A regulatory role for CHD4 in maintenance of the spermatogonial stem cell pool

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SUMMARY

Maintenance and self-renewal of the spermatogonial stem cell (SSC) population is the cornerstone of male fertility. Here, we have identified a key role for the nucleosome remodeling protein CHD4 in regulating SSC function. Gene expression analyses revealed that CHD4 expression is highly enriched in the SSC population in the mouse testis. Using spermatogonial transplantation techniques it was established that loss of Chd4 expression significantly impairs SSC regenerative capacity, causing a ~50% reduction in colonization of recipient testes. An scRNA-seq comparison revealed reduced expression of “self-renewal” genes following Chd4 knockdown, along with increased expression of signature progenitor genes. Co-immunoprecipitation analyses demonstrated that CHD4 regulates gene expression in spermatagonia not only through its traditional association with the remodeling complex NuRD, but also via interaction with the GDNF-responsive transcription factor SALL4. Cumulatively, the results of this study depict a previously unappreciated role for CHD4 in controlling fate decisions in the spermatogonial pool.

INTRODUCTION

Spermatogonial stem cells (SSCs) engage in self-renewal to provide a reservoir for the continuation of spermatogenesis and, thus, male fertility. Maintenance of the SSC population is driven by growth factors produced by somatic cells in the testicular niche microenvironment, such as glial cell-derived neurotrophic factor (GDNF). These extrinsic signals act to upregulate a network of intracellular transcription factors that subsequently drive self-renewal (Oatley et al., 2007). Despite the importance of SSC maintenance, SSCs must balance self-renewal with the formation of transit-amplifying progenitors that are poised for differentiation. In response to a retinoic acid pulse in the testis, over 95% of progenitor spermatogonia will default into a differentiating pathway (Tegelenbosch and de Rooij, 1993), signifying a committed step in which the cell is irreversibly fated to become a haploid spermatozoon.

With the development of transgenic mouse models that can delineate SSC and progenitor populations, such as the inhibitor of DNA binding 4 (Id4)-eGFP mouse (Helsel et al., 2017b), in conjunction with the advent of single-cell RNA sequencing (scRNA-seq) technology, it is now well established that SSCs, progenitors, and differentiating spermatogonia have distinct gene expression profiles (Guo et al., 2018; Helsel et al., 2017b; Hermann et al., 2018). Less well understood are the key drivers dictating the transcriptional changes that accompany these transitions. In this article, we explore the role of “chromodomain helicase DNA binding protein 4” (CHD4 or Mi-2β) as a modulator of SSC self-renewal and gatekeeper to the progenitor transition.

CHD4 is a well-characterized component of the nucleosome remodeling and deacetylase (NuRD) complex: one of four major ATP-dependent chromatin remodeling complexes. NuRD has traditionally been associated with gene repression; however, recent findings have demonstrated that this complex has a dynamic role that also encompasses gene activation (Bornelöv et al., 2018). Notably, CHD4 is known to regulate stem cell maintenance in a number of lineages. In embryonic stem cells (ESCs), CHD4 represses genes involved in lineage specification while simultaneously activating transcription of pluripotency factors, such as Nanog and Klf4 (Zhao et al., 2017). Similarly, conditional inactivation of Chd4 in hematopoietic stem cells has been shown to elicit abundant downregulation of genes involved in self-renewal, alongside an upregulation of expression of differentiating-driving genes (Yoshida et al., 2008).

A role for CHD4 in the regulation of SSCs has not previously been explored; however, preliminary data from recently published scRNA-seq experiments (Green et al., 2018) suggest that Chd4 expression in the germline is largely restricted to the undifferentiated spermatogonial pool. Here, we demonstrate that expression of CHD4 is highly enriched in self-renewing mouse SSCS with stepwise reductions in expression accompanying the progenitor and differentiating transitions. Using spermatogonial transplantation techniques, a functional role for CHD4 has been confirmed, demonstrating a significant loss of regenerative capacity in the spermatogonial pool following RNAi-mediated knockdown. Furthermore, scRNA-seq approaches were adopted to identify significant transcriptional changes in undifferentiated spermatogonia following Chd4 knockdown, which
included a reduction in expression of factors known to regulate SSC self-renewal, such as *Gfra1* (GDNF family receptor alpha 1), alongside increased expression of genes associated with a progenitor state. In assessing protein binding partners, it was discovered that CHD4 does interact with “traditional” components of the NuRD complex; however, it also interacts with the SSC self-renewal driving factor SALL4, which has been previously demonstrated to bind to the *Gfra1* promoter. Together, these findings demonstrate an analogous role for CHD4 in SSCs to that described for other stem cell types, in that it controls expression of a key suite of genes to regulate the balance between self-renewal and lineage commitment.

**RESULTS**

**CHD4 expression is highly enriched in SSCs in the mouse testis**

To form a comprehensive picture of *Chd4* expression in mouse testis cells and spermatogonia sub-populations specifically, we took advantage of the queryable scRNA-seq datasets produced by Hermann and colleagues (Hermann, 2018; Hermann et al., 2018) using the 10x Genomics Loupe Browser (v.4.0.0). In assessing the log2 fold-change value associated with each cluster it was apparent that *Chd4* expression is significantly enriched in the SSC population in both adult (p < 0.01) and postnatal day 6 (P6) (p < 0.001) testes, with a decline in expression accompanying the progenitor and differentiating transitions (Figures 1A, 1B, S1A, and S1B). In testicular somatic cell populations (Sertoli, Leydig, peritubular myoid, interstitial/perivascular, and endothelial cells), only low levels of *Chd4* expression are evident (Figures 1A, 1B, S1A, and S1B), suggesting that this remodeling protein primarily plays a role in regulating germ cell function in the testis.

To validate these findings at the protein level, immunofluorescence analysis was performed on testis sections from adult and P6 mice (Figures 1C and S2A). To identify SSC and progenitor populations, an *Id4-eGfp* transgenic mouse line was used (Chan et al., 2014; Helsel et al., 2017b), within which spermatogonia with “bright” GFP fluorescence (“ID4-eGFPBright”) constitute over 90% of the SSC population while “ID4-eGFPDim” cells have lost the capacity for self-renewal and represent progenitor spermatogonia (Helsel et al., 2017b). In the P6 testis, CHD4 expression was detected in the nuclei of spermatogonia with varying intensity, with high levels of expression being commonly associated with ID4-eGFP+ spermatogonia (Figure 1C, upper, the white arrow denotes an ID4-eGFP+ cell that is magnified in the inset image). In the adult testis, CHD4 expression was similarly restricted to a subset of spermatogonia, with co-expression between CHD4 and ID4-eGFP again being evident (Figure 1C, lower, the white arrows denote an ID4-eGFP+ cell that is magnified in inset image). Identical staining patterns were identified using an alternative CHD4 antibody targeting a different epitope, further supporting our reported patterns of expression (Figure S2A).

Following this, immunofluorescence analysis was also conducted on individual ID4-eGFPBright and ID4-eGFPDim cells isolated from P6 testes. These images suggested that CHD4 expression was most enriched in the eGFPBright SSCs (Figure 1D, white arrow, S2B); however, CHD4 fluorescence was variable in eGFPDim progenitors, with reduced expression being observed in a subset of cells (Figure 1D, yellow arrow), while others maintained higher levels of expression (Figure 1D, white asterisk). To provide a more objective and quantitative assessment of CHD4 expression in these spermatogonial sub-populations, immunoblotting and densitometry analyses were conducted on the following FACs isolated populations: ID4-eGFPBright SSCs, ID4-eGFPDim progenitors, and KIT+ differentiating spermatogonia (Lord et al., 2018). Populations were gated as described previously (Helsel et al., 2017b; Lord et al., 2018) (also depicted in Figure S2C). Immunoblotting analysis confirmed that CHD4 expression is highest in eGFPBright SSCs, with stepwise reductions in protein abundance in progenitor (eGFPDim) and differentiating (KIT+) spermatogonia (Figure 1E). These results were quantified using densitometric analysis (n = 3, Figure 1F), relative to the loading control (tubulin), to further demonstrate that levels of CHD4 expression are significantly enriched (p < 0.05) in the SSC population.

**CHD4 is integral for maintenance of the SSC population**

To assign a functional role for CHD4 in undifferentiated spermatogonia we utilized well-established small interfering RNA (siRNA) knockdown strategies (Kaucher et al., 2012) in primary cultures derived from *Id4-eGfp* mouse testes at P6–P8 (denoted “pup cultures”) and ~P90 (denoted “adult cultures”). Importantly, SSC and progenitor populations remain delineated by levels of eGFP fluorescence in the culture well (Chan et al., 2014), and stem cell content in these primary cultures remains robust up to passage 20 (Helsel et al., 2017a). Treatment of primary cultures with *Chd4* siRNA achieved a 70% reduction in transcript abundance and an equivalent reduction at the protein level at 24 h post-transfection, as depicted by qRT-PCR (p < 0.001) (Figure 2A), and immunoblotting (Figure S3A), respectively. At 6 days post-transfection, the percentage of ID4-eGFPBright spermatogonia was found to be reduced by >50% in both pup (p < 0.05) and adult (p < 0.05) cultures (Figure 2B) in response to *Chd4* knockdown. Loss of this putative SSC pool was not accompanied by a significant reduction in overall cell number in the culture well (Figure 2C), nor a
significant loss of viability (assessed using Draq7) (Figure 2D); suggesting that ID4-eGFPBright SSCs were instead stimulated to transition into a progenitor state.

To confirm the association between CHD4 expression and regenerative capacity, spermatogonia were again transfected with either non-targeting “control” or Chd4-targeted siRNA and were microinjected into germ cell-ablated recipient mice to quantify SSC content in the donor population (as described in Lord et al., 2018). These analyses identified a 46% reduction in the formation of donor-derived colonies of spermatogenesis after transplantation of adult cultures transfected with Chd4 siRNA when compared with

Figure 1. CHD4 expression is highly enriched in the SSC population in the mouse testis
(A and B) Gene expression data were mined from previously published scRNA-seq databases (Hermann, 2018: “Adult mouse sorted spermatogonia” (A) and “P6 mouse spermatogonia” (B)) to assess Chd4 expression in spermatogonial sub-populations and testicular somatic cells. *Statistical significance at p < 0.05. Accompanying feature plots are provided in Figure S1.
(C) Immunofluorescence analyses on P6 and adult mouse testis sections depicting CHD4 expression (red), overlaid with ID4-eGFP (green). DAPI was used as a nuclear stain (blue). White arrows depict CHD4+/ID4-eGFP+ cells magnified in inset image. Scale bar, 20 μm. Secondary only control and additional images provided in Figure S2A.
(D) Immunofluorescence analyses of CHD4 expression in undifferentiated spermatogonia isolated from P6 testes of Id4-eGfp transgenic mice. CHD4 expression (red) was consistently high in ID4-eGFPBright SSCs (white arrows), while expression was variable in eGFPDim and eGFP− progenitors (yellow arrows, eGFP−/CHD4−; white asterisks, eGFPDim/CHD4+). Scale bar, 20 μm. Secondary only control is shown in Figure S2C.
(E and F) Immunoblotting analysis of CHD4 expression across spermatogonial sub-populations: SSCs (ID4-eGFPBright), progenitors (eGFPDim), and differentiating spermatogonia (KIT+). Densitometry data were generated using n = 3 independent biological replicates (individual mice) and were corrected to loading control (tubulin) density. Histogram data depict normalized mean values ± SEM. *Statistical significance at p < 0.05.
the control (p < 0.05, Figure 2E), and a 39% reduction in pup cultures (p < 0.05, Figure 2F). Together, these results depict a key role for Chd4 in SSC maintenance in culture and demonstrate that loss of Chd4 expression compromises regenerative capacity of SSCs upon transplantation back into the mouse testis.

**Identification of differentially expressed genes following Chd4 knockdown**

To establish gene networks that are regulated by CHD4, scRNA-seq was performed on populations of adult undifferentiated spermatogonia in culture after transfection with either control or Chd4 siRNA (n = 3, schematic in Figure 3A). For these experiments, cells were collected at 24 h post-knockdown to capture a snapshot of gene expression when CHD4 expression was at its lowest (p < 0.05, Figures 3B and S3A), before a subsequent recovery of expression, which was observed by 48 h (Figure 3B). Analyses of scRNA-seq results were conducted on a merged dataset (Butler et al., 2018) containing 817 control and 861 Chd4 knockdown spermatogonia (biological replicates, created from different mice). (E and F) Spermatogonial transplantation was performed after transfection of adult (E) and pup (F) primary cultures of spermatogonia with control- or Chd4-siRNA. Histogram data depict mean ± SEM for n = 3 different cultures of undifferentiated spermatogonia (biological replicates, created from different mice). *Significantly different at p < 0.05 from non-targeted siRNA control.

**Figure 2. CHD4 plays a functional role in regulating SSC maintenance and self-renewal**

(A) Transfection of undifferentiated spermatogonia with Chd4 siRNA achieved a 70% reduction in transcript at 24 h. Histogram data depict mean ± SEM for n = 3 different cultures (biological replicates, created from different mice). *Significantly different at p < 0.001 from non-targeted siRNA control.

(B) Knockdown of Chd4 was performed in cultures derived from the Id4-eGfp transgenic mouse line. The percentage of ID4-eGFP Bright SSCs was significantly diminished in both adult (p < 0.05) and pup (p < 0.05) cultures when compared with controls. Histogram data depict mean ± SEM for n = 3 different cultures (biological replicates, created from different mice).

(C and D) Chd4 knockdown did not affect overall cell number (C) or cell viability (D). Histogram data depict mean ± SEM for n = 3 different cultures of undifferentiated spermatogonia (biological replicates, created from different mice).

(E and F) Spermatogonial transplantation was performed after transfection of adult (E) and pup (F) primary cultures of spermatogonia with control- or Chd4-siRNA. Histogram data depict mean ± SEM for n = 3 different cultures of undifferentiated spermatogonia (biological replicates, created from different mice). *Significantly different at p < 0.05 from non-targeted siRNA control.
Figure 3. A scRNA-seq comparison of control and Chd4 knockdown spermatogonia

(A) Schematic depicting workflow. Three primary cultures of undifferentiated spermatogonia (biological replicates, created from different mice) were established from adult mouse testes using THY1 magnetic-activated cell sorting selection. At passage 10, cells from each culture were transfected with control or Chd4 siRNA. At 24 h, the three replicates were pooled to produce one population of control cells and one population of Chd4 siRNA-treated cells for scRNA-seq analysis.

(B) scRNA-seq analysis was conducted at 24 h post-transfection, before the recovery of Chd4 expression, which is observed at 48 h. Histogram data depict mean ± SEM for n = 3 different cultures of undifferentiated spermatogonia (biological replicates, created from different mice).

(C and D) Unsupervised clustering analysis of a merged dataset containing control and Chd4 knockdown sequencing libraries (C) was projected onto tSNE plots and four distinct clusters were identified (D). Evidence of effective clustering is provided via heatmap analysis in Figure S3B.

(E) Dot plot depicts expression of germ cell markers (Zbtb16 and Ddx4), SSC-enriched genes (Gfra1, Ret, Bcl6b, and Etv5), and the progenitor-enriched gene Sohlh1 across clusters. Dot size is the percentage of cells expressing each gene; dot color is the level of expression. An extended panel of genes is provided in Figure S3D.

(F) Dot plot depicts Chd4 expression in SSCs (clusters 2 and 3 merged), transitory spermatogonia (cluster 1), and progenitor spermatogonia (cluster 0).
Effective clustering of these four populations can be appreciated by distinct expression profiles depicted in the heatmap in Figure S3B.

This scRNA-seq dataset is, to our knowledge, the first to be produced using spermatogonia from in vitro culture, thus providing a valuable resource for the field. To validate the identity of these cells, we confirmed that the spermatogonia marker zinc finger and BTB domain containing 16 (Zbtb16) was highly expressed across all clusters, as was the germ cell marker DEAD-box helicase 4 (Ddx4), as would be expected in a culture of undifferentiated spermatogonia (Figure 3E). Following this, we endeavored to associate the four clusters with established functional sub-populations within the undifferentiated pool by assessing differential expression of known SSC and progenitor markers (Figures 3E and S3D). Expression of genes associated with self-renewal, such as ETS variant 5 (Etv5), BCL6B transcription repressor (Bcl6b), RET proto-oncogene (Ret), and Gfra1, were consistently elevated in clusters 2 and 3, suggesting that these clusters align with the SSC population (Chan et al., 2014). Contrastingly, expression of self-renewal genes was reduced in clusters 0 and 1, and expression of the progenitor marker spermatogenesis and oogenesis-specific basic-helix-loop-helix 1 (Sohlh1) was increased in cluster 0, suggesting that cluster 1 aligns with a transitory cell type, while cells in cluster 0 are progenitors (Figure 3E) (Chan et al., 2014). Using gene ontology (GO) analysis, we established enriched biological pathways for each cluster (Figure S3C; Data S1). These analyses separated the two SSC clusters (2 and 3) by way of enriched translation in cluster 2, identified enriched cell-cycle and cell-division proteins in the transitory cluster (cluster 1), and enriched lipid metabolism and proteins involved in spermatogenesis in the progenitor cluster (3). Finally, to confirm the validity of using primary cultures of spermatogonia to glean information on the role of CHD4 in regulating SSC function, we verified that CHD4 does indeed show the same downward trend in expression upon the transition from SSC to progenitor in the culture well (Figure 3F, shown here with SSC clusters 2 and 3 merged together), mirroring trends in in vivo expression depicted previously (Figures 1B and 1C).

In assessing the effects of CHD4 knockdown in scRNA-seq datasets it was firstly appreciated that, after only 24 h, a trend could be identified in the dispersal of cells among different clusters: with a 25% increase in the number of cells delineated into the “progenitor” cluster in the CHD4 knockdown population when compared with the control (Figure 4A). This aligns with our previous experiments that monitored ID4-eGFP content in culture (Figure 2B) and colony formation after spermatogonial transplantation (Figures 2E and 2F), which demonstrated loss of SSCs by day 6 post-CHD4 knockdown. To further compliment the outcomes of these functional experiments, a comparison of selected genes that are known to regulate SSC maintenance (Id4, Gfra1, and Lhx1 [LIM homeobox 1]), drive the progenitor transition (Sohlh1/2 and Neurog3 [neurogenin 3]), or drive spermatogonial differentiation (Kit, Stra8 [stimulated by retinoic acid 8]), was conducted between the entire population of control cells versus CHD4 knockdown cells in the scRNA-seq database (Figure 4B). In observing changes in gene expression it was apparent that CHD4 knockdown did not stimulate terminal commitment to differentiation; however, it did instigate an increase in expression of genes associated with the SSC to progenitor transition, such as Sohlh1/2, Neurog3, and Dmrt1 (doublesex and mab-3-related transcription factor 1), which is known to drive Sohlh1 expression (Matson et al., 2010). Concomitantly, a decrease in expression of the self-renewal genes Id4, Gfra1, and Lhx1 was observed (Figure 4B). Although limitations to the sensitivity of scRNA-seq mean that detection of the lowly abundant Id4 transcript was not robust, trends toward reduced Id4 expression certainly mimic our previous findings of reduced Id4-eGFP transgene expression following CHD4 knockdown (Figure 2B). To support these data, two genes were selected for additional validation via qRT-PCR: Gfra1 and Neurog3. These experiments confirmed that CHD4 knockdown does indeed instigate a 40% reduction in Gfra1 expression (p < 0.01), and a 3-fold increase in Neurog3 expression (p < 0.05) (Figures S4A and S4B) in undifferentiated spermatogonia in culture.

Next, an analysis of differentially expressed genes (DEGs) was conducted in each cluster (SSC, transitory, and progenitor) using the “FindMarkers” function in Seurat with a p value of 0.05 and log2 fold-change value of 0.1 to detect subtle influences on the cell. Using these criteria, 299 DEGs were identified in the SSC population, 347 in the transitory population and 349 in the progenitor population (Figure 4C; Data S2). When restricted to a more stringent log2 fold-change value of 0.2, these values were 75, 69, and 71, respectively (Figure 4D). A subset of DEGs were common to multiple clusters, with 30% being identified in at least two sub-populations (Figure 4C). This included phosphatase and tensin homolog (Pten), a member of the phosphatidylinositol 3-kinase/AKT signaling pathway known to be critical for SSC maintenance (Goertz et al., 2011; Zhou et al., 2015), which exhibited downregulated expression in response to CHD4 knockdown in both the SSC (p < 0.001) and transitory populations (p < 0.001) (Data S2). Unique DEGs were, however, identified in each cluster, including 117 DEGs in the SSC population that were not identified elsewhere (Data S2; Figure 4C). This included the RNA-binding protein DAZL, which is thought to drive spermatogonial differentiation (Mikedis et al., 2020; Schrans-Stassen et al., 2001), and was significantly upregulated following CHD4 knockdown (p < 0.001) (Data S2). Upon mining a publicly
available anti-CHD4 chromatin immunoprecipitation (ChIP) dataset produced using THY1+ undifferentiated spermatogonia (de Castro et al., 2020), it was determined that 102 of the DEGs identified here are direct targets of CHD4 binding (Data S4), including Dmrt1 and Gfra1 (Figure 4B), which are known regulators of SSC function.

Figure 4. Knockdown of Chd4 causes dysregulated gene expression in undifferentiated spermatogonia
(A) Histogram data depict the allocation of cells to each cluster in scRNA-seq analysis, comparing control and Chd4 knockdown populations.
(B) Dot plot depicts expression of genes known to be enriched in SSC, progenitor, and differentiating spermatogonia populations, comparing control and Chd4 knockdown spermatogonia. Dot size is the percentage of cells expressing the gene; dot color is the level of expression.
(C) DEG lists were generated for control versus Chd4 knockdown populations within each cluster (SSC, transitory or progenitor). Thirty percent of DEGs were common to at least two clusters, and 15.5% of DEGs were common to all three cell populations.
(D) Using a cutoff of \( p < 0.05 \) and \( \log_2 \text{fold-change} > 0.2 \), over 90% of DEGs in SSC, transitory, and progenitor populations experienced downregulated expression following Chd4 knockdown.
(E) Volcano plot showing the distribution of DEGs in the SSC population specifically. Colored points have a \( p \) value < 0.05, blue points have a \( \log_2 \) fold-change > 0.1, red points have a \( \log_2 \) fold-change > 0.2. Additional volcano plots for transitory and progenitor clusters are provided in Figures S4C and S4D.
(F and G) Enriched GO terms (biological processes) in the SSC DEG list. Equivalent lists for transitory and progenitor populations are provided in Figures S4E and S4F.
In assessing DEG lists, it was apparent that a majority of affected genes were downregulated in response to Chd4
knockdown in SSC (Figures 4D and 4E), transitory (Figures
4D and S4C), and progenitor (Figures 4D and S4D) popula-
tions. This suggests that CHD4 primarily acts as an acti-

vator of gene expression in undifferentiated spermatogon-

ia. In assessing enriched GO biological processes in the

SSC population, terms relating to translation and ribo-

osomal assembly were downregulated (Figure 4F), aligning

with the defined role of CHD proteins in driving ribosomal

assembly in other cell types (Shimono et al., 2005; Zentner

et al., 2010). Other downregulated processes included tran-

scription, mRNA splicing, and protein stability, with the

latter likely being intertwined with the known interaction

of CHD4/NuRD with ubiquitination machinery within the

cell (Zhao et al., 2014). Osteoblast differentiation and sper-

matogenesis were identified as upregulated processes (Fig-

ure 4G), with “spermatogenesis”-related genes, including

Dazl and testis-expressed protein 15 (Tex15), which is

involved in meiotic recombination (Yang et al., 2008).

The assessment of enriched GO terms in transitory and pro-

genitor DEG lists returned similar results, which are pro-

vided in Figures S4E and S4F.

Characterizing CHD4 protein interactions via co-

immunoprecipitation

To further infer the function of CHD4 in undifferentiated

spermatogonia, co-immunoprecipitation (co-IP) was per-

formed to identify primary protein binding partners (Fig-

ure 5; Data S3). Immunoblotting techniques verified the

presence of well-known components of the NuRD complex

in the co-IP eluate: MBD3 and RBBP7 (Figure 5A), support-

ing previously published data (Chan et al., 2017). Beyond

this, a mass spectrometry-based proteomics approach was

adopted to produce a finalized list of 32 nuclear proteins

(abridged list in Figure S5B, full list in Data S3). GO terms en-

riched in this list included nucleosome assembly, positive

and negative regulation of transcription, chromatin modi-

fication, and spermatogenesis (Figures S5B and S5C). The

identification of numerous histone proteins was consistent

with CHD4 acting as component of the NuRD complex.

The identification of histone 3.3 (H3F3A/B) and its chap-

erone DAXX (death domain-associated protein) aligns

with previously published studies that have reported this

interaction (Kraushaar et al., 2018), and supports results

presented in Figure 4 in suggesting that the CHD4/NuRD

complex is primarily involved in gene activation (rather

than repression) in spermatogonia. Indeed, Kraushaar

et al. (2018) demonstrated that H3.3-NuRD/CHD4 co-

occupation is a feature of actively transcribed genes. As

further validation of this interaction, the presence of

DAXX in the CHD4 co-IP eluate was confirmed using

immunoblotting (Figure 5A).

Spalt-like transcription factor 4 (SALL4) is a known regu-

lator of spermatogonial function, including maintenance

of the SSC population (Chan et al., 2017; Lovelace et al.,

2016). The identification of SALL4 in the co-IP eluate is of

particular interest as it has previously been suggested that

the SALL family members recruit NuRD to specific target

sites in the genome (Lauberth and Rauchman, 2006). As

such, we elected to validate these findings further, firstly

with immunoblotting of co-IP eluates (Figure 5A), then

using a proximity ligation assay (PLA), an antibody-based

technique that produces red puncta when two proteins

reside within 40 nm of each other, suggesting protein-pro-

tein interaction. Using undifferentiated spermatogonia

from primary culture, an abundance of fluorescent puncta

were identified within nuclei (Figure 5D, white arrows),

providing further evidence of CHD4-SALL4 binding. The

interaction between CHD4 and SALL4 is also supported

by SALL4 co-IP experiments in undifferentiated spermo-

togonia that have been published previously (Chan et al.,

2017). Finally, we mined publicly available anti-SALL4

(Lovelace et al., 2016) and anti-CHD4 (de Castro et al.,

2020) ChIP datasets from THY1+ undifferentiated sper-

matogonia, and identified 282 genes that were common

targets of these proteins (Data S4). Common gene targets

included Gfra1, which we have shown to be significantly

downregulated upon Chd4 knockout (Figures 4B and

S4A), as well as 18 other genes that were shown to have dys-

regulated expression following Chd4 knockout in our

scRNA-seq experiments (Data S4).

Cumulatively, findings reported here support a circum-

stance in which CHD4 is an integral component of the

self-renewal machinery within SSCs. Although experi-

ments described here were primarily conducted in an

in vitro setting, spermatogonial dysfunction and infertility

has recently been reported following Chd4 knockout in the

germline in vivo (de Castro et al., 2020), suggesting

that the mechanisms identified in this manuscript trans-

cend to SSCs in the testicular microenvironment. Specifi-

cally, we propose that CHD4 acts within the NuRD nucle-

osome remodeling complex to activate the transcription of

genes important for SSC maintenance and self-renewal.

CHD4/NuRD is likely to be broadly involved in the activa-

tion of transcription via interaction with DAXX/H3.3 but,

more specifically, recruited to promoters of GDNF-respon-

sive genes, such as Gfra1, via interaction with SALL4

(Figure 5E).

DISCUSSION

The spermatogonial population in the testis represents a

continuum from SSC through to progenitor and differenti-

ating spermatogonia. For the continuation of male fertili-

ty, a delicate balance must be maintained between SSC self-
renewal and the transition toward a differentiating pathway. Here, we have identified the nucleosome remodeling protein CHD4 to be integral for stem cell maintenance, while also acting as a gatekeeper to the progenitor transition.

By mining scRNA-seq databases (Hermann, 2018; Hermann et al., 2018) and using antibody-based techniques, we have established that CHD4 experiences a stepwise reduction in expression upon the SSC to progenitor transition, and again upon spermatogonial differentiation. The relationship between CHD4 levels and stem cell state was consolidated using RNAi and spermatogonial transplantation techniques. Specifically, we demonstrated that a reduction in Chd4 expression instigates a ~50% loss of stem cell content. In support of the notion that CHD4 expression plays a critical role in maintenance of SSCs, a recent preprint article (de Castro et al., 2020) demonstrated that germline-specific Chd4 knockout mice are infertile, with a complete absence of germ cells in the adult testis. Notably, ablation of CHD4 expression in the germline from embryonic day 15.5 (Ddx4-Cre) did not prevent formation of the spermatogonial pool following birth. However, the maintenance of the PLZF+ population of spermatogonia was severely impaired from P4, and this
response was exacerbated with increasing age (de Castro et al., 2020). This phenotype is analogous to what would be expected to arise from a defect in SSC self-renewal, and directly complements our findings using spermatogonial transplantation.

Our data suggest that loss of the stem cell population following Chd4 knockdown is associated with the transition of these cells into a progenitor state, rather than impaired viability or mitotic capacity. Indeed, no change in overall cell number or the percentage of non-viable cells was observed in primary cultures of undifferentiated spermatogonia at 6 days following Chd4 knockdown. However, a significant shift in ID4-eGFP transgene expression was identified, with redispersal of cells away from the ID4-eGFPbright phenotype, which has previously been characterized as a highly pure population of stem cells (Chan et al., 2014; Helsel et al., 2017b). This finding was consolidated by our scRNA-seq analysis, which depicted that cells captured within the progenitor cluster were more abundant in the Chd4 knockdown population when compared with the control. These findings also align with the gene expression trends that were identified following Chd4 knockdown, in that the expression of several self-renewal genes was decreased, while progenitor genes showed increased expression.

Mechanistically we have demonstrated that CHD4 primarily functions to activate gene expression in SSCs, including that of genes known to be important for self-renewal, such as Gfra1 (He et al., 2007) and Pten (Goertz et al., 2011; Zhou et al., 2015). However, CHD4 also appears to be capable of eliciting dichotomous regulation as has been observed in other stem cell types (Yoshida et al., 2018). Beyond the regulation of genes involved in stem cell maintenance and lineage specification, assessment of DEGs in our scRNA-seq dataset suggests that CHD4 may also play a role in regulating ribosomal biogenesis in undifferentiated spermatogonia. This finding corresponds with previously published evidence demonstrating that CHD4, like CHD7 (Zentner et al., 2010), associates with rDNA and activates rRNA transcription (Shimono et al., 2005). Functionally, altered ribosomal biogenesis will clearly modify the translational landscape within a cell; however, recent studies also suggest more direct links to regulating stem cell state (Sanchez et al., 2016). In Drosophila, modulation of ribosomal assembly factors has been identified as critical for germline stem cell homeostasis, not only through regulation of protein synthesis but also by controlling stem cell cytokinesis (Sanchez et al., 2016). These data provide impetus to explore whether ribosomal biogenesis is intertwined with similar pathways in mammalian SSCs.

In conclusion, here we have characterized the nucleosome remodeling protein CHD4 as a novel regulator of SSC function. We have demonstrated that CHD4...
expression is required for SSC maintenance and self-renewal, and to prevent these cells from aberrantly defaulting into a differentiating pathway. Our findings depict that, as in a number of other stem cell lineages, CHD4 modulates gene activity in SSCs to increase expression of self-renewal driving genes while repressing expression of genes that drive the progenitor transition. These findings begin to address gaps-in-knowledge surrounding master regulators of fate decisions in the spermatogonial pool. In better understanding these fundamental mechanisms we can begin to unravel the complex underlying causes of male infertility and testicular cancers, and endeavor to develop new fertility treatments.

**EXPERIMENTAL PROCEDURES**

A detailed description of materials and methods can be found in the supplemental experimental procedures.

**Animals**

All animal procedures were approved by the Washington State Institutional Animal Care and Use Committee (IACUC), or the University of Newcastle Animal Care and Ethics Committee (ACEC). The ID4-eGFP mouse line was derived as described previously (Chan et al., 2014). For spermatogonial transplantation experiments, Rosa26LucZ mice (Jackson Laboratories, stock no. 112073) were used to establish primary cultures (donor cells), while F1 hybrids of C57BL/6J (Jackson Laboratories, stock no. 000664) and 129S1/SvImJ (Jackson Laboratories, stock no. 112073) were used as recipients. Recipients were pre-treated with 55 mg/kg busulfan (Sigma Aldrich, St Louis, MO, USA) to ablate the THY1+ testis cell population from adult (3 months) or P6–P8 testes. Cultures were maintained in 10% O2, 5% CO2 at 37°C for endogenous spermatogenesis, as described previously (Oatley and Brinster, 2006).

**Primary spermatogonial cultures**

Cultures were established using magnetic-activated cell sorting of the THY1+ testis cell population from adult (3 months) or P6–P8 testes. Cultures were maintained in 10% O2, 5% CO2 at 37°C on mitotically inactivated SIM mouse embryo-derived thioguanine and ouabain-resistant feeder monolayers (STOs) in mouse serum-free medium supplemented with the growth factors GDNF (20 ng/mL; R&D Systems, Minneapolis, MN, USA) and fibroblast growth factor (1 ng/mL; BD Biosciences, San Jose, CA, USA) as described previously (Helsel et al., 2017b). Cells were passaged every 6 days onto fresh feeders, and the medium was replaced every second day. To ensure maximal stem cell content, all experiments were performed on cultures before passage 20 (Helsel et al., 2017a).

**siRNA transfection**

Transfection was conducted overnight (16 h) in feeder-free conditions, as described previously (Kaucher et al., 2012), using lipofectamine 3000 (ThermoFisher, Waltham, MA, USA) at a volume of 2 μL per 200,000 cells. SMARTpool non-targeting (D-001810-10) and Chd4 siRNA (L-052142-00) were purchased from GE Dharmacon (Lafayette, CO, USA), and used at a concentration of 75 pmol per 200,000 cells. SMARTpool siRNAs contain four duplexes that target different regions of the mRNA transcript, minimizing the potential for off-target effects. For experiments assessing spermatogonia at 6 days post-transfection, cells were placed back on STO feeders after overnight transfection.

**scRNA-seq**

For scRNA-seq experiments, undifferentiated spermatogonia from three individual adult mouse cultures were utilized. From each biological replicate, half the spermatogonia were transfected with control siRNA, and the other half with Chd4 siRNA. Immediately before the preparation of single-cell cDNA libraries, equal numbers of cells from each replicate were pooled to create a single control and a single Chd4 knockdown population (Figure 3A). Live cells from both populations were loaded onto a Chromium Controller (10× Genomics, Pleasanton, CA, USA), and single-cell cDNA libraries were generated as per the manufacturer’s instructions. Control and Chd4 knockdown libraries were then combined and sequenced in a single lane on an Illumina HiSeq 4000 (Genomics and Cell Characterisation Core Facility, University of Oregon).Raw base call files were demultiplexed using the 10× Genomics Cell Ranger pipeline and aligned to the mouse mm10 transcriptome.

Control and Chd4 knockdown transcriptomes were imported into Seurat and merged into a single object (Butler et al., 2018). Doublets and cells with low quality transcriptomes were filtered from the dataset. The data were then normalized and scaled using Seurat. The “FindVariableGenes” function was used to identify variable genes for use in principal-component analysis. For clustering and tSNE graphing, 25 significant principle components were used (resolution set to 0.5).

**Data availability**

scRNA-seq data are available from the GEO database (accession no. GSE163027). Mass spectrometry data are available from the ProteomeXchange Consortium via the PRIDE partner repository (PXD022066 and 10.6019/PXD022066).

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2021.04.003.

**AUTHOR CONTRIBUTIONS**

Conceptualization, T.L. and J.M.O.; methodology, T.L. and J.M.O.; investigation, T.L., M.J.O., S.L.C., C.S.D.O., and D.A.S.-B.; writing – original draft, T.L.; writing – review & editing, T.L., B.N., M.J.O., S.L.C., D.A.S.-B., and J.M.O.; funding acquisition, T.L. and J.M.O.

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