Germline Stem Cell Activity Is Sustained by SALL4-Dependent Silencing of Distinct Tumor Suppressor Genes

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SUMMARY

Sustained spermatogenesis in adult males and fertility recovery following germ cell depletion are dependent on undifferentiated spermatogonia. We previously demonstrated a key role for the transcription factor SALL4 in spermatogonial differentiation. However, whether SALL4 has broader roles within spermatogonia remains unclear despite its ability to co-regulate genes with PLZF; a transcription factor required for undifferentiated cell maintenance. Through development of inducible knockout models, we show that short-term integrity of differentiating but not undifferentiated populations requires SALL4. However, SALL4 loss was associated with long-term functional decline of undifferentiated spermatogonia and disrupted stem cell-driven regeneration. Mechanistically, SALL4 associated with the NuRD co-repressor and repressed expression of the tumor suppressor genes Foxl1 and Dusp4. Aberrant Foxl1 activation inhibited undifferentiated cell growth and survival, while DUSP4 suppressed self-renewal pathways. We therefore uncover an essential role for SALL4 in maintenance of undifferentiated spermatogonial activity and identify regulatory pathways critical for germline stem cell function.

INTRODUCTION

Maintenance of male fertility is dependent on germline stem cells within the testis seminiferous epithelium. Stem cell activity in the mouse is restricted to a population of undifferentiated spermatogonia (Figure 1A), generated postnatally from gonocytes (de Rooij and Grootegoed, 1998). The undifferentiated population consists of isolated A-type spermatogonia (A single or A1) and cells remaining interconnected by cytoplasmic bridges after division; two-cell cysts are A paired (A pr), while cysts of four or more cells are A aligned (A al). Steady-state self-renewal is restricted to cells expressing glial cell line-derived neurotrophic factor receptor 1 (Gfra1), predominantly A1 and A AL (Hara et al., 2014). The majority of undifferentiated cells, particularly A4b, act as committed progenitors and express neurogenin 3 (Ngn3) and retinoic acid receptor γ (RARγ, Rarg) (Ikami et al., 2015). Switching from stem to progenitor fates involves RARγ and sensitivity to the differentiation stimulus retinoic acid (RA) (Gely-Pernot et al., 2015; Ikami et al., 2015). Differentiation is marked by induction of c-KIT plus DNA methyltransferases 3A/3B (DNMT3A/3B) and formation of A1 cells that undergo rounds of mitosis to generate A2, A3, A4, intermediate (In), and B spermatogonia that enter meiosis and form pre-leptotene spermatocytes (Figure 1A) (de Rooij and Grootegoed, 1998; Shirakawa et al., 2013).

Undifferentiated cell self-renewal requires glial cell-derived neurotrophic factor (GDNF) produced by supporting Sertoli cells, which signals via GFRα1 and c-RET receptors (Kanatsu-Shinohara and Shinohara, 2013). In the presence of GDNF and basic fibroblast growth factor, undifferentiated cells can be propagated in vitro while maintaining stem cell capacity. The transcription factor promyelocytic leukemia zinc finger (PLZF) is an intrinsic regulator of spermatogonial self-renewal (Buaas et al., 2004; Costoya et al., 2004; Hobbs et al., 2010). We have identified a connection between PLZF and the zinc-finger transcription factor spalt-like 4 (SALL4) (Hobbs et al., 2012). SALL4 is essential for development and core transcription factor in embryonic stem cells (ESCs) (Lim et al., 2008; Zhang et al., 2006). In adults, SALL4 expression is restricted to the germline and detected within undifferentiated spermatogonia (Hobbs et al., 2012). Conditional Sall4 deletion suggested a role in spermatogonial differentiation associated with an ability of SALL4 to sequester PLZF and modulate PLZF targets (Hobbs et al., 2012). Culture-based studies suggest that SALL4 and PLZF coordinately regulate genes involved in GDNF-dependent self-renewal (Lovelace et al., 2016). However, the role of SALL4 within undifferentiated spermatogonia remains unclear.

Through development of a Sall4-inducible knockout (KO) model, here we uncover a critical role for SALL4 in undifferentiated cell function and demonstrate that SALL4...
Figure 1. Expression of SALL4 in Spermatogonia of Undisturbed and Regenerating Testis

(A) Schematic illustrating mouse seminiferous epithelium, spermatogonial hierarchy, and markers of populations.

(B–D) Representative whole-mount IF of wild-type (WT) adult seminiferous tubules. Inset in (C) shows low KI67 in SALL4+ A al–A1 (arrowheads). Arrowheads in (B) and (D) indicate A al cysts. Insets in (D) show SALL4 in GFRα1+ A s and A pr. Dashed outlines indicate SALL4+ cysts.

(E) Representative whole-mount IF of untreated and busulfan-treated WT mice. Images were taken along the tubule then stitched together. Grayscale of each channel within the indicated regions are shown.

(F) SALL4 staining intensity from (E) using ImageJ. For controls, SALL4 was measured in GFRα1+ A s and A pr. Dashed outlines indicate SALL4+ cysts.

(G) Representative whole-mount IF demonstrating mutually exclusive GFRα1 and RARγ expression in cysts of regenerating tubules from (E).

Scale bars, 50 μm. Dotted lines indicate tubule profile. See also Figure S1.
Figure 2. Effects of Acute Sall4 Deletion on Spermatogonial Populations In Vivo

(A) Representative whole-mount IF of Z/EG; UBC-CreER tubules 7 days after TAM.

(B) Adult Ctrl and Sall4\textsuperscript{ TAM−/−KO} mice were treated with TAM and harvested at the indicated time points. Lower panels: representative whole-mount IF of seminiferous tubules 7 days post-TAM.

(C) Representative whole-mount IF of seminiferous tubules 5 and 7 days post-TAM. Three mice per genotype were analyzed at 5 days and seven per genotype at 7 days. Day 7 control tubules are shown. PreL denotes preleptotene spermatocytes.

(D) Representative whole-mount IF of tubules 7 and 14 days post-TAM. Seven mice per genotype were analyzed. Day 14 control tubules are shown. Insets demonstrate PLZF localization and DAPI identifies nuclei.

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suppresses tumor suppressor genes in order to maintain stem cell activity.

**RESULTS**

**Sall4 Is Dynamically Expressed during Spermatogonial Differentiation and Regeneration**

As Sall4 expression pattern in adult spermatogonia remains unclear (Gassei and Orwig, 2013; Hobbs et al., 2012), we analyzed whole-mount seminiferous tubules by immunofluorescence (IF) (Figure 1A). Spermatogenesis is a cyclic process divided into 12 stages in the mouse (I-XII) and tubules at a given stage contain cells at a specific differentiation step (Figure S1) (de Rooij and Grootegoed, 1998). Undifferentiated spermatogonia are present at all stages. To assist with cell identification, samples were counterstained for glucocorticoid-induced leucine zipper (GLZ), which marks spermatogonia and early spermatocytes (Figures 1A and S1) (Ngo et al., 2013).

Sall4 expression was compared with Plzf, which is expressed in undifferentiated spermatogonia and early differentiating cells (A1–A3), then downregulated (Figures 1A and S1) (Hobbs et al., 2012). Aal and early differentiating cells expressed Plzf and Sall4, while PLZF + Aa and Aapr had lower SALL4 (Figure 1B). At late differentiating stages (A4–IIn), PLZF was barely detectable but SALL4 levels were similar to those in Aa and Aapr. SALL4 thus marks all spermatogonia but expression peaks in progenitors and early differentiation stages (Figure 1A), consistent with RA-dependent regulation (Gely-Pernot et al., 2015). Sall4 expression in differentiating cells was confirmed by c-KIT staining (Figure 1C) (Schrans-Stassen et al., 1999). Differentiating cells were also strongly positive for KI67, demonstrating mitotic activity (Figure 1C). Importantly, self-renewing GFRZ1 + Aa and Aapr invariably expressed SALL4 although at lower levels than progenitors (Figures 1D and 1E). Sall4 expression is compatible with roles in both self-renewing and differentiating cells.

To test whether Sall4 expression in self-renewing cells was affected by cellular activity, we treated mice with the DNA-alkylating agent, busulfan, which depletes differentiating cells plus much of the undifferentiated pool and induces regeneration from remaining stem cells (Zohni et al., 2012). This response is characterized by formation of GFRZ1 + Aal of 8 and 16 cells, potentially involved in stem cell recovery (Nakagawa et al., 2010). SALL4 was upregulated in regenerative GFRZ1 + Aal compared with steady-state GFRZ1 + Aa and Aapr (Figures 1E and 1F), suggesting a role in germline regeneration. Regenerative GFRZ1 + Aal were RARγ− (Figure 1G), indicating retention of self-renewal capacity (Ikami et al., 2015).

**Differential Sensitivity of Undifferentiated and Differentiating Spermatogonia to Sall4 Ablation**

To assess SALL4 function in adults, we developed an inducible Sall4 KO by crossing floxed mice with a line expressing tamoxifen (TAM)-regulated Cre from the ubiquitin C promoter (UBC-CreER) (Ruzankina et al., 2007). While TAM treatment of Sall4flox/flox UBC-CreER mice (Sall4TAM−/−KO) induces body-wide Sall4 deletion, Sall4 expression in adults is restricted to spermatogonia, thus allowing assessment of function within these cells. To assess UBC-CreER activity, we crossed UBC-CreER mice with a Z/EG reporter that expresses GFP upon Cre-mediated recombination (Novak et al., 2000). Seven days after TAM, GFP was induced in GFRZ1 + Aa and Aapr SALL4+ progenitors and c-KIT+ cells, confirming transgene expression throughout the spermatogonial hierarchy (Figures 2A and S2A), GFP was detected in spermatocytes and spermatids but absent from Sertoli cells (Figure S2A). PLZF+ cells expressed GFP at 7 and 60 days post-TAM, demonstrating stable lineage marking of the undifferentiated pool (Figure S2B). GFP was expressed throughout the epithelium at day 60, confirming transgene expression in stem cells (Figures S2B and S2C) (Nakagawa et al., 2010).

Sall4TAM−/−KO and Sall4flox/flox control mice were treated with TAM and harvested at different time points (Figure 2B). Depletion of SALL4+ cells in Sall4TAM−/−KO testis by 7 days post-TAM indicated effective gene deletion (Figure 2B). Some SALL4+ cells remained, in agreement with mosaic UBC-CreER activity (Ruzankina et al., 2007). Consistent with a role for SALL4 in maintenance of differentiating cells (Hobbs et al., 2012), Sall4 deletion triggered almost complete ablation of c-KIT+ KI67+ spermatogonia (Figure 2C). Depletion of c-KIT+ cells in Sall4TAM−/−KO testis was evident 5 days post-TAM but not at day 2 when SALL4 was still detected (Figures 2C and S2D). While

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(E and F) Flow cytometry of testis cells from Ctrl and Sall4TAM−/−KO mice 7 days post-TAM. Populations no. 1 are undifferentiated cells, no. 2 early differentiating cells, and no. 3 late differentiating cells. Graph shows mean percentage of cells in each population ± SEM. Four mice per genotype were analyzed. ***p < 0.001.

(G) Representative flow cytometry of EdU incorporation by undifferentiated cells (PLZF+ c-KIT−) from Ctrl and Sall4TAM−/−KO mice 14 days post-TAM as in (E). Only SALL4− cells are included from Sall4TAM−/−KO. Percentages of cells EdU+ are indicated.

(H and I) Representative whole-mount IF of seminiferous tubules 7 and 14 days post-TAM. Day 7 control tubules are shown. Arrowheads in Sall4TAM−/−KO indicate Sall4 null progenitor cysts.

Scale bars, 50 µm. Dotted lines indicate tubule profiles. See also Figures S2 and S3.
Figure 3. SALL4 Is Required for Long-Term Maintenance of Spermatogonial Stem Cell Activity

(A) Representative whole-mount IF of seminiferous tubules from Ctrl and Sall4TAM−/− KO mice 7 and 30 days post-TAM. Images were taken along the tubule and stitched together. Seven mice per genotype were analyzed at 7 days and three per genotype at 30 days. Day 7 control (legend continued on next page)
control and Sall4\textsuperscript{TAM−KO} tubes were compared at similar cycle stages, staging was not always possible in KOs due to loss of differentiating cells. In contrast, PLZF+ undifferentiated cells were readily detectable in KOs up to 14 days post-TAM (Figure 2D). From sections 7 days after TAM, 91.5% ± 4.37% of PLZF+ cells were SALL4+ in controls, while 13.0% ± 5.01% were SALL4+ in Sall4\textsuperscript{TAM−KO} mice (n = 3), confirming gene deletion. Kinetics of Sall4 deletion in undifferentiated and differentiating cells post-TAM was similar (Figure S2E).

When comparing Sall4-deleted and Sall4-retaining PLZF+ cells in the KO, a shift in PLZF localization was apparent (Figure 2D). In SALL4+ spermatogonia, PLZF was predominantly nuclear but 7 days post-TAM was present in cytosol and nucleus of Sall4-deleted cells. Re-localization of PLZF to the cytosol in Sall4-deleted cells was particularly evident 14 days after TAM, indicating that SALL4 loss disrupts PLZF function (Figures 2D and S2F).

To quantify spermatogonial abundance, fixed and permeabilized testis sections 7 days post-TAM were stained for PLZF, c-KIT, and SALL4, and analyzed by flow cytometry (Figure 2E) (Hobbs et al., 2012, 2015). Undifferentiated cells are PLZF+ c-KIT− (population no. 1), early differentiating cells (A1−A2) PLZF+ c-KIT+ (no. 2), and late differentiating cells (A3−In) PLZF\textsuperscript{low} c-KIT+ (no. 3). Abundance of early and late differentiating cells was dramatically reduced upon Sall4 deletion, while the undifferentiated population was intact (Figures 2E and 2F). Comparable results were obtained 5 and 14 days after TAM (Figures S3A–S3C). Sall4-deleted undifferentiated cells at 7 and 14 days post-TAM incorporated EdU to a similar extent as controls, while 13.0% ± 9.15% were SALL4+ in controls, 4.37% of PLZF+ cells were SALL4+ in controls, 5.01% were SALL4+ in Sall4\textsuperscript{TAM−KO} mice (n = 3), confirming gene deletion. Kinetics of Sall4 deletion in undifferentiated and differentiating cells post-TAM was similar (Figure S2E).

To confirm effects of Sall4 deletion, we analyzed independent spermatogonial markers (Figure 1A). DNMT3A+ cells were depleted following Sall4 deletion, confirming loss of differentiating cells (Figure 2H) (Shirakawa et al., 2013). FOXO1+ SALL4− spermatogonia were present in Sall4\textsuperscript{TAM−KO} mice 7 and 14 days after TAM, demonstrating retention of Sall4-deleted undifferentiated cells (Figure 2H) (Goertz et al., 2011). RARγ+ SALL4− progenitors also remained following TAM, suggesting that differentiation-primed cells do not require SALL4 for maintenance while fully committed cells do (Figure 2I) (Ikami et al., 2015); consistent with abundant Sall4-deleted A\textsubscript{al} (Figures 2D and 2H).

**SALL4 Is Required for Long-Term Maintenance of Germline Stem Cell Activity**

While undifferentiated spermatogonia persisted following Sall4 deletion, steady-state stem cells comprise a minor component and the effects of SALL4 loss on stem cell function were not immediately evident. In Sall4\textsuperscript{TAM−KO} testis 7 days after TAM, Sall4-deleted GFRα1+ self-renewing cells were present, but an increased proportion were four- and eight-cell A\textsubscript{al}, resembling a regenerative response (Figures 3A and 3B). Such a response is expected given the depletion of a large fraction of spermatogonia. Kinetics of SALL4 ablation in GFRα1+ and PLZF+ cells was similar (Figures S2E and S4). As with busulfan, induction of GFRα1+ A\textsubscript{al} upon Sall4 deletion was transient and not evident 14 days post-TAM (not shown) (Nakagawa et al., 2010).

At 30 days post-TAM, KO tubules contained regions of active spermatogenesis interspersed with areas almost devoid of germ cells (Figure 3A). Repopulated areas contained SALL4+ spermatogonia, indicating that germline...
recovery was mediated by Sall4-retaining stem cells. Occasional Sall4-deleted GFrα1+ cells were found but appeared unable to contribute to regeneration. Therefore, while undifferentiated cells tolerate SALL4 loss, they are functionally compromised as they are progressively depleted and replaced by Sall4-retaining cells. Accordingly, <15% of PLZF+ cells express Sall4 in KO testis 7 days post-TAM, but ~80% are SALL4+ by day 30 (Figure 3C). Despite expansion of Sall4-retaining cells, Sall4TAM−KO testis weights at day 30 were lower than controls, indicating incomplete recovery of the seminiferous epithelium (Figures 3D and 3E).

Undifferentiated spermatogonia initially remained mitotically active following Sall4 deletion, but were gradually depleted suggesting defective self-renewal. While increased apoptosis may contribute to depletion of Sall4 KO undifferentiated cells (Hobbs et al., 2012), mitotic potential of Sall4-deleted PLZF+ cells declined over time as indicated by Ki67 staining (Figure 3F). The majority of Sall4 KO GFrα1+ cells remaining 30 days post-TAM were Ki67−, despite abundant Sall4-depleted Ki67+ GFrα1+ cells 7 days post-TAM (Figures 3A, 3G, and 3H). These data indicate that Sall4 loss disrupts long-term proliferative capacity and maintenance of undifferentiated spermatogonia.

SALL4 Function within the Spermatogonial Pool Is PLZF Independent

SALL4 is suggested to associate with target genes indirectly via interaction with PLZF and other factors (Lovelace et al., 2016). PLZF loss should thus disrupt SALL4 function. However, nuclear retention of PLZF is SALL4 dependent (Figures 2D and S2F), indicating that SALL4 loss disrupts PLZF function. To test whether SALL4 requires PLZF to exert its function and whether disruption in PLZF activity contributes to the Sall4 KO phenotype, we crossed Sall4TAM−KO mice onto a Plzf−/−−/− background and compared effects of Sall4 deletion in the presence and absence of PLZF.

Plzf−/−−/− mice are viable but spermatogonial maintenance is disrupted (Costoya et al., 2004; Hobbs et al., 2010). Adult Plzf−/−−/− tubules contained areas of active spermatogenesis interspersed with germ cell-depleted regions (Figure 4A). Spermatogenic regions occupied 53.6% ± 2.45% of the tubules (n = 3, ≥ 15 mm scored/mouse). Plzf−/−, Sall4TAM−KO, and Plzf−/−−/− controls were treated with TAM, and then

Figure 4. Analysis of Plzf KO and Plzf/Sall4 Double KO Spermatogonial Phenotype

(A) Representative whole-mount IF of seminiferous tubules from WT and Plzf−/− mice. Images taken along the tubule were stitched together. Areas of intact and germ cell-depleted (degenerated) seminiferous epithelium are indicated. (B–D) Representative whole-mount IF of Plzf−/− and Plzf−/−; Sall4TAM−KO tubules 10 days post-TAM. Three Plzf−/− and four Plzf−/−−/−; Sall4TAM−KO mice were analyzed. Arrowheads in (C) indicate Sall4-expressing (upper panels) and Sall4 KO (lower panels) undifferentiated cells. Dashed line in (D) indicates regenerative GFrα1+ Aαl. Scale bars, 50 μm. Dotted lines indicate tubule profiles.
Figure 5. Identification of SALL4 Targets Using Cultured Undifferentiated Spermatogonia

(A) Method for generating cultures of undifferentiated spermatogonia. Spermatogonia were enriched from Sall4TAM−KO testis cell suspensions by αCD9 magnetic selection and plated onto mitotically inactivated mouse embryonic fibroblasts (MEF). Colonies formed within 1–2 weeks and passaged cells were treated with vehicle (Veh) or 4-hydroxytamoxifen (TAM) then analyzed 4 days later.

(B) Representative IF of Veh and TAM-treated Sall4TAM−KO cultures as in (A). Grayscale insets show PLZF localization.

(C) Representative images of Veh and TAM-treated Sall4TAM−KO cultures as in (B). Insets show higher magnification of indicated areas. Mean numbers of cleaved-PARP+ cells per colony are in upper graph. Fifty colonies scored per culture and condition. Lower graph shows relative KI67 levels quantified using ImageJ. One hundred cells scored per culture and condition. Mean values ± SEM from three independent cultures are shown.

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spermatogenic areas (while GILZ+ germ cells) were analyzed 10 days later. While c-KIT+ differentiating cells were present in Plzf−/− tubules, Sall4 deletion in a Plzf−/− background resulted in c-KIT+ cell depletion, as occurs in a wild-type (WT) background (Figure 4B). E-cadherin+/− undifferentiated cells were largely unaffected by Sall4 deletion in a Plzf−/− background, as when Plzf is expressed (Figure 4C) (Tokuda et al., 2007). Sall4/Plzf KO undifferentiated cells were often KI67+ indicating mitotic activity. As in a WT setting, GFRα1+ stem cells persisted following Sall4 loss in a Plzf−/− background and were often A6b, suggesting a regenerative response (Figure 4D). GFRα1+ cells were primarily A6 and A6p, in Plzf−/− controls.

Acute response of spermatogonia to Sall4 deletion was similar whether Plzf was expressed or not, suggesting that SALL4 function is independent of PLZF. Effects of Sall4 deletion on long-term undifferentiated cell maintenance in a Plzf−/− background was difficult to assess as Plzf loss itself causes germline depletion. However, undifferentiated cells remained active in Plzf−/− tubules at 6–8 weeks, while undifferentiated cell function was lost within 30 days of Sall4 deletion (Figure 3A), supportive of a PLZF-independent role. In addition, alterations in PLZF activity do not contribute substantially to the Sall4 KO phenotype. Given PLZF induction during spermatogonial development (Costoya et al., 2004), Plzf−/− undifferentiated cells may be developmentally abnormal. Definitive assessment of the role of PLZF in adult undifferentiated cells awaits an inducible KO model.

Identification of SALL4 Targets in Undifferentiated Spermatogonia

Having uncovered a role for SALL4 in undifferentiated cells, we sought to define relevant targets. Cultures of undifferentiated spermatogonia were generated from untreated Sall4TAM−/KO adults that allowed Sall4 ablation in vitro (Figure 5A) (Hobbs et al., 2010; Seandel et al., 2007). Transplantation of GFP-labeled WT lines confirmed stem cell maintenance in vitro (Figure 5A). Cultured Sall4TAM−/KO cells expressed Plzf, Sall4, Gfra1 and Gilz (Figure 5B). Sall4 was deleted in >90% of cells by 4-hydroxy-TAM, while expression of Plzf, Gfra1 and Gilz, were maintained (Figure 5B). PLZF became predominantly cytosolic upon Sall4 deletion (Figure 5B), as in vivo.

A large fraction of cultured Sall4TAM−KO cells persisted following Sall4 deletion, but increased cleaved-PARP+ apoptotic cells were evident 4 days after TAM compared with vehicle-treated controls (Figure 5C). Proliferation was inhibited as demonstrated by KI67 (Figure 5C). Cleaved-PARP+ cells were observed infrequently in TAM-treated cultures from UBC-CreER mice, and KI67 levels were unaffected, excluding Cre-dependent effects (Figure 5B). While effects of Sall4 deletion were more pronounced in culture than in vivo, our data confirm that undifferentiated cell survival and mitotic activity are SALL4 dependent.

To characterize SALL4 targets, TAM-treated Sall4TAM−/KO cultures were analyzed by microarray (Table S1). Expression of 496 annotated genes was altered in Sall4-depleted cells. Identified genes are involved in metabolic, cellular, and developmental processes (Figure S5C). Altered expression of a selection was confirmed by qRT-PCR (Figure 5D). Candidates were selected according to roles in proliferation and apoptosis (Card11, Fbxw13, Fox1, Id1, Pmaip1, and Tox3), transcriptional regulation (Dmrtr, Egr1, Gfi1, and Onecut2), and signaling (Dusp4 and Inpp5a). SALL4 targets in ESCs (Ctgf, Dusp4, Ifitm1, Klf5, Tdgf1, and Upp1) and genes with roles in undifferentiated spermatogonia (Cxcl12 and Ddit4/Redd1) were included (Hobbs et al., 2010; Kanatsu-Shinohara and Shinohara, 2013; Lim et al., 2008; Rao et al., 2010). Ddx4 (Vasa) and Pou5f1 (Oct4), markers of germline and undifferentiated cells, respectively, were used as controls (Hobbs et al., 2010). While changes in candidate mRNA were confirmed, expression of some genes (Id1, Egr1, Klf5, and Onecut2) was not affected at the protein level (Figure 5E). Importantly, Dusp4, Fox1, and Gfi1 were upregulated at mRNA and protein levels in Sall4-deleted cells (Figures 5D and 5E). Transcription factor FOXL1 was low in vehicle-treated cells by IF but upregulated upon Sall4 deletion (Figure 5F). The phosphatase DUSP4 was detectable in a subset of control cells but elevated in Sall4-deleted cells (Figure 5G). TAM-treated
Figure 6. SALL4 Targets Regulate Activity of Cultured Undifferentiated Spermatogonia

(A–C) Analysis of SALL4 binding to Foxl1 (A), Dusp4 (B), and Sall3 (C) in WT cultured undifferentiated cells by ChIP-qPCR. Top panels depict promoter regions and first exon (E1) of genes. Arrows indicate transcription start sites (TSS) and red bars ChIP amplicons. Blue lines are SALL4 binding motifs and green lines PLZF motifs from cultured spermatogonia. Orange lines are SALL4 motifs from ESCs. Graphs show relative enrichment of amplicons from four independent lines normalized to negative control region of H1foo not targeted by SALL4 (-ve Ctrl). IgG controls are included. Mean values ± SEM are shown.

(D) Immunoprecipitation with αSALL4 conjugated beads

(E) Function Proteins from SALL4 IP (Mass spectrometry)

| Function                  | Proteins from SALL4 IP (Mass spectrometry) |
|---------------------------|--------------------------------------------|
| NuRD co-repressor complex | CDK1, CDK2, CDK4, CHD5, GATAD2A, GATAD2B, HDAC1, HDAC2, MBD2, MBD3, MTA1, MTA2, MTA3, RBBP4, RBBP7 |
| Transcriptional regulators | CBX3, PLZF, SALL1, SALL2, SALL3             |

(F) Input IgG SALL4

(G) Myc-tag FOXL1 Merge PLZF

(H) Colony size (fold) GFP FOXL1 DUSP4

(I) GFP

(J) KI67 level/cell GFP FOXL1

(K) cCASP-3 PLZF

(L) cCASP3+ cells/colony GFP FOXL1

(M) #1 #2

(N) P-JNK (fold) GFP DUSP4

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UBC-CreER cells did not upregulate FOXL1 or DUSP4, controlling for effects of Cre (Figures S5D and S5E).

We next validated whether Sall4 loss in vivo was associated with altered expression of candidates. Respective antibodies performed poorly in tissue IF, so undifferentiated cells were sorted from TAM-treated Sall4<sup>TAM−/−</sup> and Sall4<sup>lox/lox</sup> mice for qRT-PCR. The EpCAM+ c-KIT− α6-integrin+ testis fraction comprises a pure population of PLZF+ undifferentiated cells with transplantation capabilities (Takubo et al., 2008). We confirmed enrichment of Gfra1-expressing cells in this fraction, and also high levels of CD9, a stem cell-associated marker (Figures S5F–SSH) (Kanatsu-Shinohara et al., 2004). EpCAM+ c-KIT− α6-integrin+ cells were present as anticipated in Sall4<sup>TAM−/−</sup> knockout and Sall4<sup>lox/lox</sup> mice (Figures 5H and S5I). Genes aberrantly expressed upon Sall4 deletion in vitro (Dusp4, Egr1, Fbxw13, Foxl1, Gfi1, Id1, Inppl5a, and Onecut2) displayed similar perturbations in sorted undifferentiated cells (Figure S1J). Combined, our data revealed SALL4-regulated genes in undifferentiated spermatogonia.

SALL4 Inhibits Foxl1 and Dusp4 Expression by Binding Proximal Promoter Elements

Notable genes were aberrantly expressed in undifferentiated cells upon Sall4 loss. Foxl1 (forkhead box L1) encodes a transcription factor with pro-apoptotic and growth-suppressive roles (Chen et al., 2015; Zhang et al., 2013). Dusp4 (dual specificity phosphatase 4) encodes an inhibitor of MAPK signaling that promotes senescence (Hijiyama et al., 2016; Schmid et al., 2015; Tresini et al., 2007). Expression by Foxl1 or Dusp4 contributes to spermatogonia (Lim et al., 2008; Lovelace et al., 2016; Rao et al., 2010), we analyzed Foxl1 and Dusp4 promoters for SALL4 recognition elements. Motifs from spermatogonia were present around and upstream of transcription start sites (TSS) of both genes, while motifs from ESCs were not (Figures 6A and 6B). As SALL4 may bind genes indirectly through PLZF and DMRT1, we scanned promoters for PLZF and DMRT1 sites (Lovelace et al., 2016). The Foxl1 promoter contained one PLZF site, but that of Dusp4 did not contain motifs for either factor. To confirm that Foxl1 and Dusp4 are SALL4 targets, we performed ChIP-qPCR using WT undifferentiated cultures (Figures 6A and 6B). To control for non-specific chromatin pull-down, we measured SALL4 binding to H1foo, an oocyte-expressed gene not bound by SALL4 together with immunoglobulin G (IgG) controls (Yuri et al., 2009). SALL4 associated with regions around the Foxl1 TSS and Dusp4 promoter 0.25–1 kb upstream of the TSS. As a positive control, we analyzed SALL4 binding to known targets Sall1 and Foxl1.

(D) Identification of SALL4 interacting proteins in undifferentiated spermatogonia. Lysates were incubated with magnetic beads conjugated to SALL4 antibody or IgG (control). Immunoprecipitated proteins were identified by MS.

(E) Summary of SALL4-associated proteins from three combined runs from (D).

(F) Confirmation of SALL4-interacting proteins by SALL4 IP from WT cultures and WB. Non-specific IgG control is shown.

(G) WT cultured undifferentiated cells infected with lentivirus containing Myc-tagged Foxl1 and Dusp4. Cells were passaged 2 days after infection and allowed to form colonies for 10 days before analysis. Infection efficiency was 30–40%. Representative IF of infected cells is shown. Insets: uninfected control cells.

(H) Relative mean colony size of infected cells (+) versus uninfected cells (−) in cultures from (G). Mean values ± SEM are shown (n = 4 lines of infected cells). GFP-infected cells were included as controls.

(I) Representative IF of WT cultures infected with lentivirus containing Myc-tagged Foxl1 or GFP control constructs as in (G). Cells were stained for Myc-tag or GFP to confirm infection (inset).

(J) Relative mean Ki67 levels in infected cells from (I) quantified with ImageJ. One hundred cells were quantified from each of six infected WT cultures. Mean values ± SEM are shown.

(K) Representative IF of WT cultures infected with lentivirus containing Myc-tagged Foxl1 and GFP control constructs as in (G). Cleaved caspase-3 stained identifying apoptotic cells.

(L) Graph indicates mean number of cleaved caspase-3+ cells per infected spermatogonial colony ± SEM from cultures of (K). One hundred colonies from each of three infected WT cultures were analyzed.

(M) WB of independent WT cultures (no. 1 and no. 2) infected with lentivirus containing Myc-tagged Dusp4 and GFP as in (G). VASA and β-ACTIN were used as loading controls.

(N) Graph indicates relative levels of P-JNK from (M) corrected to total JNK and normalized to GFP-infected cells. Four independent WT cultures were analyzed. Mean values ± SEM are shown.

*p < 0.05; **p < 0.01, ***p < 0.001. Scale bars, 50 μm. See also Figure S6.
Sall3 (Hobbs et al., 2012; Lim et al., 2008; Lovelace et al., 2016). While SALL4 associated with Sall1 and Sall3 promoters plus intronic regions, gene expression was not altered upon Sall4 loss (Figures 6C, S6A, and S6B). Thus, gene binding is not invariably predictive of SALL4-dependent regulation.

Expression of Foxl1 and Dusp4 was low in controls and induced upon Sall4 deletion (Figures 5F and 5G). Accordingly, the repressive epigenetic marker, trimethyl histone H3 Lys 27 (H3K27Me3), was readily detected on Foxl1 and Dusp4 promoters in WT cells (Figures S6C and S6D). SALL4 can repress genes by recruiting the nucleosome remodeling and deacetylase co-repressor (NuRD) complex (Lauberth and Rauchman, 2006; Lu et al., 2009). Mass spectrometry (MS) of SALL4-associated proteins in undifferentiated cultures identified NuRD components plus PLZF and other SALL family proteins (Figures 6D and 6E) (Hobbs et al., 2012; Sakaki-Yumoto et al., 2006), confirmed by immunoprecipitation (IP) and western blot (WB) (Figure 6F). SALL1 was strongly enriched in SALL4 IPs, suggesting that SALL4 functions as a SALL1-SALL4 heterodimer in undifferentiated cells (Sakaki-Yumoto et al., 2006). CBX3 was identified in the MS analysis but was poorly enriched in SALL4 IPs by WB, indicating weak or indirect binding. Our data indicate that SALL4 represses Foxl1 and Dusp4 by binding promoter elements and recruiting NuRD.

**Aberrant Foxl1 and Dusp4 Expression Disrupts Activity of Undifferentiated Spermatogonia**

Increased expression of Foxl1 and Dusp4 upon Sall4 deletion was predicted to disrupt undifferentiated cell proliferation and survival (Chen et al., 2015; Schmid et al., 2015). To confirm the effects of Foxl1 and Dusp4 we transduced WT cultures with lentivirus containing Myc-tagged constructs (Figure 6G). Compared with GFP-transduced or uninfected controls, colonies from cells overexpressing Foxl1 were smaller, suggesting that FOXL1 inhibits proliferation and/or survival (Figure 6H). Accordingly, Foxl1 overexpression caused reduction in Ki67 and increased cleaved caspase-3+ apoptotic cells (Figures 6I–6L). Dusp4 overexpression did not affect colony growth despite its ability to induce cell-cycle arrest and apoptosis via MAPK suppression in other cells (Figure 6H) (Hijiya et al., 2016; Schmid et al., 2015; Tresini et al., 2007). The high growth factor environment in vitro may over-ride negative effects of DUSP4 on MAPK. However, phosphorylated (active) JNK MAPK was decreased in Dusp4-overexpressing cells (Figures 6M, 6N, and S6E). JNK is required for self-renewal downstream reactive oxygen species (ROS) (Morimoto et al., 2013; Yeh et al., 2011). Although ERK MAPK is a mediator of spermatogonial self-renewal and DUSP4 targets ERK (Hasegawa et al., 2013; Hijiya et al., 2016; Tresini et al., 2007), phospho-ERK levels in Dusp4-transduced and control cells were similar (Figure S6F). Dusp4 thus selectively disrupts self-renewal pathways while Foxl1 inhibits proliferation and promotes apoptosis. Abrupt expression of these genes could, in part, account for loss in undifferentiated cell activity following Sall4 deletion.

**DISCUSSION**

Through development of an inducible Sall4 KO model, we characterized effects of acute Sall4 deletion on adult spermatogonial function. Besides confirming a key role for SALL4 in differentiation (Hobbs et al., 2012), we find that long-term maintenance of undifferentiated cell function is SALL4-dependent and implicates targets Foxl1 and Dusp4 as stem cell regulators (Figure 7). In contrast to rapid depletion of differentiating spermatogonia following Sall4 deletion, undifferentiated cells tolerated SALL4 loss. Coincident with differentiating cell depletion, a regenerative response was initiated from Sall4 KO stem cells.

**Figure 7. SALL4-Dependent Pathways Maintaining Undifferentiated Cell Activity**

SALL4 silences Foxl1 and Dusp4 by binding promoter regions and recruiting the NuRD co-repressor. FOXL1 can inhibit spermatogonial proliferation and survival via multiple targets. DUSP4 inhibits JNK, which is required for self-renewal downstream reactive oxygen species (ROS) and non-canonical WNT. Upon Sall4 deletion, FOXL1 and DUSP4 accumulate to block proliferation plus survival and suppress JNK-dependent self-renewal, resulting in progressive stem cell failure.
characterized by formation of GFrα1+ Aml. However, Sall4-deleted GFrα1+ stem cells lost proliferative capacity over time and were depleted. Spermatogonial recovery was driven from few Sall4-retaining cells. Thus, SALL4 loss does not affect the ability of stem cells to respond to tissue damage but disrupts long-term regenerative capacity.

We characterized two SALL4 targets in undifferentiated cells, Dusp4 and Foxl1, which are tumor suppressors. DUSP4 expression is commonly lost in B cell lymphoma, promoting cell survival by de-repression of JNK (Schmid et al., 2015). Increased DUSP4 expression inhibits ERK and induces senescence, a checkpoint in tumor development (Bignon et al., 2015; Tresini et al., 2007). Both JNK and ERK are linked to spermatogonial self-renewal, although DUSP4 inhibited JNK most effectively in undifferentiated cells (Hasegawa et al., 2013; Morimoto et al., 2013; Yeh et al., 2011). FOXL1 is downregulated in multiple cancers and low expression predicts poor outcome (Chen et al., 2015; Qin et al., 2014; Yang et al., 2014; Zhang et al., 2013). Besides promoting growth arrest, FOXL1 induces TRAIL, a mediator of DNA damage-induced apoptosis of undifferentiated spermatogonia (Ishii et al., 2014; Zhang et al., 2013). Given conserved roles in spermatogonia, increased Dusp4 and Foxl1 expression upon Sall4 deletion would suppress proliferation, disrupt self-renewal, and promote apoptosis.

Recent studies highlight the complexity of SALL4-dependent gene regulation in spermatogonia (Hobbs et al., 2012; Lovelace et al., 2016). In cultures, SALL4 is co-recruited with PLZF to promoters of genes in undifferentiated cells, including Foxo1, Gfra1, Oct4, and Etv5 (Lovelace et al., 2016). However, expression of these genes was not significantly altered upon Sall4 deletion. PLZF and SALL4 co-bound genomic regions contain PLZF binding sites, indicating that PLZF recruits SALL4 to these targets (Lovelace et al., 2016). However, Sall4 deletion triggered re-localization of PLZF to the cytosol, suggesting that the ability of PLZF to regulate genes is SALL4 dependent. Moreover, response of spermatogonia to SALL4 loss was indistinguishable whether PLZF was present or not, indicating an independent role for SALL4. Further studies are necessary to define the interplay between SALL4, PLZF, and other factors. Interestingly, proximity-ligation assays of WT cultures demonstrated that interaction of SALL4 with PLZF and DMRT1 was highly variable from cell to cell despite homogeneous expression (Figures S6G and S6H), underscoring the dynamic nature of interaction.

While we identify SALL4 targets within undifferentiated cells, it is unclear whether these are relevant for SALL4 function in differentiating cells. SALL4 interacts with DNA methyltransferases, and Sall4 deletion in oocytes results in genomic hypomethylation (Xu et al., 2016; Yang et al., 2012). Given that induction of Dnmt3a and 3b and methylation of self-renewal genes are involved in transition from undifferentiated to differentiated states (Shirakawa et al., 2013), SALL4 may directly methylate of genes that need to be silenced during differentiation. Given that SALL4 is capable of interacting with multiple transcription factors in the germ line (Hobbs et al., 2012; Lovelace et al., 2016; Yamaguchi et al., 2015), it will be of interest to characterize the dynamics of SALL4-dependent networks during germ cell maturation.

**EXPERIMENTAL PROCEDURES**

**Mouse Maintenance and Treatment**

*Sall4 flox/flox and Plzf −/−* mice are described (Costoya et al., 2004; Sakaki-Yumoto et al., 2006). Ubc-CreER and Z/EG mice were from Jackson Laboratory. For gene deletion and lineage tracing, 6- to 8-week-old mice were injected for two consecutive days with 2 mg TAM (Sigma) in sesame oil intraperitoneally (Matson et al., 2010). To induce regeneration, C57BL/6 adults were injected intraperitoneally with 10 mg/kg busulfan (Cayman Chemical) (Zohni et al., 2012). To detect proliferation, mice were injected intraperitoneally with 0.4 mg EdU (Thermo Fisher Scientific) 2 hr before harvesting. Transplantation was performed using busulfan-conditioned C57BL/6/CBA F1 recipients (Seandel et al., 2007). Cultured cell suspension (10–15 μL) was microinjected via testis efferent ducts. The Monash University Animal Ethics Committee approved animal experiments.

**IF**

IF of whole mounts, sections, and cultures are as described previously (Hobbs et al., 2012, 2015). See Supplemental Experimental Procedures for details.

**Flow Cytometry**

Cell preparation for sorting and analysis has been described previously (Hobbs et al., 2012). See Supplemental Experimental Procedures for details.

**Cell Culture**

Undifferentiated spermatogonia were cultured on mitomycin-inactivated mouse embryonic fibroblast feeders (Hobbs et al., 2012). See Supplemental Experimental Procedures for details.

**Lentiviral Overexpression**

*Dus4* and *Foxl1* cDNA (Origene) was sub-cloned by PCR into pCCL-hPGK (Dull et al., 1998). Cultures were infected with lentiviral-containing supernatant (Hobbs et al., 2010). Cells infected with pCCL-hPGK-GFP were used as controls. Colony size was measured using ImageJ.

**RNA Extraction and qRT-PCR**

RNA was purified using TRIzol (Thermo Fisher Scientific) and Direct-zol Miniprep Kits (Zymo Research). Tetro cDNA synthesis kits (Bioline) were used for cDNA synthesis. qPCR was run on a Light Cycler 480 (Roche) using SYBR Premix Ex Taq II (Takara). Primers are in Table S2.
Microarray
RNA was isolated from three independent Sall4TAM–KO cultures treated with TAM or vehicle. See Supplemental Experimental Procedures for details.

ChIP
SimpleChIP Enzymatic Chromatin IP Kits (Cell Signaling Technologies) were used for ChIP of undifferentiated cultures (Hobbs et al., 2010). Primers are in Table S2. MEME was used to define binding motifs (Machanick and Bailey, 2011). We implemented a threshold for a match of a sequence to a motif by an E value <10, position p value <0.0001, and 90% sequence identity.

ColP and Western Blotting
CoIP and WB were performed as described previously (Hobbs et al., 2012). See Supplemental Experimental Procedures for details.

Mass Spectrometry
Lysates from undifferentiated cultures were prepared as described previously (Mathew et al., 2012). SALL4 complexes were immuno-precipitated with Dynabeads coupled with zSALL4 antibody using a Dynabead Coupling Kit (Thermo Fisher Scientific). Dynabeads coupled to rabbit IgG were used for controls. See Supplemental Experimental Procedures for details.

In Situ Proximity-Ligation Assay
Cultured spermatogonia on chamber slides were fixed and permeabilized then processed using Duolink In Situ Orange Kits (Sigma-Aldrich).

Statistical Analysis
Assessment of statistical significance was performed with a standard two-tailed t test. Associated p values are indicated as follows: *p < 0.05; **p < 0.01, ***p < 0.001; not significant (ns) p > 0.05.

ACCESSION NUMBERS
The accession number for the microarray data reported in this paper is GEO: GSE98991.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.08.001.

AUTHOR CONTRIBUTIONS
A.C., H.M.L., J.M.D.L., J.M., M.R., and R.M.H. conceived and designed the study. A.C., H.M.L., J.M.D.L., J.M., M.S., and R.M.H. performed experiments. A.C., H.M.L., J.M.D.L., M.E., J.M., M.R., and R.M.H. analyzed data. A.C., J.M.D.L., and R.M.H. wrote the paper.

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