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SOHLH1 and SOHLH2 coordinate spermatogonial differentiation

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Abstract

Spermatogonial self-renewal and differentiation are essential for male fertility and reproduction. We discovered that germ cell specific genes *Sohlh1* and *Sohlh2*, encode basic helix-loop-helix (bHLH) transcriptional regulators that are essential in spermatogonial differentiation. *Sohlh1* and *Sohlh2* individual mouse knockouts show remarkably similar phenotypes. Here we show that SOHLH1 and SOHLH2 proteins are co-expressed in the entire spermatogonial population except in the GFRA1$^+$ spermatogonia, which includes spermatogonial stem cells (SSCs). SOHLH1 and SOHLH2 are expressed in both KIT negative and KIT positive spermatogonia, and overlap *Ngn3*/EGFP and SOX3 expression. SOHLH1 and SOHLH2 heterodimerize with each other *in vivo*, as well as homodimerize. The *Sohlh1*/*Sohlh2* double mutant phenocopies single mutants, i.e., spermatogonia continue to proliferate but do not differentiate properly. Further analysis revealed that GFRA1$^+$ population was increased, while meiosis commenced prematurely in both single and double knockouts. *Sohlh1* and *Sohlh2* double deficiency has a synergistic effect on gene expression patterns as compared to the single knockouts. SOHLH proteins affect spermatogonial development by directly regulating *Gfra1*, *Sox3* and *Kit* gene expression. SOHLH1 and SOHLH2 suppress genes involved in SSC maintenance, and induce genes important for spermatogonial differentiation.

Keywords

Spematogonia; Sohlh1; Sohlh2; differentiation; stem cell

Introduction

Sperm are produced via spermatogenesis, a male-specific process that involves continual self-renewal and proliferation of spermatogonial stem cells followed by differentiation. Much interest has been devoted to understanding spermatogonial self-renewal and its “stemness,” yet much remains to be learned about how spermatogonia choose to differentiate, as well as the nature of spermatogonia-specific regulators that affect such critical transition.

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In the adult mouse testes, spermatogonia, including spermatogonial stem cells (SSCs), are localized in the seminiferous tubules along the peripheral basement membrane, and the differentiating offspring of spermatogonia are arranged in a sequential order towards the center of the tubule. The cycle of the seminiferous epithelium is divided into 12 stages (stage I–XII) in mice (de Rooij, 1998, 2001; de Rooij and Russell, 2000). The spermatogonial stem cell function resides in the undifferentiated type A spermatogonia, which comprise the most primitive set of spermatogonia including A-single (A_s; isolated spermatogonia), A-paired (A_{pr}; 2-chained cells), and A-aligned (A_{al}; 4 to 16 or occasionally 32-chained cells). In addition to their self-renewal capabilities as a population of cells, undifferentiated spermatogonia generate A_1, A_2, A_3, A_4, intermediate (In) and B differentiating spermatogonia. Differentiating spermatogonia enter meiosis around postnatal day 10 to form meiotic spermatocytes, which in turn give rise to haploid spermatids and spermatozoa.

Extensive work on molecular mechanisms that control spermatogenesis has elucidated marker proteins that distinguish spermatogonial cell types. Glial cell line derived neurotrophic factor (GDNF) is secreted by the Sertoli cells and acts via GDNF receptors on undifferentiated spermatogonia to exert paracrine regulation of spermatogonial self-renewal. A_s to A_{al-4} and a portion of A_{al-8} undifferentiated spermatogonia express GDNF family receptor alpha 1 (GFRA1), as well as a receptor tyrosine kinase (RET), which is part of the GDNF receptor complex (Buageaw et al., 2005; Jijiwa et al., 2008; Naughton et al., 2006; Tokuda et al., 2007). *Ngn3*/EGFP transgenic mice express the EGFP gene under the control of the *Neurog3* promoter, and mark mainly undifferentiated and differentiating spermatogonia (Yoshida et al., 2004). Undifferentiated type-A spermatogonia show functional and molecular heterogeneity, and GFRA1 and *Ngn3*/EGFP mark these subsets. GFRA1 is expressed in most primitive undifferentiated spermatogonia, and *Ngn3*/EGFP is predominantly expressed in GFRA1-negative cells, which likely represent transit amplifying spermatogonia (Nakagawa et al., 2010; Suzuki et al., 2009; Tokuda et al., 2007; Zheng et al., 2009). The transcriptional repressor ZBTB16 (also known as PLZF) and a homophilic cell-cell adhesion molecule, CADHERIN 1 (CDH1), are expressed in the entire population of undifferentiated spermatogonia (Buaas et al., 2004; Costoya et al., 2004; Toyoda et al., 2009). ZBTB16 also can be detected in differentiating spermatogonia (Suzuki et al., 2009). KIT, a tyrosine kinase receptor for KIT ligand (KITL), is another important marker which commences expression around stages VI–VII, following that undifferentiated type A spermatogonia transform to differentiating A_1 spermatogonia and KIT expression is maintained until the pre-leptotene spermatocytes stage (de Rooij, 1998; Pellegrini et al., 2003; Schrans-Stassen et al., 1999).

Tissue specific basic helix-loop-helix (bHLH) transcription factors play critical roles in cell differentiation (Davis et al., 1987; Massari and Murre, 2000; Murre et al., 1989). Previously we reported that the germ cell specific bHLH factor SOHLH1 is essential for spermatogonial differentiation (Ballow et al., 2006a). We also discovered a SOHLH1 related bHLH protein, SOHLH2 (Ballow et al., 2006b). In *Sohlh2* deficient mice, undifferentiated type A spermatogonia do not differentiate into KIT-positive spermatogonia (Hao et al., 2008; Toyoda et al., 2009). SOHLH1 and SOHLH2 are expressed in both undifferentiated spermatogonia and differentiating spermatogonia, however the precise expression pattern of these bHLH transcriptional regulators with regards to each other and the seminiferous epithelial cycle has not been studied. Moreover, the effect of *Sohlh1* and *Sohlh2* double deficiency on spermatogonial differentiation and proliferation has not been explored. Here, we examine the expression profile of SOHLH1 and SOHLH2 proteins in spermatogonial differentiation, as well as the effects of double knockout deficiency. We also investigated the direct and indirect targets of SOHLH1 and SOHLH2 to reveal genetic pathways that control spermatogonial differentiation.

Materials and Methods

Mice

All mouse experiments were performed on the C57BL6/129S6/SvEv hybrid background. All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh IACUC.

Histology, immunostaining and determination of epithelial stages in seminiferous tubules

Protocols are published in supplementary information, and primary and secondary antibodies are listed in Table S1.

Whole mount immunofluorescence of seminiferous tubule

Whole mount immunofluorescence was performed using a previously described protocol (Nakagawa et al., 2010; Suzuki et al., 2009). 0.01%TritonX-100/PBS was used for washing and dilution of antibodies. The immunostained tubules were mounted on slide glasses and enclosed with ProLong Gold Antifade reagent (Invitrogen, Carlsbad, CA). Samples were observed using confocal laser microscopy; Nikon A1 (Nikon, Tokyo, Japan).

Co-immunoprecipitation analysis and chromatin immunoprecipitation

Guinea pig anti-SOHLH1, rabbit anti-SOHLH1 (Pangas et al., 2006), guinea pig anti-SOHLH2 (Ballow et al., 2006b) and mouse anti-FLAG (M2) (Sigma, St. Louis, MO) were used for co-immunoprecipitation and chromatin immunoprecipitation experiments. Detailed protocols are published in supplementary information.

Microarray analysis and quantitative real time RT-PCR

Total RNA was isolated from 1-week-old testes. Detailed protocols are published in supplementary information.

Results

SOHLH1 and SOHLH2 are co-expressed in GFRA1-negative spermatogonia

Although *Sohlh1* and *Sohlh2* knockout phenotypes and expression patterns are similar to each other (Ballow et al., 2006a; Hao et al., 2008; Pangas et al., 2006; Toyoda et al., 2009), co-expression and genetic interaction between these two transcriptional regulators have not been studied *in vivo*. We performed whole mount immunofluorescence with adult seminiferous tubules using anti-SOHLH1 and anti-SOHLH2 antibodies. SOHLH1 and SOHLH2 were co-expressed in the vast majority of spermatogonia in the adult testis (Fig. 1). These results are not surprising given previous observations of the SOHLH1 and SOHLH2 expression patterns, and suggest functional interrelationship.

To classify SOHLH1 and SOHLH2-expressing spermatogonial cell types in adult testis, we performed multiple immunofluorescence analysis of whole mount seminiferous tubules with anti-SOHLH1, anti-SOHLH2 and markers known to be expressed in undifferentiated spermatogonia such as CDH1, ZBTB16, GFRA1, and *Ngn3*/EGFP, and differentiating spermatogonia as determined by KIT expression. In our hands, CDH1 (Fig. S1A, B) and ZBTB16 (Figs. 2A and D–G) were also detected in differentiating spermatogonia (Suzuki et al., 2009). We combined the triple immunofluorescence data with the stage of the seminiferous epithelial cycle to better define spermatogonial populations that express SOHLH proteins (Kotaja et al., 2004). The tubules were grouped into four stages; I–VI, V–VIII, IX–XI and X–III respectively (See supplementary information). SOHLH1 and SOHLH2 expressing spermatogonia co-express CDH1 and ZBTB16 in undifferentiated

spermatogonia (A_s, A_{pr}, A_{al-4}, A_{al-8}) but a subset of CDH1 or ZBTB16 positive cells expressed neither SOHLH1 nor SOHLH2 (Figs. 1A, 1B and 2 yellow arrowheads). These findings indicate that SOHLH proteins are not expressed in the most undifferentiated spermatogonial population.

We therefore investigated the expression patterns of SOHLH1, SOHLH2, GFRA1 and *Ngn3*/EGFP in the adult testis. GFRA1 is expressed in the most primitive undifferentiated spermatogonia, and *Ngn3*/EGFP is predominantly expressed in GFRA1-negative cells and likely marks more differentiated spermatogonia. The heterogeneous expression of GFRA1 and *Ngn3*/EGFP in the undifferentiated type-A spermatogonial compartment underlies the complexity of the spermatogonial stem cells, as well as their functional hierarchy (Nakagawa et al., 2010; Suzuki et al., 2009; Zheng et al., 2009). SOHLH1 and SOHLH2 proteins were predominantly expressed in the GFRA1-negative population (Fig. 1C) (Suzuki et al., 2009), and only a small subset of spermatogonia co-expressed GFRA1 with SOHLH1 and SOHLH2 (Fig. 1C, white arrowhead). Staining for SOHLH1 and EGFP in testis of *Ngn3/EGFP* transgenic mice revealed that SOHLH1 was expressed by most *Ngn3*/EGFP-positive spermatogonia. However, a small portion of the SOHLH1-positive population was *Ngn3*/EGFP-negative, suggesting that SOHLH1 expression precedes *Ngn3*/EGFP (Fig. S1C and D). The transcription factor SOX3 (SRY-related HMG-box 3) is expressed in spermatogonia, and a germ cell specific *Sox3* knockout male mouse showed similar defects in spermatogonial differentiation as *Sohlh1/Sohlh2* knockouts (Laronda and Jameson, 2011; Raverot et al., 2005). Interestingly, the expression pattern of SOX3 in undifferentiated spermatogonia was similar to SOHLH1, and *Sox3* was predominantly expressed in the GFRA1-negative population (Fig. 1D).

We also examined the expression of SOHLH1 and SOHLH2 in the differentiating spermatogonia. KIT is expressed in differentiating spermatogonial types A_1 to B, as well as leptotene spermatocytes. KIT expression is first visualized in spermatogonia at stages VI–VII; following that, undifferentiated spermatogonia transform to differentiating A_1 spermatogonia (de Rooij, 1998; Schrans-Stassen et al., 1999). SOHLH1 expression was observed in both KIT-negative undifferentiated spermatogonia (Fig. 2, white arrowheads) and KIT-positive differentiating spermatogonia (Fig. 2, magenta arrowheads). SOHLH1 expression in KIT-positive differentiating spermatogonia gradually disappeared during stages IV–VI when intermediate and type-B spermatogonia were observed (Fig. 2B and C). Our observations indicate that SOHLH proteins disappear during differentiation of intermediate to type-B spermatogonia, and are not expressed in spermatocytes. SOHLH1 and SOHLH2 expression shows that SOHLH1 and SOHLH2 are not expressed in the most primitive spermatogonia but do precede *Ngn3*/EGFP and KIT expression. The expression patterns of SOHLH1 and SOHLH2 relative to other known spermatogonial markers are shown in Figure S1E.

SOHLH1 and SOHLH2 can form homodimers and heterodimers

Basic helix-loop-helix transcription factors are known to dimerize via their HLH domains. SOHLH1 and SOHLH2 have almost identical patterns of expression and co-localize in the spermatogonial nuclei, raising the possibility that SOHLH1 and SOHLH2 interact with each other *in vivo*. We investigated whether SOHLH1 and SOHLH2 co-immunoprecipitate in the 1-week-old testis, at a time when the vast majority of spermatogonia co-express SOHLH1 and SOHLH2 (Fig. S1F). We used anti-SOHLH1 and anti-SOHLH2 antibodies on wild type testes to determine whether SOHLH2 co-immunoprecipitates with SOHLH1 and vice versa. These antibodies were developed against the highly divergent SOHLH COOH regions (Ballow et al., 2006b; Pangas et al., 2006) and do not cross react. Both anti-SOHLH1 and anti-SOHLH2 antibodies immunoprecipitated SOHLH2 and SOHLH1 proteins respectively,

as shown by Western blots on immunoprecipitated products (Fig. 3A). Our *in vivo* data suggests that SOHLH1 and SOHLH2 can heterodimerize in spermatogonia.

We also examined SOHLH1 or SOHLH2 homodimer formation. Homodimers may differ from heterodimers in binding affinity and specificity, and may affect expression of different genes. We co-expressed MYC-tagged SOHLH1 and FLAG-tagged SOHLH1 in the 293T cell line and immunoprecipitated SOHLH1 from extracts with anti-FLAG antibody, to determine if homodimers were present. We conducted identical studies with SOHLH2. Our results indicate that SOHLH1 and SOHLH2 can homodimerize (Fig. 3B, highlighted lanes). We repeated identical experiments with anti-MYC antibody and the results were consistent with the anti-FLAG data (data not shown). SOHLH1 and SOHLH2 can therefore exist both as heterodimers and homodimers.

Sohlh1/Sohlh2 double mutants are histologically indistinguishable from single knockouts

Because SOHLH1 and SOHLH2 proteins interact *in vivo*, we hypothesized that complete elimination of SOHLH1 and SOHLH2 proteins from the testes may cause a more severe developmental defect than single deficiency. We generated *Sohlh1*$^{-/-}$*/Sohlh2*$^{-/-}$ double knockout mice to investigate the effects of double deficiency on spermatogenesis. One-week-old double mutant (*Sohlh1*$^{-/-}$*/Sohlh2*$^{-/-}$) testes showed no obvious difference when compared to the wild type (Fig. 4A–D). The general anatomy, weight and spermatogonia numbers were not significantly different between the double knockout and wild-type (Fig. S2). Spermatogenesis in *Sohlh1*$^{-/-}$*/Sohlh2*$^{-/-}$ animals was disrupted postnatally at two weeks of age, and most of the seminiferous tubules contained only spermatogonia and Sertoli cells at 7 weeks of age; findings similar to single gene *Sohlh* knockouts (Fig. S3A–H). The spermatogonia in the single or double *Sohlh* mutants retained ZBTB16 expression (Fig. 4E–H) and continued to proliferate as shown by the BrdU incorporation assay (Fig. 4I–L). Consistent with BrdU staining, real time PCR quantitation using 1-week-old testes showed that *Cyclin D1*, *D2*, *D3* and *E1* expression levels were essentially normal in our mutants as compared with wild type animals (Fig. S3I). CYCLIN D2 and E1 are essential and sufficient for germline stem cell (GSC) proliferation and maintenance (Lee et al., 2009), while the function of CYCLIN D1 and D3 in spermatogenesis is currently unknown. These results suggest that combined SOHLH1 and SOHLH2 deficiency does not affect spermatogonial survival and proliferation.

SOHLH1 and SOHLH2 functions in spermatogonial differentiation and repression of precocious meiosis

At 7 weeks of age, most of the seminiferous tubules in the *Sohlh1*$^{-/-}$*/Sohlh2*$^{-/-}$ mice retained only spermatogonia and Sertoli cells (Fig. S3E–H). Whole mount immunostaining of *Sohlh1*$^{-/-}$*/Sohlh2*$^{-/-}$ seminiferous tubules showed occasional large clusters of CDH1^{+}/ZBTB16^{+}/MVH^{+}/Kit^{-} cells, which is a typical protein expression pattern of undifferentiated spermatogonia (Fig. S3J–L). The morphology of these mutant spermatogonia was abnormal, with tightly packed cells, diminished cytoplasm, and lack of fine processes (filopodia) that emanate when wild type spermatogonia are stained with anti-CDH1 (compare Figs. 1A, 1B and S3J–O). Even in the region of the tubule where spermatogonial density was not as high, $A_{al\text{-}4}$ and longer chains did not show the normal filopodia-staining pattern seen with CDH1 (Fig. S3J–O). Confocal microscopy revealed that intercellular bridges in a subset of single and double mutant spermatogonia were misshapen and wider than in the wild type (Fig. S3M–O). The wild type spermatogonia in experimentally induced cryptorchid testis did not show abnormal intercellular bridges and filopodia, indicating that these are not caused by the diminished space in the tubules or disrupted spermatogenesis (data not shown). Our observations show that undifferentiated spermatogonia in single and double *Sohlh1/Sohlh2* knockouts are morphologically abnormal, but can proliferate.

We observed that a portion of cells showed thread-like chromosome condensations, characteristic of the leptotene spermatocytes (Fig. S3E–H). We assessed whether *Sohlh1* and/or *Sohlh2* congenital deficiency affected expression of *Hormad1*, a germ cell specific component of the synaptonemal complex that is essential for early meiosis (Shin et al., 2010; Wojtasz et al., 2009). Whole mount immunostaining revealed that a portion of germ cells ($MVH^+/CDH1^-$) in adult mutants (either single or double knockout) expressed HORMAD1. HORMAD1 expressing cells were always located near the $CDH1^+$ germ cell cluster (Figs. S3L and S4A), suggesting that $HORMAD1^+$ cells emerged from such clusters. No spermatids were observed in mutant testes, and these meiotic-like germ cells were eliminated by apoptosis (Fig. S3H). We also observed HORMAD1 positive cells in 1-week-old testis, with thread-like chromosome condensations in a few seminiferous tubules of either single or double *Sohlh1/Sohlh2* mutants. HORMAD1 positive cells were not observed in the 1-week-old wild type testis (Fig. 4M–P). These observations suggest that spermatogonia in *Sohlh1/Sohlh2* mutants undergo precocious meiosis.

Transcriptional regulator DMRT1 represses spermatogonial commitment to meiosis, and DMRT1 induces *Sohlh1* expression and suppresses *Stra8* expression in spermatogonia (Matson et al., 2010). It is therefore plausible that SOHLHs deficiency leads to precocious meiosis. Transcription factor STRA8 is one of several factors required for the initiation of meiosis (Anderson et al., 2008; Baltus et al., 2006). In adult testis of either single or double *Sohlh1/Sohlh2* mutants, a portion of spermatogonia expressed STRA8, and the frequency of $STRA8^+$ tubules was considerably increased (Fig. S4D), although DMRT1 expression was still present in $CDH1^+$ spermatogonia (Fig. S4E–F). Our data suggest that *Sohlh1* and/or *Sohlh2* deficiency causes a subset of cells to precociously commit to meiosis.

SOHLH1 and SOHLH2 double deficiency has a synergistic effect on gene expression

We performed microarray analysis on 1-week-old testes to determine molecular perturbations that precede a frank pathology in double knockout versus single knockout mouse testes. SOHLH1 and SOHLH2 are co-expressed in the same spermatogonial population at this stage (Fig. S1F). Moreover, *Sohlh1* expression persists in the *Sohlh2* knockout and vice versa, and we hypothesized that *Sohlh1*$^{-/-}$/*Sohlh2*$^{-/-}$ deficiency will have a synergistic effect on molecular pathology. We analyzed the mRNA expression pattern of wild type, *Sohlh1*$^{-/-}$, *Sohlh2*$^{-/-}$ and *Sohlh1*$^{-/-}$/*Sohlh2*$^{-/-}$ testes at 1 week, and focused on genes which showed a statistically significant fold change equal to or greater than 2, as compared with the wild type. In *Sohlh1*$^{-/-}$ and *Sohlh2*$^{-/-}$, nearly identical genetic pathways were altered, as very few genes were differentially expressed between the two (Fig. 5A and S5). In *Sohlh1*$^{-/-}$/*Sohlh2*$^{-/-}$, however, the expression of 421 genes was disrupted; that is, nearly 4 times as many genes were affected in the double mutant as compared to the single mutants: 114 genes in *Sohlh1*$^{-/-}$ and 109 genes in *Sohlh2*$^{-/-}$ (Fig. 5B, Table S3– 5). Among all of the genes with altered expression, there were 71 that were commonly altered between the three mutants: 47 genes were down-regulated and 24 genes were up-regulated (Fig. 5B and Table S6). When we compared double and single knockout gene lists, 123 genes were specifically altered in *Sohlh1*$^{-/-}$/*Sohlh2*$^{-/-}$ double mutant (≧ 2-fold change as compared to single mutants). Of the 123 genes that were only altered in *Sohlh1*$^{-/-}$/*Sohlh2*$^{-/-}$ mutants, only 3 of the genes were down-regulated, while 120 genes were up-regulated (Table S7).

We confirmed the microarray data for a select group of genes using quantitative real-time PCR from 1-week-old testes. Genes known to be involved in spermatogonial differentiation such as *Kit* and *Sox3* were significantly down-regulated in single and double mutants (Fig. 5C). Interestingly, *Sohlh2* expression in *Sohlh1*$^{-/-}$ testis was up-regulated, whereas *Sohlh1* expression in *Sohlh2*$^{-/-}$ was down-regulated (Fig. 5C). Therefore, *Sohlh1* may repress

Sohlh2 expression, while *Sohlh2* may act to stimulate *Sohlh1* expression, via direct or indirect feedback mechanisms.

A number of genes involved in spermatogonial stem cell maintenance such as *Ret*, *Gfra1*, *Nanos2* and *Pou5f1* also showed altered expression levels in single and double mutants. *Ret* and *Gfra1* encode GDNF receptors and are mainly expressed in SOHLH1 and/or SOHLH2-negative undifferentiated spermatogonia. Both were up-regulated in single and double mutants, while their ligand, *Gdnf*, was not significantly altered in any of the mutants (Fig. 5D). NANOS2 is a translational regulator expressed in GFRA1^{+} spermatogonia including stem cells (Sada et al., 2009), and *Nanos2* was up-regulated in both single and double mutants. POU5F1 is a homeobox-containing transcription factor that plays critical roles in pluripotency and self-renewal of SSCs. POU5F1 protein expression is lower in portions of male germline stem cells that express high levels of SOHLH1 in a culture system (Dann et al., 2008). *Pou5f1* transcript expression is significantly up-regulated in single and double knockouts (Fig. 5D). One interpretation is that gradual expression of SOHLH1 and SOHLH2 during spermatogonial differentiation suppresses expression of genes that are involved in SSC maintenance/self-renewal, such as *Ret*, *Gfra1*, *Nanos2* and *Pou5f1*, and promotes spermatogonial differentiation.

We also examined the effects of single and double *Sohlh* deficiencies on the expression of RNA binding proteins that are critically involved at various stages of spermatogenesis. Several RNA binding proteins are highly expressed in germ cells and localize to nuage, an electron-dense structure in the cytoplasm including ribonucleoprotein (RNP) complexes such as P-bodies, stress granules and chromatoid bodies. NANOS2, which is essential for SSC maintenance in testes, localizes to P-bodies (Sada et al., 2009; Suzuki et al., 2009). NANOS3, which is essential for the development of primordial germ cells (PGCs), is similarly localized in P-bodies and stress granules, although its function in the testis is unknown (Kedersha et al., 1999; Tsuda et al., 2003; Yamaji et al., 2010). NANOS2 is expressed mainly in GFRA1^{+}/*Ngn3*EGFP^{-} spermatogonia, while NANOS3 is predominantly expressed in GFRA1^{-}/*Ngn3*EGFP^{+} spermatogonia, just like SOHLHs (Suzuki et al., 2009). In *Sohlh1/Sohlh2* single and double mutants, both *Nanos2* and *Nanos3* expression was remarkably up-regulated (Fig. 5D and E). TIA1 is another RNA binding protein localized to the stress granules, and *Tia1* expression was significantly up-regulated in the double knockout as compared to the single knockouts (Fig. 5E). LIN28A is also localized to P-bodies and stress granules, and specifically expressed in undifferentiated spermatogonia in the mouse testis. Expression pattern of LIN28A is similar to that of ZBTB16 (Balzer and Moss, 2007; Zheng et al., 2009). The functional role of LIN28A in the testes is currently unknown. Decapping enzyme, DCP2, co-localizes to P-bodies with LIN28A (Liu et al., 2005). Both *Lin28a* and *Dcp2* are significantly down-regulated in *Sohlh* single and double mutants (Fig. 5E). Other genes involved in RNA metabolism also show altered expression patterns in *Sohlh* mutants (Tables S3 and S4). These results suggest that SOHLH1 and SOHLH2 directly or indirectly affect critical RNA processing pathways during spermatogonial development.

Genes that are important for epigenetic modification were also altered in single and double mutants. PIWI-like proteins interact with short RNA molecules, piRNAs, and regulate various aspects of spermatogenesis (Chuma et al., 2009; Deng and Lin, 2002); *Piwil1/Miwi* is important for spermiogenesis and *Piwil4/Miwi2* is essential for retrotransposon silencing as well as meiosis progression (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). *Piwil1* and *Piwil4* were significantly up-regulated in 1-week-old single and double *Sohlh* mutants (Fig. 5F). On the other hand, DNMT3B, which is essential for DNA methylation, was significantly down-regulated (Fig. 5F and Table S3) (Gowher et al., 2005; Okano et al., 1999). RNA expression arrays also show premature expression of

mRNA corresponding to *Dmrtc2/Dmrt7, Spo11* and *Rec8* at 1 week, which are essential for meiosis (Fig. 5F). *Dmrtc2* appears to play a role in the sex chromatin transformation that occurs between diplonema and pachynema, while *Spo11* and *Rec8* are involved in DNA double strand break formation and homologous recombination during meiosis (Kawamata et al., 2007; Kawamata and Nishimori, 2006; Romanienko and Camerini-Otero, 2000; Xu et al., 2005). The aberrant expression pattern of these genes may lead to abnormal meiosis and apoptosis in the mutants.

Currently there are very few proteins identified as markers for undifferentiated spermatogonia. The array expression analysis on *Sohlh1/Sohlh2* mutants revealed that numerous genes were affected, including testis specific genes with unknown functions during spermatogenesis such as *Nmt2, Ly6k, 4732415M23Rik, Dmrtb1/Dmrt6, Gml, Dzip3, Tbx22,* and *Gas5* (Fig. S6). Further studies will be necessary to examine their expression pattern and functions during spermatogonial differentiation.

SOHLH1 and SOHLH2 bind chromatin upstream of genes essential for SSC maintenance and spermatogonial differentiation

We conducted chromatin immunoprecipitation (ChIP) with anti-SOHLH1 and anti-SOHLH2 antibodies to determine whether SOHLH proteins directly regulate genes critical for SSC maintenance and spermatogonial differentiation, such as *Gfra1, Sox3, Kit, Sohlh1* and *Sohlh2*. We used 1-week-old testes to examine whether SOHLH1 or SOHLH2 bind conserved E-boxes (CANNTG) in a promoter region of each gene. E-boxes are known as DNA elements, which are bound by transcription factors from the bHLH family, including SOHLH1 and SOHLH2 (Murre et al., 1989; Pangas et al., 2006). We used *Sohlh1*$^{-/-}$ or *Sohlh2*$^{-/-}$ testes as a negative control for anti-SOHLH1 or anti-SOHLH2 antibodies, respectively. Since part of the promoter region containing conserved E-boxes was deleted during the engineering of the *Sohlh2* knockout, we used wild type testes and normal IgG of rabbit (anti-SOHLH1) or guinea pig (anti-SOHLH2) as a control. ChIP analyses showed that SOHLH1 and SOHLH2 bind a subset of E-boxes in the promoter regions of *Gfra1, Sox3, Kit, Sohlh1 and Sohlh2* (Fig. 6A–E). SOHLH1 and SOHLH2 antibodies did not immunoprecipitate E-boxes upstream of *Gapdh* (−632/763, positions determined relative to the transcription initiation site), *Sohlh1* (−71 and −4422), *Sox3* (−1382/1312 and −2135), *Lin28a* (−397, −553 and −970), *Ret* (−1441 and −2507), *Nanos2* (−459/488, −654 and 823/879) and *Dmrt1* (−519/541 and −1065) (data not shown). Interestingly, SOHLH1 and SOHLH2 also bind E-box elements located in the *Neurog3* promoter (data not shown). Despite the widespread use of *Neurog3* as a marker of spermatogonia, its functional role in spermatogonial development, if any, is unknown. These results suggest that SOHLH proteins directly regulate the transcription of genes implicated in spermatogonial maintenance and differentiation, including their own expression.

SOHLH1 and SOHLH2 are important for differentiation from the most primitive set of undifferentiated spermatogonia

Microarray and follow up qRT-PCR studies showed significantly increased levels of *Ret* and *Gfra1*, two genes that encode GDNF receptors, and are mainly expressed in SOHLH1 and SOHLH2-negative undifferentiated spermatogonia. Moreover, both SOHLH1 and SOHLH2 bind E-boxes upstream of *Gfra1*, suggesting that SOHLH1 and SOHLH2 directly regulate *Gfra1* transcription. To examine the effects of SOHLH1 and/or SOHLH2 deficiency on GFRA1 expression, we took a closer look at the GFRA1-positive cell population in wild type and mutant mice. GFRA1 is expressed in the most primitive set of undifferentiated spermatogonia, including stem cells. At one week of age, the percentages of GFRA1^{+} cells in ZBTB16^{+} spermatogonia were higher in mutants as compared to the wild type (Fig. 7A). These results were consistent with the qRT-PCR data showing higher levels of *Gfra1* in

mutant testes (Fig. 5D), and suggest that *Gfra1* expression in mutant spermatogonia is partially deregulated. In the adult *Sohlh* mutant testes, the proportion of GFRA1$^+$ chains greater than $A_{al\text{-}8}$ was increased when compared to the average in wild type (Fig. 7B). In adult wild type testes, the profile of GFRA1-positive cells depended on the stage of the epithelial cycle (Fig. 7C). In *Sohlh* single and double mutants, the stage of the seminiferous epithelial cycle could not be determined since spermatogenesis was disrupted. The average morphological profile of GFRA1$^+$ cells in either single or double mutants (A_s: 39.3–43.7%, A_{pr}: 30.4–35.6% and 3 or more in the chain: 25.0–26.0%) was similar to the profile of stages X–III in wild type (A_s: 39.7%, A_{pr}: 34.2% and 3 or more in the chain: 26.1%) (Fig. 7C). Interestingly, we often observed the GFRA1$^+$ cells to be part of chains of more than 8 cells in the mutant (Fig. 7E), which was very rare in wild type even during stages X–III (Fig. 7C and D). The longer GFRA1$^+$ chains observed in *Sohlh* mutants are not solely due to the block in spermatogenesis since we did not observe such long chains in the experimentally induced cryptorchidism in wild type testes (Fig. 7F–G).

We did observe large clusters of cells showing typical protein expression patterns of undifferentiated spermatogonia, CDH1$^+$/ZBTB16$^+$/MVH$^+$/Kit$^-$, that were GFRA1$^-$ (Figs. 7E (yellow arrowhead) and S3J–L). SOHLH protein deficiency is therefore not sufficient to de-repress GFRA1 expression, and additional factors must regulate GFRA1 expression.

Discussion

Spermatogenesis is a dynamic process that transforms diploid spermatogonial stem cells into highly differentiated haploid spermatozoa. Several transcriptional regulators preferentially expressed in spermatogonia are known to affect various steps of spermatogonial development. Different stages of spermatogenesis require different transcriptional regulators. *Pou5f1*, *Taf4b* and *Zbtb16/Plzf* are important in spermatogonial proliferation and self-renewal. Few spermatogonia-specific transcriptional regulators are known to be critical in spermatogonia differentiation. *Sox3*, a member of the HMG group of transcriptional regulators, is important in brain development as well as spermatogonial differentiation. Others and we recently discovered that *Sohlh1* and *Sohlh2* are tissue-specific bHLH transcriptional regulators that play critical roles during spermatogonial differentiation. Basic helix-loop-helix transcriptional regulators are essential in the differentiation of many cell types. Often, multiple tissue-specific bHLH factors are necessary for tissue differentiation, and double deficiencies have shown synergistic effects and more profound phenotypic changes as compared to the single knockouts (Kruger et al., 2006; Zhou and Anderson, 2002). Our current study indicates that *Sohlh1* and *Sohlh2* are co-expressed in undifferentiated and differentiating spermatogonia, and interact as heterodimers and homodimers. This is different from previous studies suggesting that SOHLH2 is expressed earlier than SOHLH1 (Ballow et al., 2006a; Toyoda et al., 2009). In adult testis, the vast majority SOHLH1 and SOHLH2 is not expressed in GFRA1-positive spermatogonia, which is the most primitive set of spermatogonia and includes spermatogonial stem cells. Our result is consistent with a previous report showing that SOHLH2 was not expressed in a subset of CDH1$^+$ spermatogonia (Toyoda et al., 2009). In addition, we showed that SOHLH1 and SOHLH2 directly regulate the transcription of *Gfra1*, *Sox3*, *Sohlh1*, *Sohlh2* and *Kit* genes that are essential for spermatogonial development and differentiation. We have also noticed that SOHLH1 and SOHLH2 deletion induces expression of meiosis related genes such as *Dmrtc2/Dmrt7* and *Piwil1/Miwi*. Moreover, the frequency of tubules containing STRA8$^+$ spermatogonia without KIT expression increased in mutants and a subset of remaining germ cells showed HORMAD1$^+$ thread-like chromosome condensation approximating leptotene to zygotene stage of meiosis. STRA8 associates with the onset of meiosis and HORMAD1 is a critical component of the synaptonemal complex (Anderson et al., 2008; Baltus et al., 2006; Shin et al., 2010; Wojtasz et al., 2009). Interestingly, DMRT1,

one of the doublesex-related transcription factors, overlaps SOHLH1 expression (Fig. S4), and *Dmrt1* deficiency partially affects spermatogonial differentiation as well as induces uncontrolled meiosis in *Dmrt1* mutant germ cells (Matson et al., 2010). Although *Dmrt1* has been implicated in differentiation, its phenotype is leakier than the *Sohlh1* knockout phenotype, with differentiating spermatogonia as well as spermatocytes and spermatids still observed in *Dmrt1* mutants (Matson et al., 2010). This may or may not be due to the conditional nature of the deletion. Nevertheless, *Sohlh1* and *Dmrt1* results suggest that disruption in differentiation leads to aberrant entry along the meiotic pathways. Moreover, our findings are consistent with the interpretation that proper differentiation is necessary for complete meiosis, as we did not observe pachytene stage spermatocytes.

Redundancy of SOHLH1 and SOHLH2

Although *Sohlh2* was identified as a homologue of *Sohlh1*, their limited homology is confined to the bHLH domains (~50%). Because of the limited homology and detectable SOHLH1/SOHLH2 heterodimers, we hypothesized that these two proteins play independent roles, and that double deficiency will produce synergistic effects. The onset and extent of pathology in mice lacking both *Sohlh1* and *Sohlh2* is identical to single knockouts. These results suggest that *Sohlh1* and *Sohlh2* share a common pathway that is enabled at a specific point during spermatogonial development. Gene expression array analyses on *Sohlh1* and *Sohlh2* mutants show little differences and also support the notion that *Sohlh1* and *Sohlh2* regulate common pathways. The molecular explanation for the lack of a more severe pathology in double mutant mice could be due to the essential roles of the SOHLH1/SOHLH2 heterodimers in spermatogonial differentiation. At the gene expression level, however, we did notice synergism in the double mutants. Almost four-fold more genes were affected in the double mutant as compared to the single knockouts, yet the phenotype is practically the same. These results suggest that either SOHLH1 or SOHLH2 homodimers, or hetero-complexes with other proteins, regulate genes involved in later steps of spermatogonial development rather than earlier steps of development, which are regulated by SOHLH1/SOHLH2 heterodimers.

***Sohlh1* and *Sohlh2* deficiency and long GFRA1+ chains—**Maintenance of spermatogenesis requires active proliferation of spermatogonia and their stem cells. Several transcription factors have been implicated in spermatogonial proliferation and include *Zbtb16* and *Taf4b*, but single and double *Sohlh1/Sohlh2* mutants spermatogonia can proliferate (Fig. 4I–L) (Ballow et al., 2006a; Hao et al., 2008). Moreover, many long GFRA1$^+$ chains were observed in single and double *Sohlh1/Sohlh2* mutant spermatogonia whereas those chains are rarely visible in wild type testes. Interestingly, long GFRA1$^+$ chains are also frequently observed in busulfan-treated testes undergoing regeneration (Nakagawa et al., 2010). Such chains may accumulate as a result of perturbed balance between differentiated and undifferentiated spermatogonia and a generalized stress response. *Sohlh1* and/or *Sohlh2* deficient spermatogonia lack characteristic filopodia, and a subset of mutant spermatogonia has misshapen intercellular bridges. These morphologic changes in *Sohlh* mutants were not observed in the wild type experimentally induced cryptorchid testes, and indicate important roles for SOHLH proteins at the onset of spermatogonial differentiation and the transition from GFRA1$^+$ to GFRA1$^-$ spermatogonia.

Upstream regulators of *Sohlh1* and *Sohlh2*

Recent data indicates that transcriptional regulator DMRT1 directly regulates *Sohlh1* and *Stra8* expression in spermatogonia (Matson et al., 2010). *Dmrt1* mutant spermatogonia precociously exit the spermatogenic program and *Sohlh1* expression was extinguished in *Dmrt1* mutants. DMRT1 appears to regulate *Sohlh1* gene expression directly, as chromatin immunoprecipitation studies show DMRT1 binding to the *Sohlh1* promoter (Matson et al.,

2010). The bound region shares similarity to the DMRT1 known DNA binding consensus site. *Sohlh1* expression during spermatogenesis overlaps *Dmrt1* expression, and these data together suggest that DMRT1 directly regulates *Sohlh1*. Our immunofluorescence data indicates that DMRT1 is expressed in the double *Sohlh1/Sohlh2* knockout, and is therefore likely to be upstream of *Sohlh1*. However, the role of DMRT1 in regulating *Sohlh2* is unknown. Moreover, *Dmrt1* is unlikely to be the only regulator of *Sohlh1*, as *Dmrt1* conditional knockouts retain KIT expression and complete spermatogenesis. *Sohlh* mutants do not express KIT in adult spermatogonia, and meiotic-like cells eventually die. It is therefore likely that a sufficient quantity of SOHLH1 and SOHLH2 proteins remain in the *Dmrt1* mutant. Further studies are necessary to determine the actions of *Dmrt1* on *Sohlh2*, as well as to identify other direct regulators of *Sohlh1* and *Sohlh2*.

It is likely that both germline and somatic factors are involved in the regulation of *Sohlh1* and *Sohlh2* genes in testis. *Sohlh2* was discovered during the search for *Bmp4* inducible genes in embryonic stem cells (Hao et al., 2008). BMP4 is expressed in Sertoli cells during the first week of life, and also in spermatogonia and early spermatocytes in adult mouse testes (Baleato et al., 2005; Pellegrini et al., 2003). *Bmp4* homozygous deletion causes embryonic lethality (Winnier et al., 1995), and heterozygous males on a C57BL/6 background show compromised fertility due to degeneration of germ cells (Hu et al., 2004). SMAD proteins, transcriptional regulators that translocate to the nucleus upon ligand stimulation, relay BMP signals. SMAD1, SMAD4 and SMAD5 are expressed in spermatogonia in mouse testes, and are likely involved in BMP4-mediated induction of KIT expression in spermatogonial cultures from postnatal day 5 testes (Itman and Loveland, 2008; Pellegrini et al., 2003). In *Sohlh1/Sohlh2* single and double mutants, *Bmp4* transcripts were not significantly altered compared to the wild type. USP9X/FAM, a deubiquitinizing enzyme which promotes SMAD4 translocation to the nucleus in human cell lines (Dupont et al., 2009), was up-regulated in single and double *Sohlh* mutants, suggesting that BMP signaling is activated (Fig. S7). Future studies are necessary to determine if SMAD4 is involved in the regulation of *Sohlh1* and *Sohlh2* genes. We have summarized our current thinking on the regulators of *Sohlh1/Sohlh2* and downstream effectors of *Sohlh1/Sohlh2* in Figure 8.

SOHLH1 and SOHLH2 are key regulators of spermatogonial differentiation, and two of the few known germ cell-specific transcriptional regulators. Our study implicates SOHLH1 and SOHLH2 in the regulation of genes critical for spermatogonial development, such as *Gfra1*, *Sox3*, *Sohlh1*, *Sohlh2* and *Kit*. Moreover, our study clearly shows the stage-specific nature of spermatogonial differentiation. Future studies will be essential to determine signaling pathways and regulators that activate SOHLH1 and SOHLH2 expression and downstream pathways.

Highlight

> SOHLH1 and SOHLH2 physically interact in vivo and control spermatogenic pathways. > SOHLH1 and SOHLH2 combined deficiency disturb more pathways than single deficiency. > The deficiency of SOHLHs increased expression of genes for stem cell maintenance. > SOHLHs regulate pathways in stemness suppression and spermatogonial differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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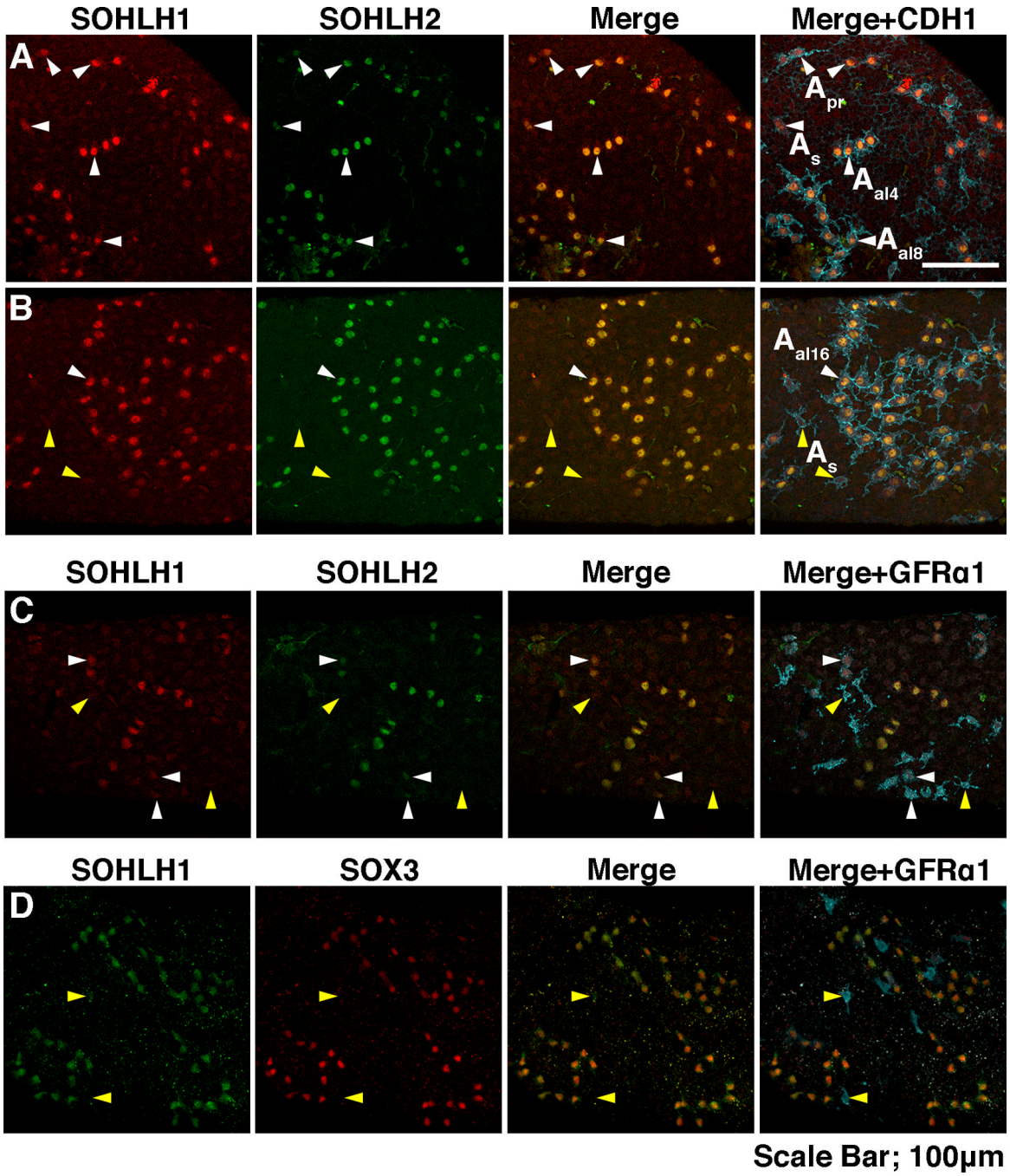

Figure 1. SOHLH1 and SOHLH2 are co-expressed in GFRA1^{-} undifferentiated spermatogonia

Whole mount immunostaining of seminiferous tubule of adult testes (7-week-old) during stages V–VIII. (A–C) Tubules were immunostained with anti-SOHLH1 (red), anti-SOHLH2 (green) and anti-CDH1(cyan) or anti-GFRA1(cyan). White arrowheads indicate spermatogonia in which SOHLH1 and SOHLH2 were co-expressed with CDH1 or GFRA1. Yellow arrowheads indicate spermatogonia devoid of SOHLH1 and SOHLH2, but positive for CDH1 or GFRA1. (D) Tubules were immunostained with anti-SOHLH1 (green), anti-SOX3 (red) and anti-GFRA1 (cyan). Yellow arrowheads indicate SOHLH1 and SOX3 negative, GFRA1 positive spermatogonia. SOX3 was co-expressed with SOHLH1 in majority of undifferentiated spermatogonia. Scale bar, 100μm.

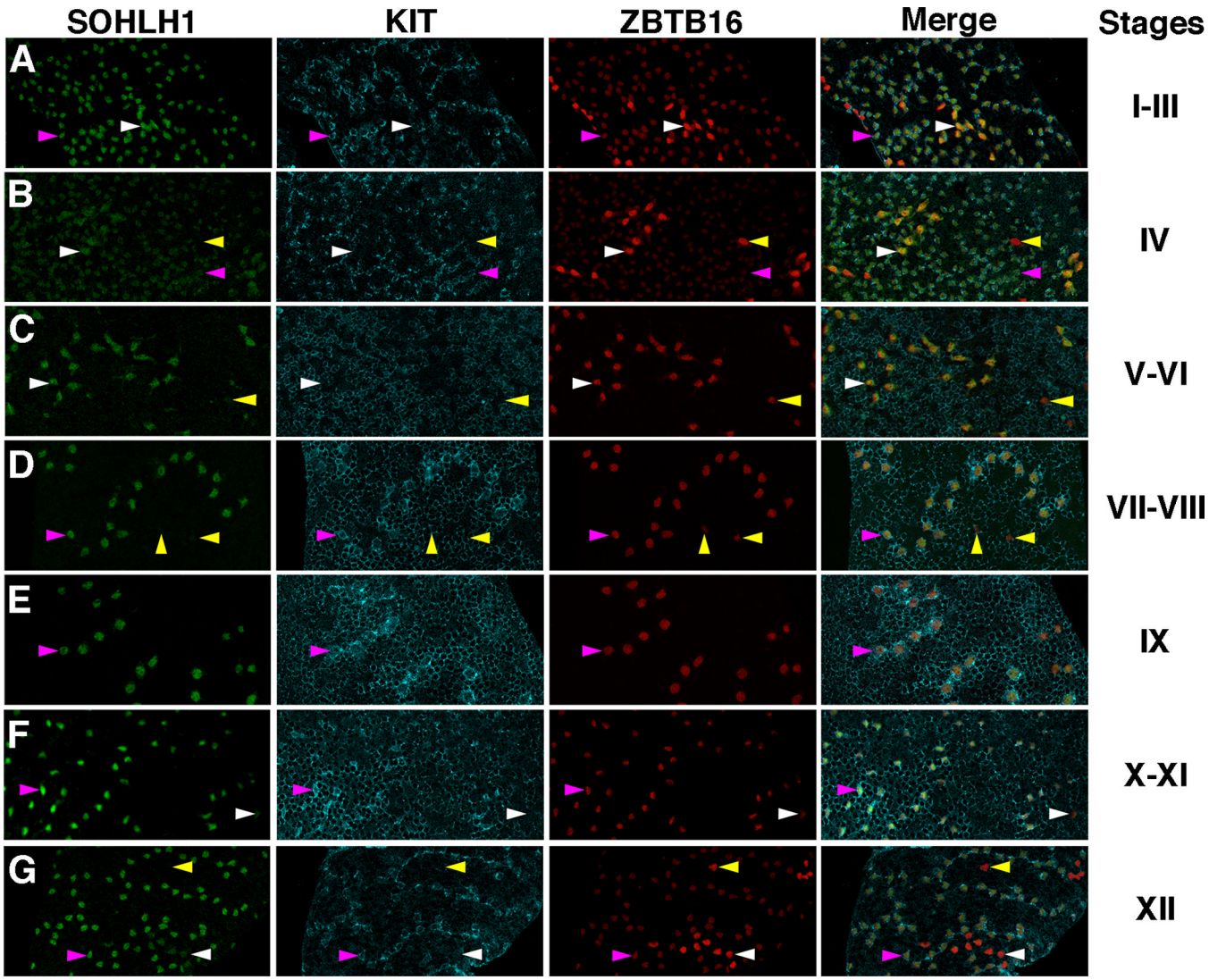

Figure 2. SOHLH1 and SOHLH2 are co-expressed in KIT-positive spermatogonia

Tubules were immunostained with anti-SOHLH1 (green), anti-KIT (cyan) and anti-ZBTB16 (red). Representative examples of the staining patterns that were observed for each group of epithelial stages are shown. (A–C) stages I–VI; (C and D) stages V–VIII; (E and F) stages IX–XI; (F, G and A) stages X–III. There is overlap of some stages among groups. The Roman numerals on the right side of the panels indicate the estimated stages of epithelial cycle from these observations (see supplementary Materials and Methods). White arrowheads indicate the SOHLH1^{+}/ZBTB16^{+}/KIT^{-} undifferentiated spermatogonia. Yellow arrowheads indicate the SOHLH1^{-}/ZBTB16^{+}/KIT^{-} undifferentiated spermatogonia. Magenta arrowheads indicate the SOHLH1^{+}/ZBTB16^{+}/KIT^{+} differentiating spermatogonia. SOHLH1 is expressed in the KIT^{+} differentiating spermatogonia but not in the spermatocytes. The same pattern of expression was observed for SOHLH2 (data not shown).

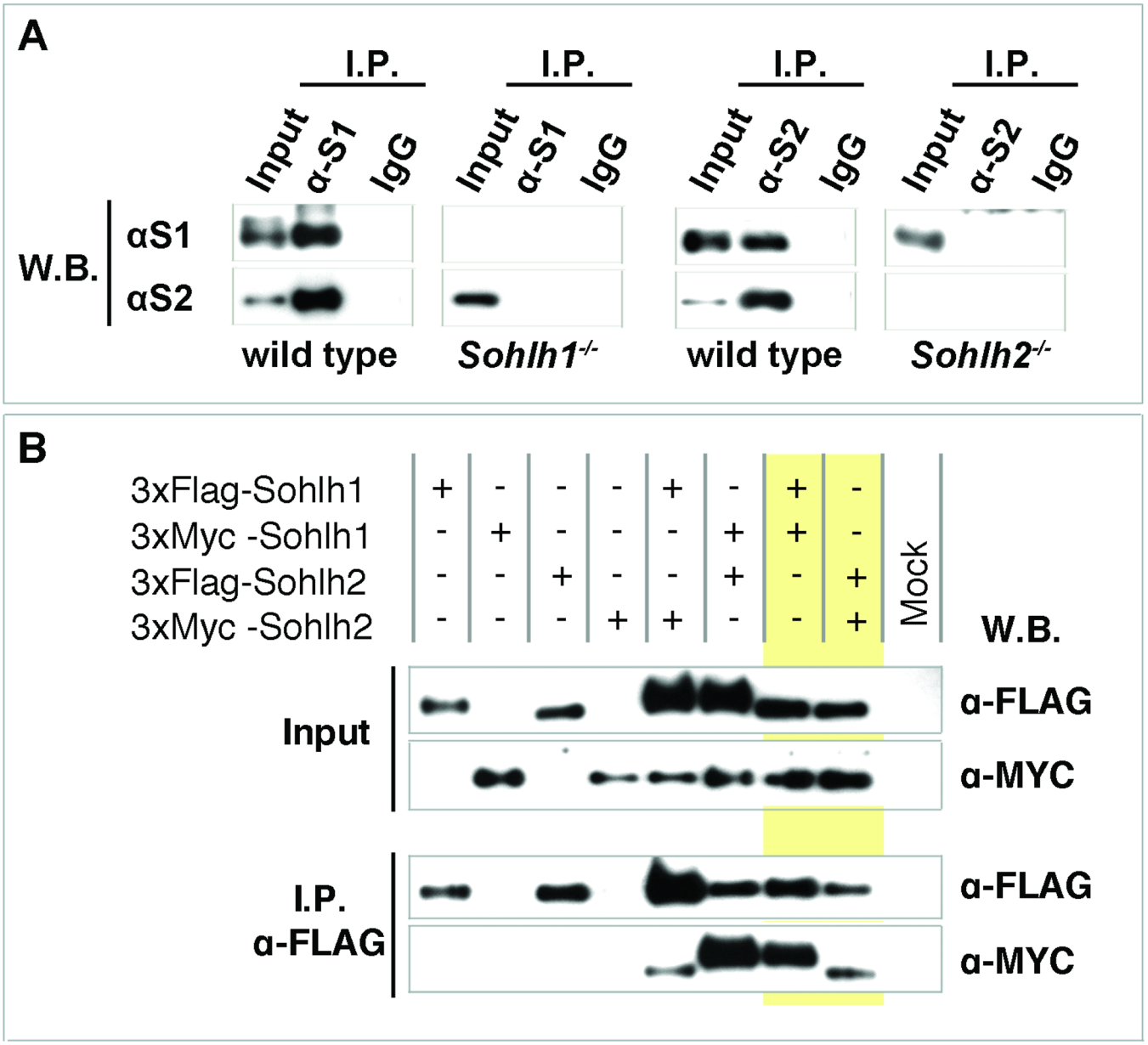

Figure 3. SOHLH1 and SOHLH2 can form homodimers and heterodimers

(A) Co-immunoprecipitation analyses with guinea pig anti-SOHLH1 (α-S1) and guinea pig anti-SOHLH2 (α-S2) antibodies using 1-week-old testes extracts from wild type, *Sohlh1*$^{-/-}$ and *Sohlh2*$^{-/-}$ mice. Each antibody used for western blot analysis (W.B.) is indicated. SOHLH2 was co-immunoprecipitated with SOHLH1 and vice versa (I.P.), indicating that SOHLH1 and SOHLH2 heterodimerize. Input: pre-immunoprecipitation testes lysate (1%), IgG: normal rabbit IgG or normal guinea pig IgG. (B) Co-immunoprecipitation analyses with anti-FLAG antibody using 293T cells over-expressing MYC- or FLAG-tagged SOHLH1 or SOHLH2 proteins. Each combination of transfected vectors and each antibody used for western blot analysis are indicated. The lanes highlighted by yellow indicate that SOHLH1 and SOHLH2 can form homodimers. 3xMYC-SOHLH1 co-immunoprecipitated with 3xFLAG-SOHLH1, and 3xMYC-SOHLH2 co-immunoprecipitated with 3xFLAG-

SOHLH2, indicating that both SOHLH1 and SOHLH2 are capable of forming homodimers. Input: pre-immunoprecipitation cell lysate (1%), Mock: pcDNA-3.1(+)-3FLAG.

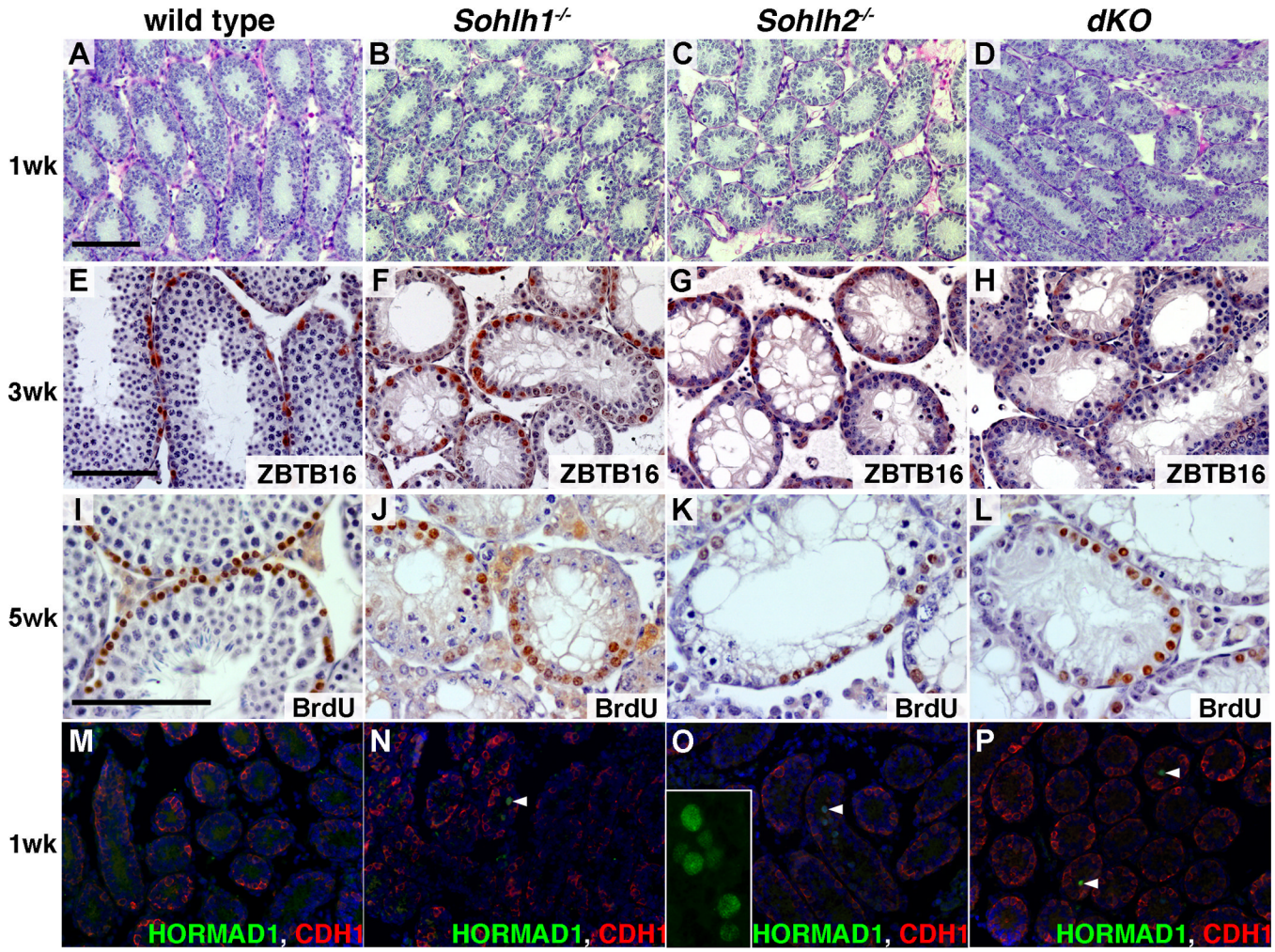

Figure 4. SOHLH1 and SOHLH2 double knockouts arrest spermatogonial differentiation and induce precocious meiosis

(A–L) Histological analyses of wild type (A, E and I), *Sohlh1*$^{-/-}$ (B, F and J), *Sohlh2*$^{-/-}$ (C, G and K) and *Sohlh1*$^{-/-}$/*Sohlh2*$^{-/-}$ (D, H and L) testes. Samples were prepared at one week (A–D), 3 weeks (E–H) and 5 weeks (I–L) after birth. (A–D) Each section was stained with Periodic Acid Schiff-Hematoxylin (PAS-H). (E–H) Each section was immunostained with anti-ZBTB16/PLZF antibody and counterstained with hematoxylin. (I–L) Samples were prepared 3 hours after BrdU injection to mice. Each section was immunostained with anti-BrdU antibody and counterstained with hematoxylin. (M–P) Immunofluorescence of wild type (M), *Sohlh1*$^{-/-}$ (N), *Sohlh2*$^{-/-}$ (O) and *Sohlh*$^{-/-}$/*Sohlh2*$^{-/-}$ (P) testes at 1 week. Each section was immunostained with anti-HORMAD1 (green) and anti-CDH1 (red), and counterstained with DAPI (blue). White arrowheads indicate meiotic cells. Inset in (O) panel shows higher magnification of cells showed thread-like chromosome condensations. Single and double knockout testes germ cells entered meiosis precociously. Scale bars, 100μm.

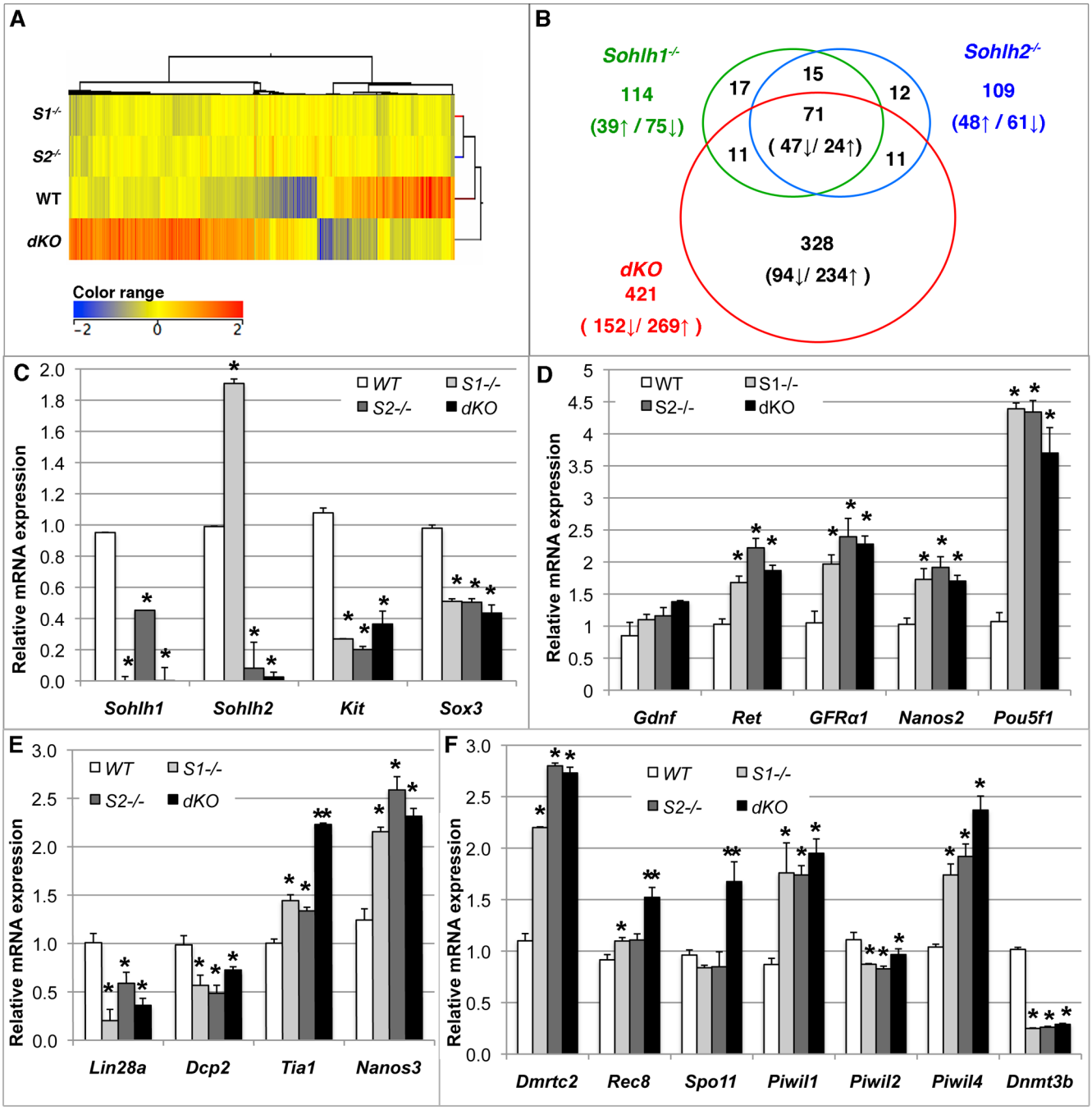

Figure 5. SOHLH1 and SOHLH2 double deficiency has a synergistic effect on gene expression

(A and B) Gene expression array data comparing single and double *Sohlh1/Sohlh2* mutant mice. (A) Hierarchical clustering analysis. Each row corresponds to a specific genotype shown at the bottom of the column, and each column corresponds to the relative expression profile of each genes. The color scheme reflects the expression level against the median of 4 genotypes, and the range bar is shown (0 is median). *Sohlh1*$^{-/-}$ (*S1*$^{-/-}$) and *Sohlh2*$^{-/-}$ (*S2*$^{-/-}$) single mutants showed overall a similar pattern of gene expression overall, while the *Sohlh1*$^{-/-}$/*Sohlh2*$^{-/-}$ double mutant (*dKO*) showed a remarkably different pattern from either the wild type (WT) or single knockouts. (B) Venn diagram of gene expression changes in *Sohlh1*$^{-/-}$ (green), *Sohlh2*$^{-/-}$ (blue) and *Sohlh1*$^{-/-}$/*Sohlh2*$^{-/-}$ (*dKO*, red). The

intersection of genotypes indicates the number of genes where expression changed in two or all *Sohlh* mutants. Upward arrows indicate numbers of genes with enhanced expression, and downward arrows indicate reduced expression. Nearly 4 times as many genes were affected in the double mutant as compared to the single mutants. (C–F) Quantitative real-time PCR analysis on 1-week-old testis for genes differentially expressed between wild type (white), *Sohlh1*$^{-/-}$ (light gray), *Sohlh2*$^{-/-}$ (dark gray) and *Sohlh1*$^{-/-}$/*Sohlh 2*$^{-/-}$ (*dKO*, black). Each gene is indicated at the bottom of the respective column. Genes involved in: (C) spermatogonial differentiation, (D) spermatogonial stem cell maintenance, (E) mRNA translational regulation, and (F) meiosis and epigenetics were evaluated. Data were normalized against *Gapdh* expression and are given as the mean relative quantity, with error bars representing the SEM. Student t-test was used to calculate P values. Significance was shown as a single asterisk when $P < 0.05$ against wild type. A double asterisk was used to show when double knockout expression significantly differed ($P<0.05$) not only against the wild type but also against *Sohlh1* and *Sohlh2* single knockouts (*Spo11*, *Rec8* and *Tia1*).

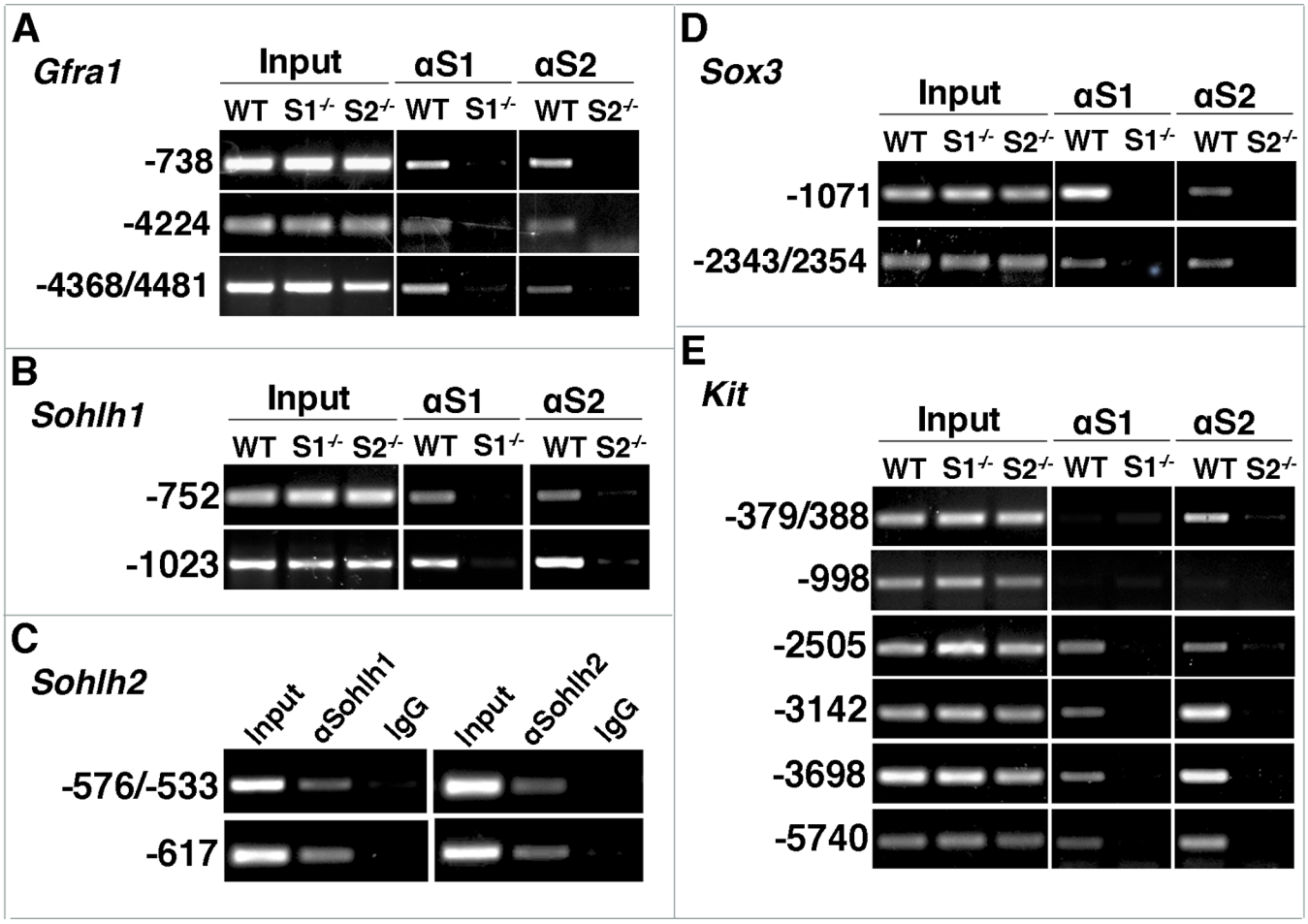

Figure 6. *SOHLH1* and *SOHLH2* bind chromatin upstream of genes essential for SSC maintenance and spermatogonial differentiation

Chromatin immunoprecipitation (ChIP) assay. Anti-SOHLH1(αS1) and anti-SOHLH2 (αS2) antibodies precipitate genomic DNA containing conserved E-boxes surrounding *Gfra1* (A), *Sohlh1* (B), *Sohlh2* (C) *Sox3* (D) and *Kit* (E) genes. *Kit* position −397/388 relative to the transcriptional initiation site is an E-box that was not immunoprecipitated significantly with anti-SOHLH1 antibodies (E), and *Kit* position −998 is an E-box that was not immmunopreicipitated with both anti-SOHLH1 and anti-SOHLH2 antibodies. One-week-old testes from the wild type (WT), *Sohlh1*$^{-/-}$ (*S1*$^{-/-}$) and *Sohlh2*$^{-/-}$ (*S2*$^{-/-}$) mice were used for ChIP. “Input” is the PCR product from chromatin pellets before immunoprecipitation (1% of the total volume used for IP). Numbers at the left of each figure indicate the E-box location from the transcription start site.

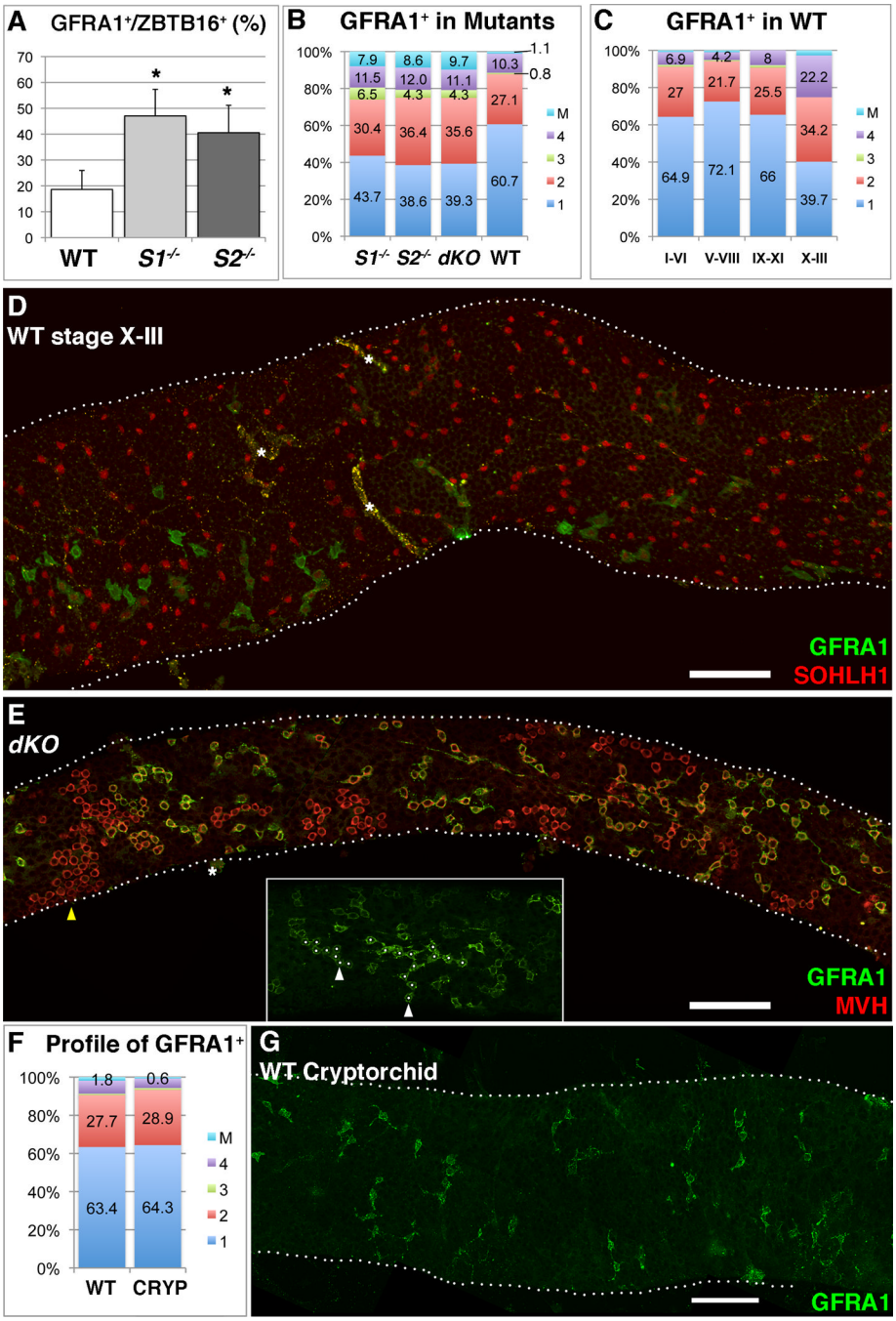

Figure 7. SOHLH1 and SOHLH2 are involved in differentiation from the most primitive set of undifferentiating spermatogonia

(A) The graph shows the percentage of GFRA1$^+$ spermatogonia in the ZBTB16$^+$ population in one-week-old testes of wild type (*WT*), *Sohlh1*$^{-/-}$ (*S1*$^{-/-}$) and *Sohlh2*$^{-/-}$ (*S2*$^{-/-}$) mice. Error bars represent the SEM. Student t-test was used to calculate *P* values. A single asterisk signifies a statistically significant ($P < 0.05$) increase in the number of GFRA1$^+$/ ZBTB16$^+$ spermatogonia as compared to the wild type. (B and C) Morphological profile of GFRA1$^+$ spermatogonia in wild type and mutant testes. A_s −1 (blue), A_{pr} −2 (red), chain of 3 cells −3 (green), $A_{al\text{-}4}$ −4 (purple) and chain of more than 4 cells -M (turquoise). Numbers displayed within each colored region indicate the percentage of the GFRA1$^+$ population in tubules.

The percentages for the green and turquoise regions in C are not shown. (B) GFRA1^{+} profile by spermatogonial morphology by genotype: *Sohlh1*$^{-/-}$ (*S1*$^{-/-}$), *Sohlh2*$^{-/-}$ (*S2*$^{-/-}$), *Sohlh1*$^{-/-}$/*Sohlh2*$^{-/-}$ (*dKO*) and wild type (WT). (C) GFRA1^{+} profile by spermatogonial morphology at different epithelial stages in the wild type testes. The stages are indicated at the bottom of its respective bar. (D and E) Whole mount immunostaining of testes seminiferous tubules. Dotted lines indicate the outline of the tubule. (D) Wild type tubules during stages X-III were immunostained with anti-GFRA1 (green), and anti-SOHLH1 (red). (E) *Sohlh1*$^{-/-}$/*Sohlh2*$^{-/-}$ (*dKO*) tubule was immunostained with anti-GFRA1 (green), and anti-DDX4/MVH (red). The yellow arrowhead indicates an unusually large cluster of GFRA1^{-} spermatogonia. White dots and arrowheads in the inset show a long GFRA1^{+} chain of more than 8 spermatogonia. Asterisks in D and E indicate non-specific staining of interstitial cells. (F) Graphic representation of the distribution of morphological profiles for GFRA1^{+} spermatogonia in the wild type (WT) and experimentally induced cryptorchid testes (CRYP) in wild type mice. A_s −1 (blue), A_{pr} −2 (red), 3 cell chains −3 (green), $A_{al\text{-}4}$ −4 (purple) and chains with more than 4 cells -M (turquoise). Numbers displayed within the blue, red and purple regions indicate the percentages of each type of cell in the total GFRA1^{+} population. There was not a significant difference between wild type (WT) and cryptorchid testis (CRYP). (G) Whole mount immunostaining of seminiferous tubules from experimentally induced cryptorchid wild type testes. Tubules were immunostained with anti-GFRA1 antibody (green). Notice the lack of long GFRA1^{+} chains, present in *Sohlh* mutants. Dotted lines indicate outline of the tubule. Scale bar, 100μm.

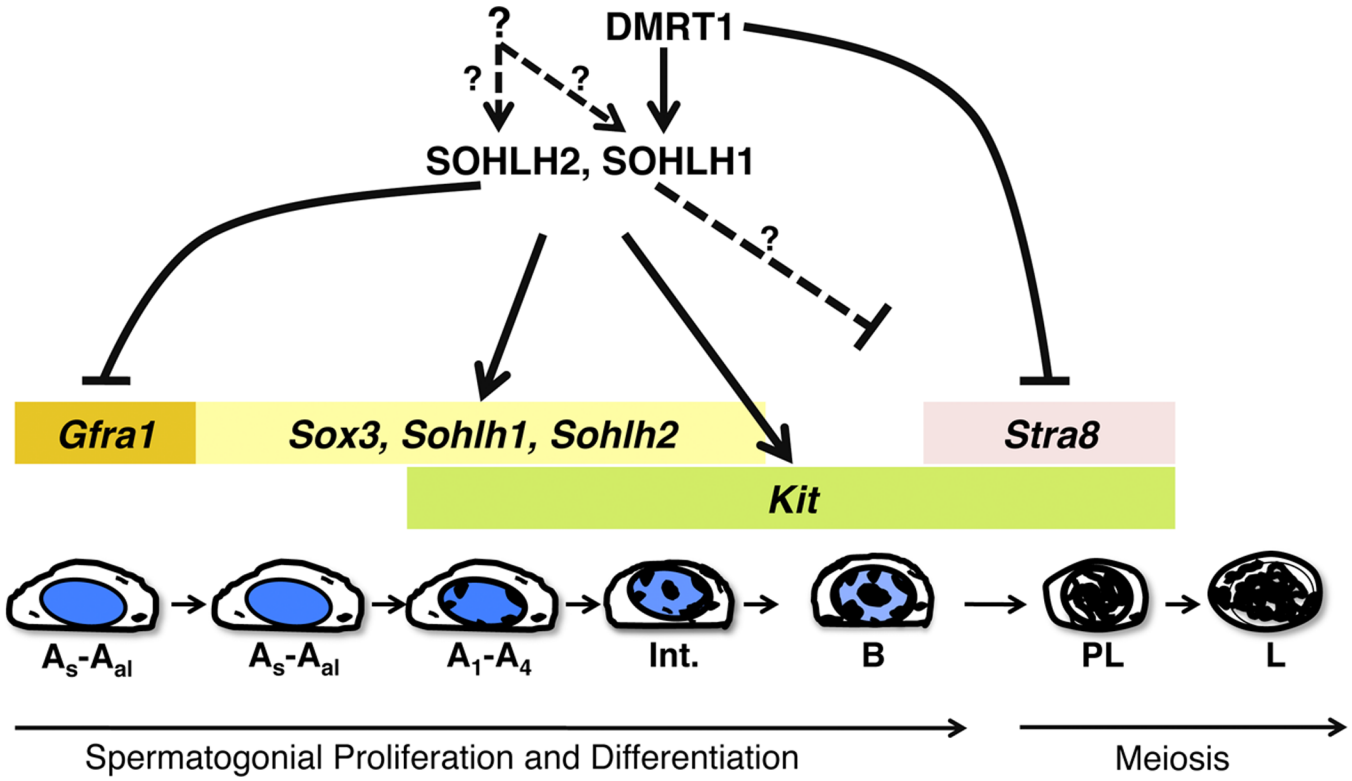

Figure 8. SOHLH1 and SOHLH2 regulation of spermatogonial development

In this figure, DMRT1 is shown as a direct regulator of SOHLH1. Current data do not support direct regulation of SOHLH2 by DMRT1 (Matson et al., 2010). DMRT1 is unlikely to be the sole regulator of SOHLH1 as spermatogonial differentiation proceeds in the DMRT1 conditional mutants (Matson et al., 2010). SOHLH1 and SOHLH2 bind the promoters of key spermatogonial molecules *Gfra1, Sox3, Sohlh1, Sohlh2 and Kit.* Solid lines indicate presumed direct transcriptional control, and dashed lines with question marks indicate undefined regulatory interactions.