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Transcription Factor GLIS3: A New and Critical Regulator of Postnatal Stages of Mouse Spermatogenesis

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Abstract

In this study, we identify a novel and essential role for the Krüppel-like zinc finger transcription factor GLI-similar 3 (GLIS3) in the regulation of postnatal spermatogenesis. We show that GLIS3 is expressed in gonocytes, spermatogonial stem cells (SSCs) and spermatogonial progenitors (SPCs), but not in differentiated spermatogonia and later stages of spermatogenesis or in somatic cells. Spermatogenesis is greatly impaired in GLIS3 knockout mice. Loss of GLIS3 function causes a moderate reduction in the number of gonocytes, but greatly affects the generation of SSCs/SPCs, and as a consequence the development of spermatocytes. Gene expression profiling demonstrated that the expression of genes associated with undifferentiated spermatogonia was dramatically decreased in GLIS3-deficient mice and that the cytoplasmic-to-nuclear translocation of FOXO1, which marks the gonocyte-to-SSC transition and is necessary for SSC self-renewal, is inhibited. These observations suggest that GLIS3 promotes the gonocyte-to-SSC transition and is a critical regulator of the dynamics of early postnatal spermatogenesis.

Keywords

GLIS3; Spermatogonial stem cell; Spermatogenesis; Gene expression

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Authors Contributions

H.S.K.: performed the experiments, conception and design, acquisition of data, analysis and interpretation of data, writing manuscript; L.Y.C., C.L.K., G.L., K.G., C.D.B.: performed the experiments, acquisition of data; H.H.C.Y., E.M.E.: analysis and interpretation of data; A.M.J.: conception and design, analysis and interpretation of data, writing manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

Introduction

GLI-similar 1-3 (GLIS1-3) proteins constitute a subfamily of Krüppel-like zinc finger transcription factors that share a conserved zinc finger domain (ZFD) consisting of five Cys_2-His_2 zinc finger motifs [1-3]. The ZFD mediates the interaction of GLIS proteins with specific DNA sequences, referred to as GLIS binding sites, in the promoter regulatory region of target genes [4, 5]. GLIS3 plays a critical role in the regulation of a number of physiological processes and is implicated in several pathologies [1, 3]. Defects in GLIS3 function in humans and mice lead to the development of neonatal diabetes, congenital hypothyroidism, polycystic kidney disease, liver fibrosis, and skeletal abnormalities [3, 6-12], while genome wide association studies identified *Glis3* as a risk locus for type 1 and type 2 diabetes, thyroid dysfunction, and Alzheimer's disease [13-19].

Glis3 mRNA is highly expressed in embryonic day 14.5 (E14.5) and early postnatal mouse testis suggesting a possible regulatory role for GLIS3 in postnatal germ cell development [20]. Spermatogenesis is a highly coordinated, multi-step process that is controlled by several intrinsic and extrinsic factors [21-25]. In mice, a few days after birth, nondividing gonocytes migrate to the basal compartment of the seminiferous tubule and differentiate into self-renewing spermatogonial stem cells (SSCs). This self-renewal capacity is essential to maintain spermatogenesis over a lifetime. The SSCs then give rise to a series of spermatogonial progenitor cells (SPCs) that have been classified as A-single (A_s; 1 cell), A-paired (A_{pr}; 2 cells), or A-aligned (A_{al4}, A_{al8}, A_{al16}; 4, 8, 16 cells) spermatogonia [21-25]. The SSCs and SPCs together are referred to as undifferentiated spermatogonia. Several proteins, including THY-1 cell surface antigen (THY1 or CD90), inhibitor of DNA binding 4 (ID4), zinc finger and BTB domain containing 16 (ZBTB16, also named PLZF), and glial cell line-derived neurotrophic factor (GDNF) family receptor A1 (GFRA1) serve as markers for undifferentiated spermatogonia subpopulations. Activation of the RET/GFRA1 signaling pathway by GDNF plays a key role in the regulation of SSC renewal, while ZBTB16 is critical for maintaining the undifferentiated state through its repression of KIT (V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog) expression, a marker of differentiated spermatogonia [21-23, 25, 26]. After extensive cell division, SPCs give rise to differentiated A spermatogonia and subsequently B spermatogonia, which after further proliferation, differentiate into meiotic spermatocytes [21, 25, 27, 28]. After meiosis is completed these cells then, via multiple steps, differentiate further into spermatids and ultimately mature male germ cells.

In this study, we examined the role of GLIS3 in postnatal testis development. We hypothesized that GLIS3 might have a critical function in the regulation of spermatogenesis and that loss of GLIS3 expression might affect the differentiation or maintenance of germ cells and the generation of mature spermatocytes. To obtain insights into the role of GLIS3 in the testis, we examined the pattern of GLIS3 protein expression during postnatal testis development in detail using *Glis3-EGFP* knock-in mice and analyzed the effect of GLIS3 deficiency on spermatogenesis in *Glis3KO2* knockout mice. Our study identifies GLIS3 as a novel and critical regulator of spermatogenesis. GLIS3 is essential for normal spermatogenesis by directly and/ or indirectly regulating the dynamics of the proliferation and differentiation of undifferentiated spermatogonia.

Materials and Methods

Mice

C57BL/6 *Glis3KO2* mice and littermate controls were obtained by mating heterozygous mice [29]. *Glis3*KO2 mice survive up to 5-6 weeks allowing us to study the function of GLIS3 postnatally. *Glis3-EGFP* mice, expressing a GLIS3-EGFP fusion protein, were generated by inserting the *EGFP* coding region into exon 10 of *Glis3*, right before the TGA stop codon [29]. All animal studies followed guidelines outlined by the NIH Guide for the Care and Use of Laboratory Animals and protocols were approved by the Institutional Animal Care and Use Committee at the NIEHS.

Histology and Immunohistochemistry

For histological analysis, testes were fixed overnight in 10% neutralized buffered formalin, washed with 1× phosphate buffered saline (PBS), and then embedded in paraffin. Subsequently, 5 μm sections were stained with hematoxylin & eosin (H&E). For immunohistochemistry, testes were fixed overnight in 4% paraformaldehyde, incubated in 30% sucrose/PBS, and then embedded in O.T.C. medium (Tissue-Tek, Hatfield, PA, https://www.emsdiasum.com/). Frozen sections (10 microns), obtained with a Leica cryostat (Leica, Buffalo Grove, IL, http://www2.leicabiosystems.com), were placed on glass slides, dried at room temperature, and then incubated with primary antibodies against GFP (1:200, Life Technology, A11122, Foster City, CA, https://www.thermofisher.com), ZBTB16 (SC-22839), CYP17A1 (SC-46081), GATA4 (SC-1237) (1:200, Santa Cruz Bio-technology, Santa Cruz, CA, http://www.scbt.com), anti-germ cell-specific antigen antibody TRA98 (1:1000, BioAcademia, Osaka, Japan, 73-003, http://www.bioacademia.co.jp/), GFRA1 (1:100, R&D systems, AF560, Minneapolis, MN, https://www.rndsystems.com/), FOXO1 (1:300, Cell Signaling Technologies, 2880, Danvers, MA, http://www.cellsignal.com), KIT (1:200, Abcam, Ab65525, Cambridge, MA, http://www.abcam.com), or heat shock 70 kDa protein 2 (HSPA2 or HSP70-2) [30]. After washing, the sections were subsequently incubated overnight at 4°C with anti-mouse, anti-rabbit, anti-goat, or anti-rat Alexa Fluor-488 or Fluor-594 conjugated secondary antibodies (1:1,000, Life Technologies, Grand Island, NY, https://www.thermofisher.com/). Apoptotic cells in testis were examined by TUNEL assay using an in situ Cell Death Detection Fluorescein Kit from Roche (Indianapolis, IN, https://lifescience.roche.com) following the manufacturer's instruction. Fluorescence was observed with a Zeiss LSM 510_UV or LSM710 confocal microscope. For quantitative analysis at least five sections of each of testes from three or more mice within each group were examined.

Cell Proliferation Assay

Cell proliferation was analyzed using the EdU (5-ethynyl-2'-deoxyuridine) incorporation assay. PND7 pups were injected intraperitoneally with EdU (25 mg/kg), 1 hour later the testis was removed, fixed, and processed as described above for immunohistochemistry. The testis sections were stained for EdU using a Click-iT Plus EdU Imaging Kit (Life Technologies) following the manufacturer's protocol; germ cells were identified by TRA98 staining.

Cell Isolation

Testes from postnatal day 1, 4, or 7 (PND1, 4, 7) mice were detunicated and dispersed by pipetting up and down in enzyme digestion solution (1 mg/ml Collagenase type IV and 1 mg/ml DNase I) [31]. The testicular cell suspension was applied on top of a 30% Percoll solution (3 ml Percoll mixed with 7 ml Hank's balanced salt solution, HBSS) and centrifuged at 600× *g* for 7 minutes to separate undigested tissue. The cell pellet was then resuspended in the HBSS for flow cytometric analysis.

Flow Cytometry

Cells (1×10^6) isolated from testis from PND7 *Glis3-EGFP* mice were incubated with either PE/Cy5-conjugated rat anti-mouse Kit (1:50, Abcam) or PE-conjugated rat anti-mouse THY1 (CD90) (1:150, Invitrogen, Carlsbad, CA, https://www.thermofisher.com) at 4°C for 30 minutes and subsequently sorted by flow cytometry to isolate populations enriched for differentiated (KIT^+) and undifferentiated ($THY1^+$ $EGFP^+$) spermatogonia, respectively [31]. Apoptotic cell death in the undifferentiated spermatogonia population was analyzed by flow cytometry using Alexa Fluor 647-conjugated Annexin V (Biolegend, San Diego, CA, http://www.biolegend.com) and PE-conjugated anti-mouse THY1 with a Becton Dickinson LSRII (BD Bioscience, San Jose, CA, https://www.bdbiosciences.com).

Microarray Analysis

Total RNA was isolated from testes of PND7 ($n = 3$) and 4 weeks old ($n = 4$) WT and *Glis3*KO2 mice using an RNeasy Mini Kit (Qiagen, Valencia, CA). Microarray analysis was carried out by the NIEHS Molecular Genomics Core using Agilent whole genome mouse oligo arrays (Agilent Technologies, Palo Alto, CA, http://www.agilent.com) as described previously [32]. Data obtained with the Agilent Feature Extraction software (v7.1) were further analyzed with OmicSoft Array Studio (V6.0) software (http://www.omicsoft.com). The micro-array data have been deposited in the NCBI's Gene Expression Omnibus under the number of GSE70196. Heatmaps were generated using the Partek Flow program (Partek Inc., St. Louis, MO, http://www.partek.com).

Quantitative Real Time PCR

Total RNA from whole testes or $EGFP^+$- or KIT^+-enriched fractions was isolated with an RNAeasy mini kit or RNAqueous® Micro RNA isolation kit (Ambion, Austin, TX, https://www.thermofisher.com), respectively, following the manufacturer's instructions. The RNA was reverse-transcribed using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, https://www.thermofisher.com). Quantitative real time PCR (QRT-PCR) reactions were carried out in triplicate in a 7300 Real Time PCR system (Applied Biosystems) using either by Taqman and SYBR Green I [33]. RNA expression in $EGFP^+$-or KIT^+-enriched fractions was normalized to the level of Gapdh mRNA, while in whole testes it was normalized to 18S rRNA or Gapdh. No significant differences in relative gene expression were observed when data were normalized to either 18S or Gapdh. PCR primer sequences are listed in Supporting Information Table 1.

Statistics

Data generated from WT and *Glis3*KO2 mice were analyzed by Graphpad Prism 6 One-way ANOVA.

Results

GLIS3 Is Expressed in Undifferentiated Spermatogonial Cells

We reported previously that at E14.5 of mouse development Glis3 mRNA is highly expressed in testis cords [20]. In this study, we show that the postnatal pattern of Glis3 mRNA expression is very similar to that of Zbtb16 (Supporting Information Fig. S1). Based on these observations we hypothesized that GLIS3 might play a critical role in the regulation of spermatogenesis. To elucidate GLIS3 function in postnatal testis, it was vital to understand its spatiotemporal pattern of expression during neonatal development. To study this, we monitored GLIS3 protein in *Glis3-EGFP* knockin mice expressing a GLIS3-EGFP fusion protein [29]. GLIS3-EGFP positive cells were observed in PND1, −5, −7, and −14 testes (Fig. 1A-1F). To determine whether GLIS3-EGFP was expressed in germ cells, its co-localization with TRA98, a pan-germ cell marker, was examined. Figure 1A shows that GLIS3-EGFP was detectable in TRA98$^+$ gonocytes located in the lumen of the seminiferous tubules of PND1 testis. In the first few days after birth, gonocytes migrate to the basal compartment of the tubules where they subsequently give rise to SSCs and SPCs [24]. In PND5 and PND7 testis, most of the TRA98$^+$ germ cells were GLIS3-EGFP positive (Fig. 1B, 1C). Similarly, E-cadherin (CDH1) positive cells, which represent undifferentiated spermatogonia, stained positively for GLIS3-EGFP (Fig. 1D) [34]. In contrast, GLIS3-EGFP was not detectable in CYP17A1$^+$ Leydig cells or GATA4$^+$ Sertoli cells (Supporting Information Fig. S2). These observations indicated that GLIS3 expression in the testis is restricted to germ cells and not detectable in somatic cells. To analyze the expression of GLIS3 in undifferentiated spermatogonia further, we compared its expression with that of ZBTB16 (PLZF), which is expressed in gonocytes and undifferentiated spermatogonia [35, 36]. Immunohistochemistry showed that at PND7 all ZBTB16$^+$ cells expressed GLIS3-EGFP (Fig. 1E); however, at PND14 many ZBTB16$^+$ cells did not stain for GLIS3-EGFP (Fig. 1F), while at PND7 and PND14 all GLIS3-EGFP$^+$ cells were positive for ZBTB16 (Fig. 1E, 1F). Quantitative analysis showed that at PND4 and PND7 nearly 100% of the ZBTB16$^+$ cells was GLIS3-EGFP$^+$, whereas at PND14, only 10% of the ZBTB16$^+$ cells expressed GLIS3-EGFP (Fig. 1G). These observations are consistent with the interpretation that GLIS3 is expressed in SSCs and in early, but not late stage ZBTB16$^+$ SPCs. This is supported by data showing that at PND4 and 5, most cells staining positively for GFRA1, which is expressed in SSCs and in A_s , A_{pr}, and A_{al4} SPCs [21-25], are GLIS3-EGFP$^+$ (Fig. 1H, 1I). However, at PND7 and later, the number of GLIS3-EGPF$^+$ cells staining positively for GFRA1 steadily decreased; at PND14 only 10% of the GLIS3-EGFP$^+$ cells were GFRA1$^+$ (Fig. 1J). These observations indicate that GLIS3 is present in most GFRA1$^+$ and ZBTB16$^+$ SSCs and SPCs; GLIS3 is repressed after the downregulation of GFRA1, but before the suppression of ZBTB16.

Immunohistochemical analysis of PND14 testis showed that cells staining positive for KIT, a marker for differentiated spermatogonia, did not express GLIS3-EGFP indicating that

GLIS3 is not expressed in differentiated spermatogonia (Fig. 2A-2D). To obtain further evidence that GLIS3 expression was restricted to undifferentiated spermatogonial cells, GLIS3-EGFP$^+$- and KIT$^+$-enriched cell populations were isolated by flow cytometry and the expression of Glis3 mRNA compared with that of two markers of undifferentiated spermatogonia, Zbtb16 and Nanos3 (Fig. 2E). QRT-PCR analysis showed that compared to the KIT$^+$ population, Glis3 mRNA was highly expressed in the GLIS3-EGFP$^+$ population as also was observed for Nanos3 and Zbtb16 (Fig. 2E). Inversely, Kit mRNA expression was expressed at much higher levels in the KIT$^+$ population than the GLIS3-EGFP$^+$ population (Fig. 2E). These data support the conclusion that Glis3 is expressed in undifferentiated spermatogonial cells, but not in differentiating spermatogonial cells. Together, these observations support the model in which GLIS3 expression is restricted to gonocytes, SSCs and early subpopulations of SPCs and is repressed at a later stage of SPC differentiation, after the suppression of GFRA1, but before the downregulation of ZBTB16 (Fig. 2F).

Spermatogenesis Is Impaired in *Glis3*KO2 Mice

To obtain insights into the function of GLIS3 in spermatogenesis, we examined the effect of loss of GLIS3 function on postnatal testis development in *Glis3*KO2 knockout mice [29]. Although there was no significant difference in the relative weight of testes at PND7, the average size and weight of testes from *Glis3*KO2 mice were considerably less at PND14 than those of WT mice (Fig. 3A). This difference became increasingly larger at 3-7 weeks (Fig. 3A, 3B). Histological analysis of tissue sections showed that the number of germ cells was significantly reduced in testes from PND7 and 4 and 7 weeks old *Glis3*KO2 mice (Fig. 3C and Supporting Information Fig. S3). At 7 weeks, testes from *Glis3*KO2 mice were almost totally depleted of spermatocytes (Fig. 3C). H&E stained sections of testes from heterozygous *Glis3*KO2 mice appeared similar to those of WT mice suggesting that spermatogenesis proceeded normally in these mice. Thus, loss of GLIS3 function leads to a severe impairment in spermatogenesis consistent with our conclusion that GLIS3 is essential for normal spermatogenesis.

As GLIS3 is expressed in gonocytes and undifferentiated spermatogonia, we examined the impact of GLIS3-deficiency on these cells at PND1, PND4, and PND7 by immunohistochemistry with TRA98 or ZBTB16 antibodies. At PND1 and PND4, the number of TRA98$^+$ cells was moderately reduced in *Glis3*KO2 testis compared to WT testis (Fig. 4A, 4C, and Supporting Information Fig. S4), but decreased dramatically in PND7 *Glis3*KO2 testis (Fig. 4B, 4C). This was supported by quantitative analyses showing that in WT testis the number of TRA98$^+$ cells/tubule increased more than sixfold from PND1 to PND7, but less than twofold in *Glis3*KO2 mice. During this stage, nondividing gonocytes migrate to the basement membrane and transit into SSCs, which then undergo extensive proliferation and differentiation into SPCs, [21, 24, 25]. The sharp reduction in TRA98$^+$ cells observed in PND7 *Glis3*KO2 testis might be due to a defect in the differentiation, survival, and/or proliferation of these cells.

To examine the effect of GLIS3 deficiency on the development of differentiated spermatogonia, the expression of KIT, a marker for differentiated spermatogonia, and HSPA2, a marker for meiotic and postmeiotic spermatogenic cells [37], was examined in

*Glis3*KO2 testis. Our data show that the number of KIT^+ and $HSPA2^+$ cells was greatly diminished in testes from, respectively, PND14 and 3 weeks old *Glis3*KO2 mice (Fig. 4D, 4E, and Supporting Information Fig. S5). These observations suggested that the development of differentiated spermatogonia and meiotic and postmeiotic spermatogenic cells was greatly impaired in *Glis3*KO2 mice consistent with our histological observations (Fig. 3C and Supporting Information Fig. S3). Together, these data support our conclusion that GLIS3 is a key regulator of early stages of postnatal spermatogenesis.

As the relative increase in the number of germ cells/ tubule in *Glis3*KO2 testis during PND4-14 was significantly smaller than that of WT testis (Fig. 4C and Supporting Information Fig. S4), we examined by TUNEL assay whether this reduction was related to increased apoptosis. As shown in Supporting Information Fig. S6A, apoptotic cell death was rarely observed in testes from both WT and *Glis3*KO2 at either PND4, -7 or -14 (Supporting Information Fig. S6B and not shown). Flow cytometric analysis indicated that the number of $THY1^+$ apoptotic (Annexin V^+) cells was less than 0.1% in both PND7 WT and *Glis3*KO2 testis (Supporting Information Fig. S6B). These results suggested that the reduction in germ cells in *Glis3*KO2 testis does not appear to involve increased apoptosis. Analysis of EdU incorporation into PND7 testes showed a reduction in EdU $^+$ $TRA98^+$ cells in *Glis3*KO2 testes compared to WT; however, the ratio of EdU $^+$ $TRA98^+$ double positive cells over $TRA98^+$ cells in *Glis3*KO2 testis was not statistically significant from that of WT testis (data not shown), which might be due to the low number of $TRA98^+$ germ cells present in Glis3KO2 testes. Although we cannot totally rule out changes in the rate of apoptosis, the reduction in undifferentiated spermatogonia might be related to a combination of the moderate decrease in the number of gonocytes at PND1 and reduced differentiation and/or proliferation of undifferentiated spermatogonia.

The Expression of SSC Markers was Greatly Reduced in *Glis3*KO2 Testis

To investigate changes in gene expression in *Glis3*KO2 testis in relation to the observed alterations in spermatogenesis, we compared the gene expression profiles of WT and *Glis3*KO2 testis from PND7 and 4-week old mice by microarray analysis. Lists and heatmaps displaying several gene clusters representative of several cell populations and different stages of spermatogenesis, are shown in Supporting Information Tables 2 and 3, and Figure 5A, 5B, respectively. Our data from PND7 testes showed that many genes associated with the regulation of SSC and SPC renewal and differentiation, as well as transposon silencing, were significantly reduced in *Glis3*KO2 testes, whereas the expression of genes associated with Sertoli and Leydig cell functions, including *CYP19A1, STAR, CYP17A1*, and *NR5A1*, or involved in retinoic acid biosynthesis or metabolism, including *CYP26B1* and *ALDH1/2*, were not or only slightly altered (Supporting Information Table 2 and Fig. 5A). The expression of the Sertoli cell marker, GATA4, was also not significantly changed in *Glis3*KO2 testis (Supporting Information Fig. S7). These findings are consistent with the expression pattern of GLIS3 and support the conclusion that GLIS3 regulates gene expression in SSCs and SPCs, but not in Sertoli and Leydig cells. The small increase in several Sertoli and Leydig cell marker genes in PND7 *Glis3*KO2 testes (Supporting Information Table 2) and the moderately relative increase in expression in 4-week old

*Glis3*KO2 testes (not shown) are likely due the reduction in the number of germ cells rather than an intrinsic increase in their expression in Sertoli and Leydig cells.

Gene profiling analysis of 4-week old testes showed that many genes associated with meiosis, spermatids, and spermatocytes, including several genes related to transposon silencing, were significantly downregulated in *Glis3*KO2 testes (Supporting Information Table 3 and Fig. 5B). These data support our conclusion that loss of GLIS3 function greatly impairs the development of later stages of spermatogenesis consistent with our histochemical observations of 4- and 7-weeks old testes (Fig. 3C and Supporting Information Fig. S3). This impairment in spermatocyte development is very likely a consequence of the effects of GLIS3 deficiency on the SSCs and SPCs.

Genes downregulated in PND7 *Glis3*KO2 testes included a set of GDNF-dependent genes, such as *Etv5, Bcl6b, Lhx1, T* (*Brachyury homolog*), and *Pou3f1*, and GDNF-independent genes, such as *Pou5f1* (*Oct3/4*). GDNF, which is produced by Sertoli and peritubular myoid cells [21, 22, 38], plays a critical role in the regulation of the proliferation and differentiation of SSCs and PGCs through its activation of the GDNF receptor complex, RET, and GFRA1 [39, 40]. The expression of Gdnf mRNA was little changed; however, the expression of Gfra1 and Ret mRNA was significantly diminished in *Glis3*KO2 testes (Fig. 5C). Examination of the expression of GFRA1 protein by immunohistochemistry showed that in PND4 WT testes, 93% of the ZBTB16$^+$ cells were GFRA1$^+$ compared to 46% in *Glis3*KO2 testes (Fig. 6A, 6B), while 28% and 1.6% of the ZBTB16$^+$ cells were GFRA1$^+$ in PND7 WT and *Glis3*KO2 testes, respectively (Fig. 6C, 6D). The change in these subpopulations would be consistent with reduced progression of the gonocyte-SSC-SPC transition in *Glis3*KO2 testis compared to WT testis [39, 41, 42]. In addition to the expression of Ret and Gfra1 mRNA, the expression of Ngn3 and Dmrt1, encoding transcription factors that play a key role in spermatogonial cell differentiation, and Nanos3, which plays a critical role in maintaining the undifferentiated spermatogonia population [24, 43-45], was also significantly reduced in *Glis3*KO2 testis (Fig. 5C). Moreover, the expression of Piwil4 (also known as Miwi2), which is expressed in gonocytes and early postnatal testis [46], and Sall4, encoding a protein that binds ZBTB16 and antagonizes its function in SSCs [47], was decreased in *Glis3*KO2 testis. QRT-PCR analysis confirmed the downregulation of Ret, Lhx1, Pou5f1, Ngn3, T, Sall4, Id4, Bcl6b, Piwil4, Zic1, Nanos3, Zbtb16, and Gfra1 expression in *Glis3*KO2 testis (Fig. 5C). These findings suggest that GLIS3 may play a key role in regulating the dynamics of undifferentiated spermatogonia proliferation and/or differentiation.

A recent study showed that the translocation of the transcription factor FOXO1 from the cytoplasm to the nucleus marks the gonocyte-to-SSC transition and that its level of expression as well as its subcellular localization can affect spermatogonial cell proliferation and differentiation [48]. Although the expression of Foxo1 mRNA was not greatly changed in PND7 *Glis3*KO2 testis, the expression of several FOXO1 target genes, including *Lhx1* and *Sall4*, was greatly (>85%) reduced (Fig. 5C). Examination of its subcellular localization showed that, as reported [48], FOXO1 was largely localized to the nucleus in the majority of germ cells in PND7 WT testis (Fig. 7A); however, in *Glis3*KO2 testis FOXO1 remained largely localized to the cytoplasm (Fig. 7A, 7B). These observations suggest that the

gonocyte-to-SSC transition might be inhibited in *Glis3*KO2 mice. This conclusion is consistent with our data showing that the expression of Id4, which was recently identi-fied as a specific marker of SSCs [49], was greatly reduced in *Glis3*KO2 testis compared to WT testis (Fig. 5C).

Discussion

In this study, we identified a novel and critical role for the Krüppel-like zinc finger transcription factor, GLIS3, in the regulation of the early stages of postnatal spermatogenesis. Post-natal spermatogenesis is a multistep process in which nonmitotic gonocytes after their migration to the basement membrane, differentiate into SSCs and SPCs, and subsequently give rise to differentiated spermatogonia and ultimately mature spermatocytes [22–25]. SSCs have an extensive self-renewal capacity and are critical in maintaining the balance between differentiation and proliferation in order to provide a life-long source for the generation of mature germ cells. A number of signaling and transcriptional pathways have been identified that are involved in the regulation of these processes [24, 25, 49, 50]. Our study of *Glis3-EGFP* mice clearly demonstrated that in the testis GLIS3 is expressed specifically in germ cells and not in somatic cells. We further show that in neonatal testis, GLIS3 protein is localized to the nucleus of gonocytes and remains present in most GFRA1$^+$ and ZBTB16$^+$ SSCs and SPCs. However, none of the KIT$^+$ cells were GLIS3$^+$ indicating that GLIS3 is not expressed in differentiated spermatogonia or at later stages of spermatogenesis. Comparison of GLIS3 expression with that of ZBTB16 and GFRA1 indicated that during the differentiation of undifferentiated spermatogonia GLIS3 expression is repressed at a later stage than the downregulation of GFRA1, but earlier than the downregulation of ZBTB16 suggesting that GLIS3 becomes repressed during the Aal stages (Fig. 2F) [21-23, 25]. The temporal window of GLIS3 expression during postnatal spermatogenesis suggests a critical role for GLIS3 in the transcriptional regulation of specific genes and biological processes associated with early neonatal spermatogenesis that may include control of self-renewal, proliferation, and/or differentiation. The small testis size (Fig. 3A, 3B) and the reduced generation of spermatocytes in *Glis3*KO2 testes indicated that GLIS3 plays a critical regulatory role in early postnatal spermatogenesis.

The selective expression of GLIS3 in gonocytes and undifferentiated spermatogonia suggested that GLIS3 might regulate gene transcription and function in these cells. Analysis of these cell populations showed that gonocytes were clearly present in PND1 *Glis3*KO2 testis and that expression of gonocyte associated genes was not significantly different between THY1-enriched populations isolated from PND1 testis of WT and Glis3KO2 mice (Supporting Information Fig. S8). The number of gonocytes/tubule was moderately decreased at PND1 suggesting a possible role for GLIS3 during prenatal testis development. Our study further demonstrated that the number of TRA98$^+$ germ cells in PND7 *Glis3*KO2 testis was greatly decreased compared WT testis (Fig. 4C). These results support the hypothesis that GLIS3 regulates spermatogenesis at early postnatal stages of spermatogenesis and that the loss of GLIS3 affects the proliferation, survival and/or differentiation of early postnatal germ cells. The greatly decreased number of KIT$^+$ cells observed in PND14 *Glis3*KO2 testis is consistent with this concept. This hypothesis was further supported by the gene expression profiling of testes from PND7 WT and *Glis3*KO2

mice. The expression of genes associated with undifferentiated spermatogonia was significantly decreased in PND7 *Glis3*KO2 testes (Fig 5A, 5C). Although the relative expression of several Leydig and Sertoli marker genes in total testis was slightly increased, this appeared to be due to the reduced contribution of germ cell RNA. The latter is consistent with our conclusion that these cells do not express GLIS3 and therefore are not direct targets for GLIS3 regulation (Fig. 5A, 5B and Supporting Information Tables 2 and 3). Comparison of gene expression profiles of testes from 4-weeks old mice showed that a number of genes associated with later stages of spermatogenesis (meiosis, spermatid, and spermatozoa) were substantially reduced in *Glis3*KO2 testis compared to WT testes. This decrease in the expression of these genes reflects the impairment in spermatocyte development observed in histochemical sections of *Glis3*KO2 testes. This impairment is very likely a consequence of defects in early spermatogenesis, the greatly reduced generation of $ZBTB16^+$ undifferentiated spermatogonia, and subsequently decrease in the generation of KIT^+ differentiated spermatogonia. Together, these findings indicate that GLIS3 is essential for normal spermatogenesis.

Early in postnatal spermatogenesis, nondividing gonocytes transition into proliferating and self-renewing SSCs that subsequently give rise to undifferentiated (progenitor) spermatogonia (SPCs). Our data clearly demonstrate that the generation of undifferentiated spermatogonia is greatly reduced in *Glis3*KO2 mice. This could be due to a less efficient transition of gonocytes into proliferative SSCs, increased apoptosis, or reduced proliferation or differentiation of SSCs and SPCs, or a combination of these possibilities. Although the increase in the number of undifferentiated spermatogonia is clearly very different between WT and *Glis3*KO2 testes, no significant differences in apoptosis and proliferation were observed. However, we may not be able to totally rule out a role for either apoptosis or proliferation, because of the low number of germ cells in Glis3KO2 testes. FOXO1, which is expressed in gonocytes and undifferentiated spermatogonial cells, plays a crucial role in gonocyte-to-SSC transition, self-renewal, and differentiation of SSCs [48]. The gonocyte-to-SSC transition is marked by the cytoplasmic-to-nuclear translocation of FOXO1. This translocation is required for self-renewal of SSCs and the induction of the expression of several genes required for SSC expansion, including Ret. Our data showed that Foxo1 mRNA expression was not very different between *Glis3*KO2 and WT testis at PND7; however, a significant reduction was observed in the nuclear localization of FOXO1 in *Glis3*KO2 testes compared to WT testes (Fig. 7). These observations suggested that GLIS3 might have a role in promoting the gonocyte-to-SSC transition and the subsequent generation of undifferentiated spermatogonia. Our observations showing that the percentage of $ZBTB16^+$ cells staining positive for GFRA1 was significantly lower in PDN4 and PND7 *Glis3*KO2 testes compared to WT testes and that substantially reduced expression of Id4 in Glis3KO2 testis compared to WT testis, are in agreement with this hypothesis. This is supported by data showing that the expression of several genes, including *Lhx1, Sall4, Id4, Ngn3*, and *Pou5f1*, which are required for SSC self-renewal and/or SPC differentiation [45], are significantly repressed in *Glis3*KO2 testes. However, as GLIS3 is also expressed in SSCs and SPCs, GLIS3 might have additional functions in regulating their proliferation and/or differentiation.

Conclusion

In this study, we demonstrate that GLIS3 is expressed selectively in mouse gonocytes and undifferentiated spermatogonial cells during postnatal testis development. Most importantly, we show that progression of spermatogenesis is greatly impaired in GLIS3-deficient mice. The observed inhibition of FOXO1 translocation to nucleus and the reduction in the expression of the SSC marker gene Id4 in GLIS3-deficient mice suggest that this involves an inhibition of the gonocyte-to-SSC transition. Together, these findings indicate that GLIS3 is essential for normal spermatogenesis. Understanding the pathways that regulate GLIS3 activity might offer new approaches to treat male infertility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance Statement

Spermatogonial stem cells have an extensive self-renewal capacity and are critical in maintaining the balance between differentiation and proliferation in order to provide a life-long source for the generation of mature germ cells. In this study, we demonstrate that the transcriptional regulator Glis3 is expressed in these cells and required for the differentiation and/or proliferation of these cells and is essential for the generation of mature germ cells. The Glis3 signaling pathway might provide new therapeutic strategies for infertility.

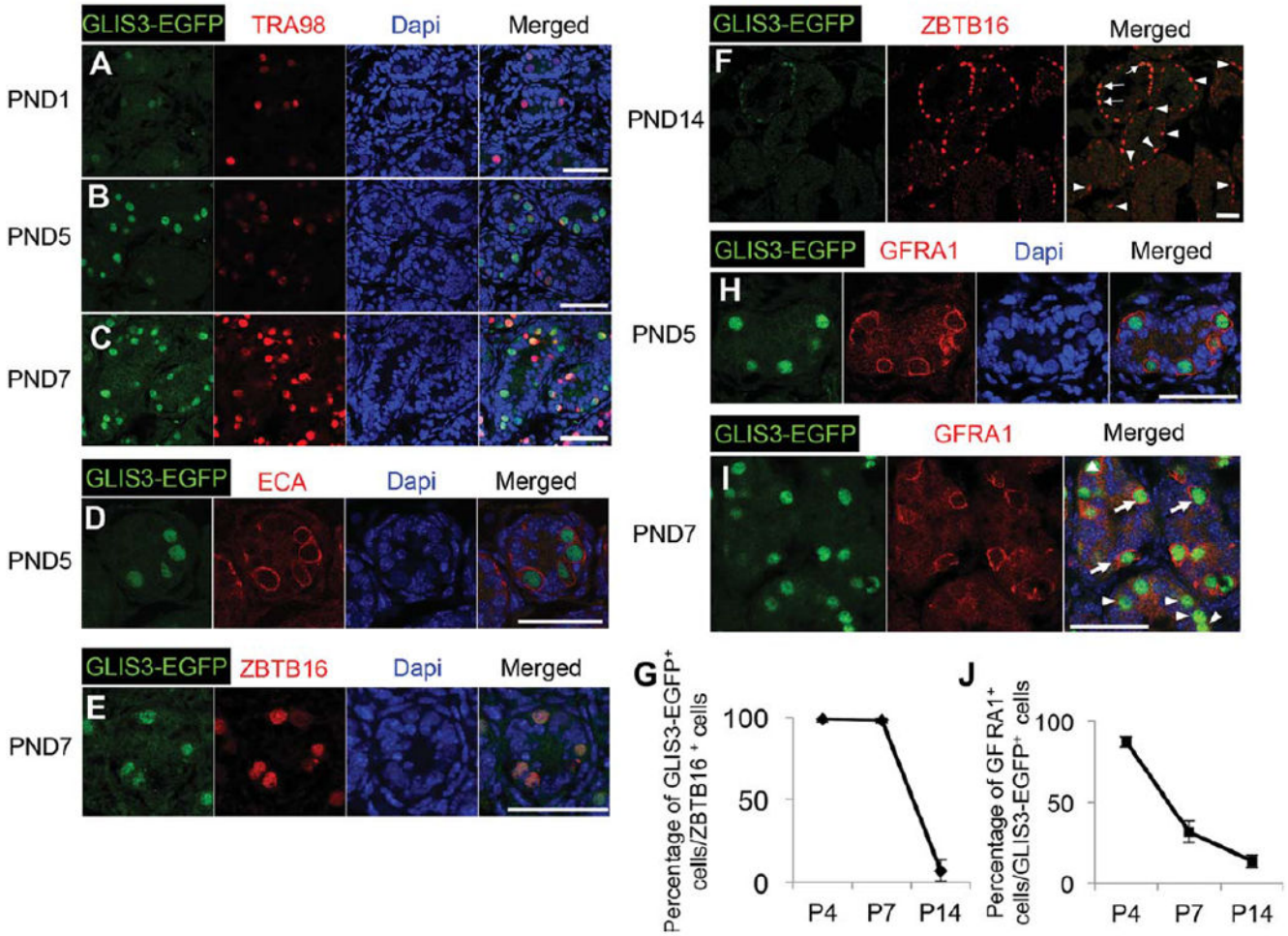

Figure 1.

GLIS3 is expressed in gonocytes and undifferentiated spermatogonial cells. *Glis3-EGFP* knock-in mouse, in which GLIS3 is expressed as a fusion protein with EGFP, was generated to analyze the pattern of GLIS3-EGFP expression. Sections of PND1 (**A**), PND5 (**B**), and PND7 (**C**) testes were examined by immunohistochemistry with antibodies against GLIS3-EGFP (green) and the pan germ cell marker TRA98 (Red). Nuclei were stained with Dapi. Merged images are indicated. (**D**): E-cadherin positive (ECAD^{+}; red) undifferentiated spermatogonia stained Glis3-EGFP^{+}. (**E, F**): Images of sections of PND7 (**E**) and PND14 (**F**) testes immunostained with antibodies against GLIS3-EGFP and ZBTB16 (PLZF) (red). Arrows indicate ZBTB16 and GLIS3-EGFP double positive cells; arrowheads indicate PLZF16 single positive cells. (**G**): Percentage of ZBTB16^{+} cells in seminiferous tubules of PND4, 7, 14 testes that were GLIS3-EGFP^{+}. (**H, I**): Representative images of sections of PND5 or PND7 testes immunostained with antibodies against GLIS3-EGFP and GFRA1 (red). Arrows in (I) indicate GFRA1 and GLIS3-EGFP double positive cells; arrowheads indicate GLIS3-EGFP single positive cells. (**J**): Percentage of GLIS3-EGFP^{+} cells in seminiferous tubules of PND4, 7, 14 testes that were GFRA1^{+}. Bars indicate 50 lm. Abbreviation: GLIS3, GLI-similar 3.

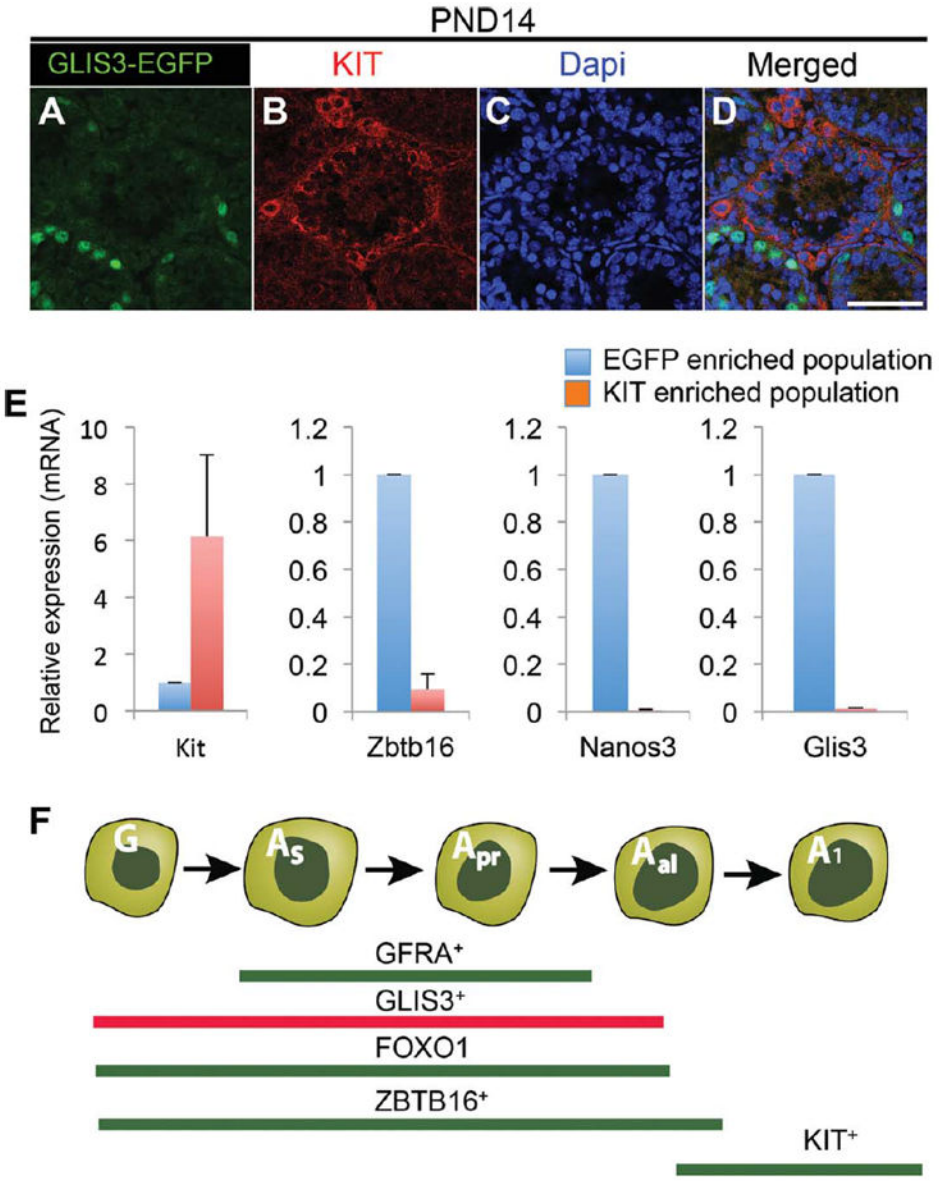

Figure 2.

GLIS3 is not expressed in differentiated spermatogonia. (**A–D**): Representative images of a section of PND14 testes stained with antibodies against GLIS3-EGFP and KIT (red; B). Nuclei were stained with Dapi. Merged images are indicated. Bar indicates 50 μm. (**E**): Cells isolated from PND7 *Glis3*-EGFP testis, were sorted by Flow cytometry to isolate EGFP- and KIT enriched cell populations as described in M&M. These subpopulations were subsequently examined for Kit, Zbtb16, Nanos3, and Glis3 mRNA expression by QRT-PCR. Data present mean ± SEM. (**F**): Proposed scheme of GLIS3 expression during early postnatal spermatogenesis. GLIS3 expression was compared to that of ZBTB16, FOXO1, GFRA1, and KIT. GLIS3 is expressed in gonocytes and most undifferentiated spermatogonia. It is repressed during the A_{al} stage after the downregulation of GFRA1, but before the downregulation of ZBTB16. G, gonocytes; A_s, spermatogonia cell A-single; A_{pr},

A-paired; A_{al}, A-aligned; A1, type A1 spermatogonia. Abbreviations: GLIS3, GLI-similar 3; QRT-PCR, Quantitative real time PCR.

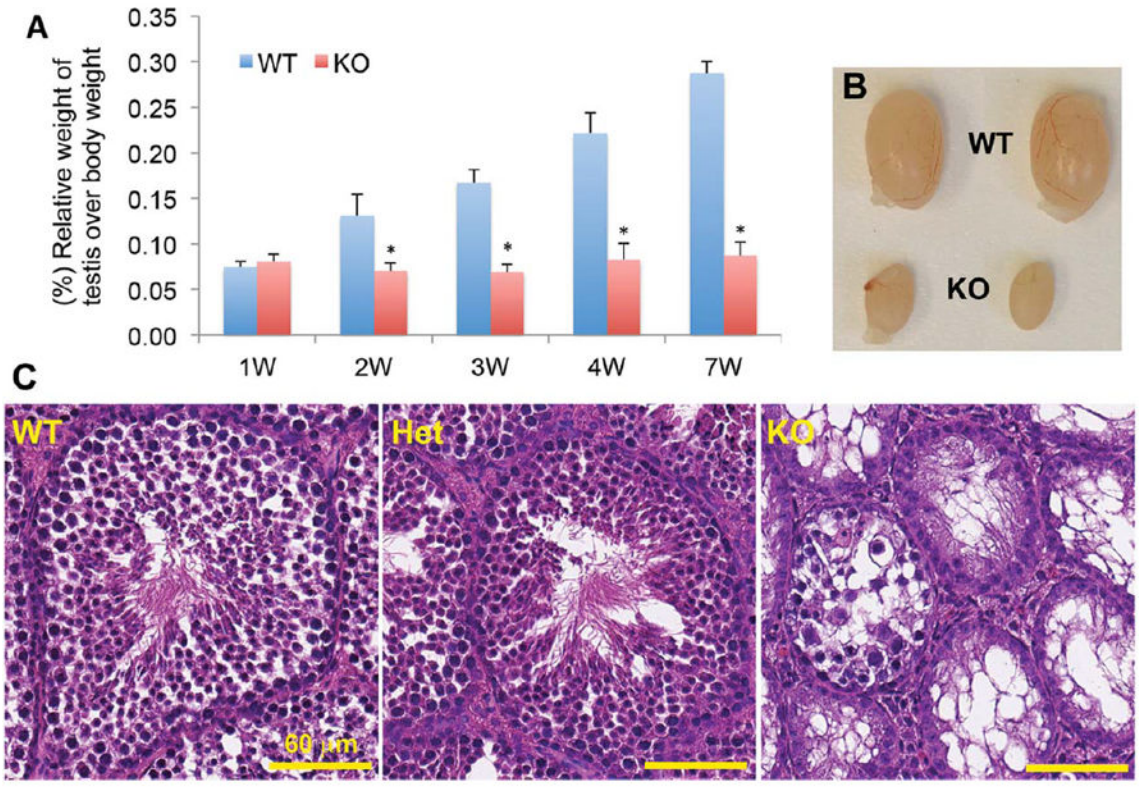

Figure 3.

GLIS3 is required for normal spermatogenesis. (**A, B**): Relative weights (A) and size (B) of testes from WT and *Glis3*KO2 mice ($n \geq 3$). Images of whole testes from 4 weeks-old WT and *Glis3*KO2 mice are shown in (B). Weight and size of *Glis3*KO2 testes are greatly reduced. Data present mean ± SEM. * $p < .05$. (**C**): Representative images of H&E stained sections of testes from 7-weeks old WT, heterozygous, and *Glis3*KO2 mice showing that spermatogenesis is greatly impaired in *Glis3*KO2 mice.

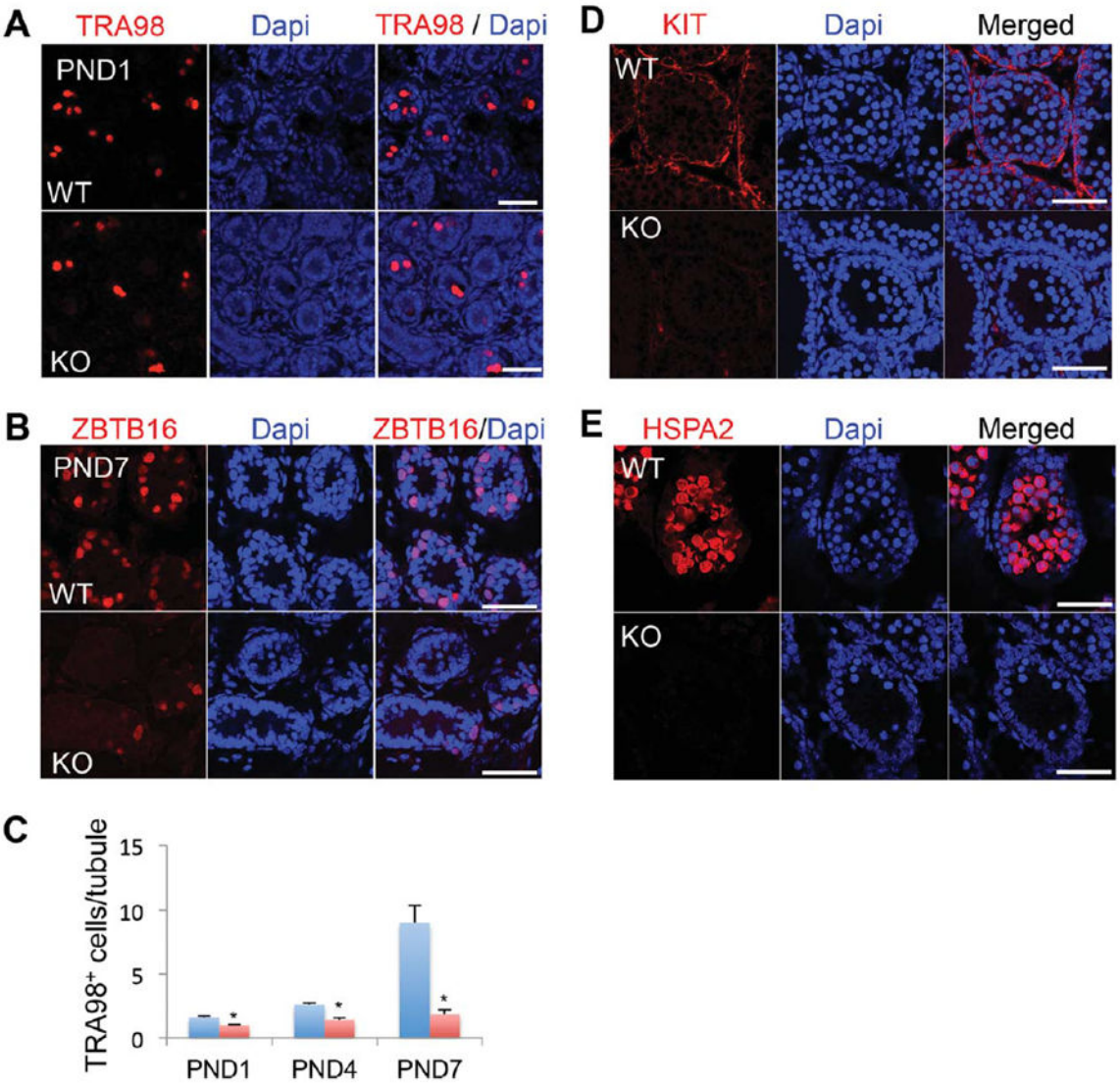

Figure 4.

The number of undifferentiated spermatogonial cells is dramatically reduced in *Glis3*KO2 testis. (**A**): Representative images of sections of PND1 WT and *Glis3*KO2 testes stained with antibodies against TRA98 (red). Nuclei were stained with Dapi. Merged images are indicated. (**B**): Representative images of sections of PND7 WT and *Glis3*KO2 testes immunostained with antibodies against ZBTB16 (red) and Dapi. (**C**): The number of $TRA98^{+}$ germ cells in seminiferous tubules of *Glis3*KO2 testes ($n \geq 4$) was greatly decreased during early postnatal spermatogenesis. (**D, E**) Representative images of sections of testes from 2-weeks old WT and *Glis3*KO2 mice stained with antibodies against KIT (red; A) and HSPA2 (red; B). Nuclei were stained with Dapi. Bars indicate 50 μm. Data present mean ± SEM. * $p < .05$.

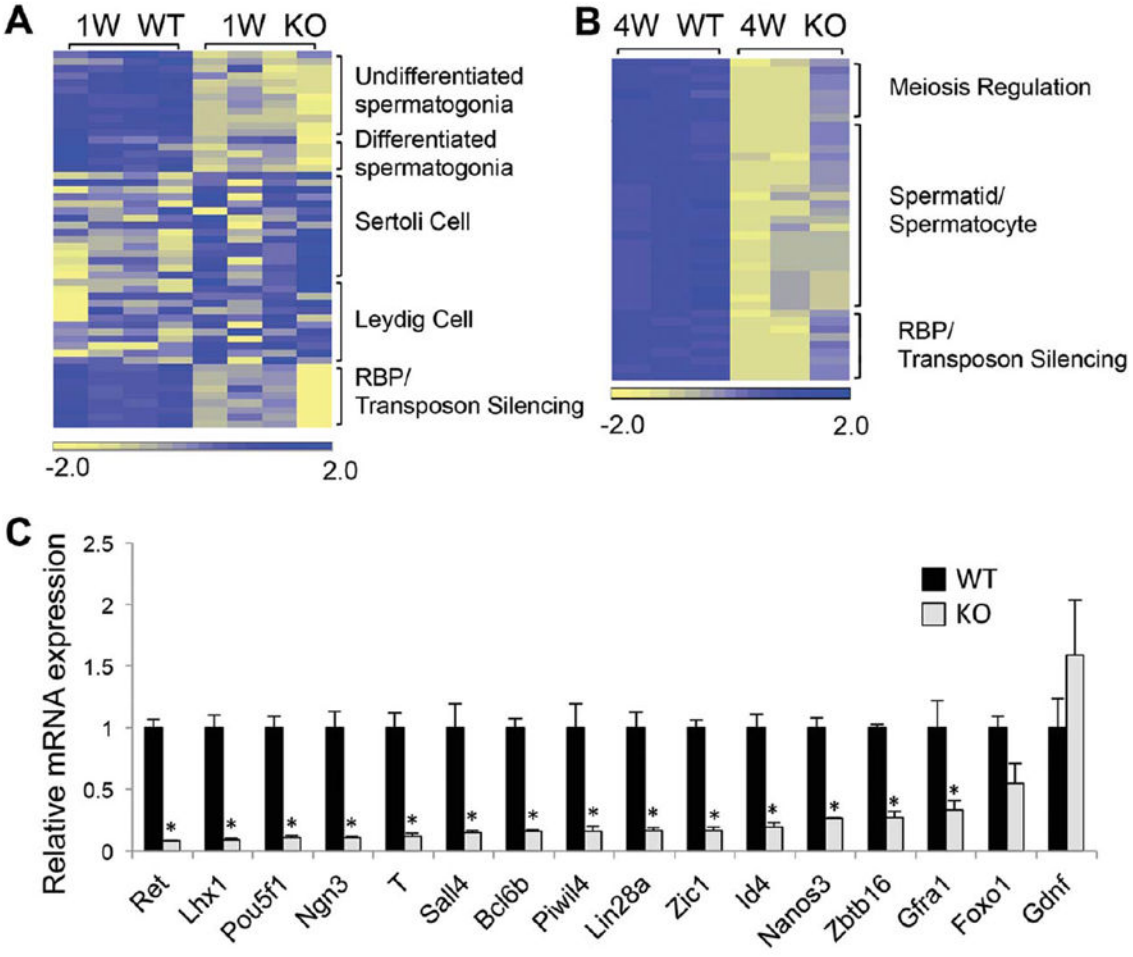

Figure 5.

GLIS3 deficiency causes dramatic changes in gene expression in testes from PND7 and 4 weeks old mice. (**A, B**): Heatmaps generated from gene expression profiles obtained by microarray analyses of testes from PND7 (A) and 4 week-old (B) WT and *Glis3*KO2 mice. Several groups of genes associated with different cell types are indicated. (**C**): The expression of several genes associated with the regulation of early spermatogenesis, in testis from PND7 WT and *Glis3*KO2 mice ($n = 3$) were compared by Quantitative real time PCR analysis. Data present mean ± SEM. * $p < .05$.

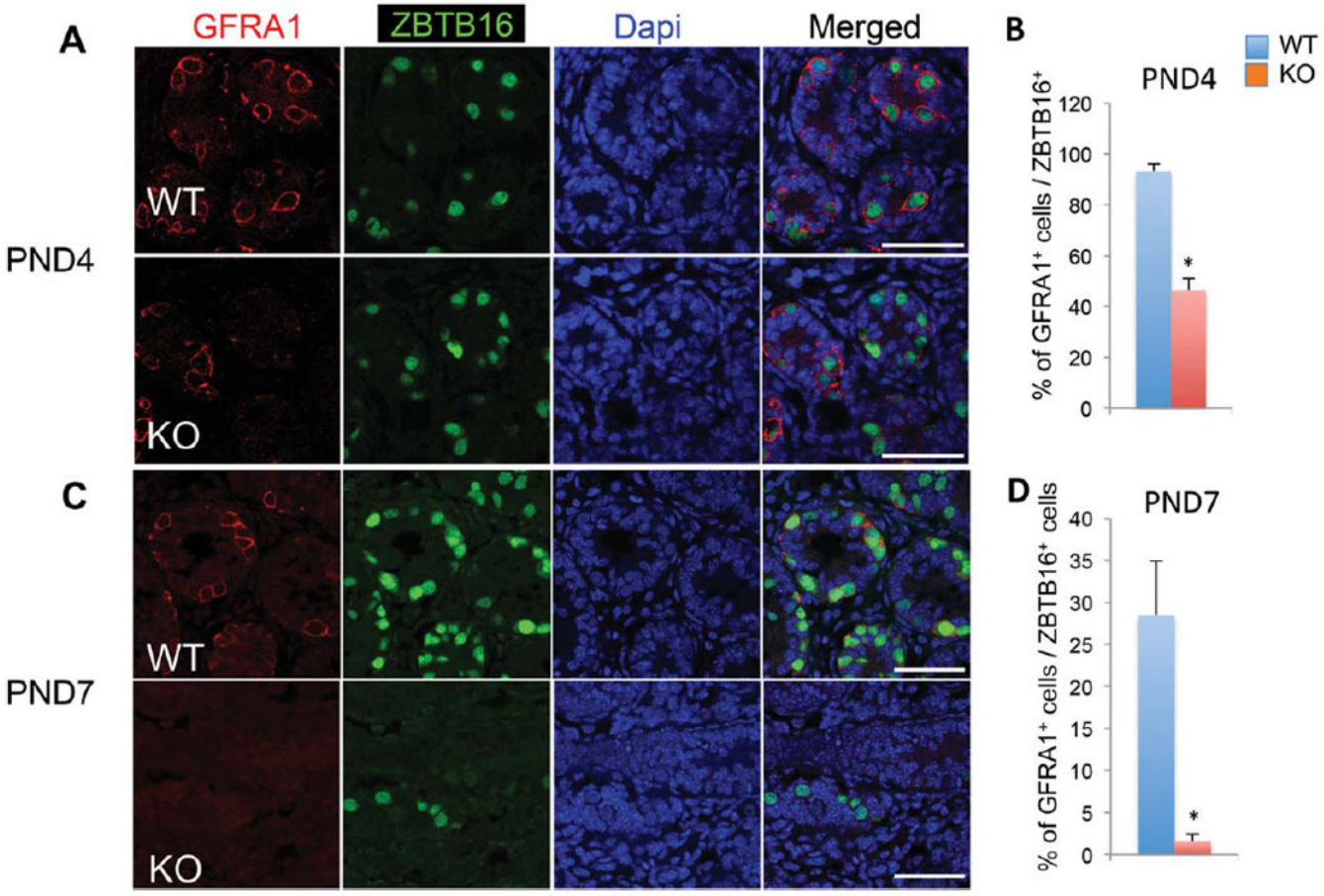

Figure 6.

GLIS3 deficiency reduces the generation of GFRA1$^+$ cells. (**A, C**): Representative images of sections of PND4 and PND7 WT and *Glis3*KO2 testes stained with antibodies against GFRA1 (red) and ZBTB16 (Plzf, green) and Dapi. (**B, D**): The percentage of ZBTB16$^+$ cells staining positively for GFRA1 in PND4 and PND7 WT and *Glis3*KO2 testes (n = 4) was calculated and plotted. Bars indicate 50 μm. Data present mean 6SEM. * p <.05.

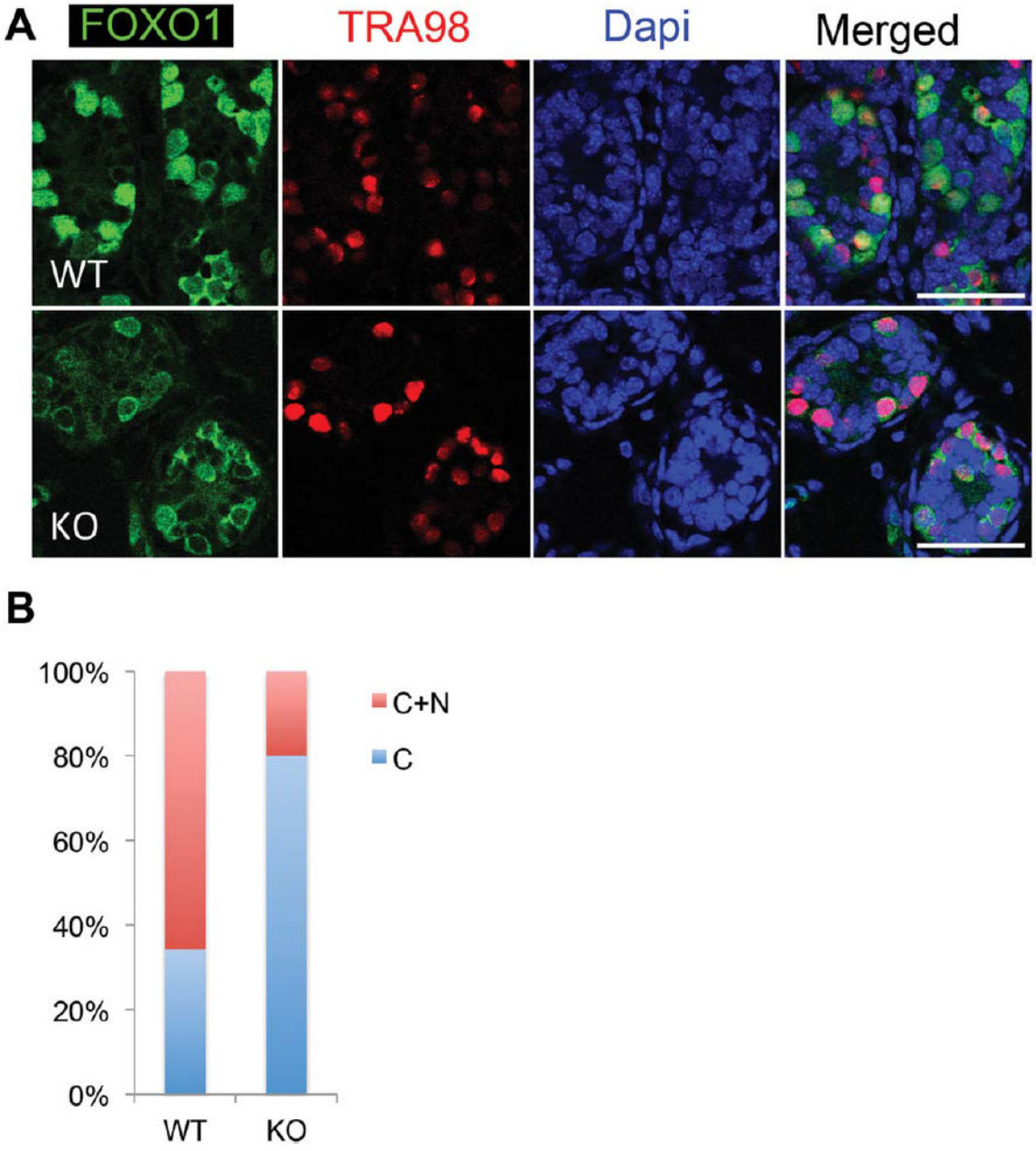

Figure 7.

GLIS3 deficiency impairs the subcellular localization of Foxo1. (**A**): Representative images of sections of PND7 WT and *Glis3*KO2 testes stained with antibodies against FOXO1 (green) and TRA98 (red), and Dapi. (**B**): The subcellular localization of FOXO1 in TRA98^{+} cells was analyzed and the number of cells expressing FOXO1 largely in the cytoplasm (C) or largely in the nucleus or about equally in both (C + N) was counted and plotted. Bars indicate 50 μm.