

# SALL4 Expression in Gonocytes and Spermatogonial Clones of Postnatal Mouse Testes

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## Abstract

The spermatogenic lineage is established after birth when gonocytes migrate to the basement membrane of seminiferous tubules and give rise to spermatogonial stem cells (SSC). In adults, SSCs reside within the population of undifferentiated spermatogonia ( $A_{undiff}$ ) that expands clonally from single cells ( $A_{single}$ ) to form pairs ( $A_{paired}$ ) and chains of 4, 8 and 16  $A_{aligned}$  spermatogonia. Although stem cell activity is thought to reside in the population of  $A_{single}$  spermatogonia, new research suggests that clone size alone does not define the stem cell pool. The mechanisms that regulate self-renewal and differentiation fate decisions are poorly understood due to limited availability of experimental tools that distinguish the products of those fate decisions. The pluripotency factor SALL4 (sal-like protein 4) is implicated in stem cell maintenance and patterning in many organs during embryonic development, but expression becomes restricted to the gonads after birth. We analyzed the expression of SALL4 in the mouse testis during the first weeks after birth and in adult seminiferous tubules. In newborn mice, the isoform SALL4B is expressed in quiescent gonocytes at postnatal day 0 (PND0) and SALL4A is upregulated at PND7 when gonocytes have colonized the basement membrane and given rise to spermatogonia. During steady-state spermatogenesis in adult testes, SALL4 expression overlapped substantially with PLZF and LIN28 in  $A_{single}$ ,  $A_{paired}$  and  $A_{aligned}$  spermatogonia and therefore appears to be a marker of undifferentiated spermatogonia in mice. In contrast, co-expression of SALL4 with GFR $\alpha$ 1 and cKIT identified distinct subpopulations of  $A_{undiff}$  in all clone sizes that might provide clues about SSC regulation. Collectively, these results indicate that 1) SALL4 isoforms are differentially expressed at the initiation of spermatogenesis, 2) SALL4 is expressed in undifferentiated spermatogonia in adult testes and 3) SALL4 co-staining with GFR $\alpha$ 1 and cKIT reveals distinct subpopulations of  $A_{undiff}$  spermatogonia that merit further investigation.

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## Introduction

Spermatogonial stem cells (SSC) are located on the basement membrane of the seminiferous tubules and drive spermatogenesis throughout adult life by balancing self-renewal and differentiation cell divisions. SSCs are generally considered to be among the cohort of undifferentiated type A spermatogonia ( $A_{undiff}$ ), the most primitive cell type in the testis (for review see [1]).  $A_{undiff}$  are arranged as single cells ( $A_{single}$  or  $A_s$ ), pairs of cells ( $A_{paired}$  or  $A_{pr}$ ) and chains of four to 16 or 32 cells ( $A_{aligned}$  or  $A_{al}$ ) [2]. Among the undifferentiated spermatogonia, the  $A_s$  spermatogonia and some  $A_{pr}$  spermatogonia are the most likely candidates to contain stem cell activity, though limited stem cell potential of larger clones has been speculated [3]. It has been estimated that in the mouse,  $A_s$  spermatogonia represent only 0.03% of germ cells in the testes [4]. The pool of adult SSCs is established after birth by the transient population of gonocytes. This cell population is quiescent after birth, but re-enters the cell cycle around postnatal day 3. The gonocytes then migrate to the seminiferous tubule basement membrane where some will establish the pool of SSCs and others will differentiate directly into cKIT-positive differentiating sper-

matogonia and give rise to the first wave of spermatogenesis. [5,6]. This phase of germ lineage development is therefore crucial to initiate and maintain male fertility.

Here we analyzed the expression of the pluripotency factor SALL4, a member of the *sal* gene family of zinc-finger transcription factors, in the male mouse germline during postnatal development and during steady-state spermatogenesis. Members of this family are highly conserved between species; homologues have been identified in *Drosophila* [7,8], *Xenopus* [9], zebrafish [10], chicken [11], mice [12] and humans [13]. In mice and humans, four *sal* proteins (SALL 1–4) have been described that are important for normal development and that are implicated in various congenital disorders [14].

During embryo development, *Sall4* regulates axis formation [15] and expression was observed in progenitor populations of brain, neural tube, heart, pituitary gland, somites and limbs, as well as in fetal hepatic stem/progenitor cells [16,17]. Proper *Sall4* action in these organs during development is crucial, as evidenced by the mutant phenotype. Mutations in the human *SALL4* locus cause Okihiro syndrome, a rare autosomal-dominant condition characterized by limb and cardiac malformations, hearing loss,

strabismus and postnatal growth retardation [18,19]. Several *Sall4* mutant mice have been developed [16,20,21,22]. Homozygous mice were embryonic lethal in these models, and haploinsufficiency mimicked the human phenotype in varying degrees, exhibiting modest to severe phenotypes and postnatal death of pups within the first weeks after birth. Although in one study the heterozygous mutants were able to produce small litters when intercrossed or backcrossed to a C57BL/6 background [20], the testicular phenotypes of the mutants have not been evaluated systematically in these studies. Conditional knock-out mice where *Sall4* was deleted in embryonic germ cells failed to establish a robust stem cell pool, resulting in many agametic tubules [23]. Postnatal deletion of *Sall4* in *Stra8*-expressing germ cells did not impair the onset of spermatogenesis, and germ cell differentiation appeared normal in young adult mice. However, increased seminiferous tubule degeneration was observed in older mice, indicating a role of SALL4 for stem cell pool homeostasis [23].

Hobbs et al. demonstrated that SALL4 can interact directly with PLZF, a transcription factor required for SSC function and proposed that the titration of these two transcription factors regulates their distribution on the chromatin and consequently target gene regulation [23]. To gain additional insights about this novel regulatory mechanism in the context of spermatogenic lineage development, we evaluated SALL4 expression in the developing testis on postnatal days 0, 3, 7 and 14, the period when gonocytes give rise the SSCs and the first round of spermatogen-

esis. In addition, we correlated SALL4 expression in adult testes with established markers of undifferentiated (*GFR $\alpha$ 1*, *PLZF*, *LIN28*) and differentiating (*cKIT*) spermatogonia as well as with clone size ( $A_s$ ,  $A_{pr}$ ,  $A_{al}$ , etc.) in whole mount preparations of seminiferous tubules.

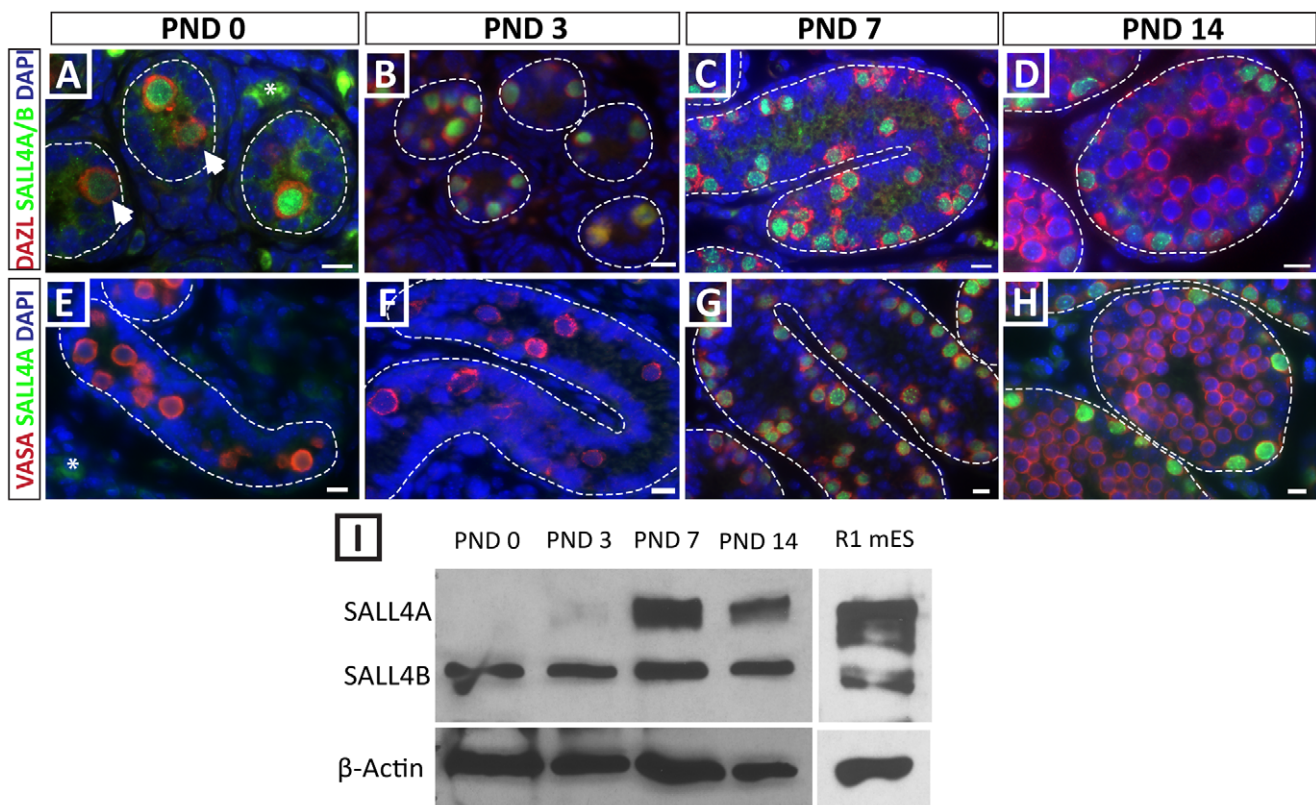
## Materials and Methods

### Animals

C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained in the laboratory animal housing facility at Magee-Womens Research Institute. All animal procedures were approved by the Institutional Animal Care and Use Committees of Magee-Womens Research Institute and the University of Pittsburgh. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Assurance # 3654-01).

### Antibodies

The following primary antibodies were used for immunoblotting, immunofluorescence staining of testis tissue sections, or whole mount seminiferous tubules: mouse anti- $\beta$  actin (No. A1978, Sigma, St. Louis, MO); mouse anti-DAZL (ab17224, Abcam, Cambridge, MA); rabbit anti-SALL4A/B (ab29112, Abcam); mouse anti-SALL4A (ab57577, Abcam), goat anti-GATA4 (SC-1237, SantaCruz); goat anti-PLZF (AF2944, R&D Systems Inc.,



**Figure 1. Differential expression of SALL4 isoforms during postnatal testis development.** (A–D) Co-staining for DAZL (all germ cells, red) and SALL4A/B (green). (A, B) Some SALL4-negative gonocytes (white arrows) were observed at PND 0. Autofluorescence was observed in the interstitium (asterisk). All germ cells strongly express SALL4 at PND 7 (C) and PND 14 (D) (E–H) Co-staining for VASA/DDX4 (all germ cells, red) and SALL4A (green). (E,F) SALL4A was absent from VASA-positive germ cells at PND 0 and 3. SALL4A was strongly expressed in all germ cells at PND 7 (G) and PND 14 (H). Scale bars = 10  $\mu$ m. (I) Western blot analysis of total testis protein isolated from different postnatal ages showed differential expression of SALL4 isoforms. Protein from R1 mouse embryonic stem cells is shown as positive control. Blots were reprobed with anti-beta actin IgG to ensure equal loading of proteins. doi:10.1371/journal.pone.0053976.g001

Minneapolis, MI); goat anti-LIN28 (AF3757, R&D Systems); goat anti-GFR $\alpha$ 1 (AF650, R&D Systems); rabbit anti-VASA/DDX4 (ab13840, Abcam); armenian hamster anti-cKIT (H2C7, [24]).

### Protein Isolation and Western Blotting

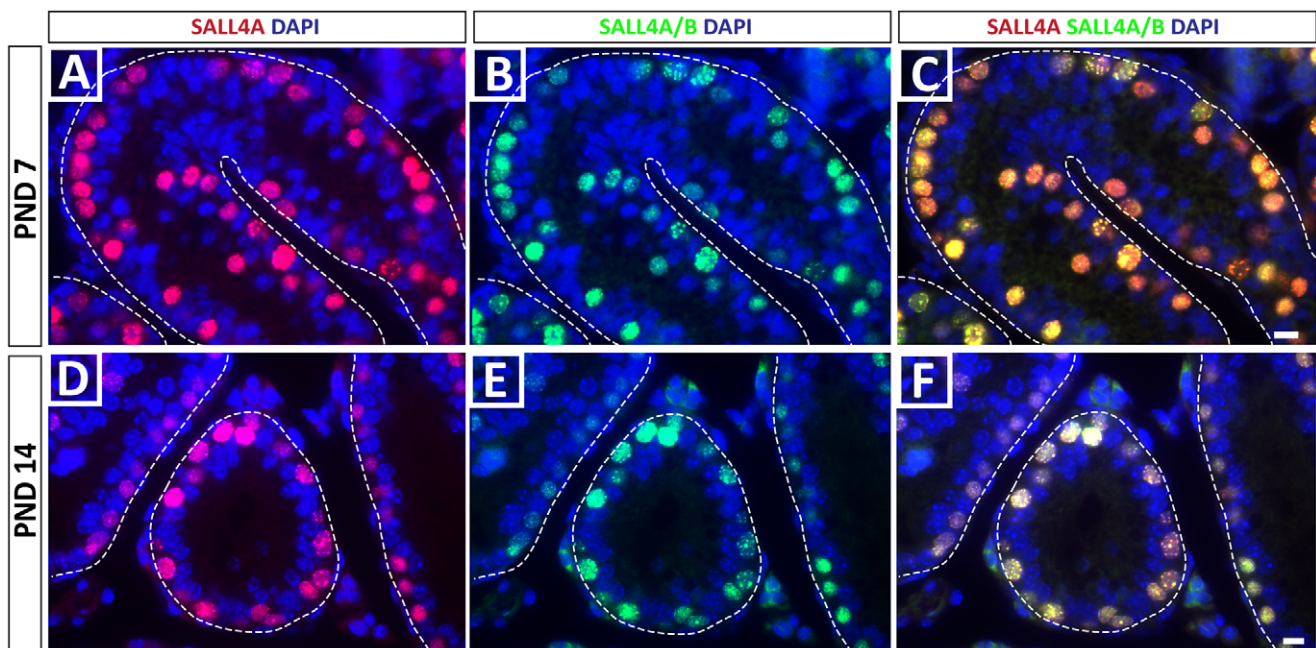
Testes from PND 0, 3, 7 and 14 mice were dissected, the tunica albuginea removed and collected in chilled RIPA buffer (150 mM NaCl, 0.1% SDS, 1% NP-40, 50 mM Tris pH 7.5, 0.5% Sodium-Deoxycholate) for preparation of whole testis protein lysates. Tissues were disrupted mechanically and incubated on ice for 30 min and lysates were cleared from residual cell debris by centrifugation for 10 min at 10,000 $\times$ g. The supernatant was collected and protein concentrations were determined by Bradford colorimetric assay (Protein Assay No.500-0006, Biorad, Hercules, CA). Protein (10  $\mu$ g) was separated by SDS-PAGE on a 10% gel (Biorad Mini-PROTEAN<sup>®</sup> TGX<sup>™</sup> Precast Gel #456-1033) and transferred onto a nitrocellulose membrane using standard techniques. Blots were blocked with 5% Blotto (No. sc-2324, Santa Cruz Biotechnology Inc., Santa Cruz, CA) in 1 $\times$  Dulbecco's Phosphate Buffered Saline (DPBS, No. 14200166, Invitrogen, Carlsbad, CA) containing 0.1% Tween-20 (P5927, Sigma) for 1 hour at room temperature. Primary antibodies rabbit anti-SALL4 (1:1000) and mouse anti-beta actin (1:10000) were incubated overnight at 4°C. After washing the blots three times for 10 min in DPBS-T, horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1:5000 dilution) were incubated for 1 hour at room temperature and protein bands were detected with Super-Signal West Pico Chemiluminescent Substrate as instructed (PI34080, Thermo Fisher Scientific, Pittsburgh, PA). ImageJ software (NIH, Bethesda, MD) was used for densitometry to evaluate relative levels of SALL4A, SALL4B and total SALL4 protein during testis development.

### Immunofluorescence Staining of Testis Tissue Sections

Testes from neonatal (postnatal day (PND) 0 and PND 3), juvenile (PND 7 and PND 14) and adult (>6 weeks of age) mice were dissected and fixed in 4% PFA for 2 hours (neonatal testes) or overnight (juvenile and adult testes) at 4°C. Fixed tissues were processed for paraffin embedding and 5  $\mu$ m serial sections were collected. Sections were deparaffinized in xylene (2 $\times$ 15 min), rehydrated in a graded ethanol series (2 $\times$ 100% for 10 min, 1 $\times$ 95% for 5 min, 1 $\times$ 80% for 5 min, 1 $\times$ 70% for 5 min, 1 $\times$ 50% for 5 min, 1 $\times$ 25% for 5 min) and rinsed in 1 $\times$  DPBS. Slides were then incubated in sodium citrate antigen retrieval buffer (10 mM Sodium Citrate, 0.05% Tween-20, pH 6) for 30 min at 97.5°C and allowed to cool to room temperature. After rinsing twice in 1 $\times$  DPBS containing 0.1% Tween-20 (DBPS-T), unspecific binding sites in tissue sections were saturated by incubation with blocking buffer (1 $\times$  DPBS containing 3% bovine serum albumin, 0.1% Triton X-100 and 5% normal serum from the host species of the secondary antibody) for 30 min at room temperature. Primary antibodies were diluted in blocking buffer and added to tissue sections for 90 min at room temperature. Isotype matched normal IgG at a comparable concentration was used instead of primary antibody in negative controls (see Figures S1 and S2). After washing the slides in DPBS-T 3 $\times$  for 5 min, samples were incubated with the appropriate secondary antibody (goat IgG or donkey IgG) conjugated to AlexaFluor-488 or AlexaFluor-568 dye (all Molecular Probes, Invitrogen) for 45 min at room temperature. Sections were washed 3 $\times$ 5 min with DPBS-T and 1 $\times$ 5 min with DPBS before mounting with Vectashield Mounting Medium with DAPI (H-1200, Vector Laboratories, Burlingame, CA).

### Immunofluorescence Staining of Whole Mount Seminiferous Tubules

Testes from adult C57/Bl6 male mice were collected in chilled DPBS and the tunica albuginea was carefully removed. The



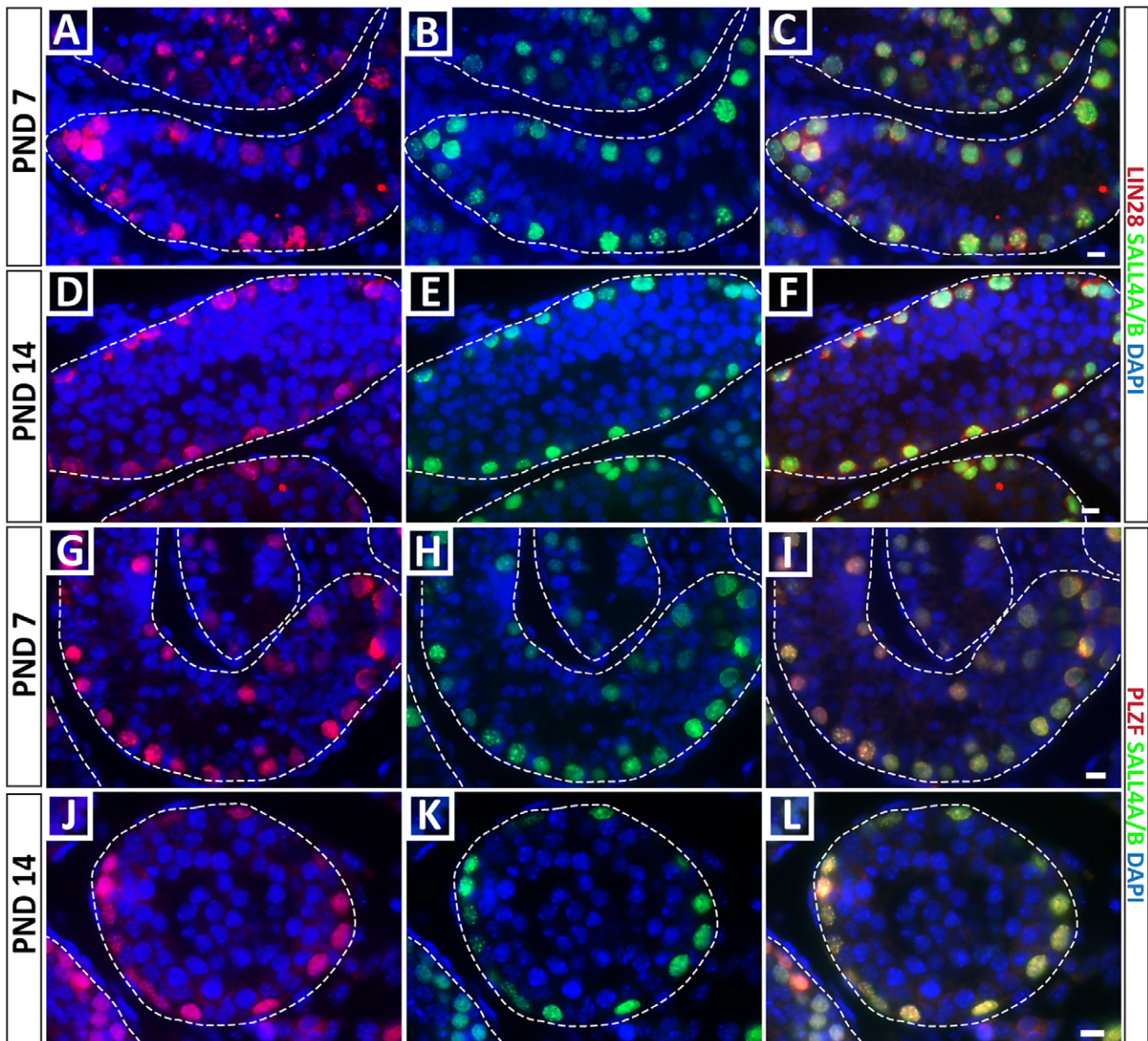
**Figure 2. SALL4A and SALL4B co-expression in juvenile germ cells.** SALL4A and SALL4B are expressed in the same population of germ cells at PND 7 (A–C) and PND 14 (D–F). Scale bars = 10  $\mu$ m.  
doi:10.1371/journal.pone.0053976.g002



seminiferous tubules were gently teased apart with fine forceps and briefly digested with 1 mg/ml DNase I (No. DN-25, Sigma) and 1 mg/ml Collagenase IV (No. C5138, Sigma) in DPBS. Tubules were fixed overnight in 4% PFA at 4°C and then extensively washed 3–4 times with DPBS at 60-minute intervals. For SALL4, PLZF and LIN28 staining, tubules were dehydrated in a graded methanol series (25%, 50%, 75%, 95% and 2× in 100% MeOH) for 10 min at room temperature. Samples were permeabilized for 3 hours at room temperature in a 4:1:1 mix of MeOH/DMSO/H<sub>2</sub>O<sub>2</sub>. Sequential rehydration was by incubation in 50% MeOH (10 min) and 25% MeOH (10 min) and DPBS (2×10 min). Samples were blocked with ice cold PBSMT blocking buffer (1×DPBS +2% blotto milk powder +0.5% Triton X-100) for 2×15 min and 1×2 hours and incubated in primary antibody at 4°C overnight. Isotype matched normal IgG at a comparable

concentration was used instead of primary antibody as a negative control (see Figure S3).

Samples were washed with ice-cold PBSMT (2×15 min, 5×1 hr) and incubated in AlexaFluor-conjugated secondary antibody overnight at 4°C. Samples were washed again with ice-cold PBSMT (2×15 min, 5×1 hr) followed by DPBS (2×10 min) and mounted on raised coverslips with Vectashield mounting medium containing DAPI nuclear stain. For co-staining of SALL4 with the surface antigens GFR $\alpha$ 1 or cKIT, tubules were only shortly dehydrated in DPBS/10% MeOH/0.1% Triton X-100 at 4°C for 60 min and blocked with PBSMT at 4°C for 60 min. Primary antibodies were diluted in PBSMT and incubated overnight at 4°C. Samples were washed 6×15 min at room temperature with DPBS/0.1% Triton X-100 (PBSTx) and incubated with donkey anti-goat Alexa568 (No. A-11057, Molec-



**Figure 3. SALL4 is expressed by undifferentiated spermatogonia in postnatal day 7 and postnatal day 14 testes.** (A–F) SALL4 expression is restricted to LIN28 positive undifferentiated spermatogonia at PND 7 and PND 14. (G–L) SALL4 expression is restricted to PLZF positive undifferentiated spermatogonia at PND 7 and PND 14. Scale bars = 10  $\mu$ m. doi:10.1371/journal.pone.0053976.g003



ular Probes, Invitrogen) or goat anti-american hamster Rhodamine-Red-X (No. 127-295-160, Jackson Immuno Research, West Grove, PA) conjugates for 1 hour at 37°C. Samples were washed 6×15 min at room temperature with PBSTx and mounted on raised coverslips with Vectashield mounting medium containing DAPI.

### Imaging and Image Analysis

Sections and tubules were imaged using an Eclipse 600 fluorescent microscope (Nikon USA, Melville, NY) equipped with an X-Cite 120 fluorescence source (EXFO Life Sciences, Ontario, Canada) and a Spot RT 2.3.1 slider camera (Diagnostic Instruments, Sterling Heights, MI). A dual-emission FITC/TRITC filter was used to observe green and red fluorescent signals. Image analysis was performed using MetaVue software (Molecular Devices, Sunnyvale, CA).

Stained tissue sections from at least three adult animals were used for each co-staining experiment and a minimum of 100 circular cross sections per animal was evaluated for quantification of co-expression patterns. Whole mount tubules were mounted in small fragments of several mm in length and a minimum of 500 spermatogonial clones were scored in at least three animals for

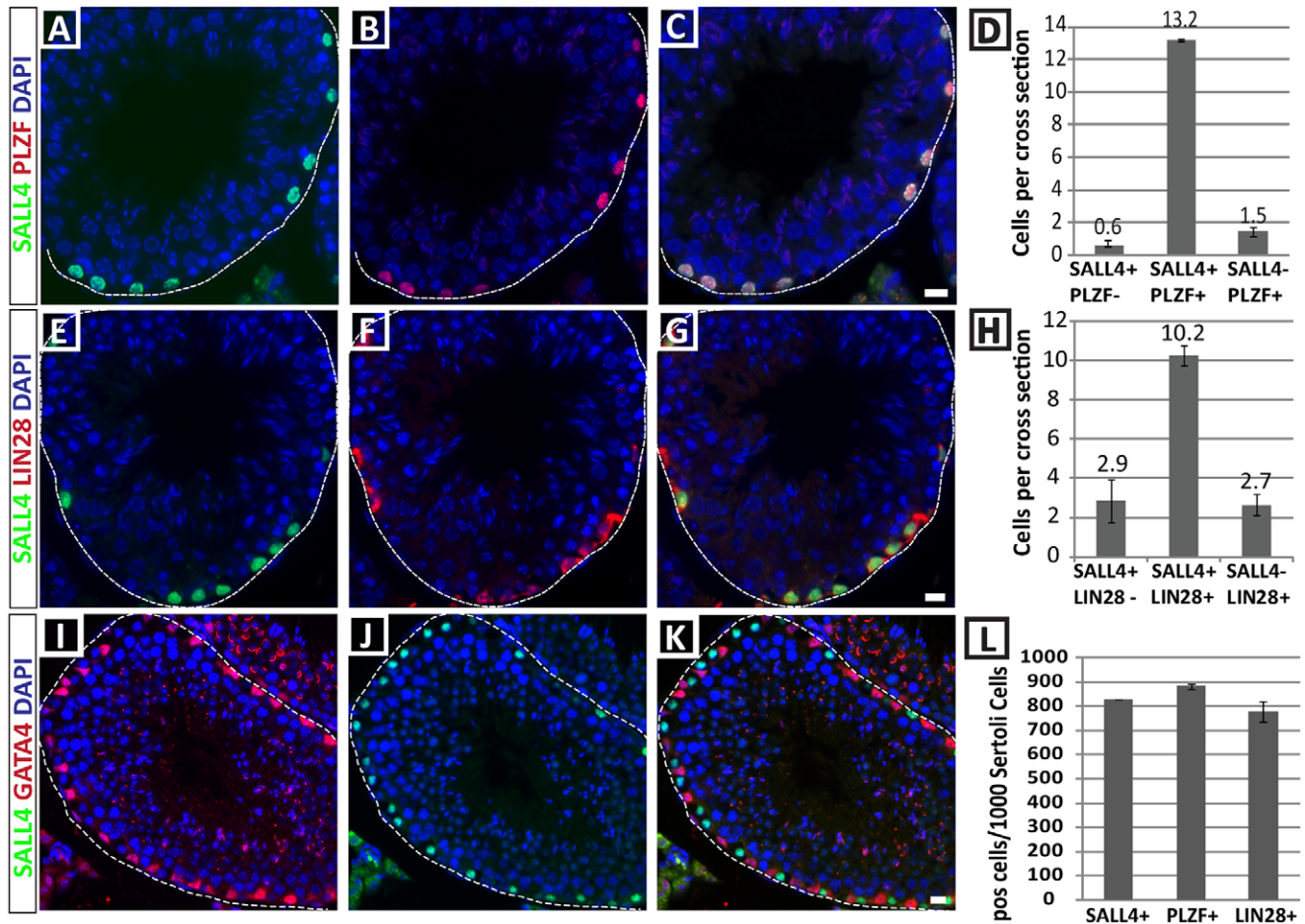
each experiment. Spermatogonial clones were defined according to Tegelenbosch and de Rooij [4]. The combined number of all single- and/or double-stained spermatogonia was used as the denominator to calculate cell ratios.

## Results

### Differential Expression of SALL4 Isoforms A and B during Postnatal Testis Development

Previous work by Wang et al. suggested that the *Sall4* gene is expressed in pre-meiotic spermatogonia in the postnatal day 7 mouse testis [25]. These observations were confirmed in the recent studies of Eildermaann et al. [26] and Hobbs et al. [23]. The first week after birth is critical for male germline development. During this time, gonocytes re-enter mitosis and migrate to the seminiferous tubule basement membrane where some will establish the pool of SSCs and others will differentiate directly into cKIT-positive differentiating spermatogonia and give rise to the first wave of spermatogenesis [6].

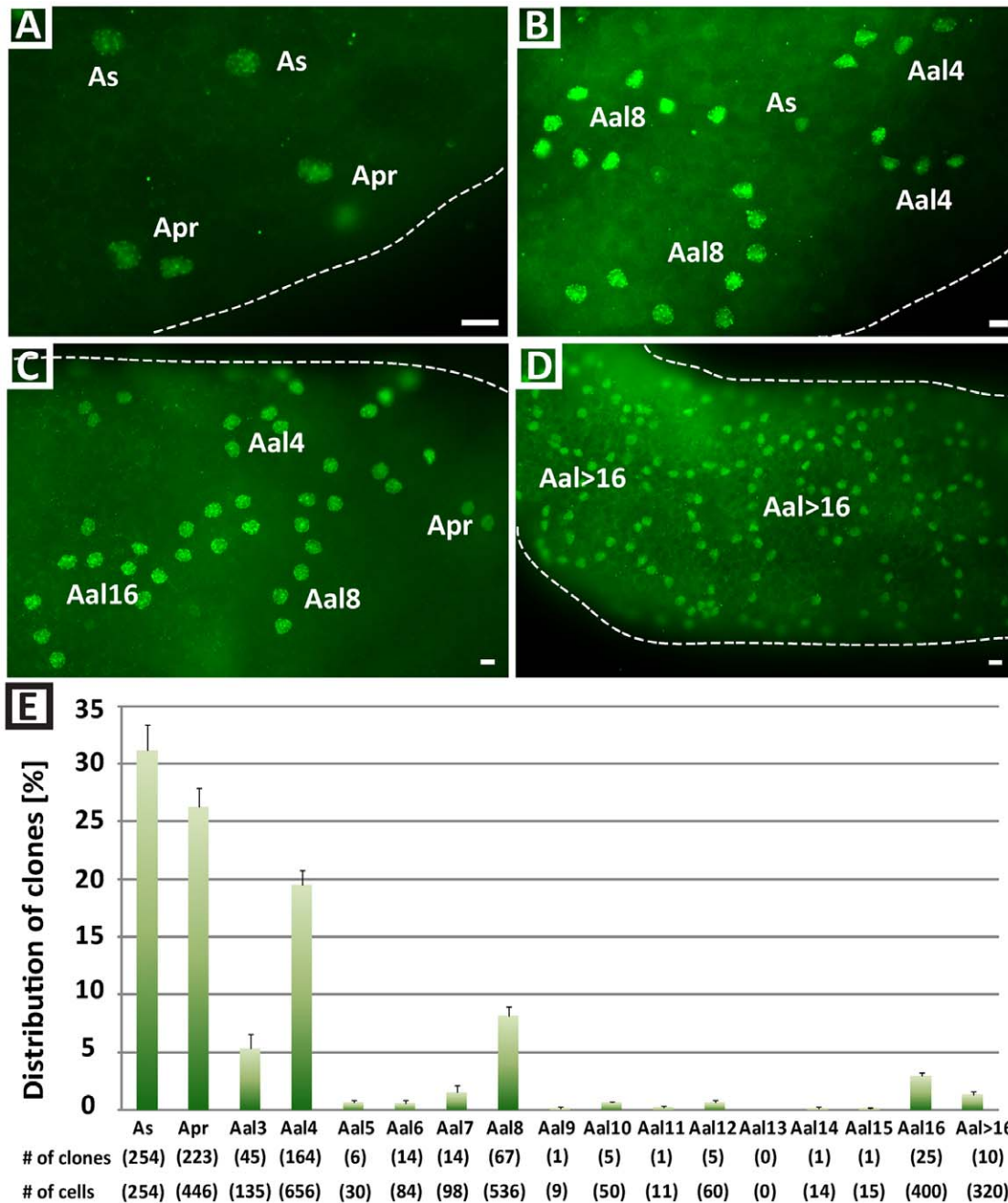
The mammalian *Sall4* gene encodes three alternative splice variants (SALL4A, SALL4B, SALL4C). SALL4A and SALL4B were recently shown to have isoform-specific target genes and to



**Figure 4. SALL4 is expressed in undifferentiated spermatogonia in adult mouse seminiferous tubules.** Double immunohistochemistry of tissue sections was used to examine the localization of SALL4 in comparison to PLZF (A–D) and LIN28 (E–H), two known markers for undifferentiated spermatogonia. (D, H) Data are expressed as the average number of single or double stained cells per tubular cross section  $\pm$  SEM. (I–K) SALL4 was not expressed in GATA4-positive Sertoli cells. (L) SALL4, PLZF and LIN28-positive cells were normalized to 1000 Sertoli cells to express the relative size of the respective spermatogonial populations. The basement membrane of each tubule is marked by a dashed line. Scale bars = 10  $\mu$ m. For quantitative analysis, 100 circular tubule cross sections in testes from at least 3 adult male mice were scored. doi:10.1371/journal.pone.0053976.g004

play different roles for pluripotency maintenance and differentiation of mouse embryonic stem cells [27]. In this context we wanted to determine which of these isoforms is present during the establishment of the spermatogonial stem cell pool and the initiation of spermatogenesis in the postnatal mouse testis. Germ cells in sections from PND 0, 3, 7, and 14 were identified by staining for either DAZL or VASA, two ubiquitous germ cell markers, that were confirmed by double-immunohistochemistry to

be expressed in all germ cells at the selected ages (data not shown). For co-staining experiments, we used a polyclonal antibody that recognizes both SALL4A and SALL4B in combination with anti-DAZL antibody, and we also used a monoclonal antibody that specifically recognizes SALL4A in combination with anti-VASA antibody. The data in Figure 1 indicate that while the SALL4A/B antibody stains DAZL+ germ cells on postnatal days 0, 3, 7 and 14 (Fig. 1A–D); the SALL4A-specific antibody stains VASA+ germ



**Figure 5. Clonal organization of SALL4 expressing cells in adult mouse seminiferous tubules.** Whole mount seminiferous tubules were isolated from adult animals and subjected to immunofluorescence staining. (A–D) SALL4 was found to be expressed in  $A_s$ ,  $A_{pr}$  and  $A_{al}$  spermatogonia. Scale bars = 10  $\mu$ m. (E) Quantification of the frequency of SALL4 positive  $A_s$ ,  $A_{pr}$  and  $A_{al}$  spermatogonial clones that were observed in adult mouse seminiferous tubules. Data are presented as average  $\pm$  SEM from three independent experiments. The number of clones counted is indicated in parentheses beneath the graph. Total cell numbers in each clone size were calculated by multiplying the number of clones by the number of cells contained in each clone of a certain size, e.g. 254  $A_s$  clones = 254 cells, 223  $A_{pr}$  clones = 446 cells, etc. Cell numbers are shown in parentheses. doi:10.1371/journal.pone.0053976.g005



cells only on days 7 and 14 (Fig. 1E–H). These results were also confirmed by Western blot with the SALL4A/B antibody that distinguishes SALL4A and SALL4B isoforms based on size (Fig. 1I).

We next tested the hypothesis that SALL4A and SALL4B might be expressed in different subpopulations of spermatogonia in the developing mouse testis by co-staining with the SALL4A/B and SALL4A antibodies. However, the data in Figure 2 indicate a complete overlap of staining with these two antibodies on days 7 and 14 of development, corresponding to times when the spermatogonial stem cell pool is established, and spermatogenesis is initiated. Therefore, SALL4A and B do not appear to mark different subpopulations of spermatogonia at these stages of development. To confirm the spermatogonial subtypes that express SALL4 in postnatal day 7 and 14 testes, we co-stained with the SALL4A/B antibody and LIN28 or PLZF, which are established markers of undifferentiated spermatogonia. We observed nearly complete overlap of SALL4 expression with both LIN28 and PLZF (Fig. 3). Therefore, SALL4 expression appears restricted to LIN28+ and PLZF+ undifferentiated spermatogonia at these stages of mouse testis development.

### SALL4 is Expressed in Undifferentiated Spermatogonia in the Adult Mouse Testis

To investigate the expression of SALL4 during steady-state spermatogenesis in the adult mouse testis, we co-stained tissue sections with SALL4 and the established spermatogonia markers, PLZF and LIN28, and the Sertoli cell marker GATA4 (Fig. 4). Co-expression analysis showed that SALL4 co-localizes with PLZF in 13.2 cells per tubular cross section ( $86.7\% \pm 1.9$  overlap) and only occasionally spermatogonia were observed that expressed either SALL4 alone (0.6 cells/cross section;  $3.8\% \pm 0.9$ ) or PLZF alone (1.5 cells/cross section;  $9.6\% \pm 1.3$ ). Similarly, co-expression of SALL4 with LIN28 was found in 10.2 cells/cross section ( $66.3\% \pm 6.3$ ), while 2.9 cells/cross section were SALL4 positive only ( $17.3\% \pm 4.7$ ) and 2.7 cells/cross section were positive for LIN28 alone ( $16.5\% \pm 1.7$ ). In contrast, SALL4 was not expressed in GATA4-positive Sertoli cells (16.6 cells/tubule cross section). Overall, 829 SALL4-positive spermatogonia, 883 PLZF-positive

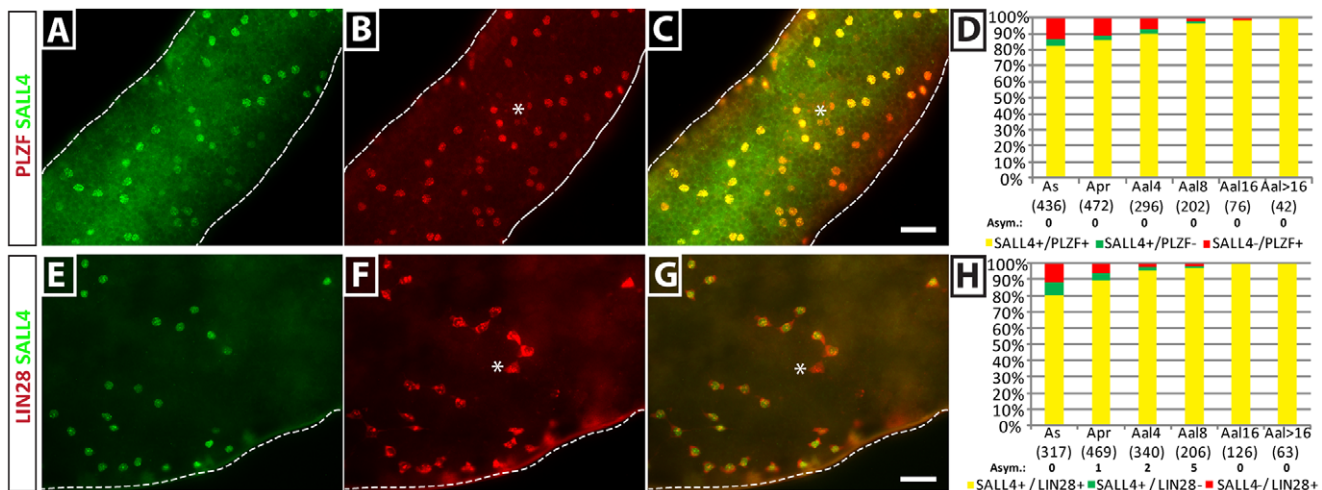
spermatogonia, and 778 LIN28-positive spermatogonia were observed per 1000 Sertoli cells in the adult testis based on these cell counts in adult mouse testicular sections (Fig. 4L). In summary, these data demonstrate that SALL4 is expressed by the majority of PLZF+ and LIN28+ undifferentiated spermatogonia during steady-state spermatogenesis.

### Clonal Organization of SALL4 Expressing Cells in Seminiferous Tubules

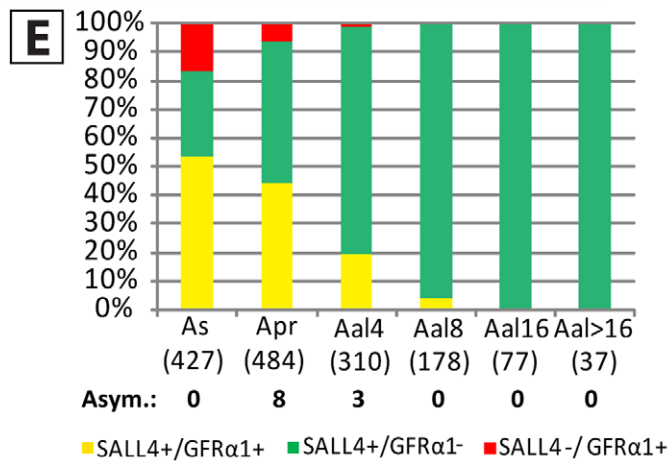
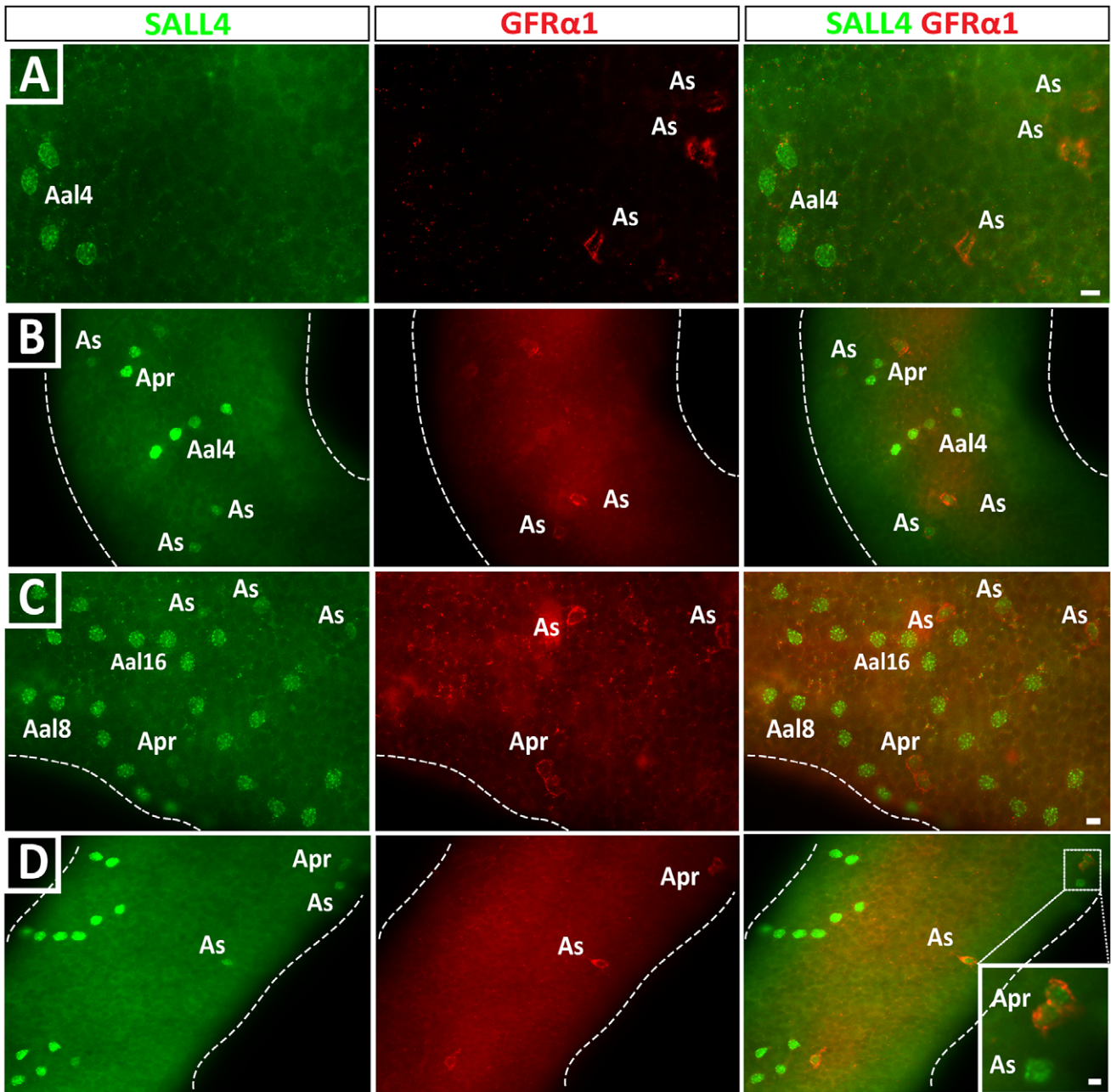
To correlate SALL4 expression with spermatogonial clone size ( $A_s$ ,  $A_{pr}$ ,  $A_{al}$ ), we performed SALL4 immunohistochemistry in whole mount preparations of seminiferous tubules (Fig. 5A–D). We found that SALL4 positive cells were arranged as  $A_{singles}$  ( $31.4\% \pm 2.2$  of SALL4 positive clones),  $A_{pairs}$  ( $26.3 \pm 1.6\%$ ), chains of  $A_{al4}$  ( $19.5 \pm 1.3\%$ ),  $A_{al8}$  ( $8.1 \pm 0.9\%$ ),  $A_{al16}$  ( $2.9 \pm 0.3\%$ ) and sometimes in longer chains of  $A_{al>16}$  spermatogonia ( $1.3 \pm 0.3\%$ ) (Fig. 5E). Clones of 3 SALL4+ cells were occasionally observed. Otherwise odd clone sizes that did not correspond to population doubling divisions (2, 4, 8, 16) were rarely observed.

### SALL4 is Co-expressed with PLZF and LIN28 in $A_s$ , $A_{pr}$ and $A_{al}$ Spermatogonia

It was previously reported that the  $A_{undiff}$  population is heterogeneous [28,29]. In order to determine the distribution of SALL4 in spermatogonial clones in relation to other known markers, we used whole mount immunohistochemical co-staining (Fig. 6). In accordance with the data from tissue section immunostaining, we found that SALL4 expression substantially overlapped with both PLZF and LIN28. In particular, PLZF was co-expressed with SALL4 in  $82.7 \pm 2.2\%$  of  $A_s$  spermatogonia,  $86.3 \pm 0.7\%$  of  $A_{pr}$  spermatogonia and  $90.3 \pm 2.0\%$  of  $A_{al4}$  spermatogonia. Co-expression further increased in chains of eight and 16 cells ( $96.6 \pm 1.7\%$  and  $98.7 \pm 1.3\%$ , respectively) and was complete in all clones observed with more than 16 cells. SALL4 and LIN28 were co-expressed in  $77.9 \pm 3.2\%$  of  $A_s$  and  $88.3 \pm 1.1\%$  of  $A_{pr}$  spermatogonia. As with PLZF, co-expression of SALL4 and LIN28 increased in  $A_{al4}$  and  $A_{al8}$  ( $95.4 \pm 1.9\%$  and  $97.2 \pm 0.7\%$ , respectively) and was complete in all chains containing 16 or more spermatogonia. Asymmetrical clones of



**Figure 6. SALL4 is co-expressed with PLZF and LIN28 in clones of undifferentiated spermatogonia.** (A–D) SALL4 and PLZF were co-expressed in most undifferentiated spermatogonia. Small fractions of  $A_s$  and  $A_{pr}$  spermatogonia showed molecular heterogeneity (\*). (E–H) SALL4 also largely overlaps with LIN28 expression, with heterogeneity of expression observed in few  $A_s$  and  $A_{pr}$  spermatogonial clones (\*). Scale bars = 50  $\mu$ m. Data obtained from  $n=3$  animals, presented as average  $\pm$  SEM. Asym: Number of clones with molecular asymmetry. doi:10.1371/journal.pone.0053976.g006





**Figure 7. Differential expression of GFR $\alpha$ 1 and SALL4 in A<sub>s</sub> and A<sub>pr</sub> spermatogonia.** Expression of GFR $\alpha$ 1 was limited to short chains of A<sub>undiff</sub> and more restricted than expression of SALL4. (A–D) Examples of GFR $\alpha$ 1 and SALL4 expressing spermatogonial clones in whole mount seminiferous tubules. Scale bars = 10  $\mu$ m. Panel D was modified from [1] with permission. (E) Quantitative evaluation of GFR $\alpha$ 1 and SALL4 expression in clones of undifferentiated spermatogonia. Among A<sub>s</sub> spermatogonia, 50% of cells co-expressed both markers, but substantial populations of SALL4-only and GFR $\alpha$ 1-only cells were also observed. The number of SALL4-only cells increased in longer chains, when GFR $\alpha$ 1 expression ceased. Asym: Number of clones with molecular asymmetry. doi:10.1371/journal.pone.0053976.g007

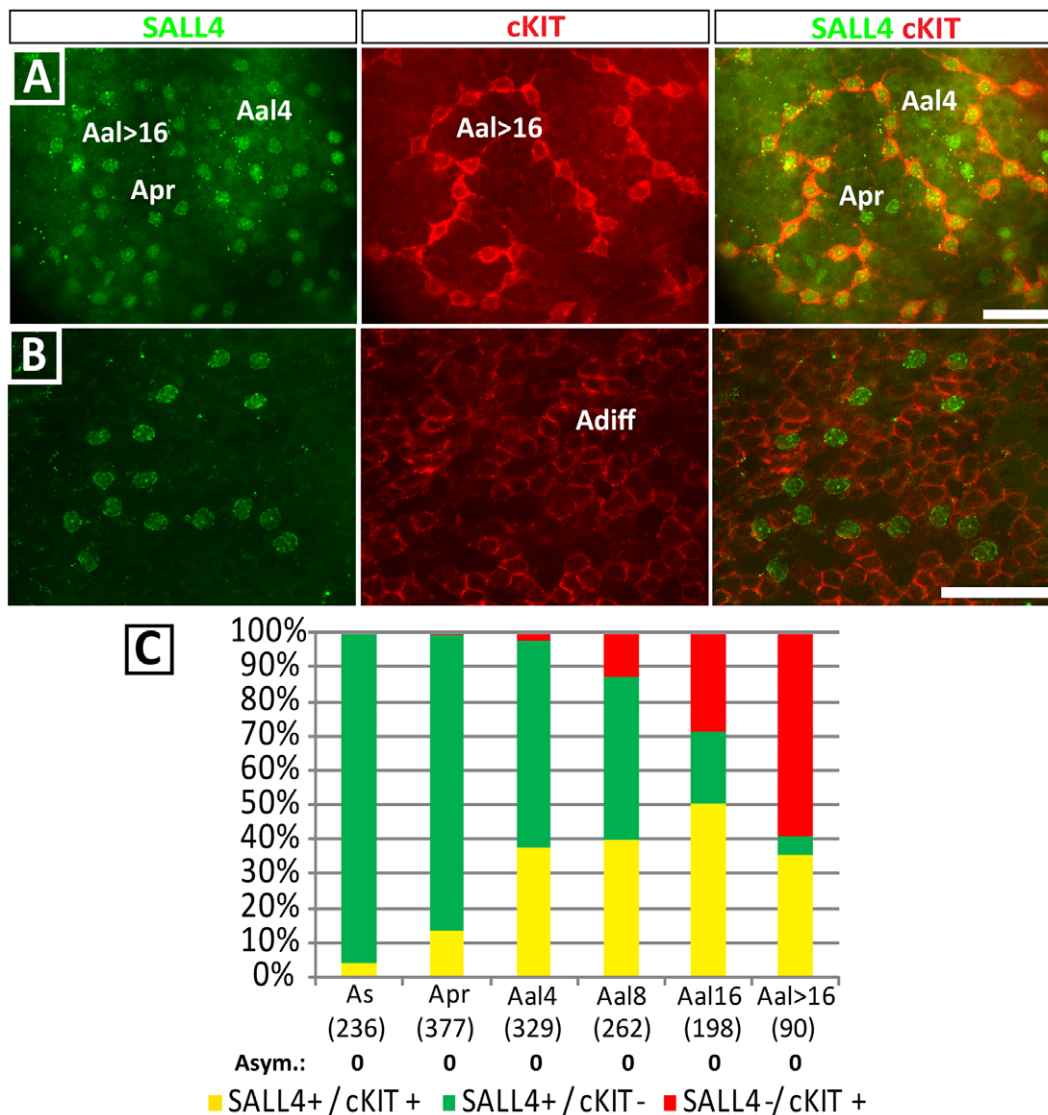
A<sub>pr</sub> and A<sub>al</sub> spermatogonia, as previously reported by others [30,31,32], were observed infrequently (<1% of clones, see Fig. 6D and H) and were counted as double stained clones because it is not possible to know whether the asymmetry is biologically relevant or an artifact caused by tubule preparation or staining procedure.

Thus, it appears that most A<sub>undiff</sub> express SALL4, but some minor populations of SALL4-only, PLZF-only and LIN28-only expressing cells were present in the seminiferous epithelium. In this regard it is interesting to note that heterogeneity of expression was greater in A<sub>s</sub> and A<sub>pr</sub> spermatogonia than in A<sub>al</sub> spermatogonia.

The biological significance of these subpopulations for spermatogonial self-renewal and differentiation remains unclear, but this observation is consistent to reports by others and will require further investigation [29,30,33].

#### Differential Expression of GFR $\alpha$ 1 and SALL4 in A<sub>s</sub> and A<sub>pr</sub> Spermatogonia

The progression of spermatogonia from A<sub>s</sub> to A<sub>al</sub> is thought to be indicative of a cell fate shift towards differentiation and thus, the



**Figure 8. SALL4 is downregulated in differentiating spermatogonia.** (A) SALL4 and cKIT are co-expressed in chained A<sub>al</sub> spermatogonia. (B) SALL4 becomes downregulated as spermatogonia differentiate. In contrast, cKIT expression persists in differentiated germ cells. (C) Quantification of SALL4/cKIT co-staining showed that SALL4 expression decreased in larger clones coincident with increased cKIT expression. Note that cKIT expression in A<sub>al16</sub> remained heterogeneous. Scale bars = 50  $\mu$ m. Asym: Number of clones with molecular asymmetry. doi:10.1371/journal.pone.0053976.g008

spermatogonial stem cell potential to self-renew is primarily attributed to  $A_s$  and sometimes  $A_{pr}$  spermatogonia. We examined this phenomenon further by evaluating the co-expression of SALL4 with GFR $\alpha$ 1, one of the most restricted markers for undifferentiated spermatogonia [3].

Glial cell line-derived neurotrophic factor (GDNF) is essential for SSC maintenance and self-renewal both in vitro and in vivo [34,35] and its receptor GFR $\alpha$ 1 is expressed on a subset of undifferentiated spermatogonia [30]. Here, we used double-immunofluorescence to investigate if SALL4 is present in the same spermatogonial population as this putative SSC marker (Fig. 7). As expected, GFR $\alpha$ 1 expression was restricted primarily to  $A_s$ ,  $A_{pr}$  and some  $A_{al4}$  spermatogonia. It was rarely observed in  $A_{al8}$ , and was never observed in chains larger than 8.

Substantial heterogeneity between SALL4 and GFR $\alpha$ 1 was observed in  $A_s$  and  $A_{pr}$  spermatogonia. In particular,  $51.7 \pm 7.8\%$  of  $A_s$  co-expressed both markers, but  $30.8 \pm 7.4\%$  of  $A_s$  were SALL4 positive only and the remaining  $17.6 \pm 3.9\%$   $A_s$  spermatogonia expressed only GFR $\alpha$ 1. Similarly,  $44.3 \pm 7.2\%$  of the  $A_{pr}$  spermatogonia co-expressed SALL4 and GFR $\alpha$ 1,  $49.7 \pm 7.0\%$  expressed SALL4 only and  $6 \pm 0.3\%$  expressed GFR $\alpha$ 1 only. In  $A_{al4}$ , only  $19.6 \pm 2.9\%$  of the cells were SALL4+/GFR $\alpha$ 1+ and the dominant phenotype was SALL4+ only ( $79.2 \pm 3.0\%$ ). GFR $\alpha$ 1/SALL4 co-expression was observed in only 4% of  $A_{al8}$  and GFR $\alpha$ 1 expression was not observed in chains of 16 or more aligned spermatogonia, resulting in a singular SALL4+ phenotype. In rare events, asymmetrical  $A_{pr}$  and  $A_{al4}$  were observed ( $<1\%$ , see Fig. 7).

### SALL4 is Downregulated in cKIT-positive, Differentiating Spermatogonia

Next, we investigated the expression of SALL4 in spermatogonia poised to undergo differentiation. The cKIT receptor tyrosine kinase is upregulated during the transition of undifferentiated spermatogonia into differentiating A1 spermatogonia and remains expressed during the amplifying pre-meiotic spermatogonial divisions [36]. cKIT expression (SALL4+/cKIT+ and SALL4-/cKIT+) was occasionally observed in the smallest  $A_s$  (4.1%) and  $A_{pr}$  (14.3%) clones, and expression increased markedly in  $A_{al4}$ , when 39.8% of spermatogonia expressed cKIT (Fig. 8). However, at this point the majority of  $A_{al4}$  ( $60.3 \pm 8.7\%$ ) were SALL4+ only.

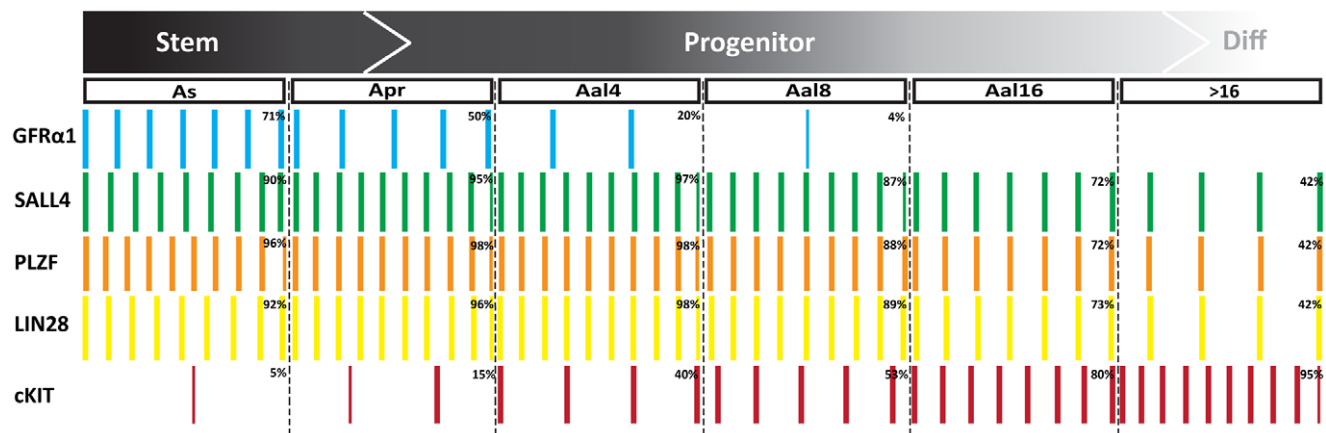
At the stage of  $A_{al8}$ , cKIT expression became more prominent, with increasing numbers of SALL4+/cKIT+ clones (40.7%) and cKIT+ only clones (12.3%) being observed. Most  $A_{al16}$  spermatogonia expressed cKIT (79.0%), but it is important to note that a substantial number of  $A_{al16}$  were SALL4+/cKIT- ( $21.0 \pm 5.14\%$ ) (Fig. 8B). Taken together, these results indicate that SALL4 expression declines in larger  $A_{undiff}$  clones coincident with increasing cKIT expression. Asymmetrical clones were not observed.

The expression data from all whole mount immunostaining experiments are summarized in Figure 9.

### Discussion

SALL4 participates in transcriptional regulatory networks during fetal development of various organs [21] and is critical for cell fate decisions and lineage specification [16]. In contrast to most organs, expression in the germline is maintained after birth [25] and is restricted to pre-meiotic germ cells in rodents, non-human primates and humans [23,26]. Although the exact function of SALL4 in spermatogonia is still unclear, increasing knowledge from studies of embryonic stem cells and hematopoietic stem cells may provide clues about possible mechanisms of SALL4 action in germ cells [37,38,39,40]. Here, we analyzed the expression pattern of SALL4 during spermatogenic lineage development and steady-state spermatogenesis in relation to clone size and established markers for undifferentiated and differentiating spermatogonia.

Our results show that SALL4 is expressed by a subpopulation of quiescent gonocytes at PND0, but is expressed by all gonocytes at PND3, which coincides with re-entry into the cell cycle and migration to the seminiferous tubule basement membrane [5]. A similar pattern has been reported for neonatal and postnatal germ cells in the marmoset [26]. The pool of perinatal gonocytes has two destinies; differentiate to produce the first round of spermatogenesis and establish the initial pool of spermatogonial stem cells [6,41]. Perhaps the heterogeneous SALL4 expression at PND0 distinguishes these two populations. However, by day 7 in our study, when gonocytes reach the basement membrane, SALL4 was expressed in all germ cells. Moreover, the observation that two SALL4 splice variants are expressed differentially during this



**Figure 9. Summary of spermatogonial marker expression in mouse spermatogonia.** Graphical summary of quantitative data from Figures 6–8 were used to calculate the ratio of cells expressing each marker within the populations of  $A_s$ ,  $A_{pr}$  and  $A_{al}$  spermatogonia. Each bar represents 10% GFR $\alpha$ 1-, SALL4-, PLZF-, LIN28- or cKIT- positive cells, respectively, and half bars represent approximately 5% positive cells. For example, co-staining revealed that 71% of  $A_s$  spermatogonia expressed GFR $\alpha$ 1, therefore 7 bars (each representing 10%), are shown in the diagram. The heterogeneity of the molecular phenotype of spermatogonia clone sizes is represented by the extent of overlap among bars across marker profiles.

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specific time when the SSC pool is established may suggest that the SALL4A and SALL4B isoforms possess different regulatory functions in germ cells. To our knowledge, this is the first study that identified differential expression of SALL4 isoforms in undifferentiated spermatogonia. It was previously shown that SALL4A and SALL4B can form homo- or heterodimers with distinct DNA target regions regulating either pluripotency genes (SALL4A/B heterodimers, SALL4B/B homodimers) or differentiating/patterning genes (SALL4A/A homodimers) [27]. Thus, the presence of SALL4B alone (in gonocytes), or SALL4A and SALL4B (in PND7 spermatogonia) may have implications for the regulation of target genes, perhaps influencing the fate toward stem cell or differentiation to produce the first round of spermatogenesis. However, we were not able to identify subpopulations of spermatogonia at PND7 or 14 with the phenotypes SALL4A only or SALL4B only; rather, all spermatogonia had the SALL4A/B co-stained phenotype. It is possible that the ratio of SALL4A to SALL4B changes on a per cell basis, but this could not be unequivocally confirmed by immunohistochemistry.

We then investigated SALL4 expression during steady-state spermatogenesis. Our data confirmed previous reports of SALL4 co-localization with PLZF, an established marker for undifferentiated spermatogonia, in tissue sections [31]. We further show that SALL4 is almost completely co-expressed with a second spermatogonia marker, the RNA-binding protein LIN28. In contrast, GATA4-expressing Sertoli cells did not express SALL4. We observed 16.6 Sertoli cells per tubular cross section, which is comparable to data reported by Oatley and coworkers [42], and using this approach we calculated that approximately 800 SALL4-positive spermatogonia per 1000 Sertoli cells are present in the adult mouse testis. It is important to note that the size of the SALL4-positive spermatogonial population was similar to the population of PLZF-positive and LIN28-positive spermatogonia. Because of the extensive overlap between SALL4, PLZF, and LIN28 expression, and almost identical population size relative to Sertoli cell number, we propose that SALL4 is expressed in undifferentiated spermatogonia.

Studies in tissue sections do not provide information about the topographical arrangement of spermatogonial clones ( $A_s$ ,  $A_{pr}$ ,  $A_{al}$ ), which might be related to stage of differentiation. It is therefore important to correlate the molecular phenotype of spermatogonia with their clonal organization by whole-mount seminiferous tubule immunofluorescence staining. Using this approach, we observed SALL4 in  $A_s$ ,  $A_{pr}$  and  $A_{al4,8,16}$  spermatogonia. Occasionally, clones with an odd number of cells ( $A_{al3}$ ,  $A_{al6}$ ,  $A_{al12}$ ) were observed. These could be the result of clone fragmentation, a phenomenon that has been described by Nakagawa et al. [3] and that was proposed as a mechanism to replenish the spermatogonial pool. Further, extensive co-localization studies in whole-mount samples revealed significant co-expression of SALL4 with PLZF and LIN28 in the majority of  $A_{undiff}$  spermatogonia.

Germ cell differentiation is regulated by endocrine and paracrine factors that signal either to the somatic component of the seminiferous tubules, the Sertoli cells, or directly to spermatogonial stem cells to elicit self-renewing or differentiation divisions. A key factor for SSC survival and renewal both in vivo and in vitro is glial cell line-derived neurotrophic factor (GDNF) [34,35] that is secreted by Sertoli cells [43] and binds to the RET/GFR $\alpha$ 1 receptor complex [44] on undifferentiated spermatogonia including SSCs [45]. Accordingly, the presence of GFR $\alpha$ 1 on the cell surface of putative SSCs is well characterized and was found to be restricted predominantly to  $A_s$ ,  $A_{pr}$  and  $A_{al4}$  [30]. In contrast to the PLZF and LIN28 results, we observed a high degree of heterogeneity among  $A_s$  spermatogonia in SALL4 and GFR $\alpha$ 1 co-

staining experiments. Grisanti et al. reported that 10% of the PLZF-expressing  $A_s$  spermatogonia are GFR $\alpha$ 1-negative and that the stem cell activity in this population is greater than in GFR $\alpha$ 1 positive cells, as determined by transplantation assay [30]. In the study mentioned above, Grisanti et al. also observed that ~5% of PLZF-positive  $A_{pr}$  spermatogonia had asymmetrical GFR $\alpha$ 1 expression. In our study, we rarely observed asymmetrical clones (either SALL4 asymmetric or GFR $\alpha$ 1 or LIN28 asymmetric, <1% of clones). The reason that we observed slightly fewer asymmetric clones than previously reported could be that Grisanti et al. made counts from images, while we counted clones directly on the microscope, which allowed us to follow clones through different focal planes.

Here, we found that only half of the  $A_s$  population co-expressed GFR $\alpha$ 1 and SALL4, while the other half consisted of SALL4 only or GFR $\alpha$ 1 only cells. The biological function and the actual stem cell potential in these different spermatogonial populations is not known and will have to be investigated in future studies, perhaps using transgenic tools.

We next performed co-staining experiments for SALL4 and the cKIT receptor tyrosine kinase, which is a marker for differentiating germ cells. cKit becomes upregulated as  $A_{al}$  transition to  $A_1$  spermatogonia, possibly through de-repression of cKIT gene expression in the absence of PLZF [46]. In this study, we observed cKIT expression in a minor fraction of  $A_s$  and  $A_{pr}$ , which increased in larger clones and was associated with a corresponding decrease in SALL4 expression in larger clones. Interestingly, a very small proportion of  $A_{pr}$  and  $A_{al4}$  clones expressed cKIT only (without SALL4), suggesting that the  $A_{al}$  to  $A_1$  transition might sometimes occur from small clones, as previously described in the Chinese hamster [47]. While the majority of  $A_{al16}$  clones expressed cKIT (either with or without SALL4), we did observe  $A_{al16}$  SALL4+ clones that were cKIT negative.

Hobbs et al. recently demonstrated a direct interaction of SALL4 and PLZF in transfected HEK293 cells and reported that the two factors can displace each other from their cognate chromatin locations depending on their relative protein levels [23]. PLZF is a repressor of the differentiation factor, cKIT, and the authors proposed that SALL4 might act as an important spermatogonial differentiation factor that functions by removing PLZF from its cognate targets (e.g. cKIT). Does this suggest that the SALL4+/PLZF-  $A_s$  and  $A_{pr}$  spermatogonia express cKIT and are destined to differentiate? In this regard, triple staining experiments would be very informative, but are difficult to execute due to availability of compatible antibodies and differences in optimal staining conditions for each marker.

Taken together, our data from cross-section and whole mount preparations of seminiferous tubules corroborate earlier reports of molecular heterogeneity among  $A_{undiff}$  spermatogonia, which may indicate functional heterogeneity among clone sizes [29,30,32,33]. The heterogeneity can only be observed when co-staining with more than one marker is performed, therefore caution is required for the interpretation of experiments that use only a single marker as a denominator for  $A_{undiff}$  spermatogonia. In this study, we report that SALL4 is a molecular marker for undifferentiated  $A_s$ ,  $A_{pr}$  and  $A_{al}$  spermatogonia, a population that includes spermatogonial stem cells, and that it can be used in combination with other molecular markers to dissect spermatogonial lineage development and steady state spermatogenesis in the postnatal testis.

## Supporting Information

**Figure S1 Immunostaining of tissue sections with anti-SALL4 and anti-DAZL IgG.** Fluorescent signal was specific for

SALL4 and DAZL (A) and no fluorescent staining signal was observed in Isotype controls (B). Scale bar = 10  $\mu$ m. (TIF)

**Figure S2 Immunostaining of adult mouse testis sections with spermatogonial markers.** Fluorescent signal was specific for SALL4, PLZF and LIN28 (A, C) and no fluorescent staining signal was observed in Isotype controls (B, D). Scale bar = 10  $\mu$ m. (TIF)

**Figure S3 Immunostaining and controls of whole mount seminiferous tubules.** Fluorescent signal was specific for SALL4, PLZF, LIN28, GFR $\alpha$ 1, or cKIT (left column) and no fluorescent staining signal was observed in Isotype controls (right column). Scale bar = 50  $\mu$ m. (TIF)

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## Author Contributions

Conceived and designed the experiments: KG KEO. Performed the experiments: KG. Analyzed the data: KG KEO. Contributed reagents/materials/analysis tools: KG KEO. Wrote the paper: KG KEO.



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