditions no trophoblast giant cells were seen in heterozygous *Sall4*^{H2bEGFP} ICM outgrowths (Fig. 4*A*). Immunostaining of *Sall4*-negative ICMs for Cdx2 or Cdh3 after 5 days of cultivation identified a lot of Cdx2/Cdh3-positive cells demonstrating that most cells of the ICM have switched on trophectoderm lineage markers which was not the case in control outgrowths (Fig. 4*B*). Extraembryonic endoderm cell lines can be obtained by culturing ICM in medium supplemented with FGF4 (5). ICMs isolated from both heterozygous and *Sall4*^{H2bEGFP}-null blastocysts started to grow readily under these conditions. In heterozygous *Sall4*^{H2bEGFP} cultures we obtained an epithelium-like cell type that formed lattice-like structures after FGF4 withdrawal, as described for extraembryonic endoderm cells (5). In contrast, cells with similar morphology were not obtained from homozygous mutant *Sall4*^{H2bEGFP} ICMs. Surprisingly, withdrawal of FGF4 from *Sall4*^{H2bEGFP}-null ICM-derived cultures yielded trophoblast giant cells (Fig. 4*C*). PCR genotyping of giant cells confirmed that they were homozygous for the *Sall4* mutant allele (Fig. 4*D*). RT-PCR expression analysis further confirmed the observed morpholog-

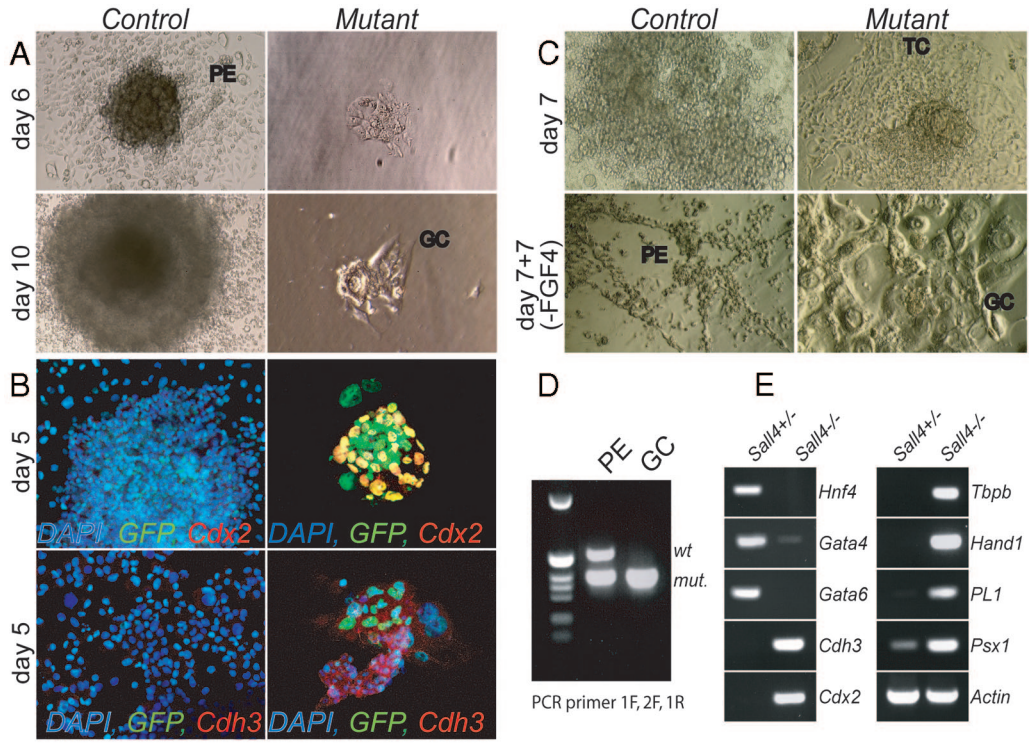

Fig. 4. *Sall4* mutant ICMs give rise to trophectoderm cultures. (*A*) Phase contrast of ICM outgrowths in ES cell medium after immunosurgery. *Sall4* mutant ICM outgrowths do not expand significantly but undergo a change in cell morphology toward a giant cell-like appearance in contrast to WT ICMs. (*B*) Immunostaining marker analysis of ICM outgrowth in ES cell medium after immunosurgery. *Sall4* mutant ICM cells are positive for the trophectoderm markers Cdx2 and Cdh3 in immunostainings. (*C*) Phase contrast of ICM outgrowth in TS medium plus FGF4 after immunosurgery. Both WT and *Sall4* mutant ICMs show robust outgrowths under these conditions. Whereas the control culture displays lattice-like structures characteristic for primitive endoderm after FGF4 withdrawal, the *Sall4* mutant outgrowths differentiate into giant cells. (*D*) PCR genotyping using primers 1F, 2F, and 1R of primitive endoderm and giant cell cultures shown in *C* demonstrating that TS cell cultures and giant cells are *Sall4*-negative. (*E*) RT-PCR marker analysis of cell cultures shown in *C* demonstrating that cells generated from *Sall4* mutant ICM outgrowths express, consistent with their morphology, trophectoderm lineage markers in contrast to the control cultures. PE, primitive endoderm; TC, trophectoderm cells; GC, giant cells.

ical differences. Primitive endoderm markers were absent in homozygous mutant *Sall4*^{H2bEGFP} cell cultures (Fig. 4*E*). This experiment demonstrates that there is a cell-autonomous requirement for Sall4 function to establish extra embryonic endoderm cell lines from primitive endoderm. Although we cannot rule out that some trophectoderm cells survived the immunosurgery, we favor the interpretation that the observed trophectoderm cell lines originate from homozygous mutant *Sall4* ICM cells (21). These results underscore the cell lineage-specific defect of *Sall4*-null blastocysts and suggest a possible switch in the developmental potential of the ICM.

To determine whether Sall4 is required cell-autonomously in the epiblast, we performed morula aggregation experiments (22). *Sall4* mutant cells contributed to the ICM in chimeric blastocysts (Fig. 5*A* and Fig. 10*A*, which is published as supporting information on the PNAS web site). Chimeric blastocysts of a *Sall4*^{H2bEGFP}*heterozygous/ROSA26* or *Sall4*^{H2bEGFP}*null/ROSA26* genotype mix were implanted independently into pseudopregnant females. Embryos recovered at 9.5 dpc were stained for β-galactosidase activity. *Sall4*^{H2bEGFP} *heterozygous/ROSA26* blastocysts resulted in embryos with various degree of chimerism (Figs. 5*B* and 10*B*). In contrast, all embryos recovered from *Sall4*^{H2bEGFP}*null/ROSA26* blastocysts were derived from *ROSA26* cells only. (Figs. 5*C* and Fig. 10*C*) demonstrating that *Sall4* is required cell-autonomously within the epiblast. In addition, epiblast-specific deletion of *Sall4* using the well established *Sox2-Cre* line (23) leads to a developmental arrest at the late primitive streak stage but clearly before somite formation (Fig. 5 *D* and *E*) despite that all four murine *Sall* family members are expressed at this stage (Fig. 6*G*). This result further demonstrates that *Sall4* is essential for epiblast development *in vivo*.

ES cells most closely resemble the properties of the epiblast *in vitro*. To assess whether Sall4 is continuously required for self-renewal of ES cells we established several independent ES cell lines from blastocyst outgrowths from *Sall4*^{floxed} heterozygous intercrosses. These ES cell lines are feeder-dependent and genotypically either heterozygous or homozygous for a *Sall4*^{floxed} allele. They express high levels of Sall4 which is down-regulated upon differentiation (Fig. 11, which is published as supporting information on the PNAS web site). Using either a pCAG-CreIRESNeo or a pCAG-CreIRESPuro vector we were unable to obtain any homozygous *Sall4*-null ES cell lines. In contrast deletion of one *Sall4* allele or both *Sall4* alleles in case a pCAG-HASall4IRESPuro rescue vector was present was successful (Fig. 11). Thus, we conclude that one functional *Sall4* allele is indispensable for maintenance of the self-renewing ES cell phenotype.

Next we asked whether *Sall4* is cell-autonomously required in the primitive endoderm. We therefore produced chimeras in which WT ES cells were introduced into mutant *Sall4* embryos (Fig. 5*F*). In this situation ES cells do not normally contribute to the primitive endoderm or trophectoderm (22, 24). Thus defects within the primitive endoderm caused by defects within the epiblast will be rescued in this constellation. *Sall4* heterozygous and homozygous mutant blastocysts were injected with *Rosa26* ES cells or R1 *CAG-LacZ* ES cells (Table 1, which is published as supporting information on the PNAS web site). All deciduae at 6.5 dpc from control and mutant *Sall4*^{H2bEGFP} genotypes were

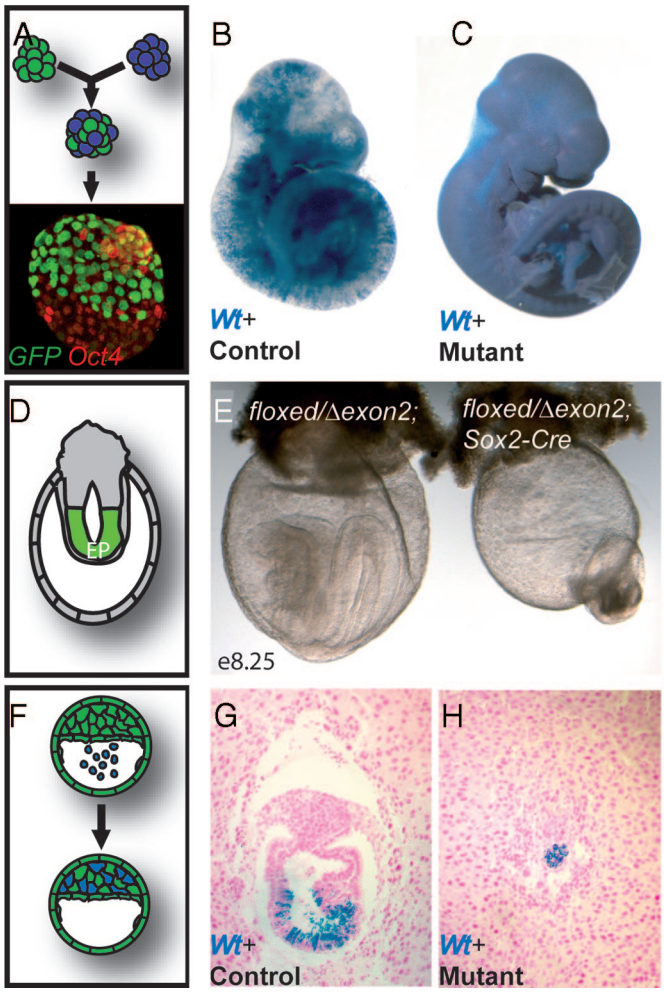

Fig. 5. Cell-autonomous requirement of *Sall4* in the epiblast and primitive endoderm. (*A*) *Sall4* heterozygous and mutant morula stage embryos were sorted according to their fluorescence intensity and aggregated with morulae heterozygous for *ROSA26*. Successful contribution of *Sall4* mutant cells to the ICM of chimeric blastocysts was checked by using GFP/Oct4 double staining. (*B* and *C*) Whole-mount β-galactosidase staining. (*B*) Representative embryo recovered from control population showing various degrees of chimerism in different organs. (*C*) Representative embryo recovered from *Sall4* homozygous mutant/*ROSA26* morula aggregations being completely blue. (*D*) Schematic drawing of the *Sox2-Cre* deletion region in green. (*E*) Epiblast-specific deletion of *Sall4* leads to an arrest of embryonic development at the late primitive streak stage before somite formation. (*F*) ES cell–embryo chimera analysis injecting WT ES cells into *Sall4* mutant blastocysts. In this situation WT *lacZ*$^+$ ES cells (blue) contribute only to the epiblast. Injection of *ROSA26* ES cells with *lacZ* inserted into the ROSA locus or R1 ES cells carrying a *CAG-LacZ* expression module in the ROSA locus into *Sall4* homozygous mutant blastocysts. (*G* and *H*) Nuclear fast red counterstained paraffin sections of whole-mount β-galactosidase-stained 6.5-dpc embryos. (*G*) Representative embryo of the control group where blue cells can be seen only in the epiblast. (*H*) The most advanced embryo found from *Sall4* homozygous mutant blastocysts injected with WT ES cells. Note that the epiblast consists of only blue cells.

stained for β-galactosidase activity followed by paraffin sectioning. Heterozygous *Sall4* blastocyst implantation sites harbored embryos of the expected developmental stage with various amount of chimerism in the epiblast. As expected no *LacZ*$^+$ blue cells contributed to extraembryonic ectoderm and primitive endoderm derivatives (Fig. 5*G*). In contrast, only embryos arrested in development at an earlier stage were found in decidual swellings resulting from injected *Sall4* mutant blastocysts (Fig. 5*H*). In these chimeric embryos all cell types appeared to be present. Interestingly, the putative epiblast consisted of only blue WT cells, suggesting that the initially present *Oct4*/*Nanog*-positive, *Sall4*-negative cells may have differentiated into trophectoderm or primitive endoderm derivatives. The developmental arrest of the WT epiblast therefore strongly suggests a cell-autonomous defect within the *Sall4* mutant primitive endoderm derivatives consistent with our observation that no extra embryonic endoderm cell lines could be established from *Sall4* mutant outgrowths.

Sall4 expression parallels expression of Oct4 and Sox2 during early mouse development but precedes the expression of Nanog and FoxD3, two other transcription factors that have been shown to govern the undifferentiated state of ES cells (11, 25). Despite the parallel expression of Oct4, Sox2, and Sall4, there are notable differences in their loss-of function phenotypes. Although homozygous mutant *Oct4*, *Sox2* and *Sall4* deciduae lack an epiblast, with only a few trophoblast cells present, blastocyst outgrowths of the three mutant genotypes show different behavior *in vitro* (refs. 9 and 12 and this work).

In contrast to *Oct4* mutant blastocyst outgrowths which only yield trophoblast giant cells, *Sall4* mutant blastocyst outgrowths contain some Oct4-positive cells and cells expressing endoderm-specific markers. However, the *Sall4* mutant outgrowths do not show any significant expansion or apoptosis under ES cell culture conditions. In this respect the observed phenotype of *Sall4* homozygous mutant blastocyst outgrowths more resembles the reported *Sox2* and *FoxD3* mutant phenotypes (9, 25). When one compares ICM outgrowths of the three mutant phenotypes the situation is reversed. In the *Oct4* mutant ICM outgrowths giant cells appear immediately, whereas prolonged cultivation of the ICMs of *Sall4* as well as *Sox2*-null mutants is required until giant cells can be observed. In contrast, when ICMs are cultivated in medium containing FGF4, *Sall4* mutant ICMs give rise to TS cell lines that readily differentiated into giant cells after FGF withdrawal. This phenotype is similar to that observed in the *Oct4* mutant situation but opposite to the *Sox2* situation where no TS cell lines could be established even from blastocyst outgrowths (9, 12). *Cdx2* has been postulated to respond in an autoregulatory manner to a yet unidentified apical signal (15). Sall4 may be required in this context to support Oct4 repression of *Cdx2* expression, consistent with the much lower expression of *Sall4* in the trophectoderm compared with the ICM. Thus, *Sall4* loss may be permissive in this situation and an additional signaling event may be required to allow the observed cell fate change. How Sall4 modulates the reported reciprocal Oct4/Cdx2 interaction remains to be explored. Consistent with this idea, we also see up-regulation of *H19* in *Sall4*-null blastocyst outgrowths pointing to another gene where Sall4 may function in a repressive mode. In addition our experiments have demonstrated that Sall4 is cell-autonomously required in the primitive endoderm. In contrast to Oct4 and Nanog, Sall4, like Sox2, is expressed later during development in various organ systems. At these later time points Sall4 expression is always correlated with uncommitted dividing stem and progenitor populations in various organ systems. Thus, the human Okihiro syndrome may result from a premature depletion of different progenitor cell pools depending on the genetic background.

In conclusion, the data presented here add Sall4 to the list of transcription factors including Oct4, Sox2, Nanog and Cdx2 that are required for early mammalian development. Further work should provide additional insights into the transcriptional cross-talk underlying murine ICM pluripotency and ES cell self-renewal.

Experimental Procedures

Generation of *Sall4*^{H2bEGFP} and *Sall4*^{floxed} Mice. Targeting strategy is described in Fig. 7. R1 ES cells were electroporated with the linearized construct, and positive clones were confirmed by Southern blot using 5′ and 3′ outside probes. *Sall4* heterozygous mice were maintained on a mixed 129/C57BL/6/CD1 background. Mice were housed in specific pathogen-free and light-controlled, temperature-controlled (21°C), and humidity-controlled (50–60% relative humidity) conditions. Food and water were available ad libitum. The procedures for performing animal experiments were in accordance with the principles and guidelines of Laboratory Animal Resources/European Molecular Biology Laboratory.

Immunohistochemistry and *in Situ* Hybridization. For immunohistochemistry, blastocysts were fixed in 2% PFA for 5 min, washed and blocked for 30 min in PBS containing 0.2% Triton X-100, 1% glycine, 3% BSA, and 5% donkey serum, followed by primary antibody overnight at 4°C. Free floating blastocysts were embedded in PBS by using spacers (Secure Seal; Molecular Probes) and analyzed immediately by confocal microscopy (TCS SP2; Leica, Wetzlar, Germany).

Sall4 peptide antibody [produced against N-terminal 16 aa of Sall4 (KQAKPQHINWEEGQGE) coupled to KLH and injected into rabbits] was used at a dilution of 1:500 after affinity purification. Troma1 antiserum (kindly provided by R. Kemler, Max Planck Institute, Freiburg, Germany) at 1:20, Oct4 antiserum (monoclonal mouse anti-Oct4, C10; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:50, Nanog antiserum (CosmoBio, Tokyo, Japan) at 1:150, antiserum against Sox2 (kindly provided by R. Lovell-Badge, MRC National Institute for Medical Research, London, U.K.) at 1:250, monoclonal anti-Cdx2 (CDX2-88; BioGenex, San Ramon, CA) at 1:100, phospho-Histone H3 antiserum (rabbit; Upstate Biotechnology, Lake Placid, NY) at 1:300, anti-Cdh3 (56C1; NeoMarkers, Fremont, CA) at 1:30. Hybridization with ^{35}S-labeled and nonradioactive antisense RNA probes was done as previously described (26).

ES Cell, TS Cell, Immunosurgery, and Outgrowth Culture Experiments. Culture and derivation of ES cell lines and of trophoblast cell lines from blastocysts was performed as described (27, 28). Immunosurgical isolation of ICMs was carried out on freshly flushed 3.75-dpc blastocysts or upon cultivation of such blastocysts in M16 medium over night (20). Unabsorbed rabbit anti-mouse T cell (Thy1) antiserum (Cedarlane, Hornby, ON, Canada) was used at 25% (vol/vol) in DMEM (Gibco) to label cells with surface exposure. Embryos were incubated for 15 min at 37°C, washed through several drops of DMEM, and incubated for 15 min at 37°C in 25% (vol/vol) Standard Guinea Pig Complement (Cedarlane) to kill all cells decorated with antibody. Efficient killing was observed microscopically by swelling of trophectodermal cells. For ES cell or TS cell outgrowth formation, blastocysts or ICMs after immunosurgery were transferred to ES cell medium or TS cell medium and documented photographically (D1; Nikon, Munich, Germany) under phase contrast optics.

Embryo Culture and Chimeric Aggregations. Two cell stage to morula stage embryos were collected by flushing oviducts with M2 medium. Blastocysts were collected, by flushing the uterine horns with M2. Collected embryos were expanded in M16 at 37°C and 5% CO_2 for 24 h (27). *ROSA26* morulae were aggregated with *Sall4*^{H2bEGFP} morulae as described (27). After transfer into pseudopregnant female mice, embryos were harvested at embryonic day 9.5. To generate ES cell–embryo chimeras, *ROSA26* and *CAG-LacZ* ES cells were injected into blastocysts obtained from *Sall4*^{H2bEGFP} heterozygous intercrosses. After transfer, embryos were harvested at embryonic day 6.5.

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