SHISA6 confers resistance to differentiation-promoting Wnt/β-catenin signaling in mouse spermatogenic stem cells

Tokue, Moe

Doctor of Philosophy
SHISA6 confers resistance to differentiation-promoting Wnt/β-catenin signaling in mouse spermatogenic stem cells

Tokue, Moe
# Contents

| Section                  | Page |
|--------------------------|------|
| Introduction             | 3    |
| Results                  | 8    |
| Discussion               | 18   |
| Materials and Methods    | 25   |
| References               | 38   |
| Figures                  | 44   |
Introduction

Stem cells support tissue homeostasis through the continual production of differentiating progeny from a pool of undifferentiated cells. A long-held and fundamental question is what mechanisms decide the fate of stem cells—whether they remain undifferentiated (self-renew) or differentiate. This is traditionally thought to be primarily dependent on asymmetric stem cell division, and this has been demonstrated in some stem cell systems (de Cuevas and Matunis, 2011). In many cases, however, the importance of environmental cues in the balance of self-renewal and differentiation has become apparent. Such tissue microenvironments, capable of housing and maintaining stem cells, are generally called “stem cell niches” (Raff, 2003). Classically, stem cell niches have been defined as a restricted local tissue microenvironment that has a physical capacity to contain only a small number of stem cells. The ovaries and testes of Drosophila and mammalian intestinal crypts represent this type of niche (Fuller and Spradling, 2007; Snippert et al., 2010).

Recently, a second class of stem cell niche, termed “facultative” or “open” niches, has been proposed; the aforementioned classical niches are referred to as “definitive” or “closed” niches (Morrison and Spradling, 2008; Stine and Matunis, 2013). In facultative niche-supported tissues, stem cells are much less constrained by tissue structure, but intermingle with differentiating progeny in apparently equal environments. Therefore, stem cells appear to behave more freely. The facultative niche system is thought to support some tissues, for example, the Drosophila midgut, mammalian interfollicular epidermis, and mouse testes. However, mechanisms of homeostatic regulation of stem cells supported by facultative niches have remained largely obscure (Ikami et al., 2015; Morrison and Spradling, 2008; Stine and Matunis, 2013; Yoshida, 2015).

In mouse testes, spermatogenesis occurs inside the seminiferous tubules that are
packed in the testicular capsule called the tunica albuginea (Fig. 1A). Undifferentiated spermatogonia, which contain a population of stem cells, are located in the basal compartment of seminiferous tubules (Fig. 1A). These cells give rise to the first step of “differentiating spermatogonia” or A₁ spermatogonia, which subsequently differentiate to become, A₂, A₃, A₄, Intermediate (In) and B spermatogonia, spermatocytes, round spermatids, elongating spermatids, and spermatozoa (Fig. 1B, 1C, and 2). This differentiation process is carried out in a periodic manner. The intervals differ by species. In mice it is 8.6 days, with 35 days needed for A₁ spermatogonia to become mature spermatozoa, whereas in rats, the interval is 12.8 days, with 58 days needed for maturation (Phillips et al., 2010). In the epithelium of seminiferous tubules (seminiferous epithelium), differentiating germ cells shift toward the luminal side in an orderly manner. Therefore, the germ cells are found aligned in four layers, where the combinations of differentiating cells change periodically. This periodic phenomenon is called the seminiferous epithelial cycle, which can be divided into stages I to XII in mice (Fig. 1A, 1C) (Oakberg, 1956; Russell et al., 1990).

There are several somatic cell types in the testes. Sertoli cells, which reside inside the seminiferous tubules, are one of the most important cell types necessary for spermatogenesis. Sertoli cells form the epithelium with a prominent network of tight junctions that divides the basal and adluminal compartments (Fig. 1A). Sertoli cells have contact with germ cells throughout their lifetimes (from stem spermatogonia to mature spermatozoa). Sertoli cells change their morphologies and gene expression patterns in accordance with the seminiferous epithelial cycle, so that they can support different steps of spermatogenesis.

Spermatogenic differentiation is associated with meiotic daughter cells connected via intercellular bridges, resulting from incomplete cytokinesis. Reflecting this property, adult testes contain both singly isolated spermatogonia (specifically termed A₅ or A_single spermatogonia) and
syncytia of spermatogonia and spermatocytes (male germ cells in mitotic and meiotic stages, respectively). Most of the syncytia are composed of $2^n$ cells (viz., 2, 4, 8, and so on, and termed $A_{pr}^{paired}$ or $A_{al-4}$, $A_{al-8}$, respectively). In the mouse spermatogenic stem cell system, $A_s$ spermatogonia have been classically considered the sole origin of spermatogenesis and, once generated, interconnections between spermatogenic cells are maintained until spermiation, which generates mature spermatozoa (Oakberg, 1971). Therefore, all of the syncytia, which are far greater in number than $A_s$ spermatogonia, have been considered to be irreversibly committed to differentiation (the “$A_s$ model”). However, recently, intravital live-imaging observations of $A_{al}$ fragmentation, which gives rise to $A_s$ and shorter syncytia, have challenged the “$A_s$ model” and raised the possibility that $A_s$ and short syncytia compose a single stem cell compartment (Hara et al., 2014; Nakagawa et al., 2010). This observation has re-ignited interests in the identity of spermatogenic stem cells and their behavior in testes.

As a general concept in stem cell biology, two cell compartments compose the stem cell population, namely, “actual stem cell (ASC)” and “potential stem cell (PSC)” populations (Potten and Loeffler, 1990). ASCs were defined as the cells that actually self-renew in an undisturbed steady state, whereas PSCs are the differentiation-destined cells that maintain their potential to self-renew and that might become PSCs when ASCs are lost. Within the population of undifferentiated mouse spermatogonia, a glial cell line-derived neurotrophic factor family receptor alpha 1 (GFRα1)$^+$ subpopulation behaves as ASCs, whereas a neurogenin 3 (NGN3)$^+$ subpopulation acts as PSCs (Fig. 2) (Nakagawa et al., 2007; Nakagawa et al., 2010). Gfra1 encodes a receptor for glial cell line-derived neurotrophic factor (GDNF), a ligand essential for the maintenance of spermatogenic stem cells both in vitro and in vivo (Kanatsu-Shinozaka et al., 2003; Meng et al., 2000). NGN3$^+$ cells are derived from GFRα1$^+$ cells and act as transit-amplifying (TA) cells in steady-state
spermatogenesis. However, NGN3⁺ cells retain the latent potential to regenerate ASCs and to self-renew, which is apparent after tissue damage or transplantation (Nakagawa et al., 2007). In contrast to GFRα1⁺ cells, the number of NGN3⁺ cells changes periodically in accordance with the seminiferous epithelial cycle (Yoshida 2004). NGN3⁺ cells express retinoic acid receptor gamma (RARγ), which allows this population to differentiate in response to RA, whereas GFRα1⁺ cells do not express RARγ or respond to RA (Ikami et al., 2015). In response to RA, which is present periodically at a particular point in the seminiferous epithelial cycle (stages VII and VIII), NGN3⁺ (RARγ⁺) cells differentiate to KIT⁺ A₁ differentiating spermatogonia in a virtually irreversible manner (Fig. 2).

WNTs are important pleiotropic signaling molecules, and most mammals, including mice, have 19 Wnt family members (Clevers and Nusse, 2012; Moon et al., 2002). This signaling is initiated by the binding of a Wnt ligand to the cell surface receptor complex, which is composed of Frizzled and single-membrane-spanning low-density receptor-related protein 5/6 (LRP5/6). Frizzleds are seven-transmembrane proteins, and 10 Frizzled proteins are present in mammals. In the Wnt/β-catenin signaling pathway, binding of a Wnt ligand to its receptor inhibits the β-catenin destruction complex (composed of AXIN, GSK3, DVL and CK1), which leads to the stabilization and translocation of β-catenin into the nucleus to activate downstream targets. This pathway, mediated by β-catenin, is known as the “canonical” Wnt pathway, whereas signaling independent of β-catenin is named the “non-canonical Wnt pathway” (Fig. 3A, B). β-catenin also interacts with the cytoplasmic domain of E-cadherin, which is a key molecule in cell adhesion, so that the majority of β-catenin is located on the cytoplasmic side of the membrane.

Wnt signaling is crucial for a wide range of biological processes. In *Xenopus*, Wnt/β-catenin signaling is required for axis formation during early development (Cadigan and Nusse, 1997). Wnt signaling is also associated with stem cell regulation. For instance, in mammalian
intestinal epithelia, WNT and its agonist R-spondin induce expression of Lgr5, which characterizes stem cells in the base of the crypt (de Lau et al., 2011; Kim et al., 2005; Sato et al., 2011). In the interfollicular epidermis and mammary glands, Wnt signaling has been implicated in stem cell maintenance (Badders et al., 2009; Choi et al., 2013; Lim et al., 2013). On the other hand, a correlation between Wnt signaling and cell differentiation has also been reported: In hair follicle regeneration, melanocyte stem cells differentiate in response to Wnt signaling (Rabbani et al., 2011). In mouse spermatogenesis, intriguingly, studies using cultured spermatogonia indicated that Wnt/β-catenin signaling could stimulate the proliferation of differentiating progenitors (Yeh et al., 2011, 2012). Similarly, in vivo, Wnt/β-catenin signaling has been implicated in the generation and/or proliferation of differentiating progenitors (Takase and Nusse, 2016). Nevertheless, the precise roles of Wnt/β-catenin signaling in the GFRα1* pool remain elusive, because of a lack of appropriate markers and the nature of current genetic tools.

For a better understanding of the stem cell system, it is important to determine how differentiating cells are produced from stem cells, while a part of undifferentiated cells remain. In my Ph.D. research, I have attempted to clarify the mechanisms that regulate the transition from GFRα1* to NGN3* cells and maintenance of GFRα1* spermatogonia without exhaustion.
Results

Searching for signaling pathways that might induce spermatogonial differentiation

To obtain gene expression profiles of GFRα1-expressing (GFRα1⁺) and NGN3-expressing (NGN3⁺) cells, I first collected these cells from adult $Gfra1^{EGFP/+}$ (Enomoto et al., 2000) and $Ngn3/EGFP^{Tg/+}$ mice (Yoshida et al., 2004), respectively, by fluorescence activated cell sorting (FACS) (Fig. 4). Using these cells, I performed a cDNA microarray analysis. Expression levels of $Gfra1$, $Ngn3$, and $Kit$ were significantly higher than those of other genes in the respective factions (Fig. 5A, 7), which verified that the microarray data appropriately reflected the gene expression profiles of these populations. Therefore, I carried out the following analyses using these data.

To elucidate the signaling pathways that induce differentiation of GFRα1⁺ cells to NGN3⁺ cells, I first identified genes that are differentially expressed between GFRα1⁺ and NGN3⁺ populations. Using the genes identified, I performed a pathway analysis using GeneSpring software and obtained a list of signaling pathways that might function differentially in GFRα1⁺ and NGN3⁺ cells. Based on these results, I focused on the Hedgehog, TGFβ, and Wnt pathways (Fig. 5B, 7).

Wnt/β-catenin signaling induces Ngn3 expression

To evaluate whether activation of Hedgehog, TGFβ, and Wnt pathways upregulates $Ngn3$ gene expression, I used germline stem (Luo et al.) cell cultures (Kanatsu-Shinohara et al., 2003). GS cells are spermatogonial cultures maintained in the presence of glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor (FGF), and epidermal growth factor (EGF), and that usually show a high level of $Gfra1$ expression, but suppressed expression of $Ngn3$ (Kanatsu-Shinohara et al., 2006). Using GS cells, I attempted to define which signal(s) acts as an inducer of the GFRα1⁺ to NGN3⁺
transition. For this purpose, GS cells were cultured in media containing recombinant WNT3a, WNT5a, BMP4/6, or SHH, which stimulate the canonical or non-canonical Wnt pathways, TGFβ, or Hedgehog pathways, respectively. After stimulation with one of these ligands for 24 hours, cells were harvested and analyzed for the expression of Ngn3 mRNA by real-time quantitative PCR (RT-qPCR) (Fig. 6). I found that only WNT3a upregulated Ngn3 mRNA. Moreover, a stronger induction of Ngn3 by WNT3a was observed when GDNF was depleted from the medium. WNT5a, BMP4/6, and SHH did not affect expression of Ngn3 mRNA (Fig. 6 and data not shown).

Many factors are necessary to transmit Wnt/β-catenin signals from the cell surface to the nucleus, including signal transducers, transcription factors, receptors, and ligands. Based on the in vivo microarray data, these factors are expressed in GFRα1+ and NGN3+ cells. In particular, Lef1, a gene encoding a transcription factor that is a downstream target of this pathway, is upregulated in Ngn3+ cells (Fig. 7) (Hovanes et al., 2001). These data indicate that both GFRα1+ and NGN3+ cells receive Wnt/β-catenin signals, consistent with a previous observation in a study that used Axin2 reporters (Takase and Nusse, 2016). The higher level of Lef1 expression in NGN3+ cells suggests that they may receive a stronger signal than GFRα1+ cells.

**Elevated Wnt/β-catenin signaling reduces the GFRα1+ cell pool in vivo**

I then asked, what is the in vivo role of Wnt/β-catenin signaling? To answer this question, I used the Ctnnb1($^{fl(ex3)}$) allele, which enabled the conditional deletion of exon 3 that encodes a critical GSK3β phosphorylation site (Fig. 9A) (Harada et al., 1999). In this mouse, β-catenin was stabilized and Wnt/β-catenin signaling was activated. To assess the specific effects of this mutation in germ cells, I used the Nanos3Cre allele (Suzuki et al., 2008) of Nanos3, a gene expressed in a germ cell-specific fashion (Fig. 8A–C). Because the following analyses were performed in a Nanos3Cre/+ background,
the genotypes will be indicated by the β-catenin alleles for simplicity.

Although the impact of stabilized β-catenin was not apparent in heterozygotes \([fl(ex3)/+]\), it was obvious in homozygotes \([fl(ex3)/fl(ex3)]\). At 8–14 weeks of age, \(fl(ex3)/fl(ex3)\) mice showed smaller testes, although body weights did not change (Fig. 9B–F). Their testes showed spermatogenesis defects (e.g., loss of or one or more exiguous germ cell layers) in about 20% of seminiferous tubule sections (Fig. 10A–D). Notably, the number of GFRα1\(^+\) cells was reduced in the homozygotes (Fig. 11A–D). A significant reduction in RARγ\(^+\) cells, largely corresponding to NGN3\(^+\) cells (Gely-Pernot et al., 2012; Ikami et al., 2015), was not observed, resulting in an increased RARγ\(^+\) cell to GFRα1\(^+\) cell ratio (Fig. 11E, F). \(fl(ex3)/fl(ex3)\) mice showed a normal testicular morphology and a normal number of GFRα1\(^+\) and MVH\(^+\) cells at postnatal day 3 (P3). MVH is a pan-germ cell marker. These findings indicate normal fetal and neonatal germ cell development in this mutant (Fig. 12A–D). To summarize, elevated Wnt/β-catenin signaling resulted in a reduction in the GFRα1\(^+\) cell pool in a dose-dependent manner.

**Deletion of β-catenin does not affect the GFRα1\(^+\) cell pool in vivo**

To study the role of Wnt/β-catenin signaling in more depth, I used a conditionally null, \(Ctnnb1\(^{fl}\)\) allele (Brault et al., 2001) (Fig. 13A). Deletion of β-catenin caused similar phenotypes as those of the stabilized β-catenin mutant mouse; both the heterozygous and homozygous mutants had smaller testes and showed spermatogenesis defects (Fig. 13B–F, 14A–D). In contrast to the stabilized β-catenin mutant, however, the number of GFRα1\(^+\) cells did not change (Fig. 15A). The RARγ\(^+\) cell number and the RARγ\(^+\) cell to GFRα1\(^+\) cell ratio decreased, consistent with the idea that GFRα1\(^+\) to NGN3\(^+\) differentiation was affected (Fig. 15B, C). This is largely in agreement with the results of a previous study (Takase and Nusse, 2016).
There were other differences between these mutants. The impact of $fl(ex3)/fl(ex3)$ seems less pronounced than that of the $fl/+$ and $fl/fl$ mutants. GFRα1$^+$ cells were found to be decreased in a spatially uniform manner; although the overall GFRα1$^+$ pool was reduced, Sertoli cell-only (Lovasco et al.) segments were rarely observed. This may reflect the weak impact of this mutation on cell-cell contact and the cytoskeletal system. On the other hand, $fl/+$ or $fl/fl$ mouse testes exhibited a more variable appearance throughout the tissue. Accumulated GFRα1$^+$ cells were observed in some parts, and SCO tubules were observed more frequently (Fig. 16A, B). I speculate that this reflects an affected differentiation process leading to the accumulation of undifferentiated cells, in addition to the overall instability (of fragility) of the SSC pool, perhaps resulting from the stronger global cellular impact of the loss of β-catenin.

**Wnt6 expression in seminiferous tubules in stages I–VIII**

By screening the expression of all mouse Wnt genes by *in situ* hybridization (ISH), I found Wnt6 expression to be predominant in Sertoli and interstitial cells (Fig. 17A–F), in agreement with a previous report (Takase and Nusse, 2016). Further, Wnt6 expression showed a seminiferous epithelial cycle-related oscillation and was highest in stages I to VI (Fig. 18A). Of note, increased Wnt6 expression coincided with a decrease in Gdnf, visualized by a Gdnf-LacZ knockin allele (Moore et al., 1996) (Fig. 17D–F, 19A), an increase in NGN3$^+$ cells, and a weak decrease in GFRα1$^+$ cells (Fig. 18B). These observations largely supported the notion that the increase in WNT6 and decrease in GDNF drive GFRα1$^+$ cells to differentiate to NGN3$^+$.

However, the spatially uniform expression of these factors over a tubule cross-section raises an additional question: why do some GFRα1$^+$ cells differentiate into NGN3$^+$ cells, while others remain GFRα1$^+$ in an apparently uniform environment? In particular, persistence of GFRα1$^+$ cells
during Wnt6 high expression stages implies that Wnt/β-catenin signaling may face interference in some GFRα1+ cells. However, this interference is unlikely to be caused by spatial unevenness in Wnt activity generated by extracellular Wnt inhibitors because GFRα1+ cells are highly migratory and extensively interspersed between NGN3+ cells (which likely receive higher levels of Wnt signaling) (Hara et al., 2014; Ikami et al., 2015). Rather, I hypothesized that GFRα1+ cells show different levels of cell-intrinsic resistance to Wnt/β-catenin signaling.

**Shisa6 is enriched in the GFRα1+ cells fraction**

I therefore searched for factor(s) that could cell-autonomously inhibit Wnt/β-catenin signaling in the population of GFRα1+ cells, with genes showing restricted expression in the GFRα1+ fraction (Fig. 19A). Although known Wnt inhibitors were not highly enriched, I focused on Shisa6, the fourth most highly enriched gene (Fig. 19B). Shisa6 is a member of the Shisa family, which comprises single-transmembrane proteins and is characterized by an N-terminal cysteine-rich domain and a proline-rich C-terminal region (Fig. 19C-D).

I hypothesized that Shisa6 could be a cell-autonomous Wnt inhibitor, because some of the Shisa family proteins (e.g., *Xenopus* (x) *shisa1*, *xshisa2*, and *xshisa3* and murine (m) *Shisa2* and *mShisa3*) are known to inhibit Wnt signaling in a cell-autonomous fashion by suppressing the maturation and cell surface expression of Wnt receptors (Chen et al., 2014; Furushima et al., 2007; Nagano et al., 2006; Yamamoto et al., 2005). However, the molecular functions of Shisa6 are relatively unclear; a recent report showed that Shisa6 stabilized AMPA receptor (AMPAR) expression in mouse brains, similar to Shisa9 (Klaassen et al., 2016; von Engelhardt et al., 2010).
**SHISA6 is expressed in a subset of GFRα1⁺ cells**

In testes sections, immunofluorescence (Shirakawa et al.) facilitated detection of SHISA6 protein in a few spermatogonia, many of which were also GFRα1⁺, on the periphery of seminiferous tubules (Fig. 20). Unfortunately, the staining conditions required for SHISA6 IF were not optimal for anti-GFRα1 antibody, and only a fraction of GFRα1⁺ cells (probably those with high levels of GFRα1) were detected in the double IF. I therefore combined fluorescence ISH (Wang et al.) for Shisa6 with IF for GFRα1 on dispersed testicular cells and found that a high level of Shisa6 expression was restricted to ~30% of GFRα1⁺ cells (Fig. 21A–B).

**Shisa6 encodes a novel cell-autonomous Wnt inhibitor**

Using *Xenopus laevis* embryos, Naoto Ueno’s group, my collaborators at National Institute for Basic Biology (NIBB), found that co-injection of mShisa6 mRNA inhibited secondary axis formation by xwnt8 (Fig. 22A–E), as previously observed for mShisa2 (Yamamoto et al., 2005). Similarly, as previous reported for xshisa1 and mShisa2, mShisa6 injection alone enlarged the cement gland (Fig. 23A–E), an indication of Wnt inhibition that has also been observed with xshisa1, xshisa2, and mShisa2 (Furushima et al., 2007; Yamamoto et al., 2005).

Furthermore, in HEK293T cells, mShisa6 inhibited activation of TCF-luc, a Wnt/β-catenin signaling reporter, via Wnt/β-catenin signaling activated by WNT3a (Fig. 24A). Using this experimental system, I investigated whether Shisa6 cell-autonomously inhibits Wnt/β-catenin signaling. For this purpose, Shisa6 and TCF-luc were co-transfected, or transfected separately, as shown in the scheme and schedule in Fig. 24B and 24C. Inhibition was observed when Shisa6 and the reporter were co-transfected, but not when these two components were transfected into separate
cells and then the transfected cells were mixed (Fig. 24D). Together, these results indicate that SHISA6 is a novel cell-autonomous Wnt inhibitor.

I then examined whether Shisa6 could also affect FGF and GDNF signaling, which both play important roles in maintenance of spermatogenic stem cells (Kanatsu-Shinohara et al., 2005), using the same experimental design. Some SHISA proteins inhibit FGF signaling (Furushima et al., 2007; Nagano et al., 2006; Yamamoto et al., 2005), and SHISA6 only weakly suppressed FGF signals in HEK293T cells (Fig. 24E). On the other hand, no inhibition of the GDNF signal by SHISA6 was observed (Fig. 24F).

**Function of Shisa6 in GS cells**

I then investigated the expression of Shisa6 in GS cells by microarray analysis. The data, normalized by the 75th percentile, indicated that Shisa6 was expressed more highly in GS cells than in GFRα1+ cells (Fig. 25A, B). IF revealed that SHISA6 was expressed in all GS cells (Fig. 25C), allowing us to test the role of SHISA6 in vitro. I assumed that SHISA6 might block GS cell differentiation driven by Wnt/β-catenin signaling. To investigate this hypothesis, GS cells were co-transfected with Shisa6 (or control) siRNA and a luciferase reporter plasmid flanked by the Ngn3 regulatory sequence. In this case, addition of WNT3a alone did not increase Ngn3-luc activity, but when both WNT3a and RSPO2 (R-spondin2, a co-factor of Wnt) were provided simultaneously, NGN3-luc activity was significantly increased (Fig. 26). These data indicated that SHISA6 suppressed the function of WNT3a to induce Ngn3 expression in GS cells.
*Shisa6* is regulated positively by GDNF and FGF and negatively by Wnt/β-catenin signaling in GS cells

Then, I examined the relationship between the expression of *Shisa6* and the GDNF, FGF, and Wnt/β-catenin signaling pathways. For this purpose, GS cells were deprived of GDNF and/or FGF2 for 3 days (Fig. 27A). RT-qPCR analysis revealed a decrease of *Shisa6* mRNA when either GDNF or FGF2 was withdrawn (Fig. 27B). This was in stark contrast to *Ngn3* mRNA, whose expression was increased when these ligands were removed (Fig. 27C). To assess a possible role of Wnt/β-catenin signaling in *Shisa6* expression, I performed a microarray analysis using GS cells. Expression of *Shisa6* was suppressed when the cells were exposed to WNT3a for 24 hours (Fig. 27D, E).

These results suggest that *Shisa6* expression is increased by GDNF and FGF, which are required for stem cell maintenance, whereas it is suppressed by Wnt/β-catenin signaling that promotes cell differentiation.

**Generation of Shisa6 knockout mice using the CRISPR/Cas9 system**

Next, I examined the function of *Shisa6* in vivo by generating *Shisa6*-null alleles using the CRISPR/Cas9 system in collaboration with the Satoru Takahashi Lab at Tsukuba University (Fig. 28A) (Cong et al., 2013). Several founders carrying disrupted “indel” alleles of the *Shsia6* gene, including *Shisa6Δ6+502* (referred to as *Shisa6KO* hereafter), were obtained (Fig. 28B). *Shisa6* mRNA was hardly detected, probably due to nonsense-mediated mRNA decay, confirming the success of the disruption (Fig. 29A) (Peltz et al., 1993). Further, SHISA6 protein was not detected in *Shisa6KO/KO* mice (Fig. 29B). However, *Shisa6KO/+* and *Shisa6KO/KO* mice were seemingly healthy, with normal body weights (Fig. 30A, B), and were fertile. *Shisa6KO/KO* mice did not show apparent defects in spermatogenesis (Fig. 30C–F) or the number of GFRα1+ cells (Fig. 30G).
SHISA6 confers resistance to Wnt/β-catenin signaling to maintain the GFRα1⁺ cell pool

Then, to clarify the relationship between SHISA6 and Wnt/β-catenin signaling, I mated Shisa6<sup>KO/+</sup> and Nanos3<sup>Cre/+</sup>;Ctnnb1<sup>fl(ex3)/+</sup> mice, and found that the Shisa6<sup>KO/+</sup>;Nanos3<sup>Cre/+</sup>;Ctnnb1<sup>fl(ex3)/+</sup> mice had smaller testes than those of the Nanos3<sup>Cre/+</sup>;Ctnnb1<sup>fl(ex3)/+</sup> mice (Fig. 31A–C). In these mice, however, the body weights also mildly reduced (Fig. 31D). In Shisa6<sup>KO/+</sup>;Nanos3<sup>Cre/+</sup>;Ctnnb1<sup>fl(ex3)/+</sup> mice, the frequency of defective tubules increased (Fig. 32A–C). While fl(ex3)/+ or Shisa6<sup>KO/+</sup> mice showed a normally sized GFRα1⁺ cell pool (i.e., the GFRα1+ cell number per seminiferous tubule cross-section), Shisa6<sup>KO/+</sup>;fl(ex3)/+ mice exhibited a significant reduction in the GFRα1⁺ pool (Fig. 33A). The number of RARγ⁺ cells did not change either, as observed in the fl(ex3)/ fl(ex3) mice. Further, the RARγ⁻ to-GFRα1⁺ cell ratio showed a trend toward an increase, although there was no significant change (Fig. 33B, C). This was unlikely due to off-target effects, because compatible phenotypes were observed for another Shisa6 null allele, Shisa6 (Fig. 35A–C).

Considering that SHISA6 is a cell-autonomous Wnt inhibitor, these observations strongly suggest that SHISA6 plays a role in maintenance of the GFRα1⁺ pool by reducing Wnt/β-catenin signaling. SHISA6 probably achieves this function through interfering with the expression of Wnt receptors on cell surfaces, a conjecture made based on the function of xshisa1 (Yamamoto et al., 2005). However, the possibility that SHISA6 acts through different mechanisms is not excluded.

Shisa6 is expressed in T (brachyury)-positive cells that show stem cell characteristics

Finally, I examined the properties of the SHISA6⁺ subset of GFRα1⁺ cells. Given the limited immunodetectability and genetic tools available, I sought to make use of gene(s) that showed expression concordant with that of Shisa6, as a second-best strategy. Among genes showing
similarly high enrichment in the GFRα1+ fraction (Fig. 19A), I focused on T (brachyury). Since an engineered T allele (TnEGFP-CreERT2) was available, in which nuclear (n)-GFP and CreERT2 (a tamoxifen-inducible Cre) are ligated to the endogenous Brachyury via 2A peptides, so that these three proteins are simultaneously generated from a single polycistronic mRNA transcribed from the T locus (Fig. 35A) (Imuta et al., 2013). This enabled reliable visualization and tracing of the T+ cells, using GFP and CreERT2, respectively. In the TnEGFP-CreERT2/+ mouse testes, T-GFP+ cells comprised ~40% of GFRα1+ spermatogonia (Fig. 35B, C). Shisa6 expression was specifically detected in a majority (~70%) of T-GFP+ cells (Fig. 36A–C). Based on this considerable concordance, properties of T+ cells were then studied to gain insights into SHISA6+ cells.

I found that the T-GFP+ population was composed of a higher percentage of Aα cells compared to T-GFP+/GFRα1+ cells (Fig. 37A) throughout the seminiferous epithelial cycle (Fig. 37B). The function of T+ cells in steady-state spermatogenesis was also examined following pulse-labeling by tamoxifen activation of CreERT2. Shortly (2 days) after induction, a fraction of T-GFP+ cells were successfully labeled by a lineage reporter, R26RH2B-mCherry (Abe et al., 2011) (Fig. 38A–C). One and 6 months later, the induced cells (labeled with GFP in this case) formed large patches occupying seminiferous tubule segments, indicating that at least a part of T+ cells contributed to long-term spermatogenesis (Fig. 39A–C). These characteristics of T+ (and probably SHISA6+) cells are relevant to its stem cell functions.
Discussion

Wnt/β-catenin signaling in mouse spermatogenesis

In many tissues, Wnt/β-catenin signaling inhibits stem cell differentiation, including in the interfollicular epidermis, small intestinal crypts, and embryonic stem cells (Kim et al., 2005; Lim et al., 2013; Sato et al., 2004). Mouse spermatogenesis illustrates the less-investigated, differentiation-promoting activity of WNT, as observed in melanocyte stem cells during hair follicle regeneration (Rabbani et al., 2011).

In this study, I showed that Wnt/β-catenin signaling drove the initial step of spermatogonial differentiation (i.e., GFRα1+ to NGN3+). This study reinforced and complemented the results of previous studies that combined in vitro cell culture with transplantation-based stem cell assays (Yeh et al., 2011, 2012) and that analyzed the in vivo impact of β-catenin deletion (Takase and Nusse, 2016). First, because GFRα1+ and NGN3+ cells both form repopulating colonies in recipients’ testes after transplantation (Grisanti et al., 2009; Nakagawa et al., 2007), it was difficult to unambiguously link the results of the transplantation assay with the states of differentiation. The present study showed that activation of Wnt/β-catenin signaling upregulates Ngn3 expression in vitro (Fig. 6). Second, because β-catenin also contributes to cell adhesion and cytoskeletal regulation, the study by Takase and Nusse (2015) inevitably leaves some ambiguity about the role of the Wnt pathway (Takeichi, 2014). The present study showed that stabilization of β-catenin—which has less impact on cell adhesion (Harada et al., 1999)—reduced the GFRα1+ pool in vivo. This was in agreement with enhanced differentiation and largely consistent with the results of β-catenin deletion (Fig. 13–15; Takase and Nusse, 2016).

Takase and Nusse concluded that Wnt/β-catenin signaling stimulates the proliferation of
PLZF+ undifferentiated spermatogonia (2016). I, therefore, examined the proliferation status of GFRα1+ cells in a Ctnnb1 mutant series; these cells did not show significant differences when compared to Ctnnb1+/+, nor did they show consistent trends suggesting the role of Wnt/β-catenin signaling (Fig. 41). Wnt/β-catenin signaling may stimulate the proliferation of NGN3+ (i.e., PLZF+/GFRα1−) cells, in addition to GFRα1+-to-NGN3+ differentiation.

Although the Ctnnb1fl(ex3) allele causes abnormalities in many other tissues of heterozygotes (Harada et al., 1999; Lee et al., 2004; Soshnikova et al., 2003), spermatogenic defects were observed only in the homozygous mice in my study. A similar phenomenon was reported by Huels et al. Within the intestinal epithelium, they observed that Ctnnb1fl(ex3)/+ mice exhibited a crypt progenitor cell (CPC)-like phenotype, with cells failing to differentiate, retaining proliferative capacity, and failing to migrate up the crypt-villus axis. On the other hand, the colon had this phenotype only in Ctnnb1fl(ex3)/fl(ex3), but not in Ctnnb1fl(ex3)/+, mice (Huels et al., 2015). The authors also showed that conditional double knockout mice (i.e., E-cadherin+/−:β-catenin+/−) showed colon abnormalities. From these results, they proposed that the observed difference between the small intestine and the colon was associated with higher expression of E-cadherin and a higher number of E-cadherin:β-catenin complexes at the membrane, because E-cadherin was acting as a sink and limiting the mutant β-catenin protein from entering the nucleus and transducing the Wnt signal. Concordant with this, mouse spermatogonia also express a high level of E-cadherin (Tokuda et al., 2007), and IF imaging using anti-non-phosphorylated β-catenin revealed strong signals near the cell membrane (Fig. 42). This may suggest that, in mouse spermatogonia, Wnt/β-catenin signaling is also suppressed by E-cadherin, hiding the phenotypic effect of the introduction of heterologous Ctnnb1fl(ex3)+ by sinking the mutant β-catenin.

It is of particular note that, in spermatogonia, suppression of the Wnt/β-catenin signal
involves other mechanisms, in addition to the aforementioned E-cadherin-related ones. Clearly, SHISA6 contributes to suppression as already discussed. Furthermore, SHISA2, which has stronger Wnt inhibitor activity than SHISA6, is also enriched in GFRα1+ cells, showing broader expression in spermatogonia (Fig. 19, 22, 23). These mechanisms may effectively protect the stem cells from Wnt/β-catenin signaling, because this signaling can elicit multiple powerful effects on target cells, affecting cell proliferation, differentiation, migration, genetic stability, and apoptosis.

**SHISA6 is a novel Wnt inhibitor that acts in a cell autonomous manner**

In this study, I report a novel Wnt inhibitor, SHISA6, a member of the Shisa family whose function had not been characterized, in cultured fibroblasts. Shisa family members are commonly characterized as single transmembrane proteins having a cysteine-rich N-terminal domain and a proline-rich C-terminal region (Pei and Grishin, 2012). The first report of a Shisa family member (xshisa) was on body pattern formation during early embryogenesis of *Xenopus laevis* (Nagano et al., 2006; Yamamoto et al., 2005). These researchers showed that xshisa inhibits both Wnt/β-catenin and FGF signaling pathways by blocking the maturation and cell surface expression of their receptor proteins, promoting head formation. Following this pioneering study, mShisa (the mouse orthologue of xshisa, also called Shisa2) was also shown to antagonize Wnt and FGF signaling (Furushima et al., 2007). Aside from these, SHISA9 (also called CKAMP44) has been shown to play an important role in AMPA receptor desensitization (von Engelhardt et al., 2010). Interestingly, Shisa6 has been recently reported to also desensitize the AMPA receptor in the central nervous system (Klaassen et al., 2016). SHISA6 is, therefore, a context-dependent dual-function protein.

This study illustrates a unique mode of Wnt inhibitor function. Many Wnt inhibitors are secreted proteins that act in a non-cell-autonomous manner and tune the spatial pattern of Wnt
activity. These include Dickkopf proteins (DKKs), secreted Frizzled-related proteins (sFRPs), and Wnt inhibitory factor 1 (WIF1) (Cruciat and Niehrs, 2013). Similarly, cell-autonomous Wnt inhibitors identified so far (viz., xshisa1, xshisa2, xshisa3, Apcdd1, Tiki1, and flop1/2) also shape the spatial patterns of Wnt activity (Miyagi et al., 2015; Nagano et al., 2006; Shimomura et al., 2010; Yamamoto et al., 2005; Zhang et al., 2012). In contrast, this study suggests that heterogeneous Shisa6 expression variegates stem/progenitor spermatogonia in terms of their sensitivity to Wnt/β-catenin signaling in a spatially uniform facultative microenvironment.

**Shisa6 expression is regulated by GDNF and FGF**

The importance of GDNF and FGF signals for the maintenance of spermatogenic stem cells is established (Ishii et al., 2012; Kanatsu-Shinohara et al., 2006; Kanatsu-Shinohara et al., 2003; Kanatsu-Shinohara et al., 2005; Kubota et al., 2004; Meng et al., 2000). However, what molecular machinery acts downstream of these factors to inhibit differentiation remains elusive. In the current study, I showed that GDNF and FGF promote the expression of Shisa6. Therefore, one of the roles of GDNF and FGF may be to increase Shsia6 expression and block differentiation-promoting Wnt/β-catenin signaling.

Therefore, the mechanisms that produce SHISA6⁺ and SHISA6⁻ cells *in vivo* should be of particular interests. However, the mechanisms remain unknown. Indeed, there are puzzling observations: Gdnf and Wnt6 expression is observed in Sertoli cells, depending on the stage of the seminiferous epithelial cycle. Despite the periodic expression of these factors that promote and inhibit the expression of Shisa6, the number of T-GFP⁺ (probably SHISA6⁺) cells was found largely constant throughout the seminiferous tubule cycle. Therefore, the expression is Shisa6 may not be a simple reflection of GDNF and WNT6. Moreover, GFRα1⁺ cells, which include SHISA6⁺ and SHISA6⁻
cells, are highly motile in the basal compartment of seminiferous tubules. Therefore, to uncover the mechanisms underlying the expression of SHSA6, future studies with intravital live-imaging experiments should be performed.

Identification of a SHISA6⁺/T⁺ and GFRα1⁺ subpopulation of GFRα1⁺ spermatogonia and behavior of these cells in vivo

Given that SHISA6 confers resistance to differentiation-inducing Wnt/β-catenin signaling, SHISA6⁺ cells should be a key population to understand this stem cell system. Although their in-depth characterization was difficult, I analyzed T (Brachyury)⁺ cells that showed considerable overlap with SHISA6⁺ cells, taking advantage of an engineered T\(^{\text{neGFP-CreERT2}}\) allele. T⁺/GFRα1⁺ cells are morphologically more biased to A⁺ cells than to T⁻/GFRα1⁺ cells (Fig. 37A), and are persist throughout the seminiferous epithelium cycle (Fig. 37B). Furthermore, pulse-labeled T⁺ cells showed a long-term contribution to spermatogenesis (Fig. 38, 39). These features of T⁺ cells are postulated to be present in stem cells. Echoing this is the suggestion that T is crucial for the self-renewing potential of cultured spermatogonia (Wu et al., 2011). I assume that SHISA6⁺ cells also showed similar stem cell-related characteristics, although I cannot exclude the possibility that only the T⁺/SHISA6⁻ cells exhibited long-term stem cell functions. To understand the detailed fates of SHISA6⁺/T⁺ cells, future investigations using live-imaging and/or pulse-labeling experiments to evaluate these scenarios should be undertaken for a fuller understanding of the dynamics of the spermatogenic stem cell system.

Identity of spermatogenic stem cells

It is not easy, however, to conclusively determine the identity of steady-state stem cells (Yoshida, in
press). A number of genes (e.g., Id4, Pax7, Erbb3, and Bmi1) have been reported to delineate subsets of GFRα1+ cells showing stem cell-related characteristics, as has been shown for T+ (and probably SHISA6+) cells in this study. Accordingly, cells expressing these genes are proposed to be the bona fide stem cells (Aloisio et al., 2014; Chan et al., 2014; Chapman et al., 2015; Komai et al., 2014). It is puzzling, however, that these genes appear to delineate different subsets of GFRα1+ cells. For example, the observed frequencies were very different, namely, 1.1 ± 0.1 Id4-GFP+ cells/tubule section (Chan et al., 2014) and about one PAX7+ cell in an entire testis cross-section, which usually contains >100 tubule sections (Aloisio et al., 2014). In addition, these genes show very different degrees of enrichment in the GFRα1+ fraction (Fig. 41). Furthermore, intravital live-imaging studies demonstrated that GFRα1+ cells continually interconvert between the Aa, Apr, and Aal states through incomplete division and intercellular bridge breakdown (Hara et al., 2014). Combined with a clonal fate analysis and mathematical modeling, the entire GFRα1+ population is proposed to comprise a single stem cell pool. Therefore, the spermatogenic stem cell system may be more complex and dynamic than it has been considered previously, with SHISA6+/T+ cells (or states) playing key roles. This possibility warrants further in-depth investigations. One hypothesis is that GFRα1+ cells may interconvert between SHISA6/T-positive and -negative states that show high and low potentials, respectively, for self-renewal.

Conclusion

In this study, based on mouse spermatogenesis, I attempted to clarify the mechanism underlying the maintenance of stem cells and production of differentiating cells in a facultative (or open) niche environment. Finally, I propose a generic mechanism that determines heterogeneous stem cell fates in facultative niche environments, in which there is no apparent definitive (or closed) niche and cells
do not divide asymmetrically. Different levels of a cell-autonomous inhibitor (SHISA6, in this case) may confer heterogeneous resistance to uniformly distributed differentiation-promoting extracellular signals (such as WNTs). In this model, stem cells exposed to higher levels of inhibitors would have a higher probability of remaining in the undifferentiated cell pool, whereas those exposed to lower levels of inhibitors would be more inclined to differentiate (Fig. 43). I believe this study provides a substantial contribution to the understanding of stem cell systems.
Materials and Methods

Animals

Ngn3/EGFP (Yoshida et al., 2006), Gfra1EGFP (Enomoto et al., 2000), Ctnnb1fl(ex3) (Harada et al., 1999), Ctnnb1f (also designated β-cateninflo or β-cateninΔex2-6f) (Brault et al., 2001), Nanos3-Cre (Suzuki et al., 2008), Gdnf-LacZ (Moore et al., 1996), CAG-CAT-EGFP (Kawamoto et al., 2000), R26R-H2B-mCherry (Abe et al., 2011), and TnEGFP-CreERT2 (Imuta et al., 2013) mice were described previously. The Shisa6 KO allele was generated as described in Supplemental Information. All mice were maintained in a C57BL/6 background (Japan CLEA or Charles River Japan). All animal experiments were conducted in accordance with guidelines of the Institutional Animal Care and Use Committee of National Institutes of Natural Sciences or as specified.

Generation of Shisa6 KO mice using the CRISPR/Cas9 system

The bicistronic expression vector (pX330; Addgene) was used to express Cas9 and an sgRNA. The sgRNA target sequence was CCAGTACGACAAGGAGTTCGAGT, taken from exon 1 of Shisa6. This expression vector was injected into fertilized eggs of C57BL/6J mice (Charles River Laboratories). The obtained mice were genotyped using genomic DNA prepared from clipped tails. PCR was carried out with one of the following primer pairs: GAGACGTGCTGGGGCTACTA and TTTGGTAGTTGGTGCACTGG or CAGCGCGACTGTTACTTACG and TGGGCCTTGTCATAGGAGAC using Extaq (#RR001A; Takara) and Thermal Cycler SimpliAmp (Applied Biosystems). These PCR products were purified using Amicon Ultra-0.5 mL filtration units (Merck Millipore) and directly sequenced with primer TTTGGTAGTTGGTGCACTGG or GTCTCCTATGACAAGGCCCA using a 3130xl Genetic Analyzer (Applied Biosystems). Thirty-two G0
founders (16 males and 16 females) were obtained. Of the 16 males, only the wild-type (WT) alleles were detected in eight mice, whereas the others had one or more mutant alleles in addition to the WT allele.

**Pulse-chase experiment**

\( T^{\text{EGFP-CreERT2}};R26R-H2B-mCherry \) or \( T^{\text{EGFP-CreERT2}};CAG-CAT-EGFP \) mice were injected intraperitoneally with 2.0 mg of 4-hydroxytamoxifen per individual (Sigma) as reported previously (Hara et al., 2014; Nakagawa et al., 2010). After specific chase periods, the testes were removed and analyzed by IF.

**Xenopus laevis embryo experiments**

Experiments using *Xenopus laevis* embryos were performed as described previously (Morita et al., 2010). Briefly, \( xwnt8, mShisa6, mShisa2 \) were subcloned into the \( pSP64T, pCSf107mT, pCSf107mT \) vectors, respectively, with which capped mRNAs were synthesized using an mMACHINE SP6 Kit (Ambion) and were purified on NICK columns (GE Healthcare). mRNAs were injected into the ventral marginal zone of four cell-stage embryos, as previously reported (Glinka et al., 1997). Injected embryos were incubated in 3% Ficoll/0.1× Steinberg’s solution at 13°C overnight, then in 0.3× Marc's Modified Ringer's solution at 13°C for 6 days until reaching stage 33–35.

**Cell sorting**

To purify GFRα1⁺, NGN3⁺, and KIT⁺ fractions, single-cell suspensions were prepared from the testes of 8–14-week-old \( Gfra1^{\text{EGFP/+}} \) and \( Ngn3/EGFP^{\text{gy/+}} \) mice, as described below. Testes were removed from the tunica albuginea, and seminiferous tubules were detangled. The tubules were incubated in
phosphate-buffered saline (PBS) with 2 mg/mL collagenase (#C5138; Sigma), 2 mg/mL hyaluronidase (#H3506; Sigma), and 0.01 mg/mL DNaseI (#D5025; Sigma) at 37°C for 15 min. Next, approximately 5 volumes of DMEM (#11885; Life Technologies) containing 10% FBS (#171012; CBC) was added, and cells were centrifuged at 300 × g. Supernatants were discarded, and the pellet was resuspended in Dulbecco’s PBS (DPBS; #14190; Life Technologies) containing 1% FBS. The cells were then passed through a 40 µm cell strainer (#352340; Falcon), and the cell concentrations were determined after staining with trypan blue (#207-17081; Wako). GFRα1+ fraction was collected as GFP+ fraction from the Gfra1EGFP/+ mice, while NGN3+ and KIT+ cells were collected as GFP+/KIT− and GFP+/KIT+ fractions, respectively, from the Ngn3/EGFPtg/+ mice, after stained with SPRD-conjugated anti-KIT antibodies (#734257; Beckman Coulter) or anti-KLH antibodies (#731675; Beckman Coulter) as an isotype control. Beckman Coulter FACS flow cytometer was used to fractionate these testicular single-cell suspensions.

To collect undifferentiated spermatogonia from Shisa6 mutant mice, cell suspensions prepared from the Shisa6+/+, Shisa6Δ6+502/+, Shisa6Δ6+502/Δ6+502, and Shisa6Δ6+502/Δ1 mice were stained with an anti-E-cadherin primary antibody (#M108; clone ECCD-2; Takara) and a PE-conjugated goat anti-rat IgG secondary antibody (#550767; BD Pharmingen), and the undifferentiated spermatogonia were purified as E-cadherin+ fractions. BD FACS Aria II was used to fractionate these testicular single-cell suspensions. Throughout, approximately 1-2 × 10⁴ cells were collected for each fraction and processed for microarray analysis. Sorted cells were lysed with 1% β-mercaptoethanol (#139-0686; Wako) in RLT buffer from an RNeasy Micro Kit (Qiagen) and stored at -80°C until analysis.
RNA isolation

Extraction of total RNA was carried out using an RNeasy Micro Kit (Qiagen) according to the manufacturer’s protocol. RNA quality and quantity was determined using a NanoDrop-1000 Spectrophotometer (NanoDrop Technologies).

Microarray analysis

cRNA synthesis from 25 ng total RNA and cyanine 3 (Cy3)-CTP labeling was carried out using a Low Input Quick Amp Labeling Kit (Agilent) according to the manufacturer’s protocol. Purification of labeled cRNA was performed using an RNeasy Mini Kit (Qiagen). The concentration and quality of labeled cRNA was determined using a NanoDrop instrument with an RNA Pico 6000 LabChip Kit (Agilent) and a 2100 Bioanalyzer (Agilent). The Cy3-labeled cRNA was hybridized to the SurePrint G3 Mouse GE 8x60K Microarray (Agilent) using a Gene Expression Hybridization Kit (Agilent). After hybridization, arrays were washed and dried according to the manufacturer’s instructions and then were scanned using an Agilent Technologies Scanner (G2505C and G2565CA) with the default settings for 8 × 60 k-format one-color arrays, or they were performed by Hokkaido System Science (Japan). Images were analyzed using Feature Extraction ver. 10.7.3.1 (Agilent). Three samples from different animals were used to analyze each spermatogonial fraction (for Ngn3+ cells, one sample was prepared by pooling three experimental compartments from two mice). Data collection and statistical analyses were performed using GeneSpring GX v11.5.1–13.0 (Silicon Genetics). Data correction was performed with the raw signal threshold set to 1.0, percent shift normalized to the 75th percentile, and no baseline transformation. The results were, in part, published previously (Ikami et al., 2015), with the dataset deposited in Gene Expression Omnibus under accession number GSE75532.
Cell culture

Established GS cells prepared from the testes of P7 mice obtained from intercrosses between C57BL/6J and ICR mice (Ogawa et al., 2004). GS cells were maintained according to a previously reported protocol (Kanatsu-Shinohara et al., 2003). Briefly, StemPro-34 SFM (#10639; Invitrogen) was used as a base medium and the following reagents were added: Stempro-34-supplement, 25 µg/mL insulin (#19251-24; Nacalai Tesque), 100 µg/mL transferrin (#205-18121; Wako), 60 µM putrescine (#P7505; Sigma), 30 nM sodium selenite (#S1382; Sigma), 6 mg/mL D-(+)-glucose (#G7021; Sigma), 200 µg/mL pyruvic acid (#P2256; Sigma), 1 µg/mL DL-lactic acid (#L4263; Sigma), 5 mg/mL bovine albumin (#810661; MP Biomedicals), 2 mM L-glutamine (#G7513; Sigma), 5 × 10⁻⁵ M β-mercaptoethanol, 1% (v/v) MEM vitamin solution (#11120-052; Invitrogen), 1% (v/v) MEM non-essential amino acids (#11140; Invitrogen), 1 × 10⁻⁴ L-ascorbic acid (#A4544; Sigma), 10 µg/mL D-biotin (#B4501; Sigma), 60 ng/mL progesterone (#P8783; Sigma), 100 U/mL penicillin-streptomycin (#15140-122; Gibco), 1% (v/v) FBS (Hyclone), 20 ng/mL recombinant mouse epidermal growth factor (EGF #354010; Corning), 15 ng/mL recombinant rat GDNF (#450-51; Peprotech), and 10 ng/mL recombinant human FGF2 (#100-18B; Peprotech). The cells were cultured at 37°C, and medium was changed every 2 to 4 days. Cells were passaged about every 7 days, depending on the proliferation state of the cells in each well and were maintained on a feeder layer of mouse embryonic fibroblasts (MEFs; Oriental Yeast, Co.), which were treated with mitomycin C (#M0503; Sigma) for 2 hr. The cells were maintained in 10% FBS/DMEM containing penicillin streptomycin and were passaged every 2 to 3 days, according to standard protocols. For IF, GS cells were fixed with 4% PFA in PBS at room temperature for 10 min, and processed for the incubation with anti-SHISA6 primary antibody and subsequent process described in the next section.
**RT-qPCR analyses**

RT-qPCR was performed with a LightCycler 480 system (Roche) and Thunderbird SYBR qPCR mix (#QPS-201; TOYOBO) after total RNA was reverse transcribed using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR primed with a mixture of oligo(dT) and random hexamers (#18080400; Invitrogen). Expression was normalized to that of β-actin. Detailed procedures and the primers used are listed below.

**IF analysis of sections and whole-mount specimens**

Excised testis samples, either perfusion-fixed with 4% PFA in PBS or removed from anesthetized animals without perfusion, were fixed in 4% PFA overnight at 4°C, embedded in Tissue-Tek OCT Compound (#4583; Sakura), and processed for cryosectioning using a cryostat (Bright OTF5000; Scilogex). After blocking with TNB blocking buffer (#FP1020; PerkinElmer) containing 4% donkey serum (DS; Jackson ImmunoResearch) and Hoechst 33342 (Invitrogen, Life Technologies) at room temperature for 1 h, the sections were incubated with primary antibodies overnight in 4% DS/TNB blocking buffer at the dilutions indicated below and then washed three times in PBS containing 0.05% Tween-20 (PBST; #1379; Sigma) for 10 min. Tissues were then incubated with secondary antibody for 1 h at room temperature. For staining with anti-SHISA6 antibody, unfixed tissues were embedded directly in OCT compound and sectioned. Samples were then immersed in methanol or acetone at room temperature for 30 min, followed by the same staining protocol.

Whole-mount IF of seminiferous tubules was performed as previously described (Nakagawa et al., 2010). After the testes were dissected in PBS, untangled seminiferous tubules were fixed for 3 hr in
4% PFA in PBS and attached to MAS-GP-coated slide glasses (Matsunami). The samples were then dehydrated through a methanol series and subsequently rehydrated in PBST. Finally, the specimens were treated as described for IF above.

The primary antibodies used in this study and their dilution factors (in parentheses) were as follows:
- anti-GFRα1 goat polyclonal antibodies (1:200-1000; #AF560; R&D Systems),
- anti-RARγ rabbit monoclonal antibody (1:200; #5916; clone#D3A4; Cell Signaling),
- anti-KIT rat monoclonal antibody (1:200; #553356; clone#2B8; BD Pharmingen),
- anti-GFP rabbit and rat polyclonal antibodies (1:300; #A11122; Invitrogen, Life Technologies; and 1:200; #04404-84; Nacalai Tesque),
- anti-SHISA6 rabbit polyclonal antibodies (1:25-50; #HPA023440; Atlas Antibodies),
- anti-DsRed rabbit polyclonal antibodies (1:200; #632496; Clontech),
- anti-MVH rabbit polyclonal antibodies (1:200; #13840; Abcam),
- anti-non-phospho (active)-Ctnnb1 antibody (1:200; #1499; clone#D13A1; Cell Signaling),
- and anti-phospho-histone H3 rabbit polyclonal antibodies (1:200; #06-570; Merck millipore).

The secondary antibodies, all manufactured by Invitrogen, were as follows (all diluted 1:1000): Alexa 488-conjugated anti-goat (#A11055), anti-rabbit (#A21206), and anti-rat (#A21208) IgG antibodies; Alexa 594-conjugated anti-goat (#A11058), anti-rabbit (#A21207), and anti-rat (#A21209) IgG antibodies; and Alexa 647-conjugated anti-rat (#A21247) and anti-goat (#A21447) IgG antibodies.

Throughout, observations and measurements were performed using a BX51 upright fluorescence microscope equipped with a DP72 CCD camera (Olympus) or a confocal fluorescence microscope DM6000CFS (Leica).

ISH

ISH with paraffin-embedded, 4% PFA-fixed testis sections was performed using digoxigenin
(Cadigan and Nusse)-labeled riboprobes and BM Purple AP Substrate (#11442074001; Roche) as previously described (Yoshida et al., 2001). Stages of the seminiferous epithelium cycle were determined on adjacent sections subjected to periodic acid-Schiff (PAS) staining using Schiff’s Reagent (Wako). Probes used for ISH were prepared as follows: Probes of all mouse Wnt ligands were prepared by using FANTOM Clones (DNAFORM) shown in the following table.

I used *E. coli* clones carrying plasmids that contained the respective cDNA sequences as templates for PCR to amplify the cDNA sequence using the primer pair CGACGTTGTAACCGACGCGCCAGTG and AGCGGATAAACATTTCACACAGGAAAC. The PCR product was purified with a Wizard SV Gel and PCR Clean-up System (#A9282; Promega) and used as a template for cRNA synthesis. A WNT3a probe harboring a full-length mouse WNT3a cording sequence was used (Roelink and Nusse, 1991). The plasmid was linearized by a *Bam*HI digestion and used as a template for cRNA synthesis. A Wnt8a probe was prepared from purchased plasmid. Using *Afi*I, the probe was linearized and became a template for cRNA synthesis. Using these templates, digoxigenin-labeled cRNA probes were synthesized using a DIG RNA Labeling Kit (Roche) according to the manufacturer’s protocol. The synthesized probes were fragmented using an alkaline treatment reagent (63 mM Na₂CO₃) containing 1 mM dithiothreitol (Nacalai Tesque).

**FISH and IF of dispersed testicular cells**

ISH of dispersed single cells was carried out using an RNAscope Fluorescent Multiplex Kit (Advanced Cell Diagnostics). Testes from 8–14-week-old WT or *T<sup>EGFP-CreER<sub>12/1</sub></sup>* mice were processed to generate single-cell suspensions as described above for cell sorting. The single-cell suspensions were applied to an MAS-GP-coated slide glasses (Matsunami) for detection of *Shisa6* RNA using a
specific target probe (#435181A; Advanced Cell Diagnostics) and HybEZ Hybridization System (#310010A; Advanced Cell Diagnostics). The same slides were processed for IF using anti-GFRα1 or anti-GFP antibodies according to the methods described above for IF. The observations and measurements were performed using a BX51 upright fluorescence microscope equipped with a DP72 CCD camera (Olympus) or a confocal fluorescence microscope DM6000CFS (Leica).

**Luciferase assay**

Luciferase assays were performed using a Dual-Luciferase Assay System (#1960; Promega). GS or HEK293T cells, which had been transfected with combinations of plasmids including firefly and Renilla luciferase reporters using Lipofectamine 2000 (#11668-019; Thermo Fisher), were washed with DPBS and dissolved in the lysis buffer. Luciferase activities of the lysates were then measured using a Glomax a 20/20 luminometer (Promega). Renilla luciferase activities derived from co-transfected plasmids were used to normalize the firefly luciferase activities.

For the experiments shown in Fig. 24D–F, the following elaborate experimental design was applied before cells were lysed for luciferase assays. Because Shisa6-transfected HEK293T cells were found to be severely damaged by the trypsinization process required for co-culturing of separately transfected cells, we used a Tet-On 3G Inducible Expression System (#631165; Clontech), in which the timing of Shisa6 expression could be controlled by the addition of doxycycline (DOX). HEK293T cells were seeded on 6-well plates at a density of 8 × 10^4 cells/well in DMEM containing 10% FBS. The next day, cells were transfected with the combinations of plasmids shown in the table below. Detailed information on the plasmids is given at the end of this section. Transfections were performed using Lipofectamine 2000 reagent as recommended by the manufacturer. Briefly, a
mixture of plasmid DNA (4.44 μg) was diluted in 100 μL Opti-MEM (#31985062; Thermo Fisher) and then mixed with Lipofectamine 2000 solution (4 μL Lipofectamine 2000 diluted in 100 μL/well Opti-MEM 5 min prior to transfection). After incubation at room temperature for 20 min, the transfection mix was poured onto the cells in place of the culture medium, followed by incubation at 37°C for 24 hr. Then, the cells were trypsinized, mixed according to the combination shown below at a ratio of 1:1, and reseeded at a density of 2.5 × 10^4 cells/well in 48-well plates. Twelve hours later, when the cells had adhered to the bottoms of wells, the medium was changed to fresh medium containing 1 μg/mL DOX (#D9891; Sigma) and the indicated concentration of ligands. After 24 hr, cells were harvested for measurement of luciferase activity. The ligands used were recombinant mouse WNT3a (#1324-WNP-010/CF; R&D Systems), human FGF2 (#100-18B; Peprotech), and rat GDNF (#450-51; Peprotech), all applied at a concentration of 200 ng/mL.

For the Shisa6 knockdown experiment in GS cells using siRNA transfection (Fig. 26), GS cells were seeded in 48-well plates at a density of 0.75 × 10^4 cells/well. The next day, the cells were transfected with Silencer Select pre-designed (Non-inventoried) siRNA for Shisa6 (#s117317; Ambion) or Silencer Select Negative Control No. 2 siRNA (#4390846; Ambion) with a reporter plasmid Ngn3-luc. After 24 hr of incubation, the medium was changed to fresh medium containing recombinant WNT3a and R-spondin2 (#51078; Sino Biological) at a concentration of 200 ng/mL. Half of the medium was changed every 24 hr two times before luciferase assay was performed. The plasmids used for luciferase assays in this study are listed below.

**Plasmids**

*Ngn3-luc* is a derivative of pGL3 (Promega) harboring a 7.0-kb genomic sequence of the mouse
Ngn3 gene upstream to the start codon (Gu et al., 2002; Yoshida et al., 2004). pT2-TCF-mini-Eluc (Shimizu et al., 2012) was used to monitor the activity of the Wnt/β-catenin signaling pathways, while pGL4.33 (luc2P/SRE/Hygro; designated as SRE-luc; # E1340; Promega) was used to monitor the activity of FGF and GDNF. pGL4.70 (hRluc; designated as renilla-luc; # E6881; Promega) was used as an internal control. pCMV6-Kan/Neo-Gfra1 and pCMV6-Kan/Neo-Ret (#MC203605and #MC206083; Origene) plasmids harboring mouse Gfra1 (Genbank: NM_010279) and Ret (Genbank: BC059012) coding sequences, respectively. These plasmids were co-transfected to reconstitute the GDNF receptor complex in HEK293T cells. pCMV-Tet3G (Clontech) was used to supply the Tet-activator protein necessary for induction of the gene of interest from pTRE3G-BI-mCherry vector (Clontech). pcDNA3-Shisa6 and pTRE3G-BI-mCherry-Shisa6, used for constitutive and
DOX-dependent expression of Shisa6, were generated by inserting a Shisa6 coding sequence (Genbank: XM_006533619) into pcDNA3 and pTRE3G-BI-mCherry, respectively. The Shisa6 coding sequence was obtained by modifying the FANTOM Clone M5C1056A20 (DANAFORM) to include exon 3 from WT mouse testes. pcDNA3-Shisa2, a constitutive Shisa2 expression plasmid, was constructed by subcloning the coding sequence from the FANTOM Clone E130308O13 (DANAFORM, Genbank: AK087512) into pcDNA3.
Table: List of all mouse Wnt ligands *in situ* hybridization probes

| Genes | Accession # | FANTOM clone ID | bp   |
|-------|-------------|-----------------|------|
| Wnt1  | AK141669    | D030017M24      | 2225 |
| Wnt2  | AK045120    | B130036G12      | 2027 |
| Wnt2b/13 | AK035653   | 9530081G10      | 3576 |
| Wnt3  | AK132516    | 4732488C12      | 3058 |
| Wnt3a | (Roelink and Nusse, 1991) |           | about 1400 |
| Wnt4  | AK012727    | 2810012H16      | 1363 |
| Wnt5a | AK036824    | 9930016B13      | 3652 |
| Wnt5b | AK049558    | C430039I17      | 2139 |
| Wnt6  | AK133954    | 5730559H23      | 2058 |
| Wnt7a | AK004683    | 1200010A19      | 3177 |
| Wnt7b | AK087499    | E130306O05      | 3402 |
| Wnt8a | BC120517 (Thermo Scientific) |           | about 1500 |
| Wnt8b | AK169459    | A630023D20      | 2875 |
| Wnt9a | AK087490    | E130305L02      | 3325 |
| Wnt9b | AK052708    | D630033J01      | 4522 |
| Wnt10a | AK013836   | 2900091F02      | 1347 |
| Wnt10b | AK029284   | 4832413L14      | 2706 |
| Wnt11 | AK077828    | 5930404H12      | 2722 |
| Wnt16 | AK054110    | E230020A13      | 1664 |

Combination of co-transfected plasmids (Ventea et al.) and co-cultured transfected cells

(bottom)

|  | 0.4 µg | 3 µg | 0.5 µg | 0.5 µg | 0.02 µg | 0.02 µg |
|---|--------|------|--------|--------|---------|---------|
| #1 | Renilla-luc | TCF-luc | pcDNA3 | | pTRE3G-Bi-mCherry | pCMV-Tet3G |
| #2 | Renilla-luc | SRE-luc | pcDNA3 | | pTRE3G-Bi-mCherry | pCMV-Tet3G |
| #3 | Renilla-luc | SRE-luc | GFRα1/pcDNA3 | Ret/pcDNA3 | pTRE3G-Bi-mCherry | pCMV-Tet3G |
| #4 | Renilla-luc | TCF-luc | pcDNA3 | | Shisa6/pTRE3G-Bi-mCherry | pCMV-Tet3G |
| #5 | Renilla-luc | SRE-luc | pcDNA3 | | Shisa6/pTRE3G-Bi-mCherry | pCMV-Tet3G |
| #6 | Renilla-luc | SRE-luc | GFRα1/pcDNA3 | Ret/pcDNA3 | Shisa6/pTRE3G-Bi-mCherry | pCMV-Tet3G |
| #7 | pcDNA3 | | | | pTRE3G-Bi-mCherry | pCMV-Tet3G |
| #8 | pcDNA3 | | | | Shisa6/pTRE3G-Bi-mCherry | pCMV-Tet3G |
*Numbers indicate mixed cell preparations transfected with the respective plasmid DNAs shown in the top table.

**Primers used for qRT-PCR**

Primers used for qRT-PCR were as follows: *Ngn3*, CGGGAGAAGTCATGCGGCGCCT and ACTTCGTGGTCCGAGGCTCCGG; *Shisa6*, AACAGACTCCAGGTGATCG and TGGCCGACGTCAGATGTTA; and β-actin, GGCTGTATTCCCCTCGATCG and CCAGTTGGTAACATGCCATGT.
References

Abe, T., Kiyonari, H., Shioi, G., Inoue, K.I., Nakao, K., Aizawa, S., and Fujimori, T. (2011). Establishment of conditional reporter mouse lines at ROSA26 locus for live cell imaging. Genesis 49, 579–590.

Aloisio, G.M., Nakada, Y., Saatcioglu, H.D., Pena, C.G., Baker, M.D., Tarnawa, E.D., Mukherjee, J., Manjunath, H., Bugde, A., Sengupta, A.L., et al. (2014). PAX7 expression defines germline stem cells in the adult testis. J Clin Invest 124, 3929–3944.

Badders, N.M., Goel, S., Clark, R.J., Klos, K.S., Kim, S., Bafico, A., Lindvall, C., Williams, B.O., and Alexander, C.M. (2009). The Wnt receptor, Lrp5, is expressed by mouse mammary stem cells and is required to maintain the basal lineage. PLoS One 4, e6594.

Brault, V., Moore, R., Kutsch, S., Ishibashi, M., Rowitch, D.H., McMahon, A.P., Sommer, L., Boussadia, O., and Kemler, R. (2001). Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. Development 128, 1253–1264.

Cadigan, K.M., and Nusse, R. (1997). Wnt signaling: a common theme in animal development. Genes Dev 11, 3286–3305.

Chan, F., Oatley, M.J., Kaucher, A.V., Yang, Q.E., Bieberich, C.J., Shashikant, C.S., and Oatley, J.M. (2014). Functional and molecular features of the Id4+ germline stem cell population in mouse testes. Genes Dev 28, 1351–1362.

Chapman, K.M., Medrano, G.A., Chaudhary, J., and Hamra, F.K. (2015). NRG1 and KITL signal downstream of retinoic acid in the germline to support soma-free syncytial growth of differentiating spermatogonia. Cell Death Discov 1.

Chen, C.C., Chen, H.Y., Su, K.Y., Hong, Q.S., Yan, B.S., Chen, C.H., Pan, S.H., Chang, Y.L., Wang, C.J., Hung, P.F., et al. (2014). Shisa3 is associated with prolonged survival through promoting beta-catenin degradation in lung cancer. Am J Respir Crit Care Med 190, 433–444.

Choi, Y.S., Zhang, Y., Xu, M., Yang, Y., Ito, M., Peng, T., Cui, Z., Nagy, A., Hadjantonakis, A.K., Lang, R.A., et al. (2013). Distinct functions for Wnt/beta-catenin in hair follicle stem cell proliferation and survival and interfollicular epidermal homeostasis. Cell Stem Cell 13, 720–733.

Clevers, H., and Nusse, R. (2012). Wnt/beta-catenin signaling and disease. Cell 149, 1192–1205.

Cruciat, C.M., and Niehrs, C. (2013). Secreted and transmembrane wnt inhibitors and activators. Cold Spring Harb Perspect Biol 5, a015081.

de Cuevas, M., and Matunis, E.L. (2011). The stem cell niche: lessons from the Drosophila testis. Development 138, 2861–2869.
de Lau, W., Barker, N., Low, T.Y., Koo, B.K., Li, V.S.W., Teunissen, H., Kujala, P., Haegebarth, A., Peters, P.J., van de Wetering, M., et al. (2011). Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. Nature 476, 293–297.

Enomoto, H., Heuckeroth, R.O., Golden, J.P., Johnson, E.M., and Milbrandt, J. (2000). Development of cranial parasympathetic ganglia requires sequential actions of GDNF and neurturin. Development 127, 4877–4889.

Fuller, M.T., and Spradling, A.C. (2007). Male and female Drosophila germline stem cells: two versions of immortality. Science 316, 402–404.

Furusshima, K., Yamamoto, A., Nagano, T., Shibata, M., Miyachi, H., Abe, T., Ohshima, N., Kiyonari, H., and Aizawa, S. (2007). Mouse homologues of Shisa antagonistic to Wnt and Fgf signalings. Dev Biol 306, 480–492.

Glinka, A., Wu, W., Onichtchouk, D., Blumenstock, C., and Niehrs, C. (1997). Head induction by simultaneous repression of Bmp and Wnt signalling in Xenopus. Nature 389, 517–519.

Grisci, L., Falciatori, I., Grasso, M., Dovere, L., Fera, S., Muciaccia, B., Fusco, A., Berno, V., Boitani, C., Stefanini, M., et al. (2009). Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. Stem Cells 27, 3043–3052.

Gu, G., Dubauskaite, J., and Melton, D.A. (2002). Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 129, 2447–2457.

Hara, K., Nakagawa, T., Enomoto, H., Suzuki, M., Yamamoto, M., Simons, B.D., and Yoshida, S. (2014). Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. Cell Stem Cell 14, 658–672.

Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M., and Taketo, M.M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. EMBO J 18, 5931–5942.

Hovanes, K., Li, T.W., Munguia, J.E., Truong, T., Milovanovic, T., Lawrence Marsh, J., Holcombe, R.F., and Waterman, M.L. (2001). Beta-catenin-sensitive isoforms of lymphoid enhancer factor-1 are selectively expressed in colon cancer. Nat Genet 28, 53–57.

Huels, D.J., Ridgway, R.A., Radulescu, S., Leushacke, M., Campbell, A.D., Biswas, S., Leedham, S., Serra, S., Chetty, R., Moreaux, G., et al. (2015). E-cadherin can limit the transforming properties of activating beta-catenin mutations. EMBO J 34, 2321–2333.

Ikami, K., Tokue, M., Sugimoto, R., Noda, C., Kobayashi, S., Hara, K., and Yoshida, S. (2015). Hierarchical differentiation competence in response to retinoic acid ensures stem cell maintenance during mouse spermatogenesis. Development 142, 1582–1592.

Imuta, Y., Kiyonari, H., Jang, C.W., Behringer, R.R., and Sasaki, H. (2013). Generation of knock-in mice that express nuclear enhanced green fluorescent protein and tamoxifen-inducible Cre
recombinase in the notochord from Foxa2 and T loci. Genesis 51, 210–218.

Ishii, K., Kanatsu-Shinohara, M., Toyokuni, S., and Shinohara, T. (2012). FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of Etv5 and Bcl6b through MAP2K1 activation. Development 139, 1734–1743.

Kanatsu-Shinohara, M., Inoue, K., Lee, J., Miki, H., Ogonuki, N., Toyokuni, S., Ogura, A., and Shinohara, T. (2006). Anchorage-independent growth of mouse male germline stem cells in vitro. Biol Reprod 74, 522–529.

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S., and Shinohara, T. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod 69, 612–616.

Kanatsu-Shinohara, M., Ogonuki, N., Iwano, T., Lee, J., Kazuki, Y., Inoue, K., Miki, H., Takehashi, M., Toyokuni, S., Shinkai, Y., et al. (2005). Genetic and epigenetic properties of mouse male germline stem cells during long-term culture. Development 132, 4155–4163.

Kawamoto, S., Niwa, H., Tashiro, F., Sano, S., Kondoh, G., Takeda, J., Tabayashi, K., and Miyazaki, J. (2000). A novel reporter mouse strain that expresses enhanced green fluorescent protein upon Cre-mediated recombination. FEBS Lett 470, 263–268.

Kim, K.A., Kakitani, M., Zhao, J., Oshima, T., Tang, T., Binnerts, M., Liu, Y., Boyle, B., Park, E., Emtage, P., et al. (2005). Mitogenic influence of human R-spondin1 on the intestinal epithelium. Science 309, 1256–1259.

Klaassen, R.V., Stroeder, J., Coussen, F., Hafner, A.S., Petersen, J.D., Renancio, C., Schmitz, L.J., Normand, E., Lodder, J.C., Rotaru, D.C., et al. (2016). Shisa6 traps AMPA receptors at postsynaptic sites and prevents their desensitization during synaptic activity. Nat Commun 7, 10682.

Komai, Y., Tanaka, T., Tokuyama, Y., Yanai, H., Ohe, S., Omachi, T., Atsumi, N., Yoshida, N., Kumano, K., Hisha, H., et al. (2014). Bmi1 expression in long-term germ stem cells. Sci Rep 4, 6175.

Kubota, H., Avarbock, M.R., and Brinster, R.L. (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 101, 16489–16494.

Lee, H.Y., Kleber, M., Hari, L., Brault, V., Suter, U., Taketo, M.M., Kemler, R., and Sommer, L. (2004). Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. Science 303, 1020–1023.

Lim, X., Tan, S.H., Koh, W.L., Chau, R.M., Yan, K.S., Kuo, C.J., van Amerongen, R., Klein, A.M., and Nusse, R. (2013). Interfollicular epidermal stem cells self-renew via autocrine Wnt signaling. Science 342, 1226–1230.

Meng, X., Lindahl, M., Hyvonen, M.E., Parvinen, M., de Rooij, D.G., Hess, M.W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., et al. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287, 1489–1493.
Miyagi, A., Negishi, T., Yamamoto, T.S., and Ueno, N. (2015). G protein-coupled receptors Flop1 and Flop2 inhibit Wnt/beta-catenin signaling and are essential for head formation in Xenopus. Dev Biol 407, 131–144.

Moon, R.T., Bowerman, B., Boutros, M., and Perrimon, N. (2002). The promise and perils of Wnt signaling through beta-catenin. Science 296, 1644–1646.

Moore, M.W., Klein, R.D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L.F., Ryan, A.M., Carver-Moore, K., and Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. Nature 382, 76–79.

Morita, H., Nandadasa, S., Yamamoto, T.S., Terasaka-Iioka, C., Wylie, C., and Ueno, N. (2010). Nectin-2 and N-cadherin interact through extracellular domains and induce apical accumulation of F-actin in apical constriction of Xenopus neural tube morphogenesis. Development 137, 1315–1325.

Morrison, S.J., and Spradling, A.C. (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell 132, 598–611.

Nagano, T., Takehara, S., Takahashi, M., Aizawa, S., and Yamamoto, A. (2006). Shisa2 promotes the maturation of somitic precursors and transition to the segmental fate in Xenopus embryos. Development 133, 4643–4654.

Nakagawa, T., Nabeshima, Y., and Yoshida, S. (2007). Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. Dev Cell 12, 195–206.

Nakagawa, T., Sharma, M., Nabeshima, Y., Braun, R.E., and Yoshida, S. (2010). Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science 328, 62–67.

Oakberg, E.F. (1956). Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. Am J Anat 99, 507–516.

Oakberg, E.F. (1971). Spermatogonial stem-cell renewal in the mouse. Anat Rec 169, 515–531.

Ogawa, T., Ohmura, M., Tamura, Y., Kita, K., Ohbo, K., Suda, T., and Kubota, Y. (2004). Derivation and morphological characterization of mouse spermatogonial stem cell lines. Arch Histol Cytol 67, 297–306.

Pei, J.M., and Grishin, N.V. (2012). Unexpected diversity in Shisa-like proteins suggests the importance of their roles as transmembrane adaptors. Cell Signal 24, 758–769.

Peltz, S.W., Brown, A.H., and Jacobson, A. (1993). mRNA destabilization triggered by premature translational termination depends on at least three cis-acting sequence elements and one trans-acting factor. Genes Dev 7, 1737–1754.

Phillips, B.T., Gassei, K., and Orwig, K.E. (2010). Spermatogonial stem cell regulation and spermatogenesis. Philos Trans R Soc Lond B Biol Sci 365, 1663–1678.

Potten, C.S., and Loeffler, M. (1990). Stem cells: attributes, cycles, spirals, pitfalls and uncertainties.
Lessons for and from the crypt. Development 110, 1001–1020.

Rabbani, P., Takeo, M., Chou, W.C., Myung, P., Bosenberg, M., Chin, L., Taketo, M.M., and Ito, M. (2011). Coordinated activation of Wnt in epithelial and melanocyte stem cells initiates pigmented hair regeneration. Cell 145, 941–955.

Raff, M. (2003). Adult stem cell plasticity: fact or artifact? Annu Rev Cell Dev Biol 19, 1–22.

Roelink, H., and Nusse, R. (1991). Expression of two members of the Wnt family during mouse development—restricted temporal and spatial patterns in the developing neural tube. Genes Dev 5, 381–388.

Russell, L.D., Ettlin, R.A., Sinha, H.A.P., and Clegg, E.D. (1990). Histological and histopathological evaluation of the testis. Cache River Press.

Sato, N., Meijer, L., Skalsounis, L., Greengard, P., and Brivanlou, A.H. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nature medicine 10, 55–63.

Sato, T., van Es, J.H., Snippert, H.J., Stange, D.E., Vries, R.G., van den Born, M., Barker, N., Shroyer, N.F., van de Wetering, M., and Clevers, H. (2011). Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature 469, 415–418.

Shimomura, Y., Agalliu, D., Vonica, A., Luria, V., Wajid, M., Baumer, A., Belli, S., Petukhova, L., Schinzel, A., Brivanlou, A.H., et al. (2010). APCDD1 is a novel Wnt inhibitor mutated in hereditary hypotrichosis simplex. Nature 464, 1043–1047.

Snippert, H.J., van der Flier, L.G., Sato, T., van Es, J.H., van den Born, M., Kroon-Veenboer, C., Barker, N., Klein, A.M., van Rheenen, J., Simons, B.D., et al. (2010). Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. Cell 143, 134–144.

Soshnikova, N., Zechner, D., Huelsken, J., Mishina, Y., Behringer, R.R., Taketo, M.M., Crenshaw, E.B., 3rd, and Birchmeier, W. (2003). Genetic interaction between Wnt/beta-catenin and BMP receptor signaling during formation of the AER and the dorsal-ventral axis in the limb. Genes Dev 17, 1963–1968.

Stine, R.R., and Matunis, E.L. (2013). Stem cell competition: finding balance in the niche. Trends Cell Biol 23, 357–364.

Suzuki, H., Tsuda, M., Kiso, M., and Saga, Y. (2008). Nanos3 maintains the germ cell lineage in the mouse by suppressing both Bax-dependent and -independent apoptotic pathways. Dev Biol 318, 133–142.

Takase, H.M., and Nusse, R. (2016). Paracrine Wnt/beta-catenin signaling mediates proliferation of undifferentiated spermatogonia in the adult mouse testis. Proc Natl Acad Sci U S A 113, E1489–1497.

Takeichi, M. (2014). Dynamic contacts: rearranging adherens junctions to drive epithelial remodelling.
Nat Rev Mol Cell Biol 15, 397–410.
Tokuda, M., Kadokawa, Y., Kurahashi, H., and Marunouchi, T. (2007). CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. Biol Reprod 76, 130–141.
von Engelhardt, J., Mack, V., Sprengel, R., Kavenstock, N., Li, K.W., Stern-Bach, Y., Smit, A.B., Seeburg, P.H., and Monyer, H. (2010). CKAMP44: a brain-specific protein attenuating short-term synaptic plasticity in the dentate gyrus. Science 327, 1518–1522.
Wu, X., Goodyear, S.M., Tobias, J.W., Avarbock, M.R., and Brinster, R.L. (2011). Spermatogonial stem cell self-renewal requires ETV5-mediated downstream activation of Brachyury in mice. Biol Reprod 85, 1114–1123.
Yamamoto, A., Nagano, T., Takehara, S., Hibi, M., and Aizawa, S. (2005). Shisa promotes head formation through the inhibition of receptor protein maturation for the caudalizing factors, Wnt and FGF. Cell 120, 223–235.
Yeh, J.R., Zhang, X., and Nagano, M.C. (2011). Wnt5a is a cell-extrinsic factor that supports self-renewal of mouse spermatogonial stem cells. J Cell Sci 124, 2357–2366.
Yeh, J.R., Zhang, X., and Nagano, M.C. (2012). Indirect effects of Wnt3a/beta-catenin signalling support mouse spermatogonial stem cells in vitro. PLoS One 7, e40002.
Yoshida, S. (2015). From cyst to tubule: innovations in vertebrate spermatogenesis. Wiley Interdiscip Rev Dev Biol 5, 119–131.
Yoshida, S., Ohbo, K., Takakura, A., Takebayashi, H., Okada, T., Abe, K., and Nabeshima, Y. (2001). Sgn1, a basic helix-loop-helix transcription factor delineates the salivary gland duct cell lineage in mice. Dev Biol 240, 517–530.
Yoshida, S., Sukeno, M., Nakagawa, T., Ohbo, K., Nagamatsu, G., Suda, T., and Nabeshima, Y. (2006). The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. Development 133, 1495–1505.
Yoshida, S., Takakura, A., Ohbo, K., Abe, K., Wakabayashi, J., Yamamoto, M., Suda, T., and Nabeshima, Y. (2004). Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. Dev Biol 269, 447–458.
Yoshida, Y. (in press). Regulatory mechanism of spermatogenic stem cells in mice: their dynamic and context-dependent behavior. In Reproductive & Developmental Strategies, K. Kobayashi, T. Kitano, Y. Iwao, and M. Kondo, eds. (Springer).
Zhang, X., Abreu, J.G., Yokota, C., MacDonald, B.T., Singh, S., Coburn, K.L., Cheong, S.M., Zhang, M.M., Ye, Q.Z., Hang, H.C., et al. (2012). Tiki1 is required for head formation via Wnt cleavage-oxidation and inactivation. Cell 149, 1565–1577.
Figure 1. Spermatogenesis in mouse

(A–C) Schema of mouse testis and seminiferous tubule. In the testis, seminiferous tubules are packed. This scheme indicates a testis with the tunica albuginea removed. The right panel is an expanded image of the insert (A). (B) Schema of mouse sperm development. (C) Schema of the mouse seminiferous epithelium cycle.
Figure 2. Construction of mouse spermatogonia

Schema of undifferentiated and differentiating spermatogonia and their differentiation process. Relationship between GFRα1, NGN3, and KIT, spermatogonial markers, expressing populations.
Figure 3. Wnt/β-catenin signaling

(A) In the absence of WNT, β-catenin, an integral E-cadherin cell-cell adhesion adaptor and signal transducers, is targeted by coordinated phosphorylation by CK1 and the AXIN/GSK-3β-complex leading to its ubiquitination and proteasomal degradation in the cytoplasm. DKKs interacts with the Wnt co-receptor LRP5/6 and inhibits Wnt signaling. WIF1 and sFRP trap Wnt ligands and inhibit Wnt signaling. (B) When WNT is associated with Frizzled, the co-receptor LRP5/6 contacts with Wnt-bound Frizzled. This leads to activation of Dishevelled (DVL) by sequential phosphorylation, poly-ubiquitination, and polymerization, which displaces GSK-3β, therefore β-catenin is released from the complex and translocates to the nucleus. In the nucleus, β-catenin forms a transcriptionally active complex with the TCF/LEF family of transcription factors, and Wnt target genes are activated.
Figure 4. Sorting of GFRα1⁺, NGN3⁺, and KIT⁺ spermatogonia from adult mouse testes by fluorescence-activated cell sorting (FACS)

FACS profiles upon fractionation of GFRα1⁺, NGN3⁺, and KIT⁺ spermatogonia. GFRα1⁺ cells were collected as the GFP⁺ fraction of testicular cells from adult (8–14-week-old) Gfra1EGFP⁺ mice (upper right panel; Enomoto et al., 2000). NGN3⁺ and KIT⁺ cells were collected from 8–14-week-old Ngn3/EGFP⁺ mice (Yoshida et al., 2004) as GFP⁻/KIT⁻ and GFP⁺/KIT⁺ fractions, respectively (lower panels). Staining for KIT was carried out using a PE-Cy5-conjugated anti-KIT antibody, and testes from WT and Ngn3/EGFP⁺ mice were stained with an isotype control as a negative control (lower right panel and data not shown).
Figure 5. Expression levels of GFRα1⁺, NGN3⁺, and KIT⁺ in sorted cells and a pathway analysis

(A) Qualification of the fractionation and microarray analysis based on the signal strengths of Gfra1, Ngn3, and Kit in our microarray data. For each fraction, means ± SEM of three independent RNA samples from different animals are shown. Signal intensities were normalized to the 75th percentile and are shown on a linear scale.

(B) Cells of candidate signaling pathways that induce differentiation signals for the GFRα1⁺-cell-to-NGN3⁺ cell transition. Results were obtained by a pathway analysis in GeneSpring 13.0. Upregulated genes with P ≤ 0.05 and fold change ≥ 2 between the GFRα1⁺ and NGN3⁺ cell compartments were analyzed by the program. Pathways with P-values ≥ 1 were shown.
Figure 6. Up-regulation of Ngn3 mRNA by Wnt/β-catenin signaling in cultured gonial cells

Expression of Ngn3 mRNA in GS cells in the presence or absence of GDNF, WNT3a, or WNT5a. GS cells cultured on laminin-coated plates for 24 h were switched to the indicated conditions and cultured for an additional 24 h, followed by RT-qPCR analysis of Ngn3 mRNA. Represented as means ± SEM (n = 3 independent experiments). *P < 0.05, **P < 0.001 (Student’s t test).
Figure 7. Expression profiles of Wnt related factors in spermatogonia and whole testis

Expression of genes related to Wnt/β-catenin signaling in GFRα1*, NGN3*, KIT* fractions and whole testis, summarized from the microarray data. For genes that have multiple probes on the microarray, the probes IDs are indicated. Signal intensities were normalized by the 75th percentile and are shown in a linear scale. Represented as means ± SEM (n = 3 microarrays, each form different mice).
**Figure 8.** *Nanos3*<sup>Cre/+</sup> expresses Cre protein in germ cell specific

(A) Schematics of the *Nanos3*<sup>Cow/+</sup> and CAG-CAT-EGFP allele, producing EGFP protein after Cre-mediated recombination. (B, C) Representative IF images of cryosectioned testes from adult CAG-CAT-EGFP<sup>Tg/Tg</sup> mice (B) and *Nanos3*<sup>Cow/+</sup>, CAG-CAT-EGFP<sup>Tg/+</sup> mice (C), stained for GFP. Seminiferous tubules are outlined by a dotted line. Note that most of the tubules were filled with GFP<sup>+</sup> germ cells in the latter, indicating efficient recombination under the *Nanos3*<sup>Cow/+</sup> background. A small portion (comprising a few percent) of germ cells was also found to be GFP negative (asterisk). Scale bar: 100 μm.
Figure 9. Impact of stabilized β-catenin on mouse testes

(A) Schematics of the Ctnnb1^{fl(ex3)/} allele, producing stabilized β-catenin protein after Cre-mediated recombination. (B-F) Representative appearance of the testes of adult mice of the indicated genotypes (B-D), and their testis weights (E) and body weights (F). Actual values from different individuals and their averages are shown by dots and columns. Scale bar: 1 mm.
Figure 10. Impact of stabilized β-catenin on spermatogenesis in vivo

(A-C) Representative histological images of testicular sections of adult mice for the indicated genotypes, stained with PAS-hematoxylin. Lower panels are the magnified images of the indicated regions in the upper panels. Open and closed red dots indicate the tubule sections with lost or exiguous germ cell layer(s) (see the lower panels, showing tubule sections at stage II-III in which pachytene spermatocytes layer is colored green), and those containing Sertoli cells only (see the lower right panel of (C)), respectively. Scale bar: 100 μm. (D) Percentages of the total defective tubules (as indicated by either the open or closed red dots in (A-C) in testes with indicated genotypes). Actual values from different individuals and their averages are shown by dots and columns. *P < 0.05, ** P < 0.01 (Student’s t test).
Figure 11. Impact of stabilized β-catenin on GFRα1+ pool

(A-C) Representative IF images of cryosectioned testes from adult Nanos3Cre+/++;Ctnnb1+/+ (A), Nanos3Cre+/++;Ctnnb1flox+ (B) and Nanos3Cre+/++;Ctnnb1flox/flox (C) mice, stained for GFRα1 and RARy. Open and filled arrowheads indicate GFRα1+ and RARy+ cells, respectively. Lower panels are the magnified images of the indicated regions in the upper panels, and seminiferous tubules are outlined by a dashed line. Scale bars: 20 μm. (D-F) Average numbers of GFRα1+ (D) and RARy+ (E) cells per tubule, and the ratio between RARy+ cells and GFRα1+ cell (F), for the indicated genotypes, based on double IF for GFRα1 and RARy on testicular sections. Only the cells located in tubule sections showing orbicular shape were counted. Throughout, mice were analyzed at the age of 8-14 weeks. Actual values from different individuals and their averages are shown by dots and columns, respectively. *P < 0.05, ** P < 0.01 (Student’s t test).
Figure 12. Impact of stabilized β-catenin on juvenile testis

(A, B) Representative IF images of cryosectioned testes from P3 *Nanos3Cre<sup>+/+</sup>;Ctnnb<sup>flox3/flox3</sup>* (A) and *Nanos3Cre<sup>tg</sup>;Ctnnb<sup>flox3/flox3</sup>* mice (B), stained for GFRα1 and MVH. Open and filled arrowheads indicate GFRα1<sup>+</sup>/MVH<sup>+</sup> and GFRα1<sup>+</sup>/MVH<sup>-</sup> cells, respectively. Seminiferous tubules are outlined by a dotted line. Scale bar: 10μm. (C, D) Numbers of GFRα1<sup>+</sup> (C) and MVH<sup>+</sup> (D) cells per tubule section. Open and filled arrowheads indicate GFRα1<sup>+</sup>/MVH<sup>+</sup> and GFRα1<sup>+</sup>/MVH<sup>-</sup> cells, respectively. Actual values from different individuals and their averages are shown by dots and columns.
Figure 13. Impact of deleted β-catenin on mouse testes

(A) Schematics of the Ctnnb1^fl allele, producing functionally null mutation (Ctnnb1^Δ) after deletions of exons 2 through 6 by Cre-mediated recombination. (B-D) Representative appearance of the testes of adult (8–14-week-old) mice of the indicated genotypes. Scale bar: 1 mm. (E, F) Testis weights (E) and body weights (F) of adult mice of the indicated genotypes. The fl/fl group includes data from the fl/Δ mice (indicated by open dots), in which one fl allele has already been deleted probably in their parent’s germline, which is also the case in Fig. 14, 15. Actual values from different individuals and their averages are shown by dots and columns.

***P < 0.001 (Student’s t test)
Figure 14. Impact of deleted β-catenin on GFRα1+ pool

(A-C) Representative histological images of testicular sections in adult (8–14-week-old) mice of the indicated genotypes, stained with PAS-hematoxylin. Lower panels are the magnified images of the indicated regions in the upper panels. Open and closed red dots indicate the tubule sections with lost or exiguous germ cell layer(s) (see the middle panels, showing tubule sections at stage II-III in which round spermatid layer is colored green), and those containing Sertoli cells only (see the bottom panels), respectively. Scale bars: 100 μm. (D) Percentages of the total defective tubules (as indicated by either the open or closed red dots in (A-C) in testes with indicated genotypes. Actual values from different individuals and their averages are shown by dots and columns. ** P < 0.01 (Student’s t test).
Figure 15. Impact of deleted β-catenin on GFRα1* pool

(A-C) Average numbers of GFRα1* (A), RARγ* (B) cells per tubule and the ratio between RARγ* cells and GFRα1* cell (C) for the indicated genotypes, based on double IF for GFRα1 and RARγ. Only cells located in tubule sections showing orbicular shape were counted. “fl/+ plus fl/fl” group includes all the fl/+, fl/fl and fl/flΔ mice examined, whose β-catenin gene is deleted in germ cells either heterogeneously or homogeneously, showing significant differences with the WT group. Actual values from different individuals and their averages are shown by dots and columns. *P < 0.05, ** P < 0.01 and ***P < 0.001(Student’s t test).
Figure 16. Uneven spermatogenesis defect and GFRα1* cells distribution in β-catenin depleted testes
(A) Percentages of Sertoli-cell-only (SCO) seminiferous tubule sections in mice of the indicated genotypes, showing that significant portions of SCO tubules were found in the fl/+ and fl/fl testes. (B) Representative IF images of adult fl/fl mice stained for GFRα1. Right panels are the magnified images of the surrounded regions in the left panel, showing the GFRα1* cells. Seminiferous tubules are outlined by dashed line. Asterisks indicate the SCO tubules; star indicates a tubule showing apparently normal spermatogenesis, harboring a larger number GFRα1* cells. Actual values from different individuals and their averages are shown by dots and columns. Scale bar: 10 μm. *P < 0.05, ** P < 0.01 (Mann-Whitney U Test).
Figure 17. Expression of Wnt6 and Gdnf-LacZ in mouse testis

(A–F) Images of adult mouse testis sections after visualizing the expression of Wnt6 by ISH (A–C) and Gdnf-LacZ by β-galactosidase staining using Gdnf-LacZ/+ mice (D–F). Asterisks indicate tubules with high expression of Wnt6 or LacZ in Sertoli cells. High-power images of Wnt6- or LacZ-high (B and E) and low/negative (C and F) tubules are also shown. Roman numerals indicate the stage of seminiferous tubules determined by PAS-stained adjacent sections. The expression of Wnt6 was also detected in interstitial cells (arrowheads in [A]), while that of Gdnf-LacZ was undeterminable due to background β-galactosidase activity. Scale bars: 100 μm.
Figure 18. Seminiferous epithelial cycle-related expression of Wnt6, Gdnf-LacZ, GFRα1, and Ngn3

(A) Percentage of Wnt6\textsuperscript{high} and Gdnf-LacZ\textsuperscript{high} tubules in each stage, in which Sertoli cells expressed Wnt6 or LacZ in a major part of the tubule circumference. A total of 18–123 tubule sections were counted for each stage from three (Wnt6) or two (Gdnf-LacZ+/+) mice. (B) Numbers of GFRα1\textsuperscript{+} and Ngn3\textsuperscript{+} cells per Sertoli cell at the indicated stages, as obtained from IF- and ISH-stained testis sections, respectively. The latter were reproduced from a previous report (Ikami et al., 2015).
Figure 19. Expression of Shisa6 in mouse testes

(A) List of genes showing the highest levels of enrichment in the GFRα1+ fraction over the NGN3+ fraction in the microarray analysis. (B) Expression profiles of Shisa2 and Shisa6 in subsets of GFRα1+ cells and/or related spermatogonial compartments between the GFRα1+, NGN3+, KIT+ fractions and the whole testis. Signal intensities were normalized to the 75th percentile and are shown on a linear scale. Represented as means ± SEM (n = 3 microarrays, each from different mice). (C, D) Phylogenetic tree of the Shisa family (C), Domain structure of SHISA proteins (D) (Pei and Grishin, 2012).
Figure 20. Expression of SHISA6 protein in mouse seminiferous tubules

Representative IF images of cryosectioned testes of adult WT mice, stained for SHISA6 and GFRα1. Arrowheads and square indicate the SHISA6⁺ spermatogonia and the magnified areas on the right panel, respectively. Seminiferous tubules are outlined by dashed lines. Scale bar: 20 μm.
Figure 21. Comparison of a Shisa6\(^+\) cell population with a GFRα1\(^+\) cell populations

(A, B) Representative image (A) and quantification (B) of dissociated testicular cells of adult mice, doubly stained for Shisa6 by FISH and for GFRα1 by IF. 835 cells from 5 mice were counted. Scale bar: 10 μm.
Figure 22. Effects of SHISA6 rescue experiment in *Xenopus laevis* embryos

(A–D) Representative appearances of *Xenopus laevis* embryos at stage 33–35, after injection of the indicated combination of mRNAs into their ventral side at the four-cell-stage. (E) Quantification of data in (A–D), classified by the presence or absence of a duplicated (second) body axis and the degree of anteriorization (e.g., with or without cement gland [CG] and/or eyes). Numbers of counted embryos are shown above.
Figure 23. Effects of SHISA6 overexpression in *Xenopus laevis* embryos

(A–D) Anterior views of stage 21 *Xenopus laevis* embryos that had been injected with the indicated amount of *mShisa6* or *mShisa2* mRNA at the four-cell-stage on their animal side. Note the enlarged cement glands (arrowheads) in (C) and (D), compared with untreated controls (A). (E) Percentages of embryos showing enlarged cement glands (i.e., more than 20% increase in either height or width). The number of embryos injected is indicated. Enlargement of the cement gland indicates anteriorization, implying inhibition of posteriorization function of WNT.
Figure 24. SHISA6 is a cell-autonomous Wnt inhibitor

(A) Effects of SHISA6 on TCF-luc activity stimulated by WNT3a. HEK293T cells were transfected with the TCF-luc reporter and the Shisa6-expression or the empty vector (Shisa6/pCNA3 or pCNA3, respectively). Twenty-four hours later, cells were stimulated with WNT3a for another 24 h before analysis. (B–F)

Cell-autonomous and non-cell-autonomous effects of SHISA6 on WNT3a, FGF2, and GDNF signaling.

HEK293T cells were transfected with a luciferase reporter plasmid (TCF-luc or SRE-luc) and Shisa6/pCNA3 either simultaneously (co-transfection) or separately. Transfected cells were then mixed as schematically shown in (B) and scheduled in (C), followed by stimulation with WNT3a (D), FGF2 (E), or GDNF (F) for 24hr. Lucerase assays were then performed. In (F), expression plasmids for GFRα1 and RET were also co-transfected with SRE-Luc reporter to reconstruct a receptor for GDNF. Each experiment was carried out at least in triplicate. Represented as means ± SEM. **P < 0.01 (Student’s t tests).
Figure 25. Expression of Shisa6 in germline stem (GS) cells

(A, B) Expression profiles of Shisa6 based on microarray data in Fig. 5 and using GS cells. GS cells were cultured as described in the schedule (A). Signal intensities were normalized to the 75th percentile and are shown on a linear scale. Represented as means ± SEM (n = 3 microarrays, each from different mice or wells).

(C) Representative IF image of GS cells stained with an anti-SHISA6 antibody. Scale bar: 20 μm.
Figure 26. *Shisa6* knockdown and stimulation by WNT3a and RSPO2 synergistically upregulate *Ngn3* in germline stem (GS) cells

GS cells were transfected with an Ngn3-luc reporter and a *Shisa6* or control siRNA expression vector and cultured for 3 days as shown in the schedule, in the presence or absence of WNT3a and R-spondin2 (RSPO2), before they were subjected to luciferase assay. Represented as means ± SEM (n = 3 independent experiments).
Figure 27. *Shisa6* expression was downregulated by GDNF and FGF

(A) Schedule of (B), (C). Germline stem cells were plated on laminin-coated plates. The next day (day 0), the medium was changed, and on days 1 and 2, half of the volume of the medium was changed. These cells were harvested at day 3. (B) The expression level of *Shisa6* was measured by RT-qPCR. The conditions for GDNF and FGF supplementation were standard (= 1). (C) The expression level of *Ngn3* was measured by RT-qPCR. The experimental schedule was the same as described in (B). (D) Schedule of (E). (E) The expression level of *Shisa6* was measured by RT-qPCR. ***P ≤ 0.0001, **P ≤ 0.001, *P ≤ 0.05* (Student’s t tests). Represented as means ± SEM. (n = 3 independent experiments).
**Figure 28. Generation of Shisa6 KO mice using the CRISPR/Cas9 strategy**

(A) Schematics of the Shisa6 locus and the location of the CRISPR/Cas9 targeting site. (B) Shisa6 alleles obtained by 'indel' mutagenesis using the CRISPR/Cas9 system. Alleles recovered in the F1 generation are associated with founder males, many of which were found to be mosaics. The Shisa6Δ6+502 allele from founder #21 was mainly used for further analysis because this allele was suggested to be functionally null and enabled unambiguous genotyping. Translated amino acids are shown below the nucleic acid sequence around the target sites. Red letters and asterisks indicate altered amino acid residues and generated stop codons, respectively.

### Table 1: Shisa6 Alleles

| Allele   | Description         | Founder |
|----------|---------------------|---------|
| WT       |                     | #21     |
| Shisa6Δ6+502 | :Shisa6 KO          | #21     |
| Shisa6Δ3+2 |                   | #27     |
| Shisa6Δ4 |                     | #28     |
| Shisa6Δ6 |                     | #27     |
| Shisa6Δ18 |                   | #28     |
| Shisa6Δ5+2 |                   | #28     |
| Shisa6Δ4+10 |                  | #28     |

**Diagram Description**

- **Primer Pair for qRT-PCR**: Indicates the location of the primer pair for qRT-PCR.
- **Target Site**: Shows the target site for CRISPR/Cas9 activity.

**Sequence Details**

- **WT sequence**: Consists of the wild-type sequence.
- **Shisa6Δ6+502 sequence**: Shows the targeted deletion of 502 bp.
- **Shisa6Δ3+2 sequence**: Indicates a deletion of 3 bp.
- **Shisa6Δ4 sequence**: Highlights a deletion of 4 bp.
- **Shisa6Δ6 sequence**: Shows a deletion of 6 bp.
- **Shisa6Δ18 sequence**: Indicates a deletion of 18 bp.
- **Shisa6Δ5+2 sequence**: Shows a deletion and a 2 bp insertion.
- **Shisa6Δ4+10 sequence**: Highlights a deletion and a 10 bp insertion.

**Translated Amino Acids**

- **Shisa6Δ6+502**: Red letters indicate altered amino acid residues.
- **Shisa6Δ3+2**: Red letters indicate altered amino acid residues.
- **Shisa6Δ4**: Red letters indicate altered amino acid residues.
- **Shisa6Δ6**: Red letters indicate altered amino acid residues.
- **Shisa6Δ18**: Red letters indicate altered amino acid residues.
- **Shisa6Δ5+2**: Red letters indicate altered amino acid residues.
- **Shisa6Δ4+10**: Red letters indicate altered amino acid residues.

**Translational Notes**

- Red letters represent altered amino acid residues.
- Asterisks indicate generated stop codons.
Figure 29. Generation of Shisa6 KO mice using the CRISPR/Cas9 strategy

(A) Expression levels of Shisa6 mRNA in undifferentiated spermatogonia of adult mice with the indicated genotypes. E-cad* cells were collected by FACS and quantification was carried out using RT-qPCR with the primers whose locations are shown in Figure 28; values are indicated relative to the average of WT mice. Each bar represents individual mice. The data indicate that mRNA derived from the Shisa6 K0 and Shisa6st alleles were hardly detected, probably due to nonsense-mediated mRNA decay (Peltz et al., 1993). (B, C) Representative double IF images of cryosectioned testes from adult Shisa6+/+ (B) and Shisa6KO/KO (C) mice stained for SHISA6 and GFRα1. Open and filled arrowheads indicate GFRα1* cells with or without the SHISA6 signal, respectively. Seminiferous tubules are outlined by dotted line. Scale bar: 20μm.
Figure 30. Disruption of Shisa6 and the testicular phenotype of Shisa6-null mice

(A, B) Representative testis weights (A) and body weights (B) of adult (8–14-week-old) mice of the indicated genotypes. (C–E) Representative histological images of PAS-hematoxylin-stained sections of the testes from Shisa6*+/+ (WT) (C), Shisa6*KO/+ (D) and Shisa6*KO/KO (E) mice at 10 weeks of age. Scale bar: 100 μm. (F) Percentages of defective tubules counted from PAS-stained sections of testes from 10-week-old mice with the indicated genotypes. (G) Average numbers of GFRα1+ cells in testes from adult (8–14-week-old) mice of the indicated genotypes. Counts were obtained from cryosections stained for GFRα1. Only tubules showing orbicular sections were counted. Actual data points and means are shown. No significant difference was detected between the genotypes in Shisa6*+/+ (WT) (C), Shisa6*KO/+ (D) and Shisa6*KO/KO (E) mice.
Figure 31. *Shisa6* confers resistance to Wnt/β-catenin signal in mouse testis

(A, B) Representative appearance of testes from adult (8–14-week-old) mice with the indicated genotypes. Scale bar: 1 mm. (C, D) Testis weights (C) and body weights (D) of adult (8–14-week-old) mice with the indicated genotypes. Actual data points and means are shown. *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t test). (C) and (D) are re-used data from Fig. 9E and F, respectively.
Figure 32. Shisa6 confers resistance to Wnt/β-catenin signal in spermatogenesis

(A, B) Representative histological images of testicular sections of adult mice of the indicated genotypes, stained with PAS-hematoxylin. Lower panels are the magnified images of the indicated regions in the upper panels. Open and closed red dots indicate the tubule sections with lost or exiguous germ cell layer(s) (see the lower panels in which tubule sections at stage I are shown with round spermatid layers colored in green), and those containing Sertoli cells only (see the lower right panel of (B)), respectively. Scale bar: 100 μm. (C) Percentages of total defective tubules in mice with indicated genotypes.

*P < 0.05, **P < 0.01, ***P < 0.001 (Student’ s t test).
Figure 33. *Shisa6* confers resistance to Wnt/β-catenin signal in GFRα1+ pool

(A-C) Average number of GFRα1+ (A) and RARY+ (B) cells per tubule, and the ratio between RARY+ and GFRα1+ cells (C) in testes with the indicated genotypes, based on double IF. These are re-used data from Fig. 11D, E, and F, respectively. *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t test).
Figure 34. Analysis of Shisa6-null and β-catenin stabilization mutations

(A-C) Representative testis weights (A) and body weights (B), and portions of degenerative tubules (C) from 4-week-old mice with the indicated genotypes. Actual data points and means are shown.

*P < 0.05 (Student’s t test).
Figure 35. T-EGFP-positive cell population is included in GFRα1-positive cell population

(A) Schematic of the structure of the TEGFP-CreERT2 allele (Imuta et al., 2013). (B, C) Representative IF image (B) and quantification (C) of whole-mount IF of seminiferous tubules of TEGFP-CreERT2 adult mice stained for GFP and GFRα1. Open and filled arrowheads indicate T-EGFP+/GFRα1+ and T-EGFP+/GFRα1+ cells, respectively. 5882 cells from 4 mice were counted.
Figure 36. Characterization of the subset GFRα1+ cells expressing Shisa6 and T
(A, B) Representative image (A) and quantification (B) of dissociated testicular cells of T-GFP-Cherry2 adult mice doubly stained for Shisa6 by FISH and for GFP. 894 cells from 3 mice were counted. (C) Schematic presentation of the relationship between Shisa6+, T-GFP+, and GFRα1+ populations, summarized from Fig. 21B, Fig. 35C, and B. Areas of each circle are proportional to the numbers of these populations, indicating their nested relationship.
Figure 37. Characterization of the T-GFP+ cells

(A) Frequency of T-GFP+/GFRα1- and T-GFP+/GFRα1+ spermatogonial units showing the morphological entities indicated below. Few syncytia consisting of cells other than 2+ cells were omitted. (B) Frequency of T-GFP+ cells along the seminiferous epithelial cycle, based on cryosections stained for GFP. Stages of each tubule section were determined by PAS-stained adjacent sections. Represented as means ± SEM. (n = 3 each from a different mouse)
Figure 38. Fate of the pulse-labeled T-positive after 2 days

(A) Schematic of the structure of T\textsuperscript{nEGFP-CreERT2} and reporter mouse. (B) Schedule of the experiment. Pulse labeling and fate analysis of T\textsuperscript{+} spermatogonia. Adult mice harboring the T\textsuperscript{nEGFP-CreERT2} allele and a lineage reporter (R26R-H2B-mCherry) were injected with 4-hydroxytamoxifen (TM) and sacrificed at two days after the labeling to analyze their testes. (C) Representative IF images of whole-mount seminiferous tubules stained for GFP and H2BmCherry detected anti-DsRed antibody. Filled and open arrowheads indicated T-EGFP-positive cells and labeled cells, respectively. Scale bar = 100\mu m.
Figure 39. Fate of the pulse-labeled T-positive after 1 or 6 months

(A) Schematic of the structure of T<sup>nEGFP-CreERT2</sup> and reporter mouse. (B) Schedule of the experiment. Pulse labeling and fate analysis of T<sup>+</sup> spermatoogonia. Adult mice harboring the T<sup>nEGFP-CreERT2</sup> allele and a lineage reporter (R26R-H2B-mCherry) were injected with 4-hydroxytamoxifen (TM) and sacrificed at 1 or 6 months after the labeling to analyze their testes. (C, D) Appearance of IF images of whole-mount seminiferous tubules at 1 month (C) and 6 months (D) after labeled. Scale bar = 100μm.
Figure 40. Expression of *Gfra1*, *Shisa6*, *T*, and reported spermatogenic stem cell makers

Expression profiles of genes reported to be expressed in subsets of GFRα1⁺ cells and/or related spermatogonial compartments in the indicated fractions summarized from our microarray data. Compared with *T* (2nd highest enrichment (74.1x) to the GFRα1⁺ fraction relative to NGN3⁺ fraction) and *Shisa6* (31.1x enrichment, ranked 4th), profiles of *Id4* (Chan et al., 2014; 4.2x enrichment, ranked 408th) and *Erbb3* (Chapman et al., 2015; 2.4x enrichment, ranked 1752th) and *Bmi1* (Komai et al., 2014); no enrichment was observed in our microarray) are shown. Signals for *Pax7* (Aloisio et al., 2014) were too low to be analyzed (not shown). *Bmi1* has multiple probes on the microarray, the probes IDs are indicated.
Figure 41. Effect of Wnt/β-catenin signaling on proliferation in GFRα1+ 
Fractions of phospho-histone H3 (pH3)⁺ cells out of the GFRα1⁺ cells in testes of the indicated genotypes. Counts were obtained from cryosections of adult mouse testes after double IF for pH3 and GFRα1. Only tubules showing orbicular sections were counted. Actual values from different individuals and their averages are shown by dots and columns, respectively. *P < 0.05 (Student’s t test).
Figure 42. Expression of β-catenin protein in mouse seminiferous tubules

Representative triple IF image of whole-mount seminiferous tubules from 11-week-old TIEGFP-CavERT2 mouse IF for β-catenin (non-phosphorylated form), GFRα1 and GFP. Open and filled arrowheads indicate GFRα1+ cells with or without the T-GFP signal, respectively. Rectangles indicate the area magnified in the middle and bottom panels. Scale bars: 10 μm.
Figure 43. Model of a stem cell system in facultative niche

In actual stem cells (ASCs), heterogeneous expression of a cell-autonomous inhibitor confers resistance to homogeneously distributed differentiation-promoting signaling. This may be generic for stem cell regulation in a facultative niche.
Acknowledgements

I am grateful to Dr. S Yoshida for my entire research project. I also thank H. Enomoto, T. Sato, T. Ogawa, R. Nishinakamura S. Takada, and R. Takada for providing mice, cells, and plasmids; N. Ueno, C. Takagi, A. Miyagi for *Xenopus laevis* experiments; S. Kobayashi, H. Hara, T. Nakagawa, Y. Kitadate, R. Sugimoto, Y. Nakamura, K. Ikami, H. Mizuguchi, Y. Nonami, M. Ishizaka, S. Hira, T. Sato, and K. Hirano for discussions; A. Maruyama, R. Ichikawa, K. Inada, and K. Nishimura for technical assistance; Y. Kuboki for secretarial assistance; the staff of the Model Animal Research Facility, NIBB Bioresource Center, for animal care; the staff of the Laboratory Animal Resource Center, including S. Mizuno, F. Sugiyama, S. Takahashi for generating *Shisa6* knockout mice; and the staff of the NIBB Core Research Facilities for their support in the microarray analysis and use of the ABI 3130xl sequencer. I would also like to present my gratitude to my family and friends for their dedicated support and warm encouragement.