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# 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 spermatogonia 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 transcriptomic 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 transcriptomic 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* promotor. 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 spermatogonial 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 10× 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-eGFP$^{Bright}$") constitute over 90% of the SSC population while "ID4-eGFP$^{Dim}$" 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-eGFP$^{Bright}$ and ID4-eGFP$^{Dim}$ cells isolated from P6 testes. These images suggested that CHD4 expression was most enriched in the eGFP$^{Bright}$ SSCs (Figure 1D, white arrow, S2B); however, CHD4 fluorescence was variable in eGFP$^{Dim}$ 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-eGFP$^{Bright}$ SSCs, ID4-eGFP$^{Dim}$ 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 eGFP$^{Bright}$ SSCs, with stepwise reductions in protein abundance in progenitor (eGFP$^{Dim}$) 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-eGFP-$^{Bright}$ 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

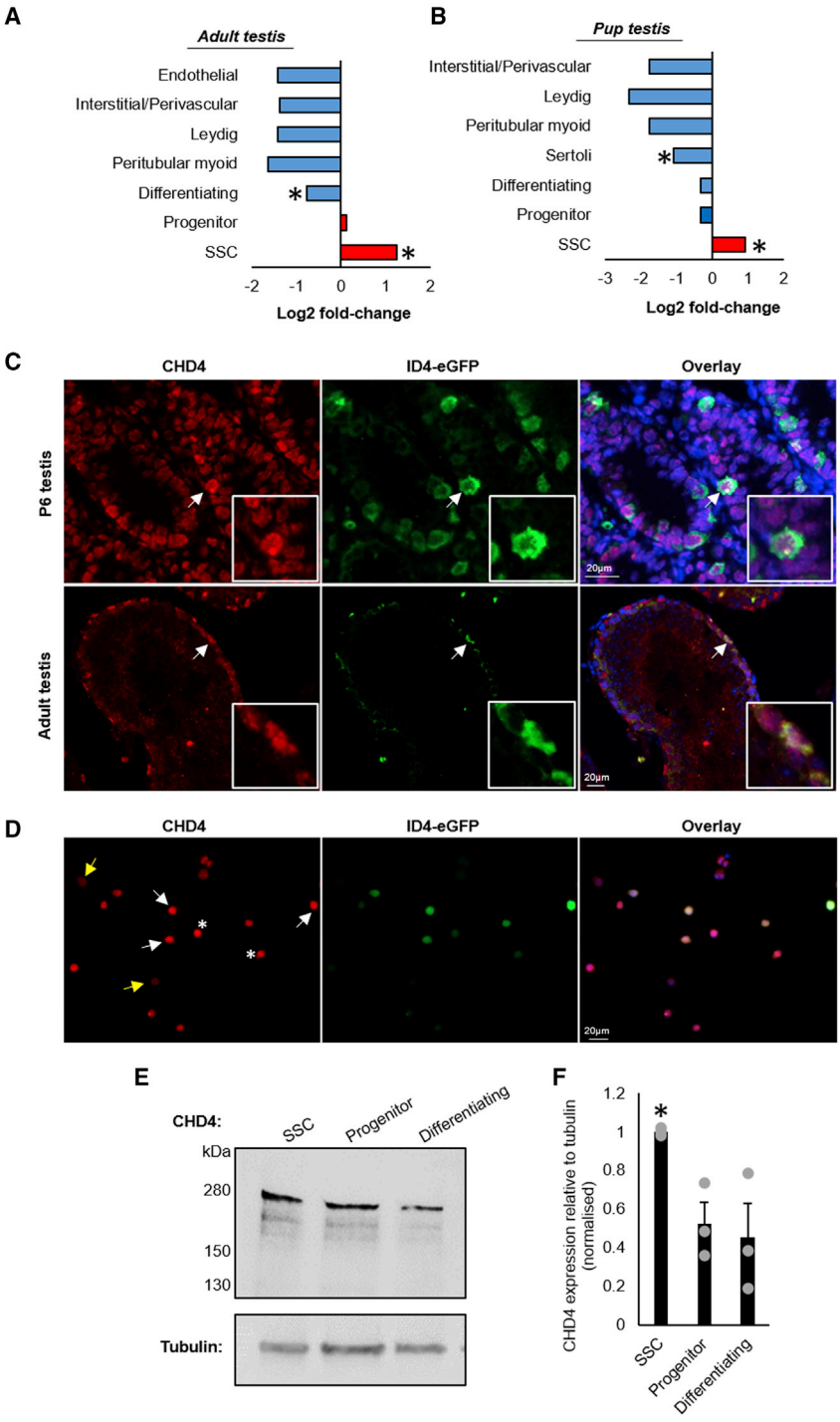


**Figure 1. CHD4 expression is highly enriched in the SSC population in the mouse testis**

(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-eGFP$^{Bright}$ SSCs (white arrows), while expression was variable in eGFP$^{Dim}$ and eGFP− progenitors (yellow arrows, eGFP−/CHD4−; white asterisks, eGFP$^{Dim}$/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-eGFP$^{Bright}$), progenitors (eGFP$^{Dim}$), 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 $p < 0.05$.

significant loss of viability (assessed using Draq7) (Figure 2D); suggesting that ID4-eGFP$^{Bright}$ 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

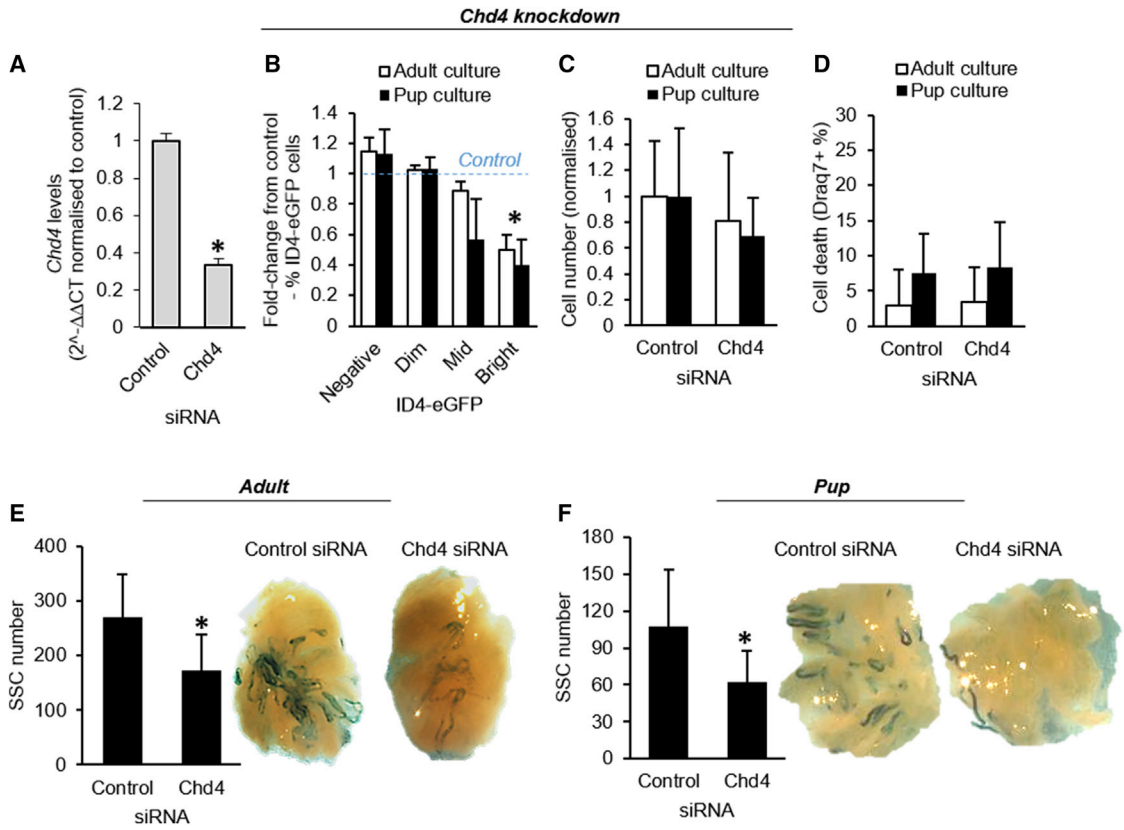


**Figure 2. CHD4 plays a functional role in regulating SSC maintenance and self-renewal**

(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.

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 (Figure 3C). Each cell from the control dataset had an average of 222,662 unique molecular indices and 4,282 median genes per cell, while these values in the *Chd4* knockdown dataset were 174,661 and 4,359, respectively. Unsupervised clustering projected onto t-distributed stochastic neighbor embedding (tSNE) analysis plots revealed four populations, here labeled "0," "1," "2," and "3" (Figure 3D; Data S1).

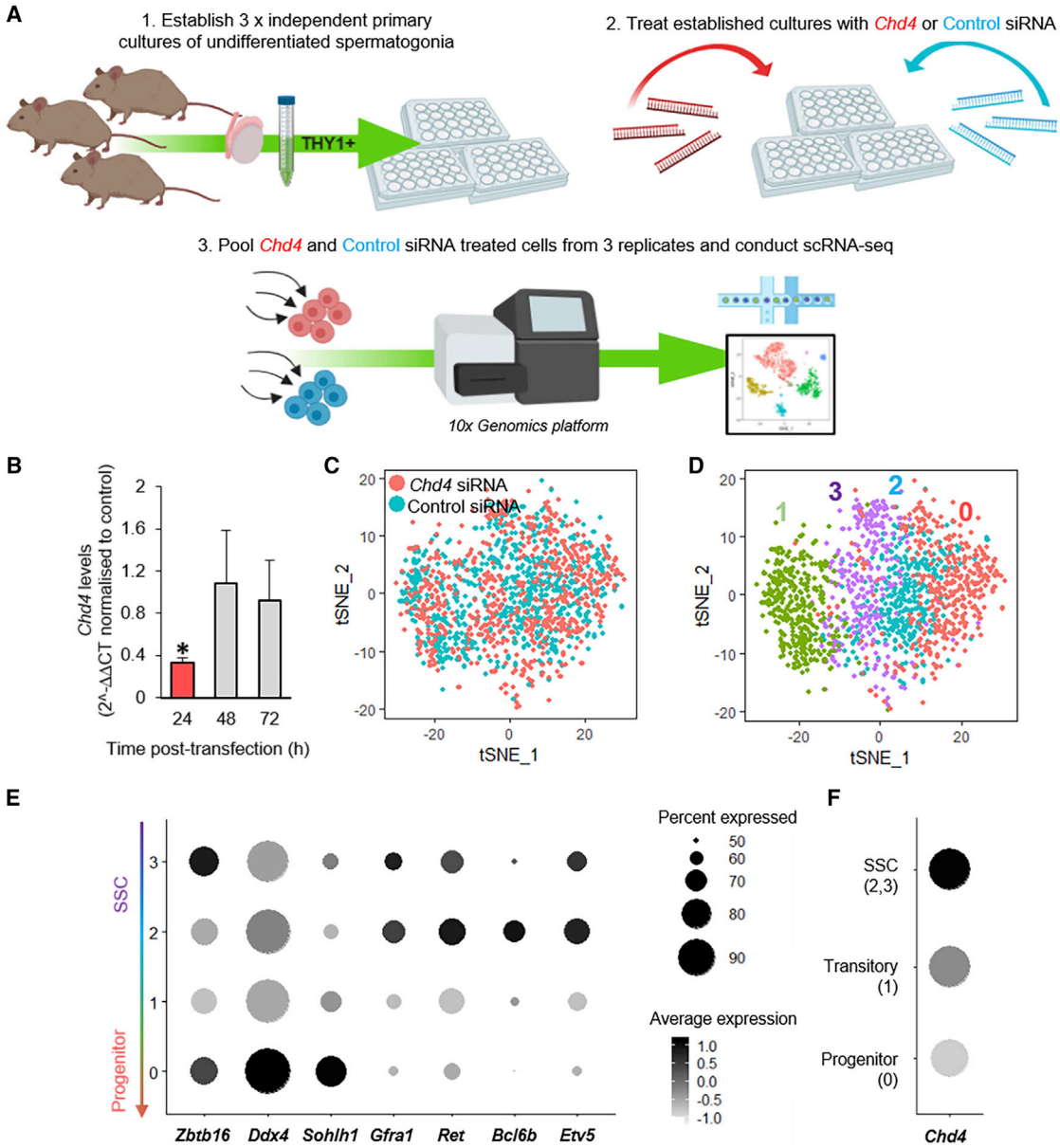


**Figure 3. A scRNA-seq comparison of control and *Chd4* knockdown spermatogonia**

(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

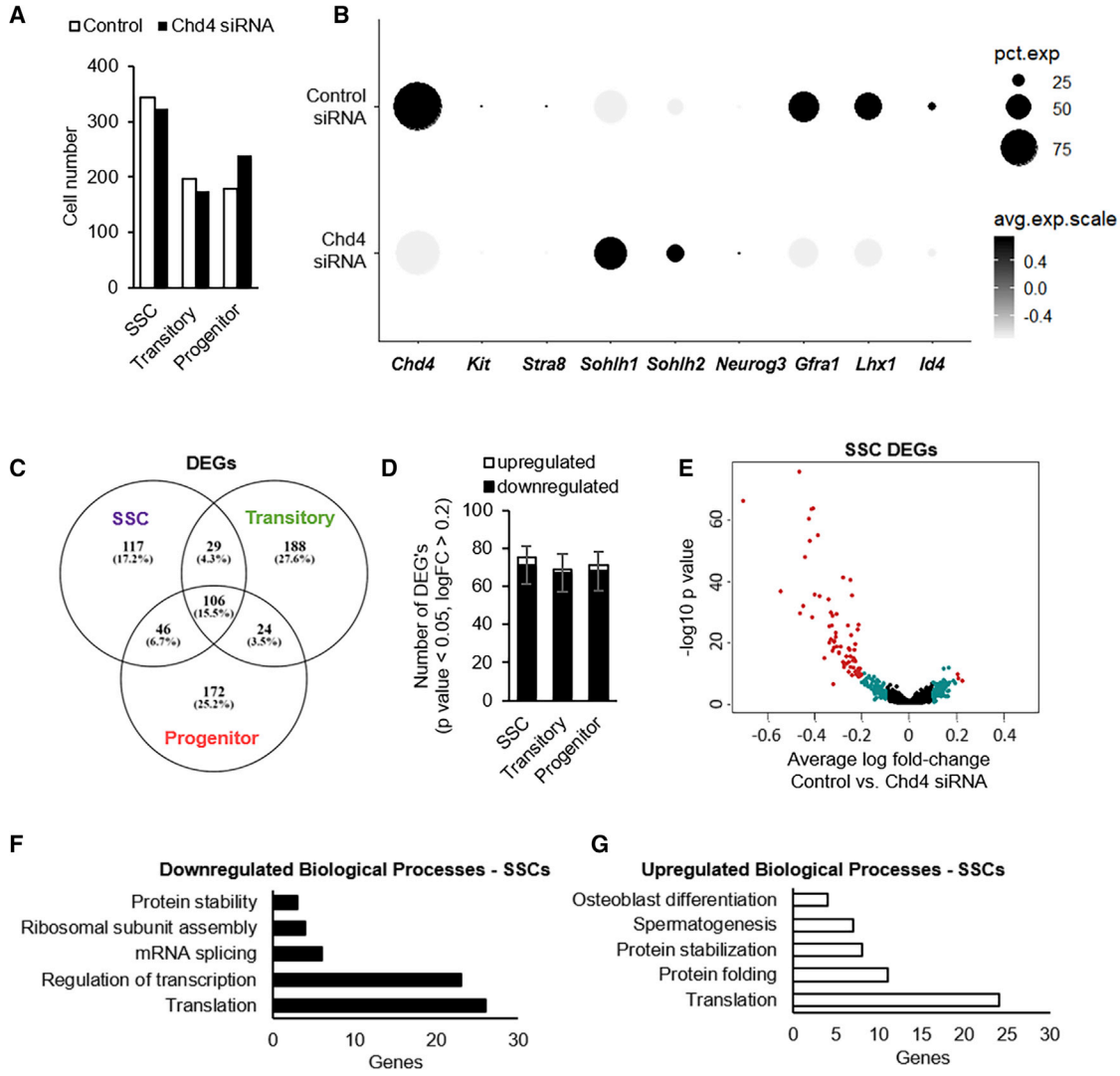


**Figure 4. Knockdown of *Chd4* causes dysregulated gene expression in undifferentiated spermatogonia**

(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 log2 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 log2 fold-change > 0.1, red points have a log2 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.

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.

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) populations. This suggests that CHD4 primarily acts as an activator of gene expression in undifferentiated spermatogonia. In assessing enriched GO biological processes in the SSC population, terms relating to translation and ribosomal 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 transcription, 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 spermatogenesis were identified as upregulated processes (Figure 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 progenitor DEG lists returned similar results, which are provided 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 performed to identify primary protein binding partners (Figure 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), supporting 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 5B, full list in Data S3). GO terms enriched in this list included nucleosome assembly, positive and negative regulation of transcription, chromatin modification, and spermatogenesis (Figures 5B and 5C). 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 chaperone 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 regulator 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-protein 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 spermatogonia 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 spermatogonia, 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* knockdown (Figures 4B and S4A), as well as 18 other genes that were shown to have dysregulated expression following *Chd4* knockdown in our scRNA-seq experiments (Data S4).

Cumulatively, findings reported here support a circumstance in which CHD4 is an integral component of the self-renewal machinery within SSCs. Although experiments 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 transcend to SSCs in the testicular microenvironment. Specifically, we propose that CHD4 acts within the NuRD nucleosome 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 activation of transcription via interaction with DAXX/H3.3 but, more specifically, recruited to promotors of GDNF-responsive 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 differentiating spermatogonia. For the continuation of male fertility, a delicate balance must be maintained between SSC self-

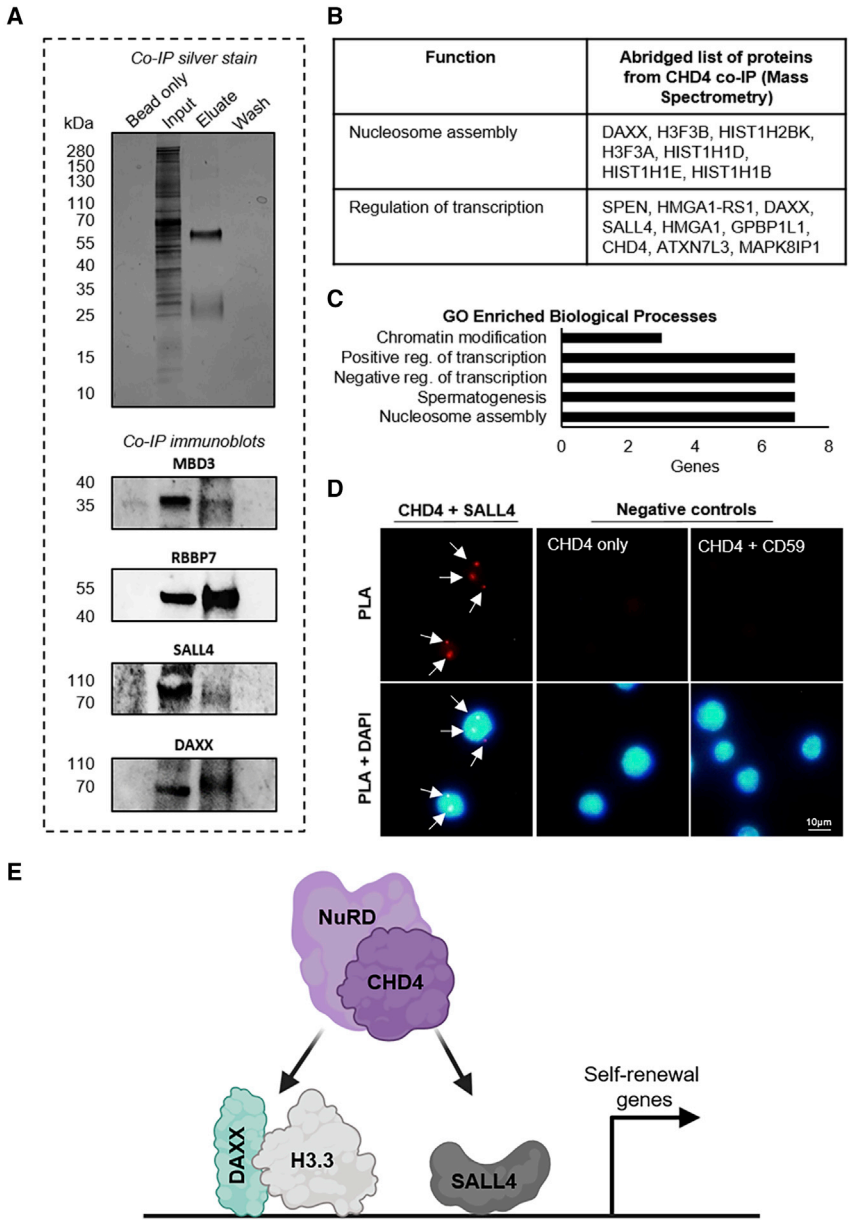


**Figure 5. Identification of CHD4 binding partners within undifferentiated spermatogonia using co-immunoprecipitation**

(A) Using CHD4 antibody as bait, several interacting proteins were "pulled down" via co-IP, as can be appreciated in the eluate following silver staining (upper). Immunoblotting (lower) was utilized to confirm the presence of MBD3, RBBP7, SALL4, and DAXX.

(B and C) Mass spectrometry was used to further identify CHD4 binding partners in undifferentiated spermatogonia. A full list of identified proteins is provided in Data S3.

(D) A duolink proximity ligation assay (PLA) was used to verify the interaction between CHD4 and SALL4 in undifferentiated spermatogonia. Red punctate fluorescence was observed (white arrows) that was absent from the negative control, confirming protein interaction. Scale bar, 10 μm.

(E) A model for the regulation of gene expression by CHD4 in undifferentiated spermatogonia.

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-eGFP$^{Bright}$ 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., 2008; Zhao et al., 2017). In support of a primary role for CHD4 in activating gene expression in undifferentiated spermatogonia, co-IP analysis revealed an interaction between CHD4 and histone 3.3 (H3F3), and its chaperone DAXX. Promotor co-occupancy by histone 3.3 and the NuRD complex has been previously shown to directly correlate with increased levels of transcription (Kraushaar et al., 2018), with DAXX being required to facilitate assembly of the histone 3.3 complex on the chromatin (Lewis et al., 2010). Thus, this interaction likely explains why >90% of DEGs ($p < 0.05$, log2 fold-change > ±0.2), including self-renewal genes, were downregulated following CHD4 knockdown due to a loss of this "activating" stimuli. Beyond this, however, more specific targeting of gene activation by CHD4/NuRD is likely to be facilitated by the interaction between CHD4 and SALL4, which was demonstrated in this study using co-IP and PLA techniques, and has been observed previously in undifferentiated spermatogonia (Chan et al., 2017). SALL4 has binding sites in the promotors of a number of GDNF-responsive genes that are critical for SSC maintenance (Lovelace et al., 2016), and SALL proteins are known to recruit the NuRD complex to target sites within the genome (Lauberth and Rauchman, 2006). Thus, it is harmonious that the GDNF-responsive gene *Gfra1* was found to be significantly downregulated following *Chd4* knockdown in our article. The notion that *Gfra1* expression is regulated by a CHD4/SALL4 complex is further supported by ChIP sequencing data produced by de Castro et al. (2020), in which CHD4 was found to bind to the *Gfra1* promotor in undifferentiated spermatogonia isolated from a P5 mouse testis.

Beyond the role for CHD4 in gene activation, we identified some evidence for CHD4 acting as part of a repressive complex within undifferentiated spermatogonia. Indeed, *Chd4* knockdown resulted in upregulated expression of several genes known to drive the progenitor transition, including *Sohlh1/2* (Suzuki et al., 2012) and *Neurog3* (Kaucher et al., 2012), as well as genes involved in later stages of spermatogenic differentiation, such as *Dazl* (Mikedis et al., 2020; Schrans-Stassen et al., 2001) and the meiotic recombination factor *Tex15* (Yang et al., 2008). Again, these findings support a role for CHD4 in maintaining an SSC state by activating genes involved in self-renewal and repressing genes required for the progenitor transition. Certainly, in ESCs, expression of the pluripotency genes *Nanog* and *Klf4* is upregulated in the presence of CHD4, while expression of *Tbx3* is repressed to inhibit lineage specification (Zhao et al., 2017). Indeed, it has been suggested that NuRD remodeling activity modulates chromatin structure to "fine-tune" gene expression, rather than activate or repress expression exclusively (Bornelöv 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, *Rosa26LacZ* 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/SvlmJ (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 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% $O_2$, 5% $CO_2$ at 37°C on mitotically inactivated SIM mouse embryo-derived thioguanine and ouabain-resistant feeder monolayers (STOs) in mouse serum-fee 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.; iknvestigation, 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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