FOXO TRANSCRIPTION FACTORS CONTROL SPERMATOGONIAL STEM CELL SELF-RENEWAL AND THE INITIATION OF SPERMATOGENESIS

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DEDICATION

Dedicated to Grandmama Devere O’Malley

For her courage and inspiration

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by

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Spermatogonial stem cells (SSCs), as the foundation for spermatogenesis, must maintain a balance of both self-renewal and differentiation. Although several factors important for these processes have been identified, the fundamental mechanisms regulating SSC self-renewal and differentiation remain essentially unknown. The work presented here describes the discovery of a role for the Foxo forkhead transcription factors in mouse spermatogenesis and SSCs. Foxo1 was found to specifically mark mouse gonocytes, and its cytoplasmic-to-nuclear translocation delineated the gonocyte-

to-SSC transition in neonatal testes. In adults, Foxo1 is specifically expressed within a subset of undifferentiated spermatogonia with stem cell potential. Genetic analyses showed that Foxo1 was required for both SSC maintenance as well as the initiation of spermatogenesis, with limited contributions from Foxo3 and Foxo4. Conditional inactivation of PI3K/Akt pathway components in the male germ line confirmed that PI3K signaling regulates Foxo1 stability and subcellular localization, revealing that the Foxos are crucial effectors of PI3K/Akt signaling in SSCs. The nuclear localization of Foxo1, indicating functional activation, was found to correlate with Gfrα1 expression and thus stem cell potential. Subsequent gene expression analyses identified a complex network of Foxo gene targets that rationalized both the maintenance of SSCs and initiation of differentiation by the Foxos. Taken together, these findings demonstrate that the Foxos, particularly Foxo1, are essential in maintaining the spermatogonial stem cell population, and regulation of the Foxos through the PI3K/Akt signaling pathway is a critical process underlying SSC self-renewal versus differentiation.

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BSA:	bovine serum albumin
cDNA:	complementary deoxyribonucleic acid
ChIP:	chromatin immunoprecipitation
DBD:	DNA-binding domain
Disp1:	dispatched homolog1
DMEM:	Dulbecco's modified eagle's medium
DNA:	deoxyribonucleic acid
dpc:	days post-coitum
Dppa4:	developmental pluripotency-associated gene 4
E:	embryonic day
FBS:	fetal bovine serum
FGF:	fibroblast growth factor
FSH:	follicle-stimulating hormone
GC:	granulosa cell
GCNA:	germ cell nuclear antigen
GDNF:	glial-derived neurotrophic factor
GSC:	germline stem cell
H&E:	hematoxylin & eosin
IF:	immunofluorescence
IGF1:	insulin-like growth factor 1

IGF1R:	insulin-like growth factor 1 receptor
IHC:	immunohistochemistry
IR:	insulin receptor
LCM:	laser-capture microdissection
LH:	luteinizing hormone
MEF:	mouse embryonic fibroblast
NES:	nuclear export sequence
NLS:	nuclear localization sequence
PBS:	phosphate buffered saline
PCNA:	proliferating cell nuclear antigen
PCR:	polymerase chain reaction
PD:	post-natal day
Pdk1:	phosphoinositide-dependent kinase-1
PF:	primordial follicle
PFA:	primordial follicle activation
PGC:	primordial germ cell
pH3:	phospho-histone H3
PI3K:	phosphoinositide 3-kinase
PIP2:	phosphatidylinositol 4,5-biphosphate
PIP3:	phosphatidylinositol 3,4,5-triphosphate
PKB:	protein kinase B (also known as Akt)
Plzf:	promyelocytic leukemia zinc finger protein
PTEN:	phosphatase and tensin homologue deleted on chromosome 10

qPCR:	quantitative polymerase chain reaction
RA:	retinoic acid
RAR:	retinoic acid receptor
RNA:	ribonucleic acid
RTK:	receptor tyrosine kinase
RXR:	retinoid X receptor
Sall4:	sal-like protein 4
SCF:	stem cell factor
SF:	spermatogonial stem cell culture media
SRY:	sex-determining region Y
SSC:	spermatogonial stem cell
Stra8:	stimulated by retinoic acid gene 8
TUNEL:	terminal deoxynucleotidyl transferase dUTP nick end labeling
WT:	wild-type

CHAPTER ONE
Introduction

REPRODUCTIVE DEVELOPMENT AND GAMETOGENESIS

Introduction

Sexual reproduction in mammals has evolved to produce genetically diverse offspring. Genetic recombination of germ cells and combination of haploid genomes at fertilization allows for great genetic variation from generation to generation, advantageous for species' survival and fitness. Obviously, gametogenesis, or the production of gametes within the gonads, must be tightly regulated to maintain the viability of future offspring.

The future gametes of the organism are specified very early in embryonic development, and based on the genetic sex of the organism, will develop into mature eggs or sperm in the adult. Gametogenesis is a cyclical process, paralleled in both males and females, with the alternation of diploid precursors producing haploid germ cells, which will combine at fertilization to again become diploid. Mitotic divisions propagate and expand the diploid precursors, but meiosis generates the haploid germ cells.

While the overall mechanism of gametogenesis is similar in both males and females, the timing and regulation of production varies greatly. The most striking difference in mammals being that spermatogenesis is continuous throughout the life of the male, while females have finite resources for oogenesis. The fate of the germ cells is

specified very early in development and subject to regulation at multiple levels, the specifics of which vary from male to female.

Specification and migration of primordial germ cells

Primordial germ cells, or PGCs, are first detectable in the mouse embryo at approximately embryonic day (E) 6.25 (Hayashi, de Sousa Lopes et al. 2007). These PGCs are designated early in development as the precursors to the gametes. The process of germ cell specification occurs through the protein Blimp1, a transcriptional repressor, which represses somatic cell gene programs and promotes germ cell gene fate in a group of pluripotent cells (Hayashi, de Sousa Lopes et al. 2007). These initial PGCs undergo expansion at E7.25, resulting in approximately 40 PGCs located in the extraembryonic mesoderm (Ohinata, Payer et al. 2005). At this time, the PGCs begin to migrate and will reach the primitive gonad at E11.5 (Molyneaux, Stallock et al. 2001). By E9.0, the PGCs are incorporated into the hindgut , and between E9.0 and E9.5, they will migrate to the genital ridge, or the gonadal precursor (Ginsburg, Snow et al. 1990). During migration, the PGC population will continue to expand, leading to nearly 25,000 stem cells in the gonad at E13 (Tam and Snow 1981). After reaching the gonad, the PGCs will continue to proliferate until around E15, and at this point they commit to a sex-specific developmental pathway, becoming either prospermatogonia or oogonia (Alberts 2002).

Some of the factors and signaling molecules needed to maintain spermatogenesis are also implicated in the survival, proliferation, and migration of PGCs (see below for roles of Kit and RA in spermatogenesis). One of the factors essential in the migration of

PGCs to the gonad is Kit, a receptor tyrosine kinase expressed on the surface of the cell (Besmer, Manova et al. 1993). Interaction of Kit on PGCs with its ligand stem cell factor (SCF) is important for their adhesion in the hindgut, as well as survival and migration to the gonad from the hindgut. Mutations in either *Kit* or *SCF* leads to decreased numbers of PGCs and erroneous localization after migration (Besmer, Manova et al. 1993). Moreover, retinoic acid (RA) may also affect the proliferation and survival of PGCs, as addition of RA to PGCs in culture significantly increases mitotic capabilities and growth (Koshimizu, Watanabe et al. 1995). Stimulated by retinoic acid gene 8 (Stra8) is known to act downstream of RA in inducing meiosis during spermatogenesis, but it may also act during embryonic development during PGC migration and colonization of the gonad. During mouse embryogenesis, Stra8 is specifically expressed within the male gonads, and may act through RA signaling to enhance PGC colonization of the developing testis (Oulad-Abdelghani, Bouillet et al. 1996).

Mammalian sex determination

Determination of male versus female gonadal development in mammals is genetically dependent. Embryos with two X chromosomes will eventually become female, while those with one X and one Y will become male. In mice, the gonadal primordium, also known as the genital ridges, arise at 10 days post coitum (dpc). At this point, there is no difference between the XX or XY gonads, both of which contain supporting cell precursors which will become either Sertoli cells or granulosa cells, respectively.

The transcription factor *Sry* (Sex-determining region Y), expressed on the Y chromosome, is the male determining factor in both mice and humans. Deletion of *Sry* from the Y chromosome causes a male-to-female sex reversal, while XX females with transgenic expression of Sry leads to formation of male gonads (Maatouk and Capel 2008). Expression of Sry in the XY genital ridge occurs between dpc10.75 and 12.5 and is restricted to the supporting cell precursors in XY gonads, eventually triggering their differentiation into Sertoli cells through regulation of *Sox9* (Maatouk and Capel 2008; Sekido 2010). Loss-of-function and gain-of-function studies with Sry revealed transcription factor *Sox9* as a target, resulting from the transient expression of Sry.

Sox9 expression begins at approximately dpc10.5 in the support cell precursors of the bipotential gonad. By 11.5 days post-coitum, Sox9 is highly expressed in the XY gonads and significantly downregulated in the XX gonads (Kent, Wheatley et al. 1996; Morais da Silva, Hacker et al. 1996). Sox9 in turn upregulates the expression of additional genes responsible for differentiation of the Sertoli cells. The differentiating Sertoli cells then envelop the germ cells and begin to form the testis cords, which eventually become the seminiferous tubules. The Sertoli cells also act to stimulate the sex-specific differentiation of the primordial germ cells, as well as the development of the Leydig cells and testis vascular cells. Additionally, through secreting anti-Mullerian hormone, they also act to suppress female reproductive development (Alberts 2002).

In XX gonads, the absence of Sry allows expression of the female-specific gene program, including Wnt4 and FoxL2, at 11.5-12.5 dpc. This then leads to differentiation of the supporting cell precursors into pre-granulosa cells as well as the production of

oocytes and primordial follicles. After birth, the pre-granulosa cells surround the germ cells and form primordial follicles.

Oogenesis

Oogenesis, or the formation of mature eggs within the ovary, begins when the PGCs reach the primitive gonad and become oogonia, or primordial follicles. The oogonia proliferate by mitosis for two to three days before differentiating into primary oocytes in the embryonic ovary (Alberts 2002). At this point, around PD2, the oocyte will begin to undergo the first meiotic division and arrest in prophase. The oocyte will remain arrested in meiotic prophase until the organism reaches sexual maturity (Alberts 2002). Because the ovary contains a finite number of oocytes, individual primordial follicles (PFs) remain quiescent until they undergo primordial follicle activation (PFA).

Upon hormonal cues through follicle stimulating hormone (FSH), the process of oocyte maturation continues and meiosis I is completed, producing a secondary oocyte and a small polar body. The second meiotic division arrests at metaphase and is completed only upon ovulation and fertilization by spermatozoa (Alberts 2002).

The mechanisms regulating the recruitment of primordial follicles to undergo PFA, resulting eventually in either atresia or ovulation, is poorly understood, though the transcription factor Foxo3 has been found to maintain quiescence in PFs (see Chapter II) (Peters, Byskov et al. 1975; Elvin and Matzuk 1998; Fortune, Cushman et al. 2000; McGee and Hsueh 2000; Castrillon, Miao et al. 2003). Once activated, and meiosis is initiated, the oocyte will grow in size and the granulosa cells (GCs) will transition from a

squamous shape surrounding the PF to a cuboidal shape surrounding the primary oocyte (Lintern-Moore and Moore 1979). The GCs will continue to proliferate as the oocyte matures, and a surge of luteinizing hormone (LH) promotes the final stages of maturation and finally, ovulation (Lintern-Moore and Moore 1979). The corpus luteum develops from the follicle after the release of the oocyte from the ovary, and its presence is indicative of normal ovulation and female fertility.

Organization of the testis

The mammalian testis consists of the interstitial compartment and the seminiferous tubule compartment. Components of the interstitial compartment include the blood vessels, which do not penetrate the seminiferous tubules, and the lymphatic vessels (Russell, Ettlin et al. 1990). However, the most functionally significant portion of the interstitium is the Leydig cell, a significant source of testosterone and other androgens in the presence of luteinizing hormone from the pituitary (LH) (Kurland, Christensen et al. 1975; Mori and Christensen 1980). The Leydig cells surround the seminiferous tubules, and in the presence of LH at sexual maturity, will produce and secrete testosterone, which is necessary for the maintenance of spermatogenesis (Zirkin 1998).

The seminiferous tubules are where spermatogenesis occurs within the testis and are convoluted loops comprising the vast majority of the testis. Both ends of an individual seminiferous tubule are connected to the rete testis, from which spermatozoa become concentrated and enter the epididymis (Russell, Ettlin et al. 1990). The cells present within the tubules include the germ cells representing each step of

spermatogenesis (see below), and the Sertoli cells, the support system necessary for maintaining spermatogenesis.

The Sertoli cells are a somatic cell population that resides within the seminiferous tubules and acts as a support system for the germ cells and spermatogenesis (Alberts 2002). The Sertoli cell extends from the basement membrane to the lumen of the tubule, with numerous attachments to other cells as well as the basal lamina (Russell and Peterson 1985). As mentioned above, the Sertoli cells play important roles in early sexual development by suppressing the female developmental program and inducing Leydig cell development, but their role also extends into adulthood and steady-state spermatogenesis. The Sertoli cells' complex array of attachments to the basement membrane, germ cells, and other Sertoli cells acts to form the blood-testis barrier and generally maintains the integrity of the tubule to allow for cell-to-cell communication. The Sertoli cells compartmentalize the seminiferous tubule into basal and adluminal compartments (Russell, Ettlin et al. 1990). The basal compartment includes the spermatogonia and early spermatocytes, which have access to substances through the lymphatic system (the vasculature does not penetrate the tubules) (Figure 1.1A) (Russell 1977; Russell 1978). The adluminal compartment includes late spermatocytes through spermatozoa, which do not have access to the lymph, as they are within the blood-testis barrier. The multiple tight junctions between Sertoli cells form this barrier, which makes the adluminal compartment immune-privileged and exposed to fewer potentially cytotoxic substances (Dym and Fawcett 1970; O'Rand and Romrell 1977). Gap junctions connect the Sertoli cell to the developing germ cells, and these allow for signaling molecules to pass from Sertoli to germ cell (Russell 1977; Russell 1977).

Spermatogenesis

In adult mammals, spermatogenesis, or the process by which mature spermatozoa are formed, is sustained by the spermatogonial stem cells (SSCs), and occurs within the seminiferous tubules of the testis. Spermatogenesis consists of three phases: 1) the proliferative phase, 2) the meiotic phase, and 3) the differentiation phase (outlined in Figure 1.1B) (Russell, Ettlin et al. 1990).

In the proliferative phase, the spermatogonia, or most immature germ cells, undergo many rounds of mitosis, with incomplete cytokinesis, to create a large pool of cells that will undergo further differentiation. The spermatogonial population includes the undifferentiated spermatogonia, a population which includes the SSCs, and the differentiated spermatogonia. In the mouse testis, the undifferentiated spermatogonia includes the A_{single} (isolated spermatogonia or A_s), A_{paired} (interconnected pairs of spermatogonia or A_{pr}), and $A_{aligned}$ (chains of 4, 8, or 16 interconnected spermatogonia or A_{al}). From here, these cells transform into the differentiated spermatogonia, which are termed A_{1-4}, B, and Intermediate spermatogonia (Russell, Ettlin et al. 1990; de Rooij 2001). The transition from A_{al} to A_1 involves very subtle morphological changes in cell shape, but coincides with the onset of Kit expression (Russell, Ettlin et al. 1990; Yoshinaga, Nishikawa et al. 1991).

In the meiotic phase, the most mature spermatogonia, Type B, divide to form preleptotene spermatocytes. Preleptotene spermatocytes are the last cells to go through the S-phase of the cell cycle, and can be distinguished from B spermatogonia by their slightly smaller size (Russell, Ettlin et al. 1990). These spermatocytes then enter a long-lasting prophase of the first meiotic division. The timeline of early prophase to late

prophase is about three weeks, and the size of the spermatocyte progressively increases during this time. There are five phases of prophase (preloptotene, leptotene, zygotene, pachytene, and diplotene) and these can be distinguished by the nuclear morphology of the spermatocyte (Russell, Ettlin et al. 1990). After this long prophase, the remainder of the first meiotic division occurs quite rapidly, producing secondary spermatocytes. From here, meiosis II is also quickly completed, as intermediary cells are rarely found in histological cross-section. This second meiotic division then produces haploid spermatids which are significantly smaller than the precursor spermatocytes (Russell, Ettlin et al. 1990).

The spermiogenic phase is the final phase of spermatogenesis, and begins when the spermatid begins to develop polarity. During this time, one of the morphological changes that occurs to the spermatid is the development of an acrosome, an organelle derived from the Golgi that caps the head of the spermatozoa. The spermatid will also develop a flagellum and undergo nuclear condensation (Russell, Ettlin et al. 1990). At this point, the spermatozoa undergo the process of spermiation. Spermiation is the release of the sperm from the Sertoli cells into the lumen of the tubule, and the Sertoli cell is an active and essential participant in the release (Fawcett 1969) . Sperm that are not released are phagocytized by the Sertoli cells (Kerr and de Kretser 1974). During spermiation, the extraneous organelles and cytoplasm that the sperm does not require are removed. These spermatozoa are mature but lack motility once released into the tubular lumen (Jones 1999). Motility is then acquired as the spermatozoa move through the epididymis (Jones 1999).

In histological cross-sections of the testis, each cell type of spermatogenesis is generally concentrically located within the seminiferous tubule (Russell, Ettlin et al. 1990). Spermatogonia are located along the basement membrane, spermatocytes are somewhat medial within the tubule, and spermatids are located near the lumen. Additionally, the specific cell types found in a tubule cross-section will always co-exist, and this specifies a stage of spermatogenesis. In the mouse, there are 12 stages of spermatogenesis, and each of these specifies a "grouping of germ cell types at a specific developmental progression" (Russell, Ettlin et al. 1990). Each stage of the cycle is defined by the specific spermatogenic cell types present within a cross-section of the seminiferous tubule.

Vitamin A and spermatogenesis

Vitamin A, in the form of retinol or retinoic acid, is essential for fertility and spermatogenesis, as vitamin A deficiency results in infertility and a complete lack of spermatogenesis (Livera, Rouiller-Fabre et al. 2002). Dietary vitamin A (retinol) is converted to its oxidized form retinoic acid (RA), which acts within the testis (Molotkov, Ghyselinck et al. 2004; Paik, Vogel et al. 2004). RA has two families of intracellular receptors known as retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both of which are expressed in various testis cell types throughout development and adulthood (Mark, Ghyselinck et al. 1999; Livera, Rouiller-Fabre et al. 2002). These receptors, upon RA activation, will then directly bind the promoters of genes regulated by vitamin A. Within the testis, the major source of RA synthesis is thought to be the Sertoli cells,

which will then disburse RA to its surrounding germ cells (Figure 1.1A) (Livera, Rouiller-Fabre et al. 2002).

As mentioned above, vitamin A deficiency in males leads to infertility and a complete lack of spermatogenesis. Histological analysis of these vitamin A-deficient rodent testes reveals the continued presence of undifferentiated spermatogonia on the basement membrane of the tubules, but a failure of spermatogenic differentiation (Coward, Howell et al. 1969; Mitranond, Sobhon et al. 1979; Unni, Rao et al. 1983). RA therefore plays a key role in initiating differentiation and meiotic entry. However, upon restoration of vitamin A to the diet, spermatogenesis resumes and any prior defects in differentiation are reversed (Morales and Griswold 1987).

The mechanism by which RA acts to promote meiosis and differentiation has been linked to the action of the RA-responsive gene *Stra8* (stimulated by retinoic acid gene 8). Induction of *Stra8* requires the action of RA, and it is found within both testes and ovaries to promote meiosis (Koubova, Menke et al. 2006; Anderson, Baltus et al. 2008). Juvenile male mice lacking *Stra8* have normal mitotic expansion of primordial germ cells and undifferentiated spermatogonia, but germ cells never exhibit the morphological hallmarks of meiotic prophase initiation, such as chromosome condensation and recombination (Baltus, Menke et al. 2006; Anderson, Baltus et al. 2008).

Spermatogonial stem cells

In the adult testis, spermatogonial stem cells (SSCs) allow for continual high productivity of spermatogenesis and the production of mature spermatozoa. The SSCs arise postnatally from the gonocyte population and persist throughout the lifetime of the animal. The gonocyte-to-SSC transition occurs between postnatal day (PD) 0 and 6, as the gonocytes migrate to the basement membrane.

Like other stem cell populations, SSCs are capable of divisions of either self-renewal or differentiation, allowing for maintenance of spermatogenesis into adulthood. The classic, or A_s model, of germline stem cells maintains that the stem cell population resides solely within the A_s population of undifferentiated spermatogonia (Huckins 1971; Oakberg 1971). Therefore, division of the germline stem cells would be symmetrical in this model, with SSC division producing either two new stem cells or two interconnected A_{pr} spermatogonia. However, asymmetric divisions of germline stem cells is a common mechanism in *C. elegans* and *Drosophila* gonads (Morrison and Kimble 2006). These opposing models of SSC divisions are both viable and possible in the mammalian gonad, but a combination of the two is the currently accepted model.

While A_s were once considered as synonymous with the stem cell population, the delineations between stem cell and differentiated cell are more enigmatic than previously thought (Morrison and Kimble 2006; Nakagawa, Nabeshima et al. 2007; Nakagawa, Sharma et al. 2010). Currently, the undifferentiated spermatogonia containing A_s to A_{al} are the smallest spermatogonial population proven to contain the stem cells (Russell, Ettlin et al. 1990; de Rooij and Russell 2000; de Rooij 2001). However, differentiating cells are not necessarily committed to continue differentiating and have been found to

have the ability to revert to a stem cell state (Nakagawa, Sharma et al. 2010). Such elastic delineations of the stem cell population would allow for a greater potential for recovery upon damage. Obviously, the mechanisms governing the spermatogonial stem cell population are complex, though not necessarily as rigid as previously thought.

Most adult stem cell populations, including the SSCs in *Drosophila*, are supported by a stem cell niche, a specific microenvironment that provides the essential factors for SSC maintenance (Spradling, Drummond-Barbosa et al. 2001; Scadden 2006). As the cells divide and move away from the niche, the absence of the particular niche factors helps contribute to these cells' differentiation. However, there is conflicting evidence over presence of a stem cell niche *per se* in the mammalian testis. The undifferentiated spermatogonia, including the SSCs, reside on the basement membrane of the tubules, with the basal membrane on the basal side and the Sertoli cell barrier on the other. After SSC division, the daughter cells remain between the basement membrane and Sertoli cell junctions (Russell, Ettlin et al. 1990). Once the differentiating daughter cells begin the process of meiosis and become spermatocytes, they move through the Sertoli cell barrier and away from the basement membrane (Russell, Ettlin et al. 1990). Thus, if present, the stem cell niche would presumably be present near the basal membrane. Many consider the intra-tubular Sertoli cells as a major contributor to the niche, while recent evidence points to a more distinct microenvironment involving the Leydig and peritubular myoid cells (Yoshida, Sukeno et al. 2007). Yoshida *et al*. (Yoshida, Sukeno et al. 2007) observed through *in vivo* imaging the accumulation of As, A_{pr}, and A_{al} spermatogonia near blood vessels, especially where these vessels branch. While *in vivo* studies have produced data supporting the presence of the niche

microenvironment, conflicting data has recently emerged from *in vitro* studies of SSCs derived from rat testis. Wu *et al.* showed that these SSCs are able to both self-renew and differentiate in culture in a cell-autonomous manner, regardless of microenvironment (Wu, Luby-Phelps et al. 2009).

While the presence and physical location of the SSC niche in the testis remains to be definitively proven, the importance of particular molecular factors in maintaining the stem cells is clear. GDNF (glial-derived neurotrophic factor) is produced by the Sertoli cells, and is essential in the maintenance and self-renewal capacity of SSCs (Meng, Lindahl et al. 2000). The GDNF receptor is a heterodimer of Ret and Gfrα1 and resides on A_s and A_{pr} undifferentiated spermatogonia. Upon binding of GDNF, the receptor activates Akt and other signaling pathways to promote self-renewing divisions (Meng, Lindahl et al. 2000; Lee, Kanatsu-Shinohara et al. 2007). While the presence of Ret or Gfrα1 on spermatogonia does not necessary indicate stemness, staining for these receptors is often used to mark the putative spermatogonial stem cell population (Figure 1.1A).

Nanos2 is a zinc-finger RNA-binding protein which, similarly to Ret and Gfrα1, is predominantly expressed in A_s and A_{pr} spermatogonia (Sada, Suzuki et al. 2009). Studies in multiple model species have discovered the importance of Nanos proteins in maintaining the germ line and stem cells (Mochizuki, Sano et al. 2000; Jaruzelska, Kotecki et al. 2003; Wang and Lin 2004; Draper, McCallum et al. 2007). Lineage-tracing analyses by Sada *et al*. (Sada, Suzuki et al. 2009) followed the specific population of undifferentiated spermatogonia expressing Nanos2 in mice and found that the $Nanos2^+$ cells undergo both divisions of self-renewal and divisions leading to differentiated cells.

These studies provide further evidence that the model for SSC division is a complex balance of both symmetric and asymmetric stem cell divisions. Additionally, conditional knockout of *Nanos2* in mice testes led to severe defects in spermatogenesis that increased with age, due to rapid and irreversible loss of the SSC population (Sada, Suzuki et al. 2009). Conversely, overexpression of Nanos2 led to an significant increase in the numbers of undifferentiated spermatogonia populating the testis, along with a decrease in Kit^+ differentiating spermatogonia (Sada, Suzuki et al. 2009). It is currently thought that Nanos2 acts within the mammalian germ line stem cells to suppress differentiation programs, and that its expression and activity acts downstream of GDNF signaling (Saga 2010; Sada, Hasegawa et al. 2012).

Finally, Plzf (promyelocytic leukemia zinc finger protein) is a transcriptional repressor which is expressed in all undifferentiated spermatogonia (A_s through A_{al}), along with the transcription factor Oct4 (Buaas, Kirsh et al. 2004; Dann, Alvarado et al. 2008). Ablation of *Plzf* in mice causes progressive spermatogenesis defects, with young mice able to produce limited numbers of spermatozoa, and adult mice entirely infertile. Transplantation experiments of the juvenile germ cells confirmed the depletion of SSCs in *Plzf* testes, indicating a *bona fide* stem cell phenotype (Buaas, Kirsh et al. 2004). Similarly, knockout of *Oct4* in SSC cultures severely reduced their ability to colonize seminiferous tubules through transplantation, and induced differentiation programs *in vitro* (Dann, Alvarado et al. 2008). Conditional knockout of *Oct4 in vivo* has not yet been done to verify these results, but these data provide the initial evidence that Oct4 is also necessary to maintain SSC self-renewal capabilities.

Although the SSCs are a critical part of spermatogenesis and fertility, the precise molecular characteristics and signaling pathways defining this highly-specialized cell population remains to be fully understood. The molecules described above were each found to be essential for stem cell self-renewal, but how they relate to each other in the complex process of SSC maintenance is unknown (Figure 1.1A).

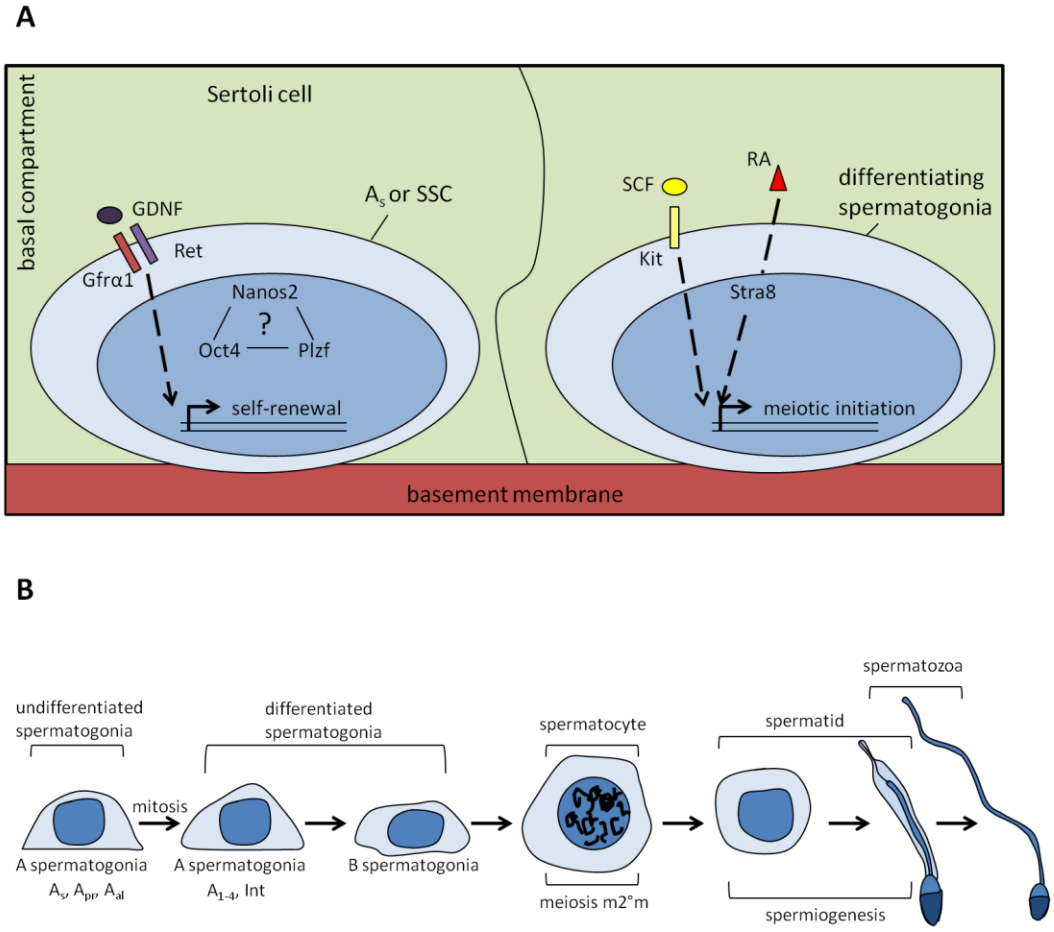

Figure1.1: Spermatogonial stem cells undergo either self-renewal or meiotic initiation and spermatogenic differentiation

(A) Representation of spermatogonia on the basement membrane of the seminiferous tubule. Specific factors have been found to be required for stem cell self-renewal and differentiation, though their interrelationship and functional hierarchy is poorly understood. GDNF = glial cell line-derived neurotrophic factor; A_s = A single spermatogonia; SSC = spermatogonial stem cell; SCF = stem cell factor. (B) Process of germ cell differentiation. Undifferentiated spermatogonia expand through mitosis before differentiation and meiosis begins.

CHAPTER TWO
Introduction

PI3K PATHWAY AND FOXO TRANSCRIPTION FACTORS IN REPRODUCTION AND GAMETOGENESIS

FOXO forkhead transcription factors

The Foxos are a highly conserved family of transcription factors, all of which share a forkhead DNA binding domain. While first identified in *Drosophila* (fkh) (Weigel, Jurgens et al. 1989), subsequent studies have since identified closely related Foxo members in all animal species examined, including *C. elegans*, zebrafish, rat, mouse, and human (Lin, Dorman et al. 1997; Ogg, Paradis et al. 1997; Nakae, Park et al. 1999; Furuyama, Nakazawa et al. 2000; Biggs, Cavenee et al. 2001). Highly conserved throughout evolution, the Foxos function in multiple cellular processes including cell cycle arrest, tumor suppression, organismal longevity, and apoptosis (Birkenkamp and Coffer 2003; Hannenhalli and Kaestner 2009; Zhang, Tang et al. 2011).

Four FoxO members have been identified in the mouse to date: Foxo1, Foxo3, Foxo4, and Foxo6. The three principal Foxos, Foxo1, Foxo3, and Foxo4, share some overlapping expression patterns as well as an identical DNA binding consensus sequence. However, individual knockout of each of these Foxos gives a distinct phenotype in the mouse, evidence of functional differences (Hosaka, Biggs et al. 2004). Disruption of Foxo1 causes embryonic lethality at around E10.5 due primarily to defects in the vasculature, reflective of the high expression of Foxo1 in normal embryonic

cardiovasculature. Conversely, Foxo4 knockout mice are have no overtly discernible phenotype, though have been described to have increased atherosclerosis in cardiac tissue (Zhu, Zhang et al. 2011). These distinct phenotypes resulting from single ablation of each of the Foxos indicates the potential for functional redundancy among the Foxo proteins. Foxo3 disruption, though pups are viable and indistinguishable from wild-type littermates, causes an interesting fertility phenotype. Foxo3-null females are infertile due to premature follicle depletion (see below for more detail of Foxos' role in reproduction) (Castrillon, Miao et al. 2003; Hosaka, Biggs et al. 2004).

PI3K/Akt/Foxo signaling pathway

The Foxos canonically act downstream of the PI3K/Akt pathway in response to insulin and insulin-like signaling (signaling pathway outlined in Figure 2.1.). PI3K itself can be activated by different cell receptors, most commonly G-proteins and receptor tyrosine kinases (Vanhaesebroeck and Alessi 2000). Upon receptor activation, PI3K is recruited to the membrane where it phosphorylates the inositol ring of phosphatidylinositol to generate PIP2 (phosphatidylinositol 4,5-biphosphate) and PIP3 (phosphatidylinositol 3,4,5-triphosphate) (Vanhaesebroeck and Alessi 2000). These molecules then activate downstream signaling kinases Pdk1 (phosphoinositide-dependent kinase-1) and Akt (Vanhaesebroeck and Alessi 2000). However, this process can be reversed by the protein PTEN (phosphatase and tensin homologue deleted on chromosome 10), which acts to dephosphorylate PIP2 and PIP3, and thereby prevent activation of Pdk1 and Akt (Vanhaesebroeck and Waterfield 1999).

Akt, also known as PKB, is a serine/threonine kinase consisting of an N-terminal pleckstrin homology domain, a catalytic domain, and a C-terminal hydrophobic motif (Scheid and Woodgett 2003). Generation of PIP2 and PIP3 by PI3K recruits Akt to the plasma membrane, where it is then phosphorylated and activated by kinase Pdk1 (Alessi 2001; Lawlor and Alessi 2001; Scheid and Woodgett 2003). Once Akt is active, it moves from the plasma membrane and translocates to the nucleus, where it may directly phosphorylate specific target proteins, including the Foxos (Lawlor and Alessi 2001). The Foxo proteins each contain three conserved Akt recognition/phosphorylation motifs (RXRXXS/T), phosphorylation of which leads to nuclear export of the Foxos, and thus their functional inactivation (X represents any amino acid) (Alessi, Caudwell et al. 1996; Biggs, Meisenhelder et al. 1999; Brunet, Bonni et al. 1999).

The Foxo proteins contain both a nuclear localization sequence (NLS) as well as a nuclear export sequence (NES). A NLS is required for proteins to be maintained within the nucleus while a NES maintains proteins within the cytosol. Therefore, it is through interactions of the Foxos with other regulatory proteins that determines its ultimate localization in the cell. All of the Foxos have an Akt phosphorylation motif (see above) within the NLS, and it is thought that Akt action upon the NLS introduces a negative charge which potentially hinders the function of the NLS, allowing transport to the cytoplasm (Alessi, Caudwell et al. 1996; Brownawell, Kops et al. 2001). Once in the cytoplasm, the phosphorylated Foxo protein is then targeted for degradation by the ubiquitin-proteasome system (Matsuzaki, Daitoku et al. 2003; Plas and Thompson 2003).

Well-established activators of the PI3K/Akt/Foxo signaling pathway are insulin and insulin-like growth factors. Inhibiting insulin signaling in *C. elegans* and *Drosophila*

keeps the Foxo orthologs, DAF16 and FOXO, respectively, within the nucleus and actively transcribing Foxo targets (Lin, Dorman et al. 1997; Ogg, Paradis et al. 1997; Wang, Bohmann et al. 2005). Similarly, in mammals, during a status of insulin resistance, Foxo1 accumulates within the nucleus, and ablating Foxo1 expression can reverse the effects of insulin resistance (Kitamura, Nakae et al. 2002). Foxo1 in particular is essential in insulin-sensitive tissues in regulating cell proliferation and transcribing genes involved in glucose and energy metabolism (Accili and Arden 2004; Glauser and Schlegel 2007; Kitamura and Ido Kitamura 2007). Signaling from insulin and insulin-like growth factors (IGFs) activate the PI3K/Akt pathway through activation of transmembrane tyrosine kinase receptors, insulin receptor (IR) and insulin-like growth factor receptor (IGFR). Similar in functions, there is cross-talk between insulin, IGF1, and IGF2, with receptors IR and IGF1R in particular (Ward and Lawrence 2009).

Role of PI3K and Foxos in the invertebrate gonad

In the *Drosophila* testis, the germline stem cells (GSCs) reside at the tip of the testis and receive signals from the adjoining hub cells to maintain their stem cell identity. The stem cell niche and mechanism of differentiation is paralleled in the *Drosophila* ovary as well. As the stem cell divides asymmetrically, the distal daughter cell leaves the niche and undergoes differentiation. Signaling from insulin and insulin-like peptides from the hub cells has been reported as essential in maintaining the GSC population and subsequent sperm production (Ueishi, Shimizu et al. 2009). Inhibition of insulin

production and signaling leads to decreased GSC division and GSC number with age, as insulin signaling promotes cell division at G2/M in the male GSC population (Ueishi, Shimizu et al. 2009). However, whether this action of insulin signaling on the GSCs acts specifically through PI3K and dFOXO has yet to be determined. Similarly, insulin signaling in the *Drosophila* ovary also has essential roles in maintaining the female GSC niche (Hsu and Drummond-Barbosa 2009). Recent work identified neural insulin-like peptides as acting through the PI3K pathway and dFOXO to control the G2 phase in GSC division in the Drosophila ovary (Hsu, LaFever et al. 2008). Additionally, insulin signaling, also acting through PI3K and dFOXO, is necessary to maintain the niche itself, acting within the cap cells to maintain their competency (Hsu and Drummond-Barbosa 2011). These studies reveal the importance of PI3K and dFOXO, acting within both testis and ovary, in promoting GSC competence and cell divisions.

In the mosquito species *Ae. aegypti*, there were six Fox proteins found to be expressed within the fat body, and knockdown each of these six Fox proteins through RNAi revealed its conserved function in maintaining reproduction. Reduction of Foxo expression led to a significant decrease in the number of eggs laid by these mosquitoes, though the molecular mechanism of this reduced fertility has yet to be determined (Hansen, Sieglaff et al. 2007). Similarly, Foxo and insulin signaling were also found to act within the ovary of the mosquito *Culex pipens*. RNAi knockdown of the insulin receptor in this species caused arrest of primary follicles, and knockdown of FOXO reversed the developmental arrest phenotype, indicative of FOXO acting downstream of insulin signaling in maintaining follicles (Sim and Denlinger 2008).

In parallel to its function in *Drosophila*, the insulin signaling pathway acts to promote germline proliferation in *C. elegans* as well. The development of the germline in *C. elegans* involves similar processes to mammalian germline development, with early proliferation of primitive germ cells forming a population of stem cells or progenitors that will eventually undergo differentiation (Hirsh, Oppenheim et al. 1976). Insulin and IGF-like receptor signaling pathways have been implicated in *C. elegans* fertility, but more recently, Michaelson *et al*. found that this pathway acts in a germline autonomous manner through the canonical PI3K pathway and Foxo (Daf16), necessary for the initial proliferation of the germline (Tissenbaum and Ruvkun 1998; Michaelson, Korta et al. 2010). PI3K components of the insulin-like signaling pathway in *C. elegans*, known as daf-2 and age-1, regulate the activity of daf-16, and mutations in daf-2 and age-1 also cause fertility defects (Kahn 1994; Morris, Tissenbaum et al. 1996; Tissenbaum and Ruvkun 1998). Insulin-like signaling and the PI3K pathway act through daf-16 and its target genes to regulate reproduction in the germline of *C. elegans* (Tissenbaum and Ruvkun 1998). These data reinforce the conserved role of PI3K signaling and the Foxos in maintaining the germline throughout species. Not only essential for reproductive development and germline maintenance, insulin signaling and daf-16 also play a specific role in fertilization. This signaling pathway promotes the metabolism of fatty acids into prostaglandins which guide sperm to the site of fertilization (Edmonds, Prasain et al. 2010). Activation of FOXO by reduced insulin signaling causes transcriptional repression of germline genes critical for delivery of prostaglandins to the oocyte.

Foxos in the Mammalian Gonad

The PI3K/Akt signaling pathway and Foxo3 in particular has a role in primordial follicle activation within the mammalian ovary. Primordial follicle activation (PFA) is the metered process by which primordial follicles are selected from the reserve pool into the growing follicle pool (McLaughlin and McIver 2009). In the mouse ovary, Foxo3 is highly expressed only within the oogonia and oocytes and undergoes a cytoplasmic to nuclear translocation shortly after birth (John, Gallardo et al. 2008). At postnatal day (PD) 1, Foxo3 is entirely located in the cytoplasm, but moves to the nucleus by PD3, consistent with active Foxo3 suppressing primordial follicle activation. Accordingly, ablation of Foxo3 within the primordial follicles leads to global follicle activation and premature sterility, and this role of Foxo3 was found to be downstream of PI3K/Akt signaling (Castrillon, Miao et al. 2003; John, Shirley et al. 2007; John, Gallardo et al. 2008). Artificial activation of PI3K and inhibition of PTEN in cultured mouse ovaries also leads to increased nuclear exclusion of Foxo3, and transplantation of these ovaries led to normal maturation of eggs and embryonic viability (Li, Kawamura et al. 2010), further confirming Foxo3 downstream of PI3K signaling and the importance of nuclear Foxo3 in maintaining a quiescent state. Conversely, constitutively active Foxo3 in the mouse oocyte also leads to infertility due to slowed oocyte growth, further indication of Foxo3 acting to suppress oocyte growth (Liu, Rajareddy et al. 2007). In more recent studies, this specific ovarian expression and activity of Foxo3 and PI3K pathway components were found to be conserved in both pigs and cows, as siRNA knockdown of Foxo3 in dissected ovarian tissues led to follicle activation after xenograft (Ding, Wang et al. 2010; Moniruzzaman, Lee et al. 2010; Bao, Hayakawa et al. 2011).

The receptor and ligand interaction responsible for activating the PI3K/Akt pathway in the context of PFA has yet to be determined, though SCF and Kit have been studied. Employing a knock-in mutation of Kit that specifically abrogates its signaling through PI3K, PFA occurs normally and females are fertile (Kissel, Timokhina et al. 2000; John, Shidler et al. 2009). However, abnormalities in the progression from primary to secondary follicles reveals the role of Kit in later steps of oogenesis, though dispensable for normal PFA through Foxo3 (John, Shidler et al. 2009). Interestingly, cultures of neonatal mouse ovaries implicated testosterone as upstream of Foxo3 translocation (Yang, Zhang et al. 2010). Addition of testosterone to the cultured ovaries led to shuttling of Foxo3 from the nucleus to the cytoplasm, and concurrently increased the number of activated follicles (Yang, Zhang et al. 2010). While testosterone canonically acts through the androgen receptor, this particular study, along with others, confirmed the ability of testosterone to activate PI3K/Akt signaling (Baron, Manin et al. 2004; Kang, Cho et al. 2004; Cinar, Mukhopadhyay et al. 2007).

Conversely, while these studies have implicated nuclear Foxo3 in maintaining the primordial follicle reserve and preventing PFA through cell cycle arrest, Sui *et al*. reported that in neonatal rat ovaries, nuclear Foxo3 is indicative of naked oocyte apoptosis (Sui, Luo et al. 2010). Foxo3 staining was at its highest in PD2 ovaries, and TUNEL-positive oocytes were also Foxo3-positive, with possible downstream targets including *FasL, Bim,* and *p27KIP1*. However, these observations were solely correlative, based on Foxo3 staining in $TUNEL^{+}$ apoptotic primordial follicles. The expression of Foxo3 in all primordial follicles confounds these results, and promoter occupancy of these pro-apoptotic targets was not confirmed.

Not only important in oocyte development, the Foxos have also been reported to have essential functions within the granulosa cells. Foxo1 is known to be highly expressed in granulosa cells of many mammalian species (Richards, Sharma et al. 2002; Cunningham, Zhu et al. 2003; Park, Maizels et al. 2005). Further evidence of the high conservation between species, transcripts of all three Foxos are detected in human luteinized granulosa cells, though Foxo1 is the most highly expressed of the three (Pisarska, Kuo et al. 2009). This high granulosa cell-specific Foxo1 expression is regulated by the actions of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Richards, Sharma et al. 2002; Hosaka, Biggs et al. 2004). Granulosa expression of Foxo1 is induced by FSH and estradiol and down-regulated by LH. Potential targets of Foxo1, acting downstream of FSH, were elucidated by Liu *et al*. through the use of a constitutively active Foxo1 (Liu, Rudd et al. 2009). Microarray analysis, taking advantage of the increase in Foxo1 transcription and target transcripts, found a distinct class of target genes within the granulosa cells. Genes involved in the synthesis of cholesterol and fatty acids were increased in the constitutively active Foxo1 samples, and accordingly decreased within Foxo1 DNA-binding domain mutant (DBD) samples. Additionally, Foxo1 has also been reported to be associated with granulosa cell apoptosis (Kajihara, Uchino et al. 2009). Ovaries from hyperinsulinemic rats were examined for defects in follicular development, since insulin resistance is associated with polycystic ovary syndrome (Burghen, Givens et al. 1980). The hyperinsulinemic ovaries had increased numbers of atretic follicles, and co-staining with Foxo1 and TUNEL showed a correlation between nuclear Foxo1 and apoptotic granulosa cells (Kajihara, Uchino et al. 2009). However, further experiments confirming a causative role of Foxo1 actually

initiating apoptosis were lacking, such as the presence of pro-apoptotic Foxo1 transcription targets.

As mentioned above, Foxo3 acts within the oocyte to suppress primordial follicle activation (Castrillon, Miao et al. 2003). However, work from Matsuda *et al*. recently postulated that Foxo3 is also acting within the granulosa cells to promote follicular atresia in porcine ovaries (Matsuda, Inoue et al. 2011). Foxo3 has previously been found to play a tissue-specific role in apoptosis, through transcription of pro-apoptotic targets such as Fas ligand (FasL), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and Bim (Brunet, Bonni et al. 1999; Ghaffari, Jagani et al. 2003; Gilley, Coffer et al. 2003; Sunters, Fernandez de Mattos et al. 2003; Barthelemy, Henderson et al. 2004). Levels of Foxo3 mRNA transcripts were detected in granulosa cells of porcine ovaries *in vivo*, and expression increased during atresia. Additionally, while Foxo3 protein overlapped with apoptotic cells using IHC, further evidence of Foxo3 directly inducing follicular atresia *in vivo* was lacking, and so additional work is needed to confirm this concept (Matsuda, Inoue et al. 2011).

The role of the Foxos in the mammalian ovary was first uncovered in animal model studies. However, these discoveries of Foxo function in reproduction, with strong conservation between species, hint that the PI3K/Akt pathway and the Foxos may also be important in human reproduction, and when disrupted, may lead to female infertility.

Dissertation objective

Prior to the studies outlined in this thesis, the function of the Foxos within the mammalian testis was entirely unknown. Immunohistochemistry of the three Foxos on mouse testis revealed high expression of Foxo1 in seminiferous tubules, while Foxo3 and Foxo4 were undetectable. Foxo1 germ cell-specific expression paralleled Foxo3 expression within oocytes, prompting further investigations into the specific role of Foxo1 in the testis. The primary focus of this dissertation discusses the discovery of Foxo1 as an essential factor in maintaining spermatogonial stem cells and spermatogenesis. Employing various germ cell-specific knockouts, Foxo1 was found to act downstream of PI3K/Akt signaling in spermatogonia, confirming the mechanistic conservation of germ line maintenance between species as described above (Figure 2.1).

Additionally, experiments elucidating the downstream targets of Foxo1 in the context of spermatogenesis, and the mechanism responsible for Foxo1 nuclear to cytoplasmic shuttling are outlined below. Potential targets include both pluripotency genes, most notably Ret, as well as genes necessary for differentiation, though nuclear and active Foxo1 was strongly correlated with the stem cell population. Described above, insulin signaling is responsible for reproductive maintenance through the Foxos in multiple species, but the upstream signaling factors in the mouse testis remain to be uncovered.

Finally, Foxo3 is required for maintaining primordial follicles, but the process of PFA is still relatively poorly understood. Here, conditional ablation in oocytes of various ovarian genes was undertaken in order to identify additional PFA and oogenesis factors. These experiments found that Disp1, a mediator of hedgehog signaling, is not required

for oogenesis, and ruled out insulin signaling through insulin and insulin-like growth factors as upstream of PI3K/Akt and Foxo3 in the context of PFA. Together, the work presented in this dissertation provides the first evidence of the requirement of Foxo1 in spermatogenesis, and further elucidation of the role of PI3K signaling in the maintenance of the germ line.

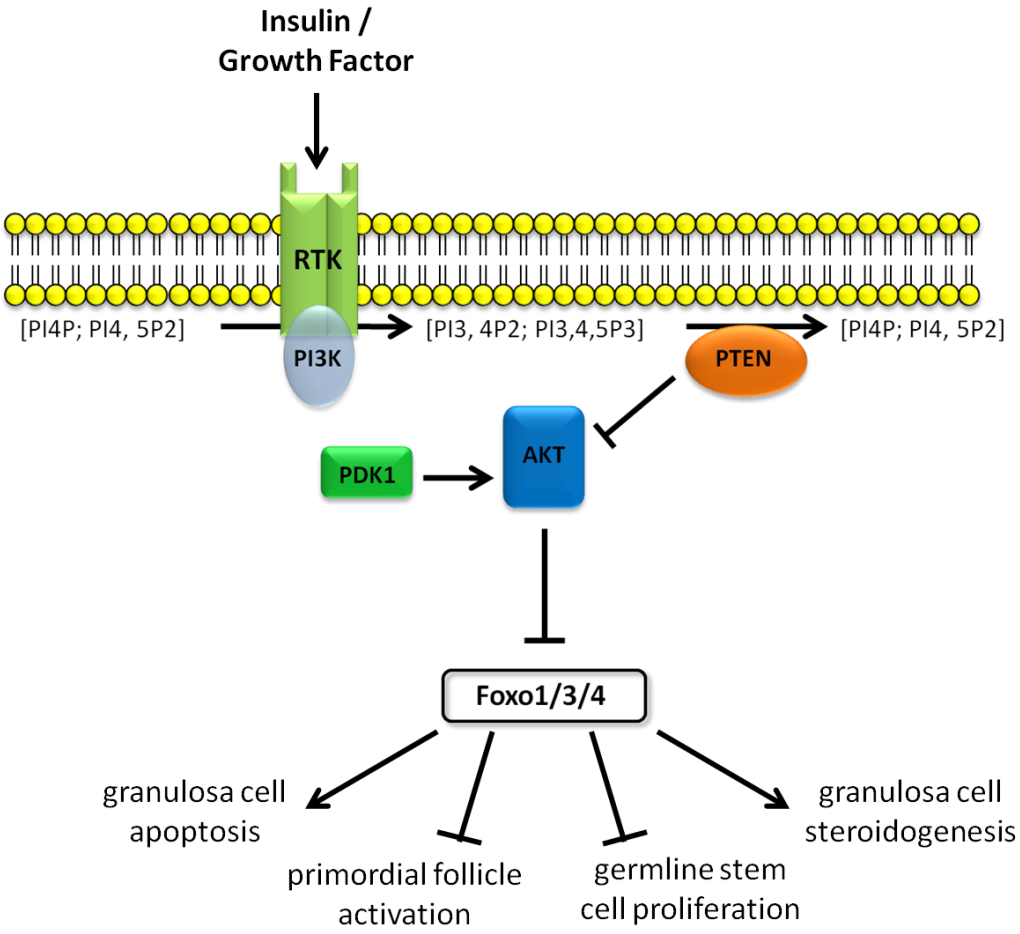

Figure 2.1: PI3K/Akt/Foxo pathway in reproduction
Binding of ligand to the receptor tyrosine kinase leads to activation of PI3K, which converts PIP2 to PIP3. These molecules then activate downstream signaling kinases Pdk1 (phosphoinositide-dependent kinase-1) and Akt. This process can be reversed by the protein PTEN (phosphatase and tensin homologue deleted on chromosome 10), which acts to dephosphorylate PIP2 and PIP3, and thereby prevent activation of Pdk1 and Akt. Akt, a serine/threonine kinase, directly phosphorylates three conserved residues on Foxo, which leads to its nuclear export. The Foxos have been found to play highly conserved germline-autonomous roles in reproduction between species, inhibiting germline stem cell proliferation in *C. elegans* and *Drosophila*, and primordial follicle activation in mammalian models.

CHAPTER THREE
Methodology

Mouse strains, breeding, and analysis

This study was approved by the UTSW Institutional Animal Care and Use Committee. All alleles were in an FVB/n background (backcrossed at least n=10 generations). Generation of the *Vasa-cre*, *Vasa-cre*^{ert2}, *Pten*^{L}, *Pdk1*^{L}, *Kit*^{Y719F}, *Disp1*^{L}, *Fgf8*^{L}, *IGF1R*^{L}, *IR*^{L}, and *Foxo1*^{L}/*3*^{L}/*4*^{L} alleles and genotypic protocols were previously described (Meyers, Lewandoski et al. 1998; Kissel, Timokhina et al. 2000; Li, Robinson et al. 2002; Bluher, Kahn et al. 2003; Kondo, Vicent et al. 2003; Mora, Davies et al. 2003; Tian, Tenzen et al. 2004; Gallardo, Shirley et al. 2007; John, Gallardo et al. 2008). *Vasa-FK2A3* transgenic mice were created through substitution of alanine at threonine 32, serine 253, and serine 325. Genotyping protocols are outlined in Table 3.1. Young *Vasa-cre* males (<10 weeks of age) were used for 2nd generation cross to avoid potent maternal effects observed with females (Gallardo, Shirley et al. 2007; John, Gallardo et al. 2008) and global recombination sometimes observed with older males. Testes from n=3 experimental and control animals (siblings not inheriting *Vasa-cre*) were evaluated as biological replicates for each timepoint in tissue-based analyses. Vitamin A-deficient mice (VAD) were generated by weaning PD21 pups onto a specific vitamin A-deficient diet (Harlan), which they were kept on for six-seven months as indicated in the text.

Tissue processing , immunohistochemistry, and immunofluorescence

For immunohistochemistry, tissues were fixed in 10% buffered formalin overnight, embedded in paraffin, and cut into 5 micron sections. Slides were deparaffinized in xylene, hydrated in an ethanol series, subjected to antigen retrieval by boiling in 10 mM sodium citrate, and cooled at RTx20 min. The detection system was Immpress (Vector, Burlingame, CA). Tissue sections from experimental and control samples were placed on the same slide to ensure identical processing. For whole mount immunofluorescence, seminiferous tubules were mechanically dissociated in PBS on ice and fixed overnight in 4% paraformaldehyde. Tubules were then dehydrated in a series of methanol washes and stored at -20C. To rehydrate and permeabilize, tubules were put through a series of methanol/PBS+0.1% Tween-20 washes. Tubules were blocked with 1% BSA/PBS at RT (Thermo Blocker #37525) for 2 hours, and primary antibody was added in 0.5% BSA/PBS with 0.02% sodium azide and incubated at 4C overnight. Tubules were washed 3x10 minutes in PBS at RT. Secondary antibody (Alexafluor 555 α-mouse cat# A-21422 or Alexafluor 488 α-rabbit cat# A-11008) was added at 1:1000 in 0.5% BSA for 2 hours at RT. Tubules were placed on glass slides and mounted in Vectashield (Vector, Burlingame, CA). The following protocol describes IF on cultured cells: after blocking with Image-iT FX signal enhancer (Invitrogen, cat#136933) for 30 minutes, cells were blocked with PBT/2% normal goat serum for an additional 30 minutes RT. Primary antibody was diluted in PBT/0.5% goat serum and added to cells for overnight incubation at 4C. After washing in PBT, secondary antibody (Alexafluor 555 α-mouse cat# A-21422 or Alexafluor 488 α-rabbit cat# A-11008 (Invitrogen 1:500) in PBT/0.5% goat serum) was added for 1 hr at RT, followed by DAPI (Pierce cat#

46290; 1:10,000 in PBS). Microscopy was performed with a Leica TCS SP5 confocal microscope with a 40x oil immersion lens using 543-nm HeNe laser (26% full power) and 488-nm Ar laser (15% full power) excitation.

Antibodies for IF, IHC, and western blots

Antibodies and titers used were: Foxo1 (Cell Signaling #2880; 1:200 IHC, 1:50 IF, 1:1000 WB), Kit (Cell Signaling #3074; 1:200 IHC, 1:50 IF, 1:1000 WB), Plzf (Calbiochem #OP128; 1:150 IHC, 1:50 IF), GCNA (1:150 IHC) (Enders and May 1994), Ret (IBL America #18121; 1:20 IHC, 1:50 IF, 1:50 WB), p-AKT (Cell Signaling #9271S; 1:100 IHC), Gfrα1 (R&D Systems #AF560; 1:100 IF), Sall4 (Abnova #H00057167-M03; 1:100 IHC), Dppa4 (Santa Cruz Biotechnology #sc-74616; 1:100 IHC), phospho-H3 (Cell Signaling #9706S; 1:100 IF), and PCNA (Cell Signaling #2586; 1:100 IF).

Testicular germ cell counts and differentiation index

After GCNA immunohistochemistry (see above), testis sections were analyzed by counting GCNA+ cells per tubule (N=3 testes). The average number of GCNA-positive germ cells per tubule was determined per animal in each genotype. A minimum of 50 tubules were included per genotyope of PD1-PD7 animals, or 75 tubules per genotype of PD14-adult animals. Empty tubules were defined by the complete absence of GCNA-positive cells. The differentiation index was employed as a measure of the percentage of tubules showing multilayer spermatogenesis, defined as the presence of any

viable germ cells forming an additional layer not associated with the tubular basement membrane. At least 100 tubules were counted per testis and testes from at least two animals were analyzed for each genotype. Student's two-tailed *t* test was used to assess statistical significance, defined as $p \leq 0.05$.

Ovarian follicle counts

Follicle counts of primordial and primary follicles were performed on H&E stained tissue sections of entirely serially-sectioned ovaries, on every 5th section. Only follicles where the oocyte nucleus was in the plane of section were counted. Equal numbers of sections were counted between control and experimental ovaries.

Real-time PCR

cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems #4368814). qPCR was performed as previously described (Bookout, Cummins et al. 2006) using Taqman Gene Expression Assays (Applied Biosystem ID#s: Mm01343388_g1 Dppa4; Mm00490671_m1 Foxo1; Mm00436304_m1 Ret; Mm00456650_m1 Egr2; Mm00842279_g1 Egr4; Mm01240680_m1 Sall4; Mm00521776_m1 Lhx1; Ha99999901_s1 Eukaryotic 18s rRNA). Additional information regarding the probe target sequences are available through the Applied Biosystems website (http://www.appliedbiosystems.com) or through the NCBI TaqMan probe database

(http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/ProjTaqMan.shtml). PCR reactions were set up using Taqman Gene Expression Master Mix (Applied Biosystems #4369016).

Expression profiling

Testes were dissected from PD4 mice and immediately homogenized in 1 ml Tripure (Roche). RNA was prepared per the manufacturer's instructions and resuspended in RNase-free water (30 µl/testes). Quality and purity of the RNA was checked by spectrophotometry and gel electrophoresis. RNA samples were submitted to the UTSW Microarray Core for labeling and hybridization per standard protocols. Each genotype was submitted in triplicate using independent testis samples and hybridized to the Illumina Mouse-6 V2 BeadChip. Analysis was performed using BeadStudio software with quantile normalization. Microarray data on mouse Kit expression during postnatal development was obtained from the National Center for Biotechnology Information, Gene Expression Omnibus Profiles, Series GSE640 (Schultz, Hamra et al. 2003).

Spermatogonial stem cell cultures

Spermatogonial stem cell cultures from mouse testes were derived as previously described (Falciatori, Lillard-Wetherell et al. 2008). Testes were dissected from FVB mice at PD21 and digested with dispase (BD Biosciences, 354235) at 32° C for 30 minutes until visibly disassociated. After centrifugation, the tissue was washed with

DMEM and filtered through a nylon mesh cell strainer (40 μm pore), resuspended in SF medium (recipe outlined in Table 3.1), and plated onto a gelatin-coated plate overnight at 37° C to remove somatic cells. Floating cells were then recovered and plated in SF medium on irradiated MEF feeder cells. Established SSC cultures were fed with SF medium every two days, and continually passaged onto fresh feeder cells.

To prepare feeder cells, DR4-MEFs (Applied Stem Cell, ASF-1002) were first grown in DMEM with 10% heat-inactivated FBS to confluence. Cells were then collected into 50 ml conical tubes, resuspended in DMEM with 10% FBS, and irradiated with 12,000 rads. Irradiated cells were then centrifuged, resuspended in Recovery Cell Culture Freezing Medium (Invitrogen, 12648-010), and aliquoted and stored in liquid nitrogen. Feeder cells were plated at a density of 4.25×10^4 cells per cm^2 on gelatin-coated plates at least 24 hours before addition of SSCs.

To prepare gelatin-coated plates, a 0.2% gelatin solution was made with type A gelatin from porcine skin (Sigma, G-1890) and water, and then autoclaved and stored at 4°C. To coat tissue culture plates, solution was added and plates were incubated at 32°C for at least one hour. Solution was then removed and plates were washed with PBS before use.

Reverse transcription PCR

RNA was isolated using Tripure reagent and resuspended in 11 μl H_2O. Samples were mixed with 1 μg random hexamers, heat denatured at 70° for 10 min, and then

chilled on ice. cDNA was synthesized using the Superscript II enzyme in 20 µl at 42° for 1 hr and then treated with 1 µl RNaseH and incubated at 42° for another 20 min. Samples were first normalized to relative amounts using *Gapdh*. All PCRs were performed using HotStart Taq (Perkin Elmer) for 28 cycles.

Statistical methods

Data were graphed and analyzed using GraphPad Prism 5. Two-tailed Student's t tests were used to evaluate significance and calculate P values, with threshold values as described in text or figure legends. Error bars represent standard deviation of mean values.

Table 3.1: PCR primers and conditions for genotyping*

Gene	Primers 5'-3'	Annealing Temp (°C)	Products
VasaCre	1.CACGTGCAGCCGTTTAAGCCGCGT 2.TTCCCATTCTAAACAACACCCTGAA	55	200 (+)
Foxo1	1.GCTTAGAGCAGAGATGTTCTCACATT 2.CCAGAGTCTTTGTATCAGGCAAATAA 3.CAAGTCCATTAATTCAGCACATTGA	60	115 (+) 149 (L) 190 (-)
Foxo3	1.AACAACCTCACACATGTGCC 2.AGTGTCTGATACCGAAGAGC 3.CATGCAGTCCGAGAGATTTG	55	170 (+) 208(L) 246 (-)
Foxo4	1.GCTTTCTTAGTGAAGGATGGGAAA 2.AATCCTTCCCTTTTCACCCACT 3.CTTCTCTGTGGGAATAAATGTTTGG	55	95 (+) 216 (-)
Foxo4	1.CTTCTCTGTGGGAATAAATGTTTGG 2.CTACTTCAAGGACAAGGGTGACAG	60	313 (+) 555 (L)
Kit Y719F	1.TCTTGGACATAGACTCCTCATACGG 2.GATGGTGAACACAGCCACGAAG	55	381 (+) 400 (-)
Pten	1.AAGCACTCTGCGAACTGAGC 2.TTGCCAGACATGCTCCGAAG 3.GCTTGATATCGAATTCCTGCAGC	58	400 (+) 650 (L) 370 (-)
Pdk1	1.ATCCCAAGTTACTGAGTTGTGTTGGAAG 2.TGTGGACAAACAGCAATGAACATACACGC	60	202 (+) 246 (L)

Gene (cont'd)	**Primers 5'-3'**	**Annealing Temp (°C)**	**Products**
Pdk1	1.ATGTGCTCTGTGTGGAGAA 2.GCCTTCAATCTCAGCACTTG	60	400 (-)
Disp1	1.CCATCGAGGACAGGGGAAATGAAG 2.TCCTGACCATCTCCTCCCTTCTGG	61	150 (+) 250 (-)
Fgf8	1.CTGCAGAACGCCAAGTACG 2.AGCTCCCGCTGGATTCCTC	60	200 (+) 300 (L)
IGF1R	1.ATGAATGCTGGTGAGGGTGTGCTT 2.ATCTTGGAGTGGTGGGTGTGTTTC	59	250 (+) 300 (L)
IR	1.GATGTGCACCCCATGTCTG 2.CTGAATAGCTGAGACCACAG	60	250 (+) 300 (L)
FK2A3	1.CACGTGCAGCCGTTTAAGCCGCGT 2.TTCCCATTCTAAACAACACCCTGAA	55	200 (+)

*All genotyping PCR reactions were performed using Promega GoTaq polymerase (#3001) and reagents. Programs were 95°C 3min; 94°C 30sec, AnnealTemp 30sec, 72°C 30sec (35 cycles); 72°C 7min.

Table 3.2: **Components of SF medium and final concentrations**

Component	**Supplier**	**Catalog No.**	**Concentration**
StemPro-34 SFM base	Invitrogen	10639011	1x to final volume
40x StemPro-34 supplement	Invitrogen	10639011	1x
Bovine serum albumin	Calbiochem	126609	5 mg/mL
d-(+) Glucose	Sigma	G7021	6 mg/mL
L-Glutamine	Invitrogen	25030-149	2 mM
100x Antibiotic-antimycotic	Invitrogen	15240-062	1x
100x MEM vitamins	Invitrogen	111420-052	1x
100x MEM non-essential amino acids	Invitrogen	11140-050	1x
d-Biotin	Sigma	B4639	10 µg/mL
Insulin	Sigma	I1882	25 µg/mL
Sodium pyruvate	Sigma	P4562	30 µg/mL
dl-Lactic acid (60% solution)	Sigma	L7900	0.06%
Ascorbic acid	Sigma	A4034	100 µM
Sodium selenite	Sigma	S5261	30 nM
Putrescine	SIgma	P5780	60 µM
Bovine apo-transferrin	Sigma	T1428	100 µg/mL
Progesterone	Sigma	P8783	60 ng/mL
B-Estradiol	Sigma	E8004	30 ng/ml

Component (cont'd)	**Supplier**	**Catalog No.**	**Concentration**
2-Mercaptoethanol	Sigma	M3148	10 µM
Fetal bovine serum	Hyclone	SH30071	1%
Recombinant human basic FGF	R&D Systems	233-FB	10 ng/mL
Recombinant rat GDNF	R&D Systems	512-GF	10 ng/mL

CHAPTER FOUR
Results

REQUIREMENT OF FOXO1 IN SPERMATOGONIAL STEM CELLS FOR THEIR MAINTENANCE AND THE INITIATION OF SPERMATOGENESIS

The work presented in this chapter has been published in the *Journal of Clinical Investigation*, volume 121, pages 3456-3466.

Introduction

Spermatogenesis is a dynamic process driven by specialized stem cells, termed spermatogonia, which in mammals reside on the basement membrane of seminiferous tubules and maintain a single specialized lineage with many cellular intermediates (see Chapter I). In addition to a capacity for self-renewal, A_s spermatogonia progress through transit-amplifying stages (A_{pr}, A_{al}, A_{1-4}) into B spermatogonia, which differentiate into spermatocytes that proceed through meiosis to generate round spermatids. An elaborate program of cytoskeletal rearrangement and chromatin condensation (Sassone-Corsi 2002) known as spermiogenesis then produces elongated spermatozoa. The concentric geometric arrangement of these cellular intermediates as spermatogenesis proceeds from the periphery towards the lumen combined with the ability to identify diverse stages by simple histologic methods makes the testis an ideal model system for studies of adult stem cell maintenance and differentiation (Oatley and Brinster 2008). Furthermore, spermatogenesis is not required for life, greatly facilitating the generation of mutants and analysis of phenotypes.

Glial cell line-derived neurotrophic factor (Gdnf) serves essential functions in SSC maintenance (see Chapter I). Mutant mice with one null *Gdnf* allele undergo SSC depletion, whereas transgenic males overexpressing Gdnf accumulate undifferentiated spermatogonia (Meng, Lindahl et al. 2000). Gdnf is produced by Sertoli cells (the somatic cells within the seminiferous tubules) and acts through a cell surface receptor heterodimer of Ret and Gfrα1, both coexpressed in a subset of undifferentiated spermatogonia (Naughton, Jain et al. 2006). Gdnf is the critical growth factor permitting limitless expansion of rodent SSCs in culture, although additional growth factors are also needed (Kanatsu-Shinohara, Ogonuki et al. 2003). These cultures consist of A_s, A_{pr} and A_{al}-like spermatogonia capable of self-renewal, immortal growth, and can reconstitute spermatogenesis following engraftment in stem cell transplantation assays (Oatley and Brinster 2008).

The Foxos function at the interface of cellular growth and organismal longevity (see Chapter II) (Salih and Brunet 2008). They are downstream of growth factor and nutrient stimuli and coordinate various responses including cell cycle arrest and programmed cell death. Foxo1, Foxo3, and Foxo4 are coordinately regulated by Akt-dependent phosphorylation, which leads to their export from the nucleus to the cytoplasm, and thus, their functional inactivation (Brunet, Bonni et al. 1999). Foxos promote organismal longevity in invertebrates, and in humans, single nucleotide polymorphisms are associated with extreme longevity, implying important roles in tissue homeostasis and hence adult stem cell function throughout life (Kenyon 2010). Consistent with this idea, the Foxos regulate self-renewal in hematopoietic and neural stem cells (Paik, Kollipara et al. 2007; Tothova, Kollipara et al. 2007; Renault, Rafalski

et al. 2009). With regards to reproduction, our previous studies in the mouse showed that neither *Foxo3* nor *Foxo4* are essential for male fertility (Castrillon, Miao et al. 2003; John, Shirley et al. 2007; John, Gallardo et al. 2008). In contrast, *Foxo1* nullizygosity results in embryonic lethality at ~e10.5, leaving open the possibility of a unique role of Foxo1 in spermatogenesis (Hosaka, Biggs et al. 2004; Paik, Kollipara et al. 2007).

On the basis of the role of the Foxos in aging and stem cell function, the cell-type specific nature of Foxo action, and our group's discovery of the role of Foxo3 in female germline maintenance, we sought to gain an in-depth understanding of the specific functions of the Foxos in the male germline, particularly in SSC self-renewal and differentiation. Here, we demonstrate through genetic, cell biological, and gene expression analyses that, uniquely among the Foxos, Foxo1 is a specific marker of gonocytes and SSCs in vivo and controls SSC differentiation and self-renewal through a specific network of transcriptional targets including Ret. We further show through genetic means that PI3K signaling controls both SSC maintenance and differentiation and that the Foxos are pivotal intermediaries of this balance.

Essential role of Foxo1 in SSC self-renewal and differentiation

To explore the role of the Foxos in spermatogenesis we first conditionally inactivated *Foxo1* (Paik, Kollipara et al. 2007) in the male germline with *Vasa-cre* (a.k.a *Ddx4-cre*). *Vasa-cre* induces recombination in germ cells between e15-e18 and is expressed in all spermatogenic cells postnatally (Gallardo, Shirley et al. 2007). *Vasa-cre; Foxo1*$^{L/L}$ (hereafter referred to as *Foxo1*) testes contained normal numbers of gonocytes at

postnatal day (PD) 1. However, spermatogonia were reduced by PD7, revealing a defect in the proliferative expansion that normally occurs by this timepoint, and testes were small by PD21 (Figure 4.1A-D). This defect was not due primarily to increased cell death (Figure 4.2A). Histologic analyses clearly showed distinct defects in SSC self-renewal and differentiation. Most tubules in *Foxo1* adults contained spermatogenic cells of diverse stages, albeit at reduced numbers. No spermatozoa were present in adult *Foxo1* epididymides despite the presence of round and elongating spermatids in most tubules (Figure 4.2B-C), and as expected given these findings, males were sterile. Some tubules exhibited spermatogonial arrest and failure of meiotic initiation, despite abundant spermatogonia (Figure 4.1D). Still other *Foxo1* tubules were devoid of germ cells (Figure 4.2D), a phenotype never observed in controls, and which implies a defect in SSC self-renewal (Buaas, Kirsh et al. 2004). Older *Foxo1* males exhibited similar phenotypes with the exception that empty tubules were not observed (Figure 4.2D-F). Thus, Foxo1 controls multiple stages of spermatogenesis from SSC proliferation and self-renewal to the progression of spermatogenesis including meiosis.

Triple Foxo knockout more clearly exposes Foxo roles in SSC self-renewal and the initiation of spermatogenesis

These genetic analyses demonstrated that *Foxo1* is necessary for male fertility and is the most important Foxo in spermatogenesis (since Foxo3 and Foxo4 were previously shown to be dispensable for male fertility (Castrillon, Miao et al. 2003; John, Shirley et al. 2007; John, Gallardo et al. 2008). However, it remained possible that

Foxo3 and Foxo4 partially compensated for Foxo1 deficiency (Paik, Kollipara et al. 2007). *Vasa-cre; Foxo1$^{L/L}$; Foxo3$^{L/L}$; Foxo4$^{L/L}$* (hereafter referred to as *Foxo1/3/4*) males had normal gonocytes numbers at birth, but exhibited significant testicular hypotrophy by PD14, earlier than *Foxo1* males, indicating a more severe phenotype (Figure 4.3A-B). Compared to *Foxo1*, *Foxo1/3/4* testes contained fewer germ cells and more tubules depleted of germ cells at all timepoints (Figure 4.3C-E). It is unclear why some tubules become devoid of germ cells while others do not, but such variation has been documented in other mutants that affect the long-term renewal of SSCs such as *Plzf* and *Taf4b* (Buaas, Kirsh et al. 2004; Costoya, Hobbs et al. 2004; Falender, Freiman et al. 2005). Strikingly, there was also a complete spermatogonial arrest with failure of spermatogenic differentiation and meiotic initiation evidenced by the absence of multilayer spermatogenesis including spermatocytes and spermatids in most tubules, even those with abundant spermatogonia (Figure 4.3E, Figure 4.4, Figure 4.5A). Thus, Foxo3/4 partially compensate for Foxo1 in spermatogenesis and simultaneous inactivation of all three Foxos more clearly revealed their collective, essential roles in SSC long-term self-renewal and the initiation of spermatogenesis in the first and subsequent waves of spermatogenesis. Notably, Foxo1/3/4 deficiency phenocopies the failure of spermatogenic initiation and meiotic progression observed in *Kit* mutants such as *Kit^{Y719F}*, which abrogates signaling through PI3K (Kissel, Timokhina et al. 2000).

Foxo1 is regulated during the gonocytes-SSC transition and is required for the induction of Kit

To begin to understand how Foxo1 controls SSC self-renewal and differentiation, immunolocalization was performed with a Foxo1-specific antibody. Foxo1 was expressed in all gonocytes at PD1-3, while in PD21 to adult animals, Foxo1 was confined to basal intratubular cells consistent with spermatogonia (Figure 4.6A; see also next section). Strikingly, Foxo1 underwent cytoplasmic to nuclear translocation during normal development. At PD1, the protein was exclusively cytoplasmic, but nuclear translocation began at PD3 and increased by PD7. At PD3 to PD7 the translocation of Foxo1 protein was not perfectly synchronized, with Foxo1 being distinctly nuclear in some gonocytes but cytoplasmic in others (Figure 4.6A-B). The biological basis of this variation is unclear, but likely reflects differences in rates of gonocytes maturation, with the gonocytes exhibiting nuclear Foxo1 being more mature. By PD21, Foxo1 was nuclear and remained so in adults (Figure 4.6A-B, Figure 4.5B). Thus, Foxo1 is a novel marker of gonocytes whose cytoplasmic-to-nuclear translocation demarcates the gonocytes-to-SSC transition. *Foxo1* testes served as negative controls in these studies, demonstrating the specificity of immunodetection. Also of note, the complete absence of $Foxo1^{+}$ cells in *Foxo1* testes by PD7 proved there was no mosaicism with regards to *Vasa-cre* mediated recombination (Figure 4.6A).

Intriguingly, this translocation of Foxo1 into the nucleus preceded the normal induction of Kit. Consistent with previous studies (Manova, Nocka et al. 1990), Kit was first detectable in wild-type testes at PD3, when Foxo1 first entered the nucleus. Kit increased with continued cytoplasmic-to-nuclear translocation of Foxo1 (Figure 4.6A-B).

This induction of Kit protein matched closely with Kit mRNA levels, suggesting that Kit is regulated transcriptionally (Figure 4.6C). Kit was greatly diminished in *Foxo1* and *Foxo1/3/4* differentiated spermatogonia at PD7 and adulthood (Figure 4.6A, Figure 4.5D-E). The presence of diverse steps of spermatogenesis in *Foxo1* testes argues that Kit underexpression in these testes is not simply explained by the absence of differentiated spermatogonia that normally express Kit. $Plzf^{+}$ (undifferentiated) and $Plzf^{-}$ (differentiated) spermatogonia were present in *Foxo1/3/4* testes, further arguing against a maturational block as the basis for Kit underexpression (Figure 4.5C). However, it remains possible that some skipping of developmental steps occurs in *Foxo* mice, as has recently been described in another spermatogenesis mutant, with loss of transcription factor Dmrt1 (Matson, Murphy et al. 2010). The attenuated induction of Kit in *Foxo* mutants rationalizes the observed defects in meiosis, given that Kit signaling is vital for this process (Kissel, Timokhina et al. 2000).

The converse possibility that Kit regulates Foxo1 (creating a feedback loop) also appeared plausible given that Kit can activate PI3K-Akt, which in turn regulate (inactivate) the Foxos. We analyzed mice homozygous for Kit^{Y719F}, a knock-in mutation that abrogates Kit signaling through PI3K. As previously reported, Kit^{Y719F} testes were populated by numerous SSCs that failed to differentiate and initiate meiosis (Kissel, Timokhina et al. 2000). However, there was no alternation in the expression or subcellular distribution of Foxo1 in Kit^{Y719F} males at PD1-21 (Figure 4.11). Thus, Foxo1 is required for the induction of Kit expression in differentiating spermatogonia, but the regulation appears unidirectional without evidence of a feedback loop (i.e. Kit is downstream of Foxo1 and acts through signaling intermediaries other than Foxo1 itself).

Foxo1 is a specific marker of undifferentiated spermatogonia in steady-state adult spermatogenesis

Co-labeling studies were conducted to further define the spermatogenic cells expressing Foxo1 in the adult testis. In wild-type testes, Foxo1 and Plzf were always coexpressed (Figure 4.7A), demonstrating that in steady-state spermatogenesis, Foxo1 expression is restricted to undifferentiated spermatogonia. Next we studied Gfrα1, which is expressed in single and paired (A_s and A_{pr}) spermatogonia. Only a subset of Foxo1$^+$ cells were also Gfrα1$^+$, but all Gfrα1$^+$ cells were Foxo1$^+$ and furthermore, Foxo1 protein in these cells was predominantly nuclear (Figure 4.7B). These results are consistent with strong Foxo1 activation in SSCs. Furthermore, the abundance of Foxo1 protein in undifferentiated spermatogonia rationalized the unique genetic requirement for *Foxo1* in spermatogenesis, as Foxo3 and Foxo4 were undetectable immunohistochemically in spermatogonia or any other cell type in testis sections (unpublished observations). Finally, Foxo1 and Kit were expressed in distinct cells, as expected given that they mark undifferentiated and differentiated spermatogonia respectively (Figure 4.7C). Thus, it appears unlikely that Kit is a direct transcriptional target of Foxo1; instead, Foxo1 must act through some less direct mechanism to regulate Kit expression in progenitor cells. These results are summarized in Figure 4.7D.

PI3K-Akt signaling operates through the Foxos in the control of SSC homeostasis

Previous studies implicated PI3K-Akt signaling in SSC function (Lee, Kanatsu-Shinohara et al. 2007; Lee, Kanatsu-Shinohara et al. 2009), prompting us to examine if

Foxo1 was an effector of this pathway in spermatogenesis. If so, then *Pten* inactivation should at least partially phenocopy *Foxo1/3/4* since Pten inhibits Akt, which in turn inhibits the Foxos (Brunet, Bonni et al. 1999). *Vasa-cre; Pten*$^{L/L}$ (hereafter referred to as *Pten*) testes contained normal numbers of gonocytes at PD1. There was an initial wave of SSC expansion as in *Foxo1* and *Foxo1/3/4* testes; however, severe defects in SSC self-renewal and differentiation became evident later, resulting in testicular hypotrophy and sterility (Figure 4.8A). Germ cell numbers were normal up to PD7, but decreased by PD21, and there was a significant age-dependent increase in the number of empty tubules (Figure 4.8B-D). There was a complete absence of post-meiotic round spermatids (Figure 4.8D). By PD21 no germ cells remained attached to the basement membrane, demonstrating exhaustion of the SSC pool. Testes were almost entirely depleted of germ cells by 4 weeks (Figure 4.8D). Thus, Pten shares with the Foxos essential roles in SSC self-renewal and spermatogenesis.

Pten loss led to Akt hyperphosphorylation and cytoplasmic localization of Foxo1 by PD7, when Foxo1 is normally predominantly nuclear. Based on its weak staining intensity, cytoplasmic Foxo1 also appeared to be rapidly degraded. This *Pten*-mediated functional inactivation of Foxo1 was associated with defective Kit induction at PD7 (Figure 4.8E). Thus, Pten acts at least in part through PI3K-Akt and the Foxos to regulate SSC maintenance and differentiation. The more severe phenotype of *Pten* vs. *Foxo1/3/4* suggests that additional effectors mediate other actions of PI3K-Akt in the male germline.

To further investigate the control of the Foxos by the PI3K pathway, we conditionally inactivated *Pdk1*, which is required for Akt activation and thus has a

biochemical role opposite to that of *Pten*. Whereas Pten promotes Akt hyperactivation and Foxo inactivation, Pdk1 loss conversely results in Akt hypoactivation and constitutive Foxo activation (Figure 4.9A). *Vasa-cre; Pdk1*$^{L/L}$ (hereafter referred to as *Pdk1*) males were sterile and their testes were small due to decreased germ cell numbers (Figure 4.9B-D). Histologic analyses revealed a complete failure of multilayer spermatogenic differentiation. Notably, the subcellular distribution of Foxo1 was dramatically altered in *Pdk1* spermatogonia. Foxo1 was nuclear in virtually all *Pdk1* spermatogonia at timepoints when it is normally cytoplasmic (Figure 4.9E, $p<0.0001$ at PD3 and 14), an effect opposite to *Pten*. Also in sharp contrast to *Pten* and *Foxo*, *Pdk1* testes did not exhibit an age-dependent SSC self-renewal phenotype. Whereas *Pten* males were depleted of germ cells by 4 weeks resulting in empty tubules, *Pdk1* males maintained large numbers of SSCs, which continued to proliferate as evidenced by abundant Ki67 positivity. Spermatogonia were numerous even at 5 months of age and were Plzf^{+} or Plzf^{-}, consistent with undifferentiated and differentiated spermatogonia (Figure 4.9D). In conclusion, although the signals that regulate Foxo in spermatogonia are not fully known, these findings strongly suggest that they act through PI3K-Akt. Furthermore, our results demonstrate that PI3K-Akt signaling must be carefully titrated *in vivo* to maintain SSC self-renewal and differentiation, and argue that the Foxos are pivotal intermediaries of this balance.

Gene expression analysis identifies Ret and other stem cell factors as Foxo1 targets

Kit underexpression in spermatogonia offered a plausible mechanism for the observed defects in SSC differentiation and meiotic initiation in *Foxo* mutants. However, our phenotypic analyses also uncovered a discrete Foxo role in SSC self-renewal, a process unaffected by Kit loss-of-function (Kubota, Avarbock et al. 2009; Morimoto, Kanatsu-Shinohara et al. 2009). Therefore, Foxo1 must regulate distinct factors relevant to SSC self-renewal. To uncover these, genome-wide transcriptomic analyses were performed on *Vasa-cre; Foxo1*$^{L/L}$ vs. sibling control *Foxo1*$^{L/L}$ testes (n=3 animals per genotype). PD4 was selected because at this timepoint, *Foxo1* testes were morphologically normal without the cell ratio alteration characterizing later timepoints. Only 31 genes were differentially expressed per stringent criteria. *Foxo1* itself was among the differentially-expressed genes and several genes were represented by non-overlapping probe sets, suggesting that these targets were physiologically valid. The relatively small number of genes suggests that most may be direct targets (Table 4.1). Quantitative PCR confirmed mRNA level alterations consistent with the microarray data for all loci tested (Figure 4.10A).

Many of these factors are well-known regulators of stem cell identity in embryonic or adult stem cells, including Gata2, Dppa4, Sall4, Lhx1, and Ret (Zhang, Tam et al. 2006; Maldonado-Saldivia, van den Bergen et al. 2007; Oatley and Brinster 2008; Bresnick, Lee et al. 2010; Tanaka, Yamaguchi et al. 2010), while others are key regulators of spermatogenic progression and meiosis, including Egr2 and Egr4, and Tex19 (Tourtellotte, Nagarajan et al. 1999; Ollinger, Childs et al. 2008; Hogarth, Mitchell et al. 2010). That many Foxo1 targets are themselves transcription factors

(underlined) suggests that Foxo1 sits atop a regulatory hierarchy controlling SSC maintenance and differentiation. The identification of Lhx1 is particularly interesting, given that it is directly involved in SSC maintenance (Oatley, Avarbock et al. 2007). Also of note, the Foxo1 transcriptome in male germ cells was distinct from that reported in other cell types, where genes regulating oxidative stress resistance have been identified as major targets (Tothova, Kollipara et al. 2007; Paik, Ding et al. 2009; Renault, Rafalski et al. 2009).

Ret appeared to be particularly significant because its ligand Gdnf is essential for SSC maintenance. In addition, a prior genome-wide screen identified Lhx1 and Egr2 among only six genes whose expression was strongly Gdnf-dependent in SSCs (Oatley, Avarbock et al. 2006), suggesting we identified a bona fide Gdnf/Ret-associated transcriptional signature. Ret protein was obviously reduced in *Foxo1* and *Foxo1/3/4* spermatogonia, confirming that Ret expression is regulated by Foxo1 (either directly or indirectly) *in vivo* (Figure 4.10B).

To further explore the physiologic significance of these findings, Ret was analyzed in *Pten* and *Pdk1* testes, where the Foxos are constitutively inactivated or activated, respectively. Similar to *Foxo* testes, and as expected, Ret was significantly reduced in *Pten* testes. However, Ret was strikingly elevated in *Pdk1* testes (Figure 4.10C). These findings strongly argue that the PI3K pathway regulates Ret expression in SSCs, and furthermore that this regulation occurs via the Foxos. Given the requirement for Ret in SSC maintenance (*Ret* loss-of-function mutations result in germ cell depletion in mice), the control of Ret by the PI3K pathway via Foxo1 rationalizes the SSC self-renewal phenotype observed in our Foxo mutants (Jijiwa, Kawai et al. 2008).

Moreover, the transcription factors Sall4 (sal-like protein 4) and Dppa4 (developmental pluripotency-associated gene 4) were both reduced in *Foxo1* testes as seen by IHC (Figure 4.10D-E). Sall4 is required for embryonic stem cell pluripotency, potentially through interaction with stem cell factor Nanog (Wu, Chen et al. 2006; Zhang, Tam et al. 2006). Likewise, Dppa4 is a marker of pluripotency in embryonic stem cells as well as germline stem cells, and its expression disappears with differentiation (Babaie, Herwig et al. 2007; Maldonado-Saldivia, van den Bergen et al. 2007). The decrease in transcripts and protein expression in *Foxo1* testes of both Sall4 and Dppa4 further confirms regulation by Foxo1, either directly or indirectly. Given what is known of their functions, loss of Sall4 and Dppa4 would further explain the defects in SSC self-renewal and maintenance seen in *Foxo1* and *Foxo1/3/4* testes.

Discussion

This study demonstrates that the Foxos, particularly Foxo1, serve critical and nonredundant roles in SSC homeostasis both *in vitro* and *in vivo*. In the absence of the Foxos, SSCs exhibit a failure to both self-renew and differentiate, leading to infertility. Given these data, the Foxos share similar functions within the male and female germline in maintaining gametogenesis (see Chapter II). Ablation of either Foxo3 or Foxo4 causes no spermatogenesis phenotype in the male, while single knockout of Foxo1 leads to infertility, demonstrating that Foxo1 is the most highly expressed and functionally important of the Foxos in the testis. On the other hand, Foxo3 is highly expressed in primordial oocytes within the ovary (John, Gallardo et al. 2008), where Foxo1 and Foxo4

are less abundant and functionally relevant. Foxo1 acts to maintain the SSC pool and regulate the onset of differentiation in males, while Foxo3 acts to maintain quiescence of the primordial follicles in females. The unique genetic requirement for *Foxo1* in males and *Foxo3* in females mirrors their high expression at discrete cellular stages in spermatogenesis or oogenesis. Moreover, both Foxo1 and Foxo3 undergo relocalization from the cytoplasm to the nucleus postnatally, suggesting that the timing of their activation is critical for normal germline development and function in juveniles. Taken together, these studies suggest that the Foxos have evolved to adopt distinct but controlling roles in oogenesis and spermatogenesis, two processes with many similarities but also important differences.

One of the more interesting and potentially unexpected findings in this study is that Foxo1 controls various aspects of spermatogenesis, from long-term SSC self-renewal to the initiation of meiosis and differentiation. The pleiotropic *Foxo1* phenotype showed various spermatogenic defects between seminiferous tubules, with some tubules containing spermatogonia through spermatids. However, no tubules contained elongating spermatids or mature spermatozoa, indicating a possible requirement of Foxo1 in spermatid polarization and potentially spermiogenesis. Expression of Foxo1 is detectable only within the undifferentiated spermatogonia, but these defects in later steps of spermatogenesis could be due to low Foxo1 expression in these cell types, or the action of Foxo1 in spermatogonia could initiate a cascade of events that influence later steps of spermatogenesis.

The underexpression of Kit in *Foxo* and *Pten* mutants, evident by IHC, provides a plausible explanation for the defects in differentiation and initiation of meiosis seen in

these testes. However, since Foxo1 contributes to diverse and functionally opposing steps of spermatogenesis, both SSC self-renewal and differentiation, further investigations will be needed to understand how transcription of the individual Foxo targets identified in this study is regulated. The Foxos join a growing network of transcription factors, including *Plzf, Taf4b,* and *Nanog*, which control early steps of spermatogenesis including SSC self-renewal (Buaas, Kirsh et al. 2004; Falender, Freiman et al. 2005; Kuijk, van Mil et al. 2010). Further dissection of the functional interrelationships among these factors and the upstream factors that contribute to their function in SSCs is warranted. Additionally, further studies analyzing the transcription targets of the Foxos in SSCs are needed to validate the results of the gene expression analysis outlined in this study, and to formally distinguish between direct promoter occupancy and indirect downstream targets. The more severe phenotype seen in the *Foxo1/3/4* testis hints that the three Foxos each act upon distinct targets, and utilization of single Foxo mutants, *in vivo* and *in vitro*, will provide additional information in understanding any functional redundancy.

Similarly to Foxo3 in primordial follicles and consistent with prior observations (Brunet, Bonni et al. 1999; John, Gallardo et al. 2008), the developmental relocalization of Foxo1 during the gonocytes-SSC transition implies that its subcellular localization is an important determinant of biological activity. Generation of *Pdk1* and *Pten* mutants showed that the PI3K-Akt pathway is the principal pathway regulating Foxo1 localization and activity in the context of spermatogenesis (see Chapter V for further analysis of Foxo1 subcellular localization). Foxo1 is highly expressed and in an active (nuclear) state in SSCs, indicating its important link with the "stem state" in spermatogonia. For

example, in *Pten* testes, Foxo1 was cytoplasmic and inactivated, and SSCs were rapidly depleted. In contrast, in *Pdk1* testes, Foxo1 remained activated and nuclear, and SSCs were never depleted. These findings also imply that the inactivation of Foxo1 that occurs physiologically triggers the initiation of spermatogenesis. However, the upstream trigger initiating PI3K/Akt signaling and Foxo translocation in spermatogenesis remains to be found. Candidates are a variety of growth factor ligands that bind cognate receptor tyrosine kinases or G-protein coupled receptors that act via PI3K, such as insulin and insulin-like growth factors which are known to be important in Foxo signaling (Lin, Dorman et al. 1997; Ogg, Paradis et al. 1997; Wang, Bohmann et al. 2005). The studies of the Kit^{Y719F} mutant argue that Kit ligand is not critical in upstream activation of the pathway. In addition, the nuclear and active state of Foxo1 in Gfrα1$^+$ A_s and A_{pr} spermatogonia further argues that the Gdnf-Gfrα1/Ret signaling axis is not the principal regulator of Foxo activity *in vivo*. If that were the case, pathway activity would drive Foxo1 out of the nucleus. Further experiments and discussion of this may be found in the following chapter.

Ablation of PI3K signaling components, Pdk1 and Pten, revealed their respective requirements for spermatogenic differentiation. Both *Pdk1* and *Pten* testes exhibited an absence of multilayer spermatogenesis; however, their respective phenotypes reflected their opposing function within the pathway itself. Pten inactivation caused complete SSC depletion by 4 weeks of age, while conversely, Pdk1 inactivation led to absence of differentiation, but the SSCs persisted and proliferated into adulthood. *Pten* testes show hyper-phosphorylated Akt and Foxo1 degradation, whereas *Pdk1* testes have increased nuclear activity of Foxo1. Finally, Pten deletion led to significantly decreased expression

of Ret, a putitive Foxo1 target, and Pdk1 deletion caused over-expression of Ret. These data reveal the importance of PI3K/Akt signaling within the SSCs, where excessive activity leads to stem cell loss, and low activity leads to a failure to differentiation and enter meiosis.

Recent studies have implicated the Foxos in maintenance of additional stem cell populations. The Foxos, particularly Foxo3, coordinately regulate neural stem cell homeostasis through genes influencing stress responses and oxygen metabolism (Paik, Ding et al. 2009; Renault, Rafalski et al. 2009). The Foxos also regulate HSC differentiation and assist long-term maintenance by protecting against oxidative stress. Moreover, Foxo1 is required to maintain pluripotency of both human and mouse embryonic stem cells, presumably through transcription of Oct4, though was not found to be acting downstream of the PI3K/Akt pathway (Zhang, Yalcin et al. 2011). The transcriptomic analyses presented here did not yield targets relevant to oxygen metabolism or cellular stress responses, but instead more closely parallel Foxo1 function in ESCs. However, the Foxos appear to control a network of genes unique to spermatogenesis, consistent with diverse studies showing that Foxo functions are biologically numerous and highly context-dependent (Salih and Brunet 2008).

The identification of Ret as a possible downstream Foxo1 target rationalizes the observed defects in long-term SSC maintenance seen in *Foxo1* testes. By IHC, Ret was undetectable in both *Foxo1* and *Foxo1/3/4* spermatogonia and was highly expressed in *Pdk1* spermatogonia. GDNF signaling through receptors Ret and Gfrα1, and the PI3K/Akt pathway, acts to promote SSC survival and proliferation (Oatley, Avarbock et al. 2007; Oatley and Brinster 2008). *Ret* inactivation results in severe defects in SSC

proliferation and differentiation by PD7, leading eventually to SSC depletion (Naughton, Jain et al. 2006; Jijiwa, Kawai et al. 2008). The similarity of the Ret and Foxo phenotypes and these previous studies together strongly argue that Ret downregulation accounts for the observed Foxo phenotypes in SSC maintenance.

The studies presented here, which were all based on phenotypic analyses of multiple *in vivo* indicate that Foxo1 expression is strongly tied to stem cell potential. They also argue that the Foxos function in a germ cell-autonomous manner to maintain the SSC population. Future dissection of the role of the Foxos and the PI3K pathway in the regulation of SSC long-term maintenance would likely benefit from the derivation and utilization of *in vitro* SSC models. Derivation and culturing of SSCs from testes conditionally-null for the Foxos and other PI3K pathway components is currently underway, allowing for more in-depth biochemical analyses of enriched SSCs (see Chapter V for Foxo1 in WT SSC cultures). Additionally, these *in vitro* models may be used to confirm direct Foxo targets in SSCs through ChIP-Seq or EMSA without contaminating somatic cell populations present *in vivo*. The combination of these *in vitro* and *in vivo* models will lead to further insights into the diverse molecular mechanisms by which the PI3K-Foxo pathway regulates the long-term maintenance and differentiation of SSCs.

In conclusion, the Foxos are pivotal regulators of SSC self-renewal and differentiation. Like other adult stem cells, SSCs represent a finite reserve that needs to be maintained throughout life. The importance of the Foxos in organismal aging (Kenyon 2010), their role in maintaining other stem cell populations (Tothova, Kollipara et al. 2007; Zhang, Yalcin et al. 2011), along with this discovery of their function in SSC

self-renewal all suggest that the Foxos function throughout life to protect this finite resource. Because the SSC population represents the entire foundation of spermatogenesis and fertility, abnormalities in SSC maintenance or differentiation may account for clinical cases of male infertility, while overexpansion of SSCs may lead to testicular cancers (Looijenga 2009). Thus, these findings and future investigations of the PI3K-Akt-Foxo pathway in SSCs should lead to important insights into the etiology of these clinically-important but poorly understood conditions.

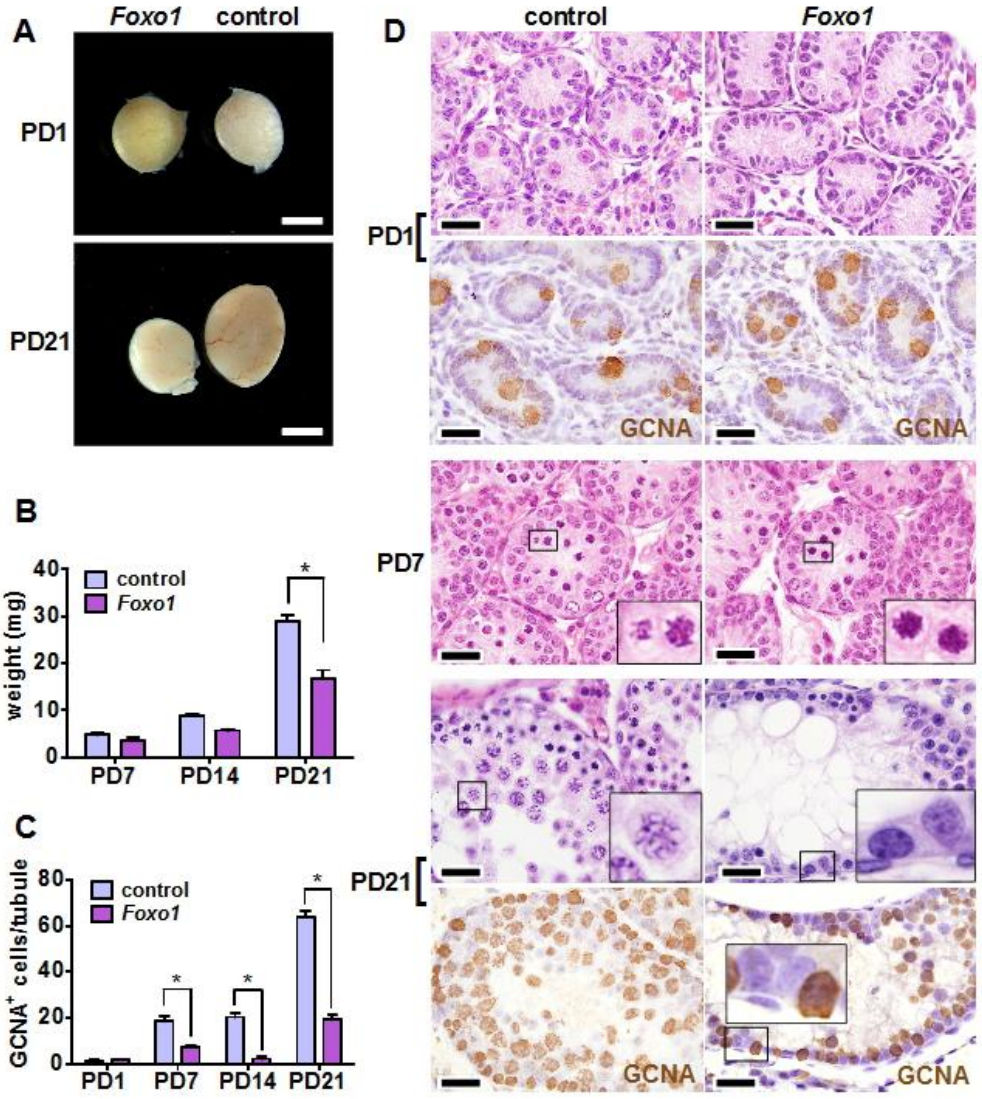

Figure 4.1: Foxo1 in SSC maintenance and differentiation *in vivo*
(A) Intact testes, bar=3 mm. (B) Testis weights, asterisk indicates $p<0.005$. (C) Germ cell numbers per immunostaining for pan-germ cell marker GCNA. Counts shown are $GCNA^+$ cells per tubule, asterisks indicate $p<0.0001$. (D) Testis histology at PD1, 7, and 21 in control sibling and *Foxo1* mice. For PD1 and PD21, representative GCNA-stained sections are shown. PD7 insets show spermatogonia in mitosis. For PD21, inset on control testis shows pachytene spermatocytes. PD21 *Foxo1* panel shows example of tubule with spermatogonial arrest. Bar=20 μm.

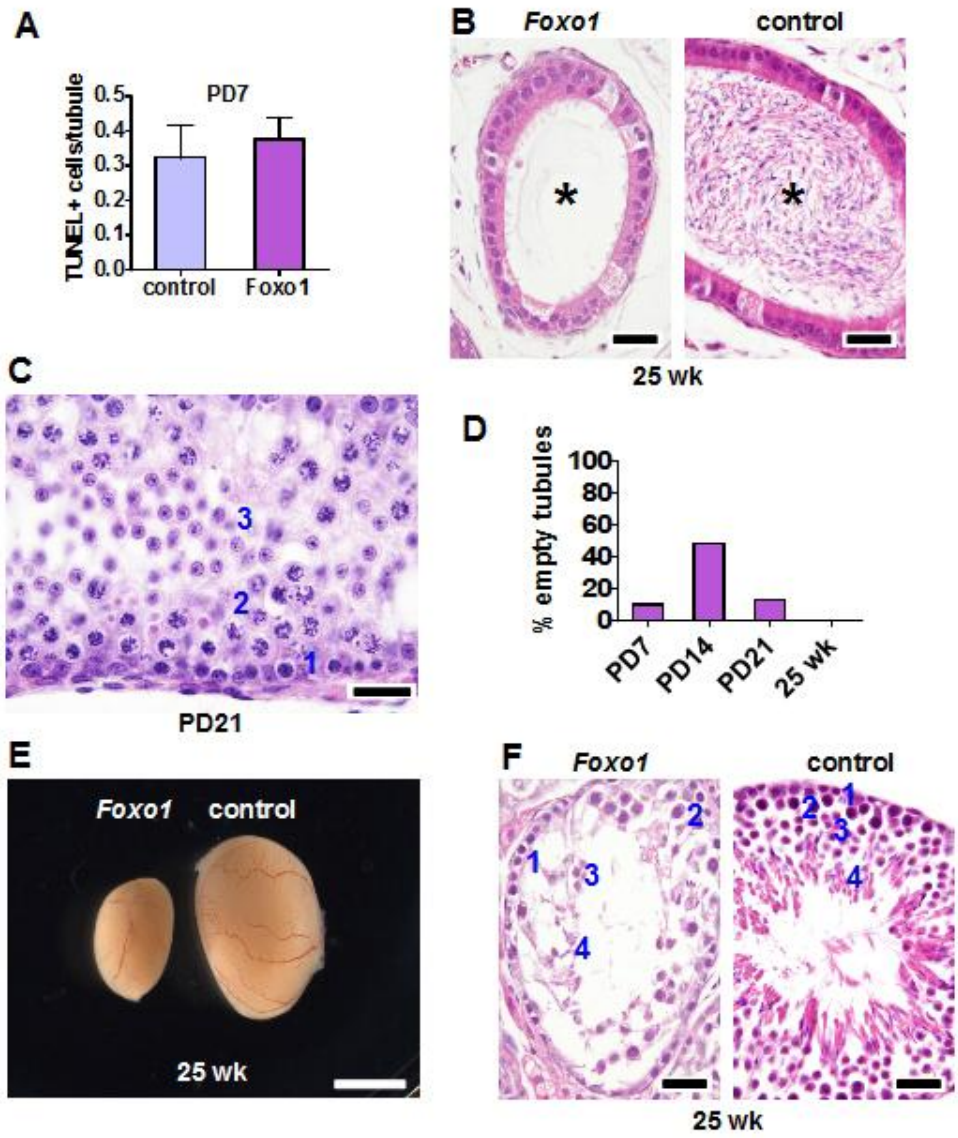

Figure 4.2: Further evaluations of *Foxo1* testes
(A) TUNEL positive cells normalized to number of GCNA positive cells. (B) Tissue sections at 25 weeks of age. No sperm were present in epididymides (asterisks) or in epididymal caudal fluid (not shown) at 25 wks or any other age; bar=20 μm. (C) *Foxo1* phenotype is variable within tubules; shown is relatively unaffected tubule at PD21 with multilayer spermatogenesis including all steps normally present at PD21 including spermatogonia (1), premeiotic spermatocytes (2) and postmeiotic round spermatids (3); bar=20 μm. (D) Percent of empty tubules harboring no germ cells per GCNA immunostains. Controls contained no empty tubules at these timepoints. (E) *Foxo1* and

control testes at 25 wks, bar=3 mm. (F) Adult (25 weeks) *Foxo1* tubules have markedly diminished spermatogenesis but all stages of spermatogenesis including spermatogonia (1), spermatocytes (2), spermatids (3), and elongated spermatozoa (4) are observed; bar=20 μm.

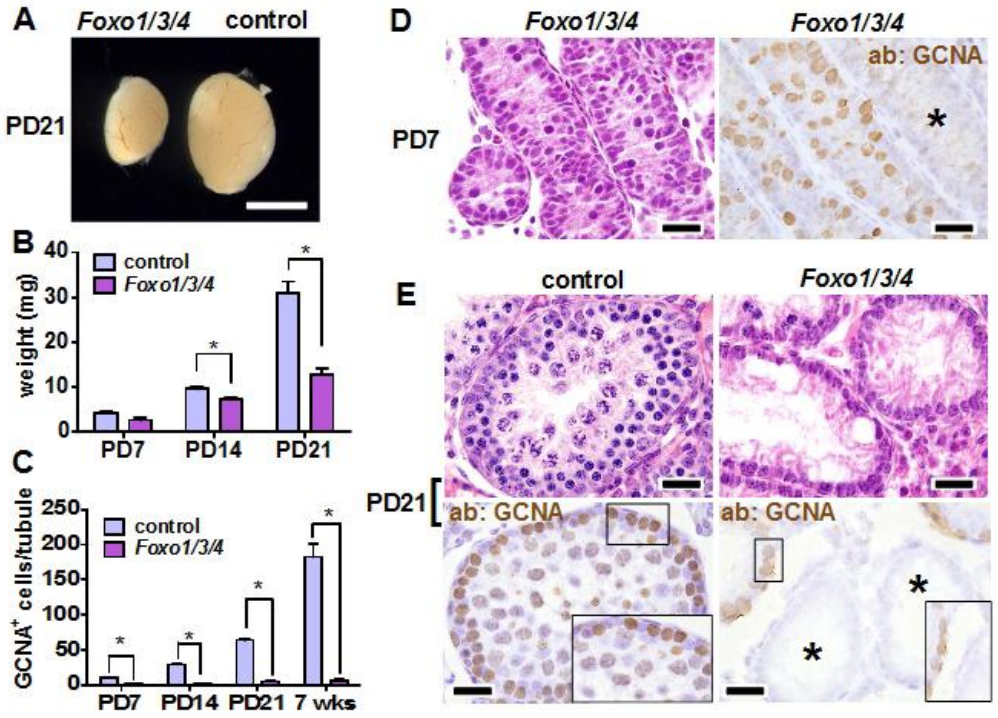

Figure 4.3: More severe SSC maintenance and meiotic initiation phenotypes in *Foxo1/3/4* male mice

(A) Intact testes, bar=3 mm. (B) Testis weights, asterisk indicates $p<0.05$. (C) Germ cell numbers by counting $GCNA^+$ cells per tubule, asterisks indicate $p<0.0001$. (D) PD7 testes, H&E and GCNA immunostain demonstrating empty tubules harboring no germ cells. Control testes contained no empty tubules at these timepoints. (E) Testis histology for mutant or wild-type controls, H&E or immunostains as indicated. Controls have multilayer spermatogenesis whereas *Foxo1/3/4* tubules have many spermatogonia (confirmed by GCNA) but no multilayer spermatogenesis. Asterisks indicate empty tubules at PD7 and PD21. Insets at PD21 highlight basal layer of spermatogonia and complete failure of spermatogenic initiation. Bar=20 μm.

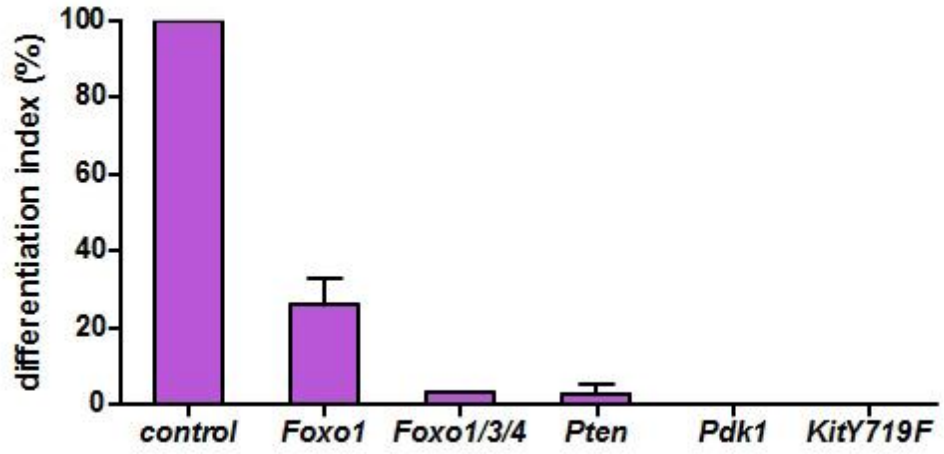

Figure 4.4: Differentiation indices
Differentiation index (percentage of seminiferous tubules showing multilayer spermatogenesis) for each mutant in this study and wild-type controls at PD21.

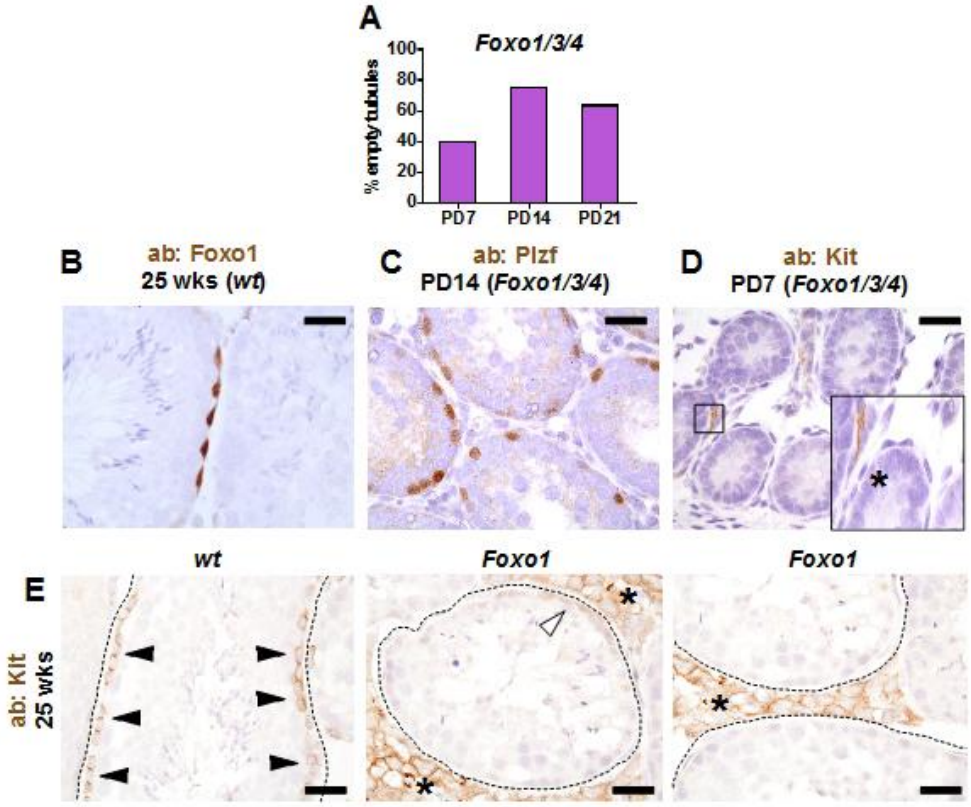

Figure 4.5: Additional studies of *Foxo1*, *Foxo1/3/4*, and control testes

(A) Empty tubules harboring no germ cells in *Foxo1/3/4* tissue sections immunostained with GCNA. Control testes contained no empty tubules at these timepoints. (B) Foxo1 immunostain of wild-type adult mouse. Cell clustering (consistent with a portion of germline cyst in the plane of section) is sometimes observed. (C) $Plzf^{+}$ and $Plzf^{-}$ spermatogonia in *Foxo1/3/4* testes at PD14. (D) Kit underexpression in *Foxo1/3/4* testes at PD7. Inset shows Kit in Leydig cells (asterisk) as internal positive control. Bar=20 μm for B-D. (E) Kit underexpression in adult *Foxo1* testis (25 weeks), tubule borders highlighted by dashed lines. In wild-type control (left panel), Kit is prominent in subset of tubules in a stage-dependent manner (arrowheads); this is not observed in *Foxo1* testis. Middle panel shows tubule with minimal focal Kit expression (arrowhead); right panel shows tubules with no Kit expression. Kit^{+} Leydig cells are prominent in *Foxo1* testes (Leydig cell hyperplasia secondary to failure of spermatogenesis, asterisks). Bar=20 μm.

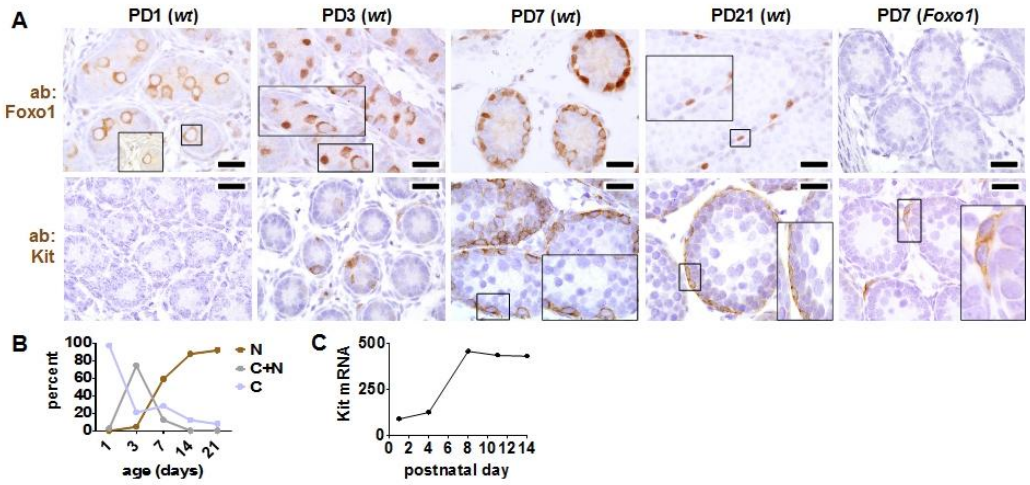

Figure 4.6: Foxo1 is specifically expressed in gonocytes and undifferentiated spermatogonia and regulates Kit

(A) Kit and Foxo1 immunostains at timepoints and genotypes as shown. PD7 *Foxo1* testis serves as negative control for Foxo1 immunostaining; Kit inset below shows Kit expression in Leydig cells as internal positive control. Other insets illustrate subcellular distributions of Foxo1 and Kit; bar=20μm. (B) Subcellular distribution of Foxo1 in spermatogonia, C=cytoplasmic; N=nuclear. (C) Developmental profile of Kit mRNA levels in mouse testis by microarray; arbitrary units on y-axis.

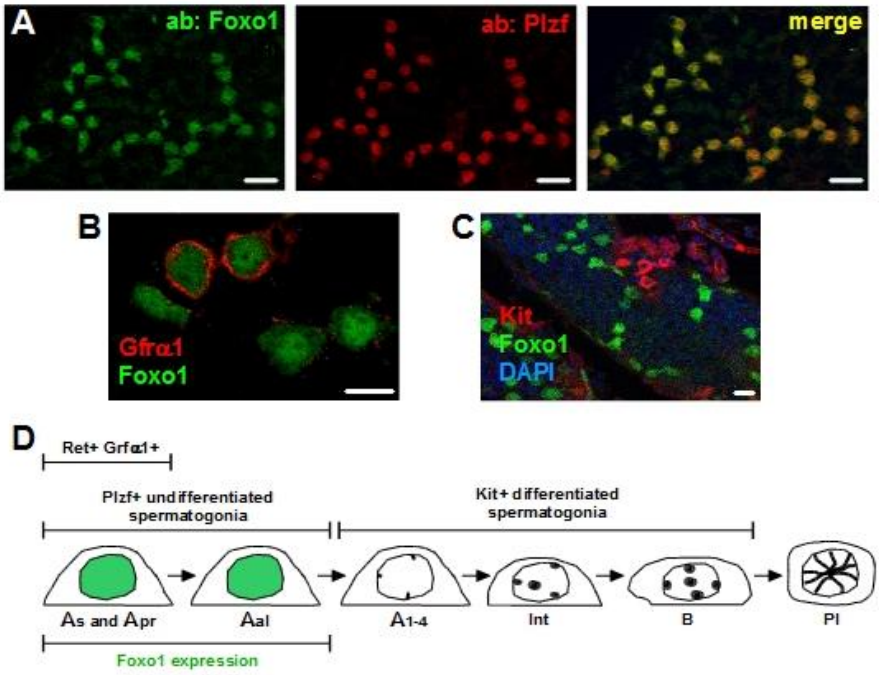

Figure 4.7: Foxo1 is specifically expressed in undifferentiated spermatogonia in adult testis

Panels show confocal images of intact tubules. (A) Foxo1 and Plzf co-expression. Shown are two 16-cell clusters. Bar=20 μm. (B) Gfrα1 and Foxo1 expression. Bar=10 μm. (C) Kit and Foxo1, non-overlapping expression. Bar=20 μm. (D) Schematic illustrating subset of spermatogonia expressing Foxo1. A_s-B spermatogonia to preleptotene (Pl) spermatocyte are shown.

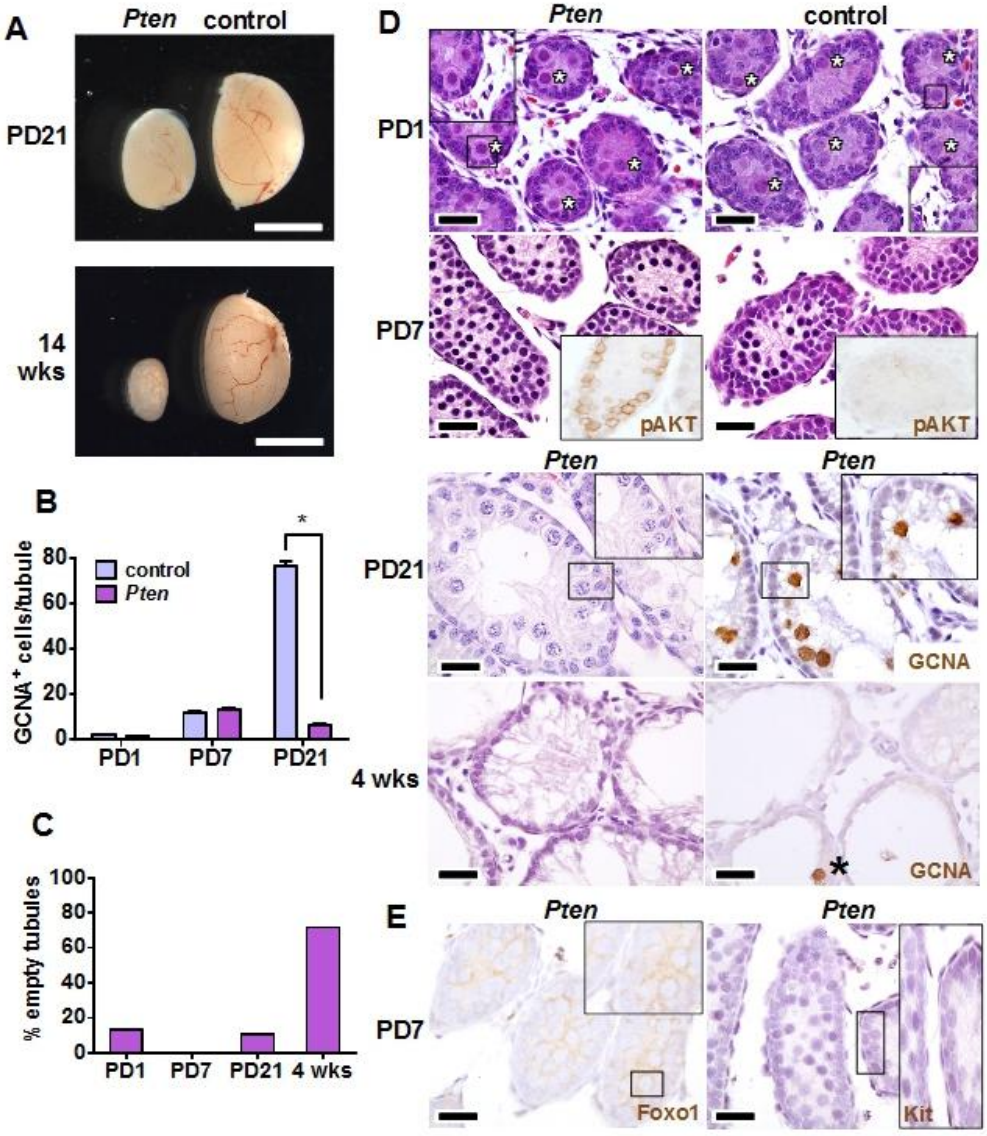

Figure 4.8: Regulation of Foxo1 by PI3K-Akt pathway, *Pten* and control testes
(A) Intact testes, bar=3 mm. (B) $GCNA^{+}$ cells per tubule, asterisk indicates $p<0.0001$. (C) Empty tubules harboring no germ cells per GCNA. Control testes contained no empty tubules at these timepoints. (D) Testis histology, H&Es or immunostains as indicated. Asterisks at PD1 highlight gonocytes, which were morphologically normal. Asterisk at 4 wks shows rare residual germ cell. (E) Foxo1 and Kit immunostains of *Pten* testes at PD7. Bar=20 μm for D-E. See Figure 3.3 for predominantly nuclear localization of Foxo1 and expression of Kit in controls.

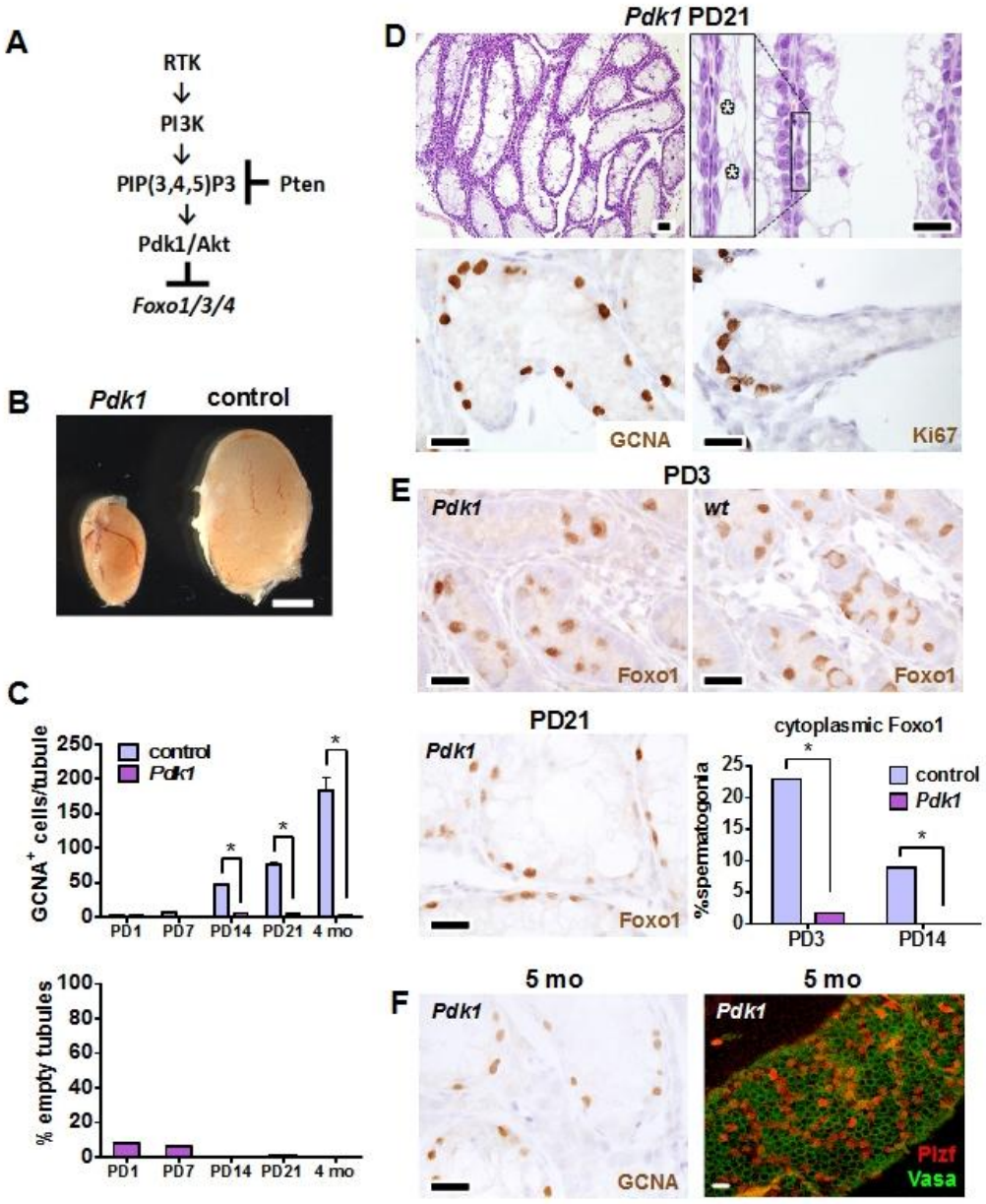

Figure 4.9: Regulation of Foxo1 by PI3K-Akt pathway, *Pdk1* and control testes
(A) Schematic of PI3K-Akt-Foxo pathway. A receptor (typically a receptor tyrosine kinase, RTK) results in PI3K activation and synthesis of 3-phosphoinositides (PIP3). Pten metabolizes PIP3 and acts as a pathway inhibitor. (B) *Pdk1* testes were small by PD14. Testes from 4 month-old males, bar=3 mm. (C) $GCNA^+$ cells per tubule, asterisks indicate $p<0.0001$. Bottom graph shows tubules devoid of germ cells per

GCNA. (D) Testis histology (H&E) and immunohistochemistry with antibodies as shown. Inset shows alternating Sertoli cells and spermatogonia (asterisks) on basement membrane with complete absence of differentiation and multilayer spermatogenesis. Ki67 demonstrates that spermatogonia are actively proliferating; the Ki67 index in spermatogonia was >10% (not shown). (E) Foxo1 localization in *Pdk1* testes at various ages as shown. *Pdk1* inactivation promotes shift of Foxo1 protein from cytoplasm to nucleus at PD3. Asterisks in graph indicate $p<0.0001$. (F) Persistence of SSCs in aged (5 month-old) males. GCNA shows abundant spermatogonia and absence of empty tubules. Plzf and Vasa staining of intact tubules (confocal microscopy) shows presence of both undifferentiated ($Plzf^+$) and differentiated ($Plzf^-$) $Vasa^+$ spermatogonia in aged *Pdk1* testes. Bar=20 μm for all panels in D-F.

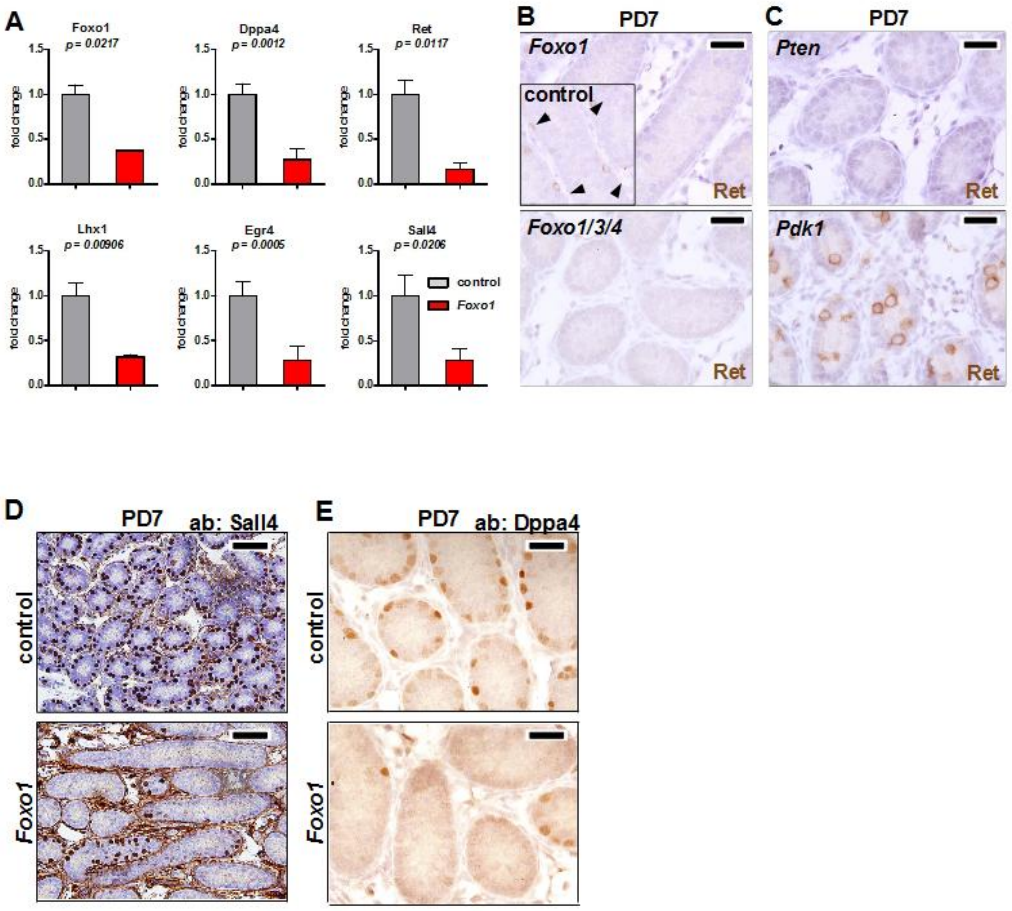

Figure 4.10: ***In vivo*** **transcriptomic analysis identifies Foxo1 targets including Ret**
(A) Relative mRNA levels in control vs. *Foxo1* testes (n=3 biological replicates) by qPCR. (B) Decreased Ret protein in *Foxo1* and *Foxo1/3/4* testes by immunohistochemistry, slides counterstained with hematoxylin. In control testis (inset), Ret is detectable on cell surface of a subset of undifferentiated spermatogonia (arrows). (C) Ret is decreased in *Pten* but increased in *Pdk1* testes. (D) Decreased Sall4 protein in PD7 *Foxo1* testes by immunohistochemistry, slides counterstained with hematoxylin. (E) Decreased Dppa4 protein in PD7 *Foxo1* testes by immunohistochemistry, slides counterstained with hematoxylin. Bar=20 μm for B,C, and E. Bar=100 μm for D.

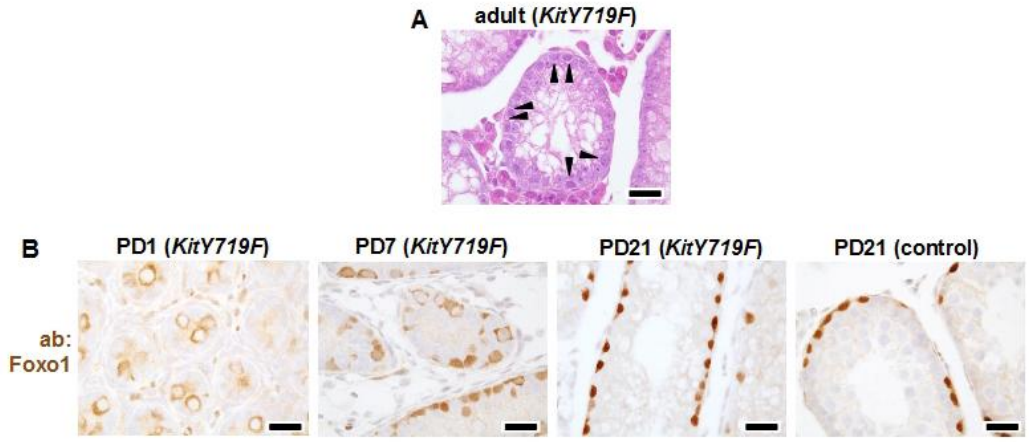

Figure 4.11: Foxo1 expression and its subcellular localization in gonocytes and undifferentiated spermatogonia are not regulated by Kit-PI3K signaling
(A) H&E-stained section from adult Kit^{Y719F} male showing failure of spermatogenic initiation manifest by absence of multilayer spermatogenesis (spermatogonial arrest). Multiple spermatogonia are present and can be distinguished from Sertoli cells by their distinct chromatin pattern and lack of prominent nucleoli (arrowheads). (B) Immunostaining for Foxo1 in Kit^{Y719F} testis at various ages. A PD21 wild-type control testis is shown on the right; see Figure 3.3 for additional control timepoints. Bar=20 μm for A and B.

Table 4.1: Expression profiling of Foxo1 vs. control testes at PD4

GENE SYMBOL	GENE NAME	FOLD CHANGE
Cdh16	cadherin 16	4.02
Krt14	keratin 14	3.11
Tacstd2	tumor-associated calcium signal transducer 2	2.82
Actg2	actin, gamma2, smooth muscle	2.77
Igfbp2	insulin-like growth factor binding protein 2	2.77
Igfbp2	insulin-like growth factor binding protein 2	2.73
Serpina3n	serine peptidase inhibitor	2.71
CN716893		2.24
Gata2	GATA binding protein 2	2.24
Moxd1	monooxygenase, DBH-like 1	2.04
Fgl1	fibrinogen-like protein 1	-2.03
Tuba3a	tubulin, alpha 3A	-2.03
CN716893		-2.06
Ela2a	elastase 2A	-2.07
Foxo1	forkhead box O1	-2.09
Igf2bp1	insulin-like growth factor 2 binding protein 1	-2.09
Fbxo41	F-box protein 41	-2.12
Sult4a1	sulfotransferase family 4A	-2.13
Lhx1	LIM homeobox 1	-2.14
Actn2	actinin, alpha 2	-2.15
Nudt6	nudix type motif	-2.2
Col9a3	collagen, type IX, alpha 3	-2.22
Pkd2l1	polycystic kidney disease 2-like 1	-2.24
1700006H03Rk		-2.25
Trim71	tripartite motif-containing 71	-2.26
Rbm35a	RNA binding motif protein 35A	-2.34
Tex19	testis expressed gene 19	-2.35
Egr2	early growth response 2	-2.78
Dppa4	developmental pluripotency associated 4	-3.13
Ret	ret proto-oncogene	-3.24
Sall4	sal-like 4	-3.26
Egr4	early growth response 4	-3.44
Fgl1	fibrinogen-like protein 1	-3.47
Tcl1	T-cell lymphoma breakpoint 1	-3.86

CHAPTER FIVE
Results

SUBCELLULAR LOCALIZATION OF FOXO1 IN UNDIFFERENTIATED SPERMATOGONIA AND SPERMATOGONIAL STEM CELLS

Introduction

Foxo1 canonically acts downstream of the PI3K/Akt pathway in multiple cell types, including spermatogonia as outlined in Chapter III. Upon phosphorylation by active Akt, the Foxos are exported from the nucleus into the cytoplasm and are thus functionally inactive (Brunet, Bonni et al. 1999). Therefore, the distinction between nuclear and cytoplasmic Foxo1 is dependent on its cell-type dependent targets and the upstream signals acting through the pathway.

The importance of Foxo1 in maintaining spermatogenesis in the mouse testis has recently been elucidated along with its striking localization within the neonatal testis (Chapter IV) (Goertz, Wu et al. 2011). Foxo1 was expressed in all gonocytes at PD1-3, while in PD21 to adult animals, Foxo1 expression was confined to undifferentiated spermatogonia on the basement membrane of the seminiferous tubules, a population that includes the spermatogonial stem cells (Figure 4.5). As previously mentioned, Foxo1 underwent cytoplasmic to nuclear translocation during normal development. At PD1, the protein was exclusively cytoplasmic, but nuclear translocation began at PD3 and increased by PD7. Interestingly, among the Foxo1-positive spermatogonia in the adult, about 80% are nuclear and 20% cytoplasmic, and this ratio stays consistent through the

life of the animal (Figure 5.1). The distinction between these Foxo1^{+} spermatogonia, in which Foxo1 is either nuclear and active, or cytoplasmic and inactive, has yet to be determined.

Foxo1 localization and retinoic acid signaling

In vivo, the majority of Foxo1^{+} spermatogonia are Foxo1 nuclear. Conversely, SSC cultures in defined media (see Chapter III) have nearly 100% cytoplasmic Foxo1 (Figure 5.1C). Addition of retinoic acid to the culture medium induced shuttling of Foxo1 into the nucleus (work of Zhuroru Wu, data not shown). This opened up the possibility that vitamin A and retinoic acid signaling was acting *in vivo* as well to determine Foxo1 activation.

The importance of vitamin A and retinoic acid signaling in spermatogenesis and fertility has been well established (see Chapter I, (Livera, Rouiller-Fabre et al. 2002)). To explore the possibility that retinoic acid signaling may induce Foxo1 shuttling, 3 week old mice were weaned onto a vitamin A-deficient diet for six to seven months. As expected, mice exhibited defects in eyesight and generally weighed less than mice fed a normal diet (data not shown).

Histological analysis of vitamin A-deficient testes revealed significant spermatogenic defects, though undifferentiated spermatogonia persisted on the basement membrane as seen by Foxo1^{+} immunohistochemistry (Figure 5.2A). If retinoic acid was in fact activating Foxo1 *in vivo*, numbers of nuclear versus cytoplasmic Foxo1 spermatogonia would be reversed from wild-type, with a majority of cytoplasmic Foxo1.

Quantification of nuclear versus cytoplasmic Foxo1 in these testes found no significant difference in numbers as compared to testes from mice on the control diet (Figure 5.2C). Foxo1 staining did, however, reveal changes in the nuclear structure of undifferentiated spermatogonia. Early studies in the effects of vitamin A deficiency on spermatogenesis found definitive ultrastructural changes to occur in the remaining cells which may account for the phenotype seen here (Mitranond, Sobhon et al. 1979; Sobhon, Mitranond et al. 1979). In nuclear Foxo1^{+} cells, Foxo1 looked to be excluded from a subcellular structure, most likely the nucleosome. While this may be due to a staining artifact or secondary to dietary deficiency, the possible implication of this exclusion should be further explored. Nevertheless, based on nuclear versus cytoplasmic quantification of Foxo1^{+} cells, retinoic acid signaling does not influence the subcellular localization of Foxo1 in undifferentiated spermatogonia.

Foxo1 localization and the cell cycle

The Foxos have previously been found to regulate cell cycle progression in different cell types (Alvarez, Martinez et al. 2001; Martinez-Gac, Marques et al. 2004). To determine if the subcellular localization of Foxo1 was related to the cell cycle in the undifferentiated spermatogonia, co-labeling studies were done with cell cycle markers in whole tubules. Double staining with Foxo1 and phospho-histone H3 (pH3), a marker of M phase, showed no distinct correlation between subcellular localization of Foxo1 and mitosis (Figure 5.3B). Less than 10% of both Foxo1 nuclear and Foxo1 cytoplasmic spermatogonia were positive for pH3. The majority of Foxo1^{+} cells also pH3^{+} showed

widespread localization of Foxo1 throughout both the nucleus and the cytoplasm (Figure 5.3A-B). Rather than a midpoint of translocation, this is most likely due to nuclear breakdown concurrent with cell division. Additionally, co-localization with PCNA (proliferating cell nuclear antigen), which is most highly expressed during S phase, showed a trend toward correlation with Foxo1 localization (Figure 5.3C, p=0.03). Cytoplasmic Foxo1 was more highly associated with S phase and PCNA positivity than nuclear Foxo1. This may indicate cytoplasmic and inactivated Foxo1 correlates with cell-cycle progression, though this needs to be further investigated with additional experiments. Taken together, these data indicate that, in the context of spermatogenesis, Foxo1 subcellular localization does not show a relationship with cell cycle, at least with the markers used in this study. These studies do not entirely exclude the possibility that Foxo1 shuttling in vivo is cell cycle related.

Foxo1 localization and clone size

As mentioned earlier, expression of Foxo1 is restricted to the undifferentiated spermatogonia, with Plzf being expressed in all Foxo1+ cells and vice-versa (Figure 4.7). Gfrα1, which is expressed in single and paired (A_s and A_{pr}) spermatogonia, is also expressed in all Foxo1$^+$ cells. Only a subset of Foxo1$^+$ cells were also Gfrα1$^+$, but all Gfrα1$^+$ cells were Foxo1$^+$ (Figure 5.4A). To explore the possibility of Foxo1 subcellular localization correlating with Gfrα1$^+$ expression and therefore stemness, whole mounts were co-stained and quantified (Figure 5.4). In this manner the distribution of Foxo1

(nuclear vs. cytoplasmic) could be analyzed in cysts of different sizes and in GFrα1^{+} versus GFRα1^{-} cells.

First, quantification of Gfrα1 and Foxo1 co-localization in the context of spermatogonial cyst size was done with A_s through A_4 spermatogonia, since occasional A_4 cysts expressed Gfrα1, while no A_8 cysts did so. Somewhat unexpectedly given previous results (Meng, Lindahl et al. 2000; Kubota, Avarbock et al. 2004), some small A_s and A_{pr} clones (Foxo1^{+}) did not express Gfrα1. In A_s, A_{pr}, and A_4 clones, cytoplasmic Foxo1 was only found in A_s and A_{pr} spermatogonia, and these were always negative for Gfrα1 (Figure 5.4B). Since Gfrα1^{+} spermatogonia purportedly contain the stem cell population, these results may indicate that cytoplasmic Foxo1 is not consistent with stemness. Further analysis of nuclear Foxo1 and Gfrα1 co-expression showed no overt correlation with spermatogonial stage (Figure 5.4B). However, all spermatogonia expressing Gfrα1 had nuclear Foxo1 (Figure 5.4C). Cytoplasmic Foxo1 and Gfrα1 were never co-expressed, and approximately 40% of all Foxo1 nuclear spermatogonia expressed Gfrα1. While nuclear Foxo1 was not always associated with Gfrα1 positivity, nuclear status of Foxo1 in Gfrα1^{+} spermatogonia is consistent with strong Foxo1 activation in SSCs.

Constitutively active Foxo3 causes testicular hypotrophy and infertility

The importance of Foxo1 in SSC maintenance and differentiation was described in Chapter IV. Ablation of either Foxo3 or Foxo4 has no effect on male fertility, but

given the more severe phenotype in the *Foxo1/3/4* testes, Foxo3 and Foxo4 are expressed in the testis and contribute to spermatogenic self-renewal and differentiation.

A constitutively nuclear, and therefore active, Foxo3 transgenic line was created to explore the nuclear versus cytoplasmic function of the Foxos in spermatogenesis. Using the *Vasa* promoter, allowing for germ cell-specificity, the three conserved Akt phosphorylation sites on Foxo3 (see Chapter II) were changed to alanine (hereafter referred to as *FK2A3*). These sites are critical in the nuclear to cytoplasmic translocation of Foxo3, and without them, Foxo3 will remain active in the nucleus.

FK2A3 males were sterile, and testes exhibited extreme testicular hypotrophy which increased with age (Figure 5.5A). H&E-stained sections of 16-week testes showed an absence of multilayer spermatogenesis in the majority of tubules, though the severity differed slightly between tubules (Figure 5.5B). Consistent with a lack of spermatogenesis and infertility, these testes also exhibited Leydig cell hyperplasia (Figure 5.5B). Comparison of $TUNEL^{+}$ cells in seminiferous tubules between 16-week *FK2A3* and sibling control showed an obvious increase in apoptosis in *FK2A3* testes (Figure 5.5C). Whether this is due to an increase in *bona fide* pro-apoptotic transcriptional targets of Foxo3 or an artifact of Foxo3 over-expression remains to be seen, but this transgenic model provides further insight into the Foxo nuclear versus cytoplasmic distinction in spermatogenesis.

Discussion

The experiments outlined here have served to narrow down the functional distinction of Foxo1 in its subcellular localization. Within the adult mouse testis, the majority of undifferentiated spermatogonia have nuclear localization of Foxo1, consistent with its transcriptional activation. The cytoplasmic to nuclear translocation present in the neonatal testis signifies the gonocyte-SSC-transition, indicating the importance of Foxo1 in establishing and maintaining the SSC population. However, in adult steady-state spermatogenesis, the presence of cytoplasmic Foxo1 in approximately 20% of spermatogonia is not easily explained.

Retinoic acid signaling regulates the differentiation and meiotic entry of undifferentiated spermatogonia (see Chapter I). In contrast to the translocation seen in SSC cultures, deprivation of vitamin A produced no difference in the nuclear to cytoplasmic ratio of $Foxo1^{+}$ cells in the mouse testis. Hence, the predominant nuclear localization of Foxo1 does not necessarily reflect an imminent onset of differentiation and meiosis. Gene expression analysis of potential Foxo1 targets did uncover factors known to promote differentiation, but also factors required for stem cell identity and pluripotency (Chapter IV), indicating that transcriptional activity of Foxo1 is complexly regulated to determine cell fate. In SSC cultures, Foxo1 is entirely cytoplasmic in defined SF media, but shuttles to the nucleus upon addition of retinoic acid. The anomalous Foxo1 localization in these cultures may be attributed to the artificial culture conditions containing high concentrations of multiple growth factors, including insulin. The effects of RA seen in SSC cultures, translocating Foxo1 to the nucleus, may also be an artifact. The *in vivo* results indicate that RA does not directly interact with Foxo1 and

its functional activity, but translocation *in vitro* could be attributed as secondary to induced differentiation. More physiologically relevant culture conditions may more closely resemble *in vivo* nuclear to cytoplasmic Foxo1 ratios, allowing for further analysis of this functional distinction.

Surprisingly, analysis of cell cycle and Foxo1 status showed no distinct correlation. The Foxos have been well described as regulators of cell cycle in multiple cell types, transcribing targets such as cyclins and p27 (Dijkers, Medema et al. 2000; Medema, Kops et al. 2000; Alvarez, Martinez et al. 2001; Ramaswamy, Nakamura et al. 2002; Stahl, Dijkers et al. 2002; Martinez-Gac, Marques et al. 2004). However, in spermatogonia, co-localization of Foxo1 with markers of M phase, pH3, and S phase, PCNA, showed similar numbers of Foxo1 nuclear and Foxo1 cytoplasmic spermatogonia undergoing each relative phase of mitosis. PCNA degradation overlaps with subsequent phases of mitosis, and so may not specifically and accurately delineate S phase. Supplementary co-localization studies with additional cell cycle markers, covering each phase of mitosis, will confirm the role of Foxo1 and cell cycle in spermatogonia. These studies uncovered a $Foxo1^{+}$ spermatogonial population in which Foxo1 is diffuse throughout the cell, present in both the nucleus and the cytoplasm. All of these cells were found to be undergoing mitosis, and so the diffuse localization of Foxo1 is attributed to nuclear envelope breakdown, leaving no intracellular barriers to confine Foxo1 localization. However, further investigations are required to rule out the possibility that these cells represent a transition between nuclear and cytoplasmic status.

Described in the previous chapter, Foxo3 and Foxo4 also contribute to the maintenance of spermatogenesis, though to a lesser degree than Foxo1. The constitutive

activation of Foxo3, *FK2A3*, led to testicular hypotrophy and spermatogenic defects, attributed to increased apoptosis. Whether this phenotype is attributed to increased expression of *bona fide* pro-apoptotic Foxo targets or an artifact of forced overexpression is unclear. Nevertheless, the constitutively active Foxo3 model does provide some insight into the effects of nuclear Foxos. Future experiments employing constitutively active Foxo1, the primary testicular Foxo, will provide more physiologically relevant *in vivo* data clarifying subcellular localization. Clearly, the Foxos themselves must be tightly regulated, as both increased and decreased Foxo expression and activation lead to various spermatogenic defects.

The most intriguing finding presented here is the nuclear status of Foxo1 in all Gfrα1^{+} spermatogonia. First, this co-expression suggests that GDNF signaling through Gfrα1/Ret does not control Foxo1 localization through the PI3K/Akt pathway (i.e. if Ret were active, Foxo1 should be cytoplasmic). Also, Foxo1 is nuclear and active within the stem cell population, validating the importance of its transcriptional targets, such as Ret. Chapter IV outlined potential Foxo1 targets in spermatogonia found through gene expression analysis, which included both well-known regulators of stem cell identity as well as factors required for differentiation and meiotic entry. Within the SSCs, Foxo1 may be actively transcribing factors necessary to maintain pluripotency and self-renewal capabilities, such as Ret, Sall4, and Dppa4. However, Foxo1 targets also include factors necessary for differentiation and meiotic entry, which are presumably not expressed within the Gfrα1^{+} stem cells. What then determines the Foxo gene targets to be expressed? The presence of both nuclear Foxo1 and Gfrα1 may represent the subpopulation of Foxo1+ spermatogonia with pluripotency-associated transcription

targets. The nuclear status of Foxo1 alone is not necessarily indicative of stemness, since the majority of Foxo1^{+} nuclear spermatogonia did not express Gfrα1, a putative stem cell marker. Additional studies differentiating these two populations of Foxo1^{+} nuclear spermatogonia are needed to validate any differences in transcriptional targets.

Canonically, nuclear Foxo1 is actively transcribing target genes, while cytoplasmic Foxo1 is functionally inactive and targeted for degradation. In this case, 80% of undifferentiated spermatogonia have active Foxo1, while the remaining 20% have active signaling through the PI3K pathway resulting in Foxo1 inactivation. However, cytoplasmic Foxo1 has more recently been discovered as crucial in autophagy (Zhao, Yang et al. 2010). Autophagy is the process whereby intracellular proteins and organelles are degraded through delivery to the lysosome (Stromhaug and Klionsky 2001). Therefore, cytoplasmic Foxo1 is not necessarily synonymous with inactive Foxo1. SSCs, as the foundation for spermatogenesis throughout the lifetime of the organism, must be diligently maintained, and the translocation of Foxo1 from the nucleus to the cytoplasm may simply indicate requirement for autophagy of defective cellular organelles or misfolded proteins.

While Gfrα1 positivity was found to associate with nuclear Foxo1, the converse was not always true, and the molecular distinction of cytoplasmic Foxo1 remains to be found. A major difficulty in differentiating these Foxo1^{+} populations lies in their relatively low numbers per seminiferous tubule, and so derivation and utilization of SSC cultures allows for relative enrichment of undifferentiated spermatogonia and will prove vital in future experiments. Additionally, live-cell imaging will contribute crucial information about the fate of nuclear versus cytoplasmic Foxo1^{+} spermatogonia. The co-

localization experiments undertaken in this project have identified three sub-populations of Foxo1^{+} spermatogonia: Foxo1 nuclear and Gfrα1^{+}, Foxo1 nuclear and Gfrα1^{-}, and Foxo1 cytoplasmic. Given its diverse transcriptional targets, these sub-populations may correspond to SSCs (Foxo1 transcribing SSC targets such as *Ret*, *Sall4*, *Dppa4*), undifferentiated spermatogonia (Foxo1 transcribing differentiation targets such as *Egr2*, *Egr4*, *Tex19*), and differentiating spermatogonia (Foxo1 cytoplasmic and inactive), respectively. Taken together, Foxo1, which is required for both SSC self-renewal and differentiation, is transcriptionally active within SSCs, and lies atop a complex hierarchy of transcription factors in determining cell fate.

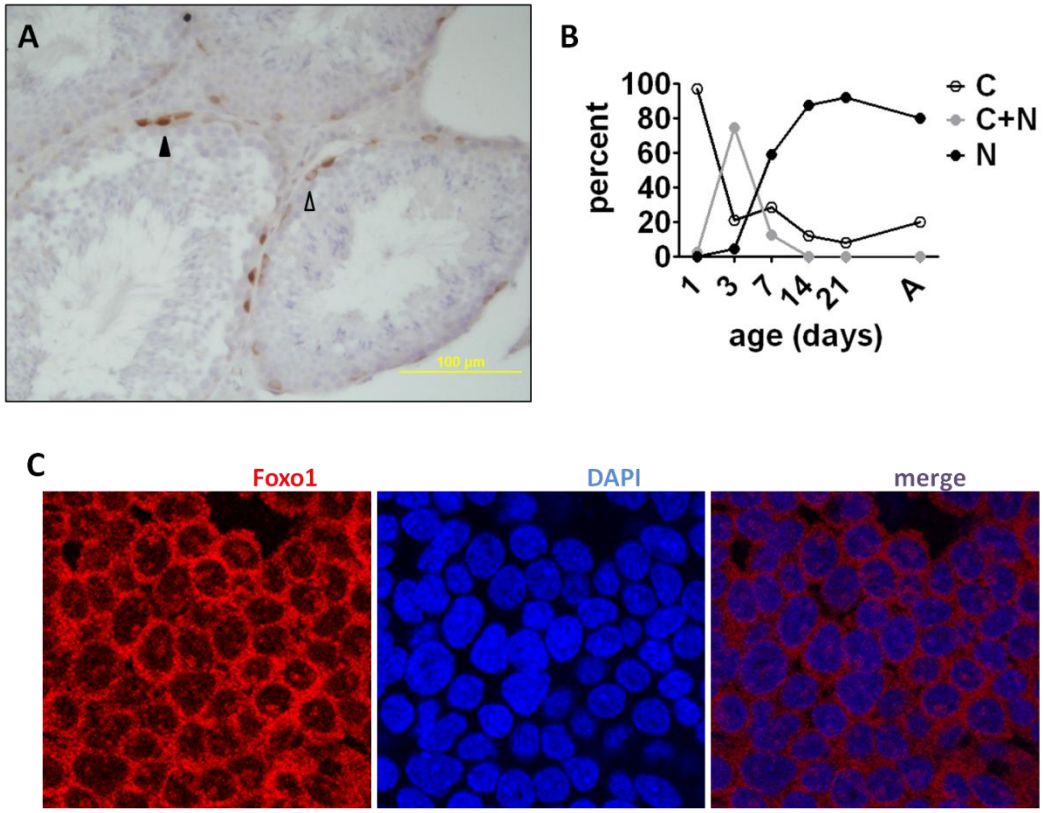

Figure 5.1: Subcellular localization of Foxo1 in undifferentiated spermatogonia of adult mice

(A) Immunohistochemistry for Foxo1 on adult testis (3 month). Localization of Foxo1 in spermatogonia is either entirely nuclear (closed triangle) or entirely cytoplasmic (open triangle). (B) Subcellular distribution of Foxo1 in spermatogonia, C=cytoplasmic; N=nuclear. (C) IF on WT SSC cultures shows predominant cytoplasmic localization of Foxo1.

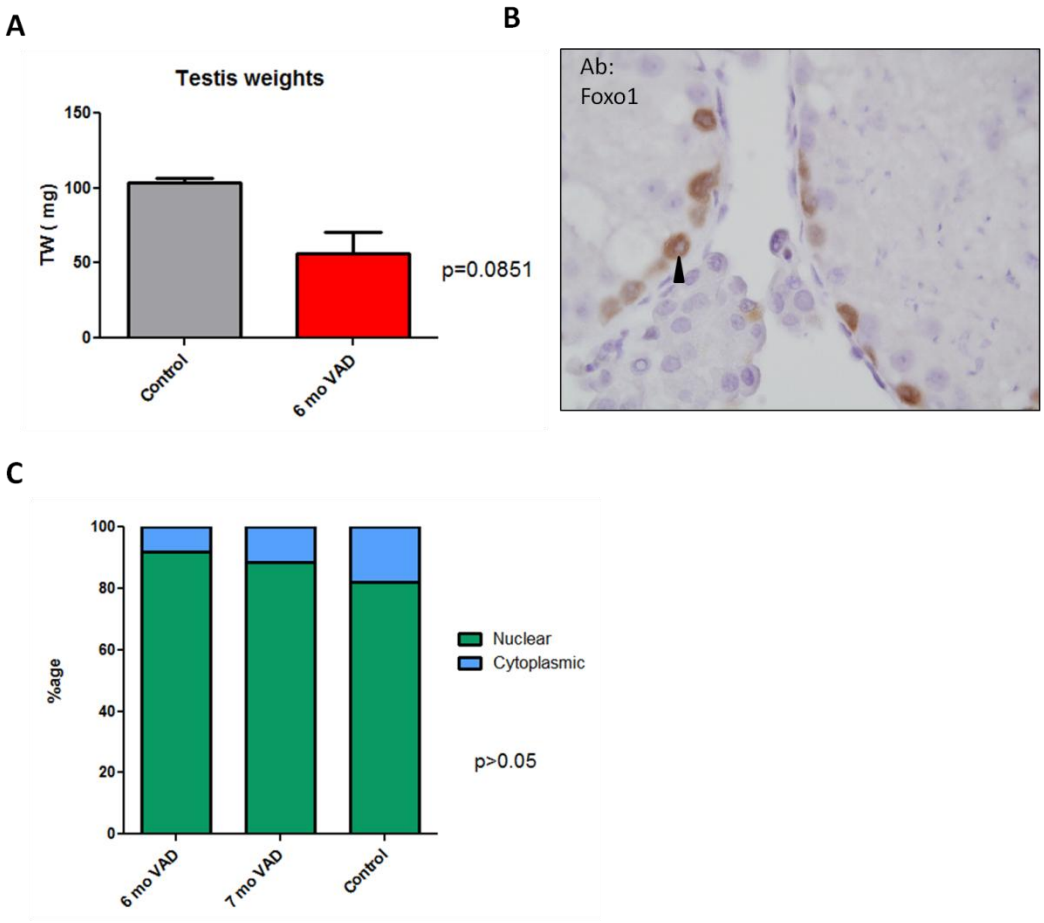

Figure 5.2: Vitamin A deficiency does not affect localization of Foxo1
(A) Relative testis weights from control diet and 6 months VAD diet mice. N=4 (B) Example of Foxo1 IHC on vitamin A deficient testes (6 months on VAD diet). Triangle shows possible nucleosomal exclusion of Foxo1. (C) Quantification of nuclear versus cytoplasmic Foxo1 localization in vitamin A-deficient animals compared to animals on control diet. P value represents t test comparison of cytoplasmic Foxo1 in VAD versus control testes. VAD = vitamin A deficient diet. Error bars represent SEM.

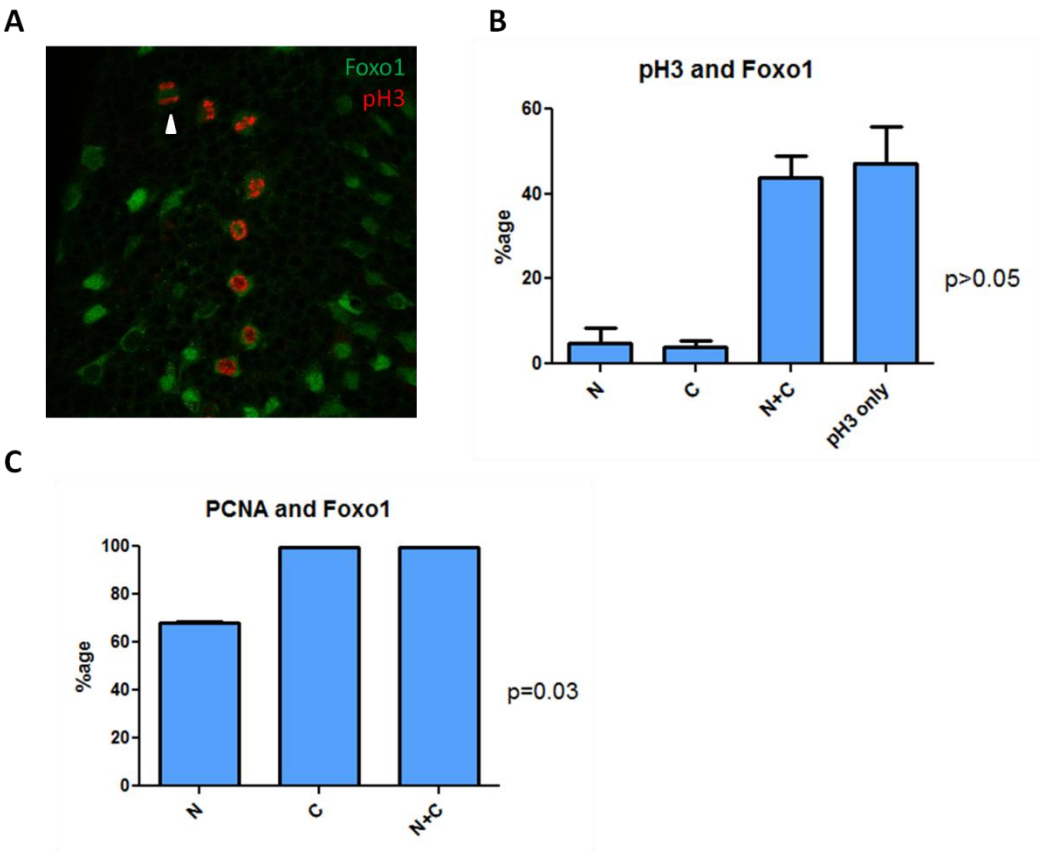

Figure 5.3: Foxo1 subcellular localization and cell cycle
(A) Whole mount IF in adult tubules co-staining for Foxo1 and pH3. Triangle points to one mitotic cell in a cyst in which Foxo1 is not discretely localized, consistent with nuclear breakdown. (B) Foxo1 localization and pH3 positivity. Cells that were positive for pH3 were then scored for the subcellular localization of Foxo1. Cells scored as N+C likely represent cells in mitosis that have undergone nuclear envelope breakdown. (C) Foxo1 localization and PCNA positivity. Cells that were positive for PCNA were then scored for the subcellular localization of Foxo1. N=nuclear; C=cytoplasmic. Error bars represent SEM of N=3 replicates, at least 100 cells counted per replicate. P value represents student's t test N vs C.

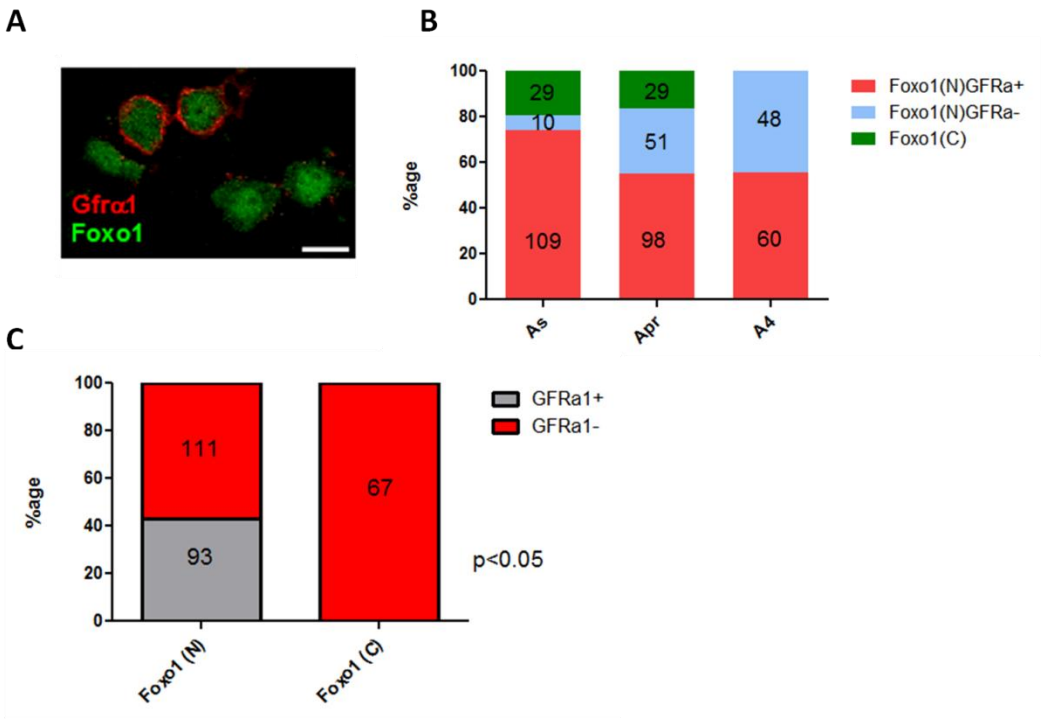

Figure 5.4: Nuclear Foxo1 associated with clone size and Gfrα1 positivity
(A) Co-localization of Foxo1 and Gfrα1. Bar=10 µm. (B) Quantification of Foxo1 localization, Gfrα1 positivity with spermatogonial chain length in 8 week wild type testes. Whole tubules were stained for Foxo1 and Gfrα1; all Foxo1+ spermatogonial cysts (N or C) were scored for Gfrα1 positivity. Cyst numbers counted per category are indicated on graph. (C) Only nuclear Foxo1 co-localizes with Gfrα1 in undifferentiated spermatogonia. Whole tubules were stained for Foxo1 and Gfrα1; all Foxo1+ cells (N or C) were scored for Gfrα1 positivity. Cell numbers counted per category are indicated on graph. These counts are aggregate of all Foxo1^{+} cells and so are irrespective of cyst size. N=nuclear; C=cytoplasmic.

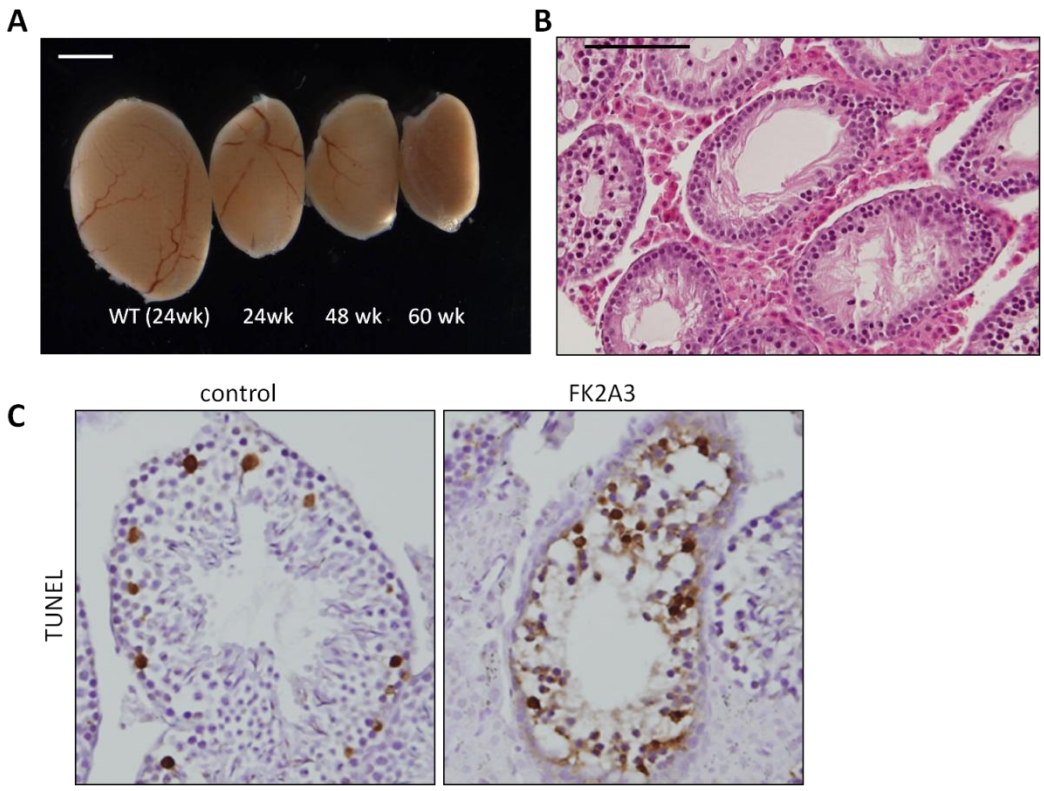

Figure 5.5: Constitutively active Foxo3 leads to infertility and increased apoptosis (A) Transgenic FK2A3 males show increased testicular hypotrophy with age. Bar=3 mm. (B) H&E of 16 wk old FK2A3 testis reveals near-complete absence of spermatogenesis (stages late spermatogonia through spermatozoa). Bar=100 µm. (C) TUNEL on WT (left) and experimental (right) shows increased germ cell apoptosis in FK2A3 seminiferous tubules.

CHAPTER SIX
Results

ROLE OF OVARIAN AND OOCYTE-SPECIFIC GENES FOR OOGENESIS AND FERTILITY

Introduction

The forkhead transcription factor Foxo3 is a master regulator of primordial follicle activation (PFA) (see Chapter II) (Castrillon, Miao et al. 2003; Hosaka, Biggs et al. 2004). Primordial follicle (PF) assembly is normal in female mice bearing a null *Foxo3* mutation (John, Shirley et al. 2007). However, immediately after follicle assembly, PFs undergo global activation and grow in a synchronized manner, resulting in ovarian hypertrophy by postnatal day (PD) 14. *Foxo3* females are fertile until follicles are depleted at ~4 months of age, demonstrating that subsequent steps of follicle maturation (ovulation, fertilization, zygotic development, etc.) are unaffected (Castrillon, Miao et al. 2003).

Discovery of the requirement for Foxo3 in oocyte maintenance marked a breakthrough in understanding the mechanisms behind PFA. However, the additional ovarian factors needed for this process were as yet elusive. This was due, in part, to the difficulties present in gene discovery with ovaries and purified oocytes. While these efforts have led to discovery of multiple oocyte-specific factors, it was still highly probable that many ovarian fertility genes were yet to be uncovered (Dong, Albertini et al. 1996; Dube, Wang et al. 1998; Soyal, Amleh et al. 2000; Rajkovic, Yan et al. 2001;

Herrera, Ottolenghi et al. 2005; Small, Shima et al. 2005; Evsikov, Graber et al. 2006; Yoon, Kim et al. 2006).

To identify such fertility genes, a microarray-based approach was undertaken, including profiling of *Foxo3* ovaries (Gallardo, John et al. 2007). Since *Foxo3* ovaries have global activation of primordial follicles, these ovaries would be enriched for transcripts important for early follicle growth. Through this approach, many ovarian factors were discovered, including *Disp1*, *IGF1R*, and *IR*. Each of these genes had an interesting expression profile, with relatively high expression in postnatal ovaries and oocytes. To address whether these genes were necessary in early follicular development and fertility, each was conditionally ablated in germ cells and ovaries were assessed for defects in oogenesis.

Disp1is dispensable for normal oogenesis and fertility

Dispatched1 (Disp1) is a ubiquitously expressed transmembrane protein that mediates cellular secretion and long-range signaling of sonic hedgehog (Shh) (Kawakami, Kawcak et al. 2002; Ma, Erkner et al. 2002; Etheridge, Crawford et al. 2010). Expression analysis revealed that Disp1 had relatively low expression in the somatic tissues profiled. However, expression in neonatal ovaries from PD1 to PD14 was elevated, with high levels in laser-captured microdissected (LCM) oocytes, indicating a potential cell-autonomous role in early oocyte maintenance (Figure 6.1B). Semi-quantitative RT-PCR confirmed the expression in neonatal ovaries, with highest expression at PD14 (Figure 6.1A).

Based on its expression pattern, Disp1 (see Chapter III for allele information) was reduced in oocytes using Vasa-Cre, a germ-cell specific deletor (see Chapter IV, (Gallardo, Shirley et al. 2007)). The Disp1 allele used in this study is hypomorphic, generating N-truncated protein (Tian, Tenzen et al. 2004). Ovaries from *Vasa-Cre; Disp*$^{L/L}$ (hereafter referred to as *Disp1*) females exhibited normal gross ovarian size and morphology at birth as compared to wild-type littermates, and this stayed true into adulthood (Figure 6.2A). Histological analysis at 3 weeks of age showed the continued presence of quiescent primordial follicles through more mature eggs (Figure 6.2C), and quantification of primordial and primary follicles in these ovaries revealed no significant difference in numbers from wild-type siblings (Figure 6.2B). Additionally, sections from 7 week ovaries showed the presence of corpora lutea, indicating normal ovulation (Figure 6.2C), and females were fertile with normal litter sizes (data not shown). Therefore, based on these data, reduced expression of Disp1does not affect normal reproductive function in female mice. However, the effect of complete *Disp1* ablation within the germ cells has not been studied, and may reveal a requirement of Disp1 and Shh signaling in oogenesis.

Insulin signaling through IGF1R and IR in oogenesis

Insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF1R) are transmembrane receptor tyrosine kinases which are activated by insulin and IGF1/2, respectively, though these ligands and receptors are often interchangeable (Jones and Clemmons 1995; LeRoith, Werner et al. 1995; Ward and Lawrence 2009). Expression

analysis of IGF1R showed relatively high expression in juvenile ovary as well as adult ovary, both in oocytes and the somatic compartment (Figure 6.3A). IR was also expressed in the postnatal ovary, with highest expression at PD1, and though expressed in the adult oocyte, was highest in the somatic compartment (Figure 6.3B). Given the established role of insulin signaling potentially acting upstream of the Foxos (see Chapter II), it was also possible that IGF1R and/or IR was signaling through the PI3K pathway and Foxo3 in the context of oogenesis.

IGF1R (see Chapter III for $IGF1R^{L}$ allele information) was conditionally deleted in the germ line with Vasa-Cre, and knockout ovaries were studied for defects in oogenesis or fertility. Ovaries from *Vasa-Cre;* $IGF1R^{L/L}$ (hereafter referred to as *IGF1R*) females were of normal size and morphology at birth as compared to control siblings, and ovaries showed no obvious phenotype into adulthood (Figure 6.4A). Histology and analysis of H&E stained ovarian sections at 3 weeks of age showed the continued presence of quiescent primordial follicles through more mature eggs (Figure 6.4C). Sections from 6 week old ovaries revealed the presence of corpora lutea, indicative of ovulation and fertility (Figure 6.4C). Follicle counts of primordial and primary follicles in 6 week old ovaries revealed no significant difference in numbers as compared to wild-type littermates (Figure 6.4B). Additionally, testes from *IGF1R* were examined for any defects in spermatogenesis. Testes from animals at seven weeks showed a slight decrease in overall testis size in *IGF1R* testes (Figure 6.4D). However, histology at seven weeks showed no discernible defects in spermatogenesis (Figure 6.4E). Tubules contained all spermatogenic cell types, spermatogonia through spermatozoa.

IR was conditionally ablated within oocytes, also using Vasa-Cre (see Chapter III for IR^L allele information). Ovaries from *Vasa-Cre;* $IR^{L/L}$ (hereafter referred to as *IR*) females exhibited normal gross ovarian size and morphology at birth as compared to wild-type littermates, and this stayed true into adulthood (Figure 6.5A). Histological analysis at 3 weeks of age showed the continued presence of quiescent primordial follicles through more mature eggs (Figure 6.5C) and quantification of primordial and primary follicles in these ovaries revealed no significant difference in numbers from wild-type siblings (Figure 6.5B). Additionally, sections from 6 week ovaries showed the presence of corpora lutea, indicating normal ovulation and likely fertility (Figure 6.5C). Therefore, activation of either IR or IGF1R singly, by insulin and insulin-like growth factors, is not required for normal oogenesis and fertility in the mouse. Also, while these receptors do act through the PI3K/Akt signaling pathway, they are not acting upstream of Foxo3 in the context of oogenesis and maintenance of primordial follicles.

Discussion

The ovarian genes found prior to this study (Gallardo, John et al. 2007), many of which were previously unknown, provided a rich source of potential factors required for oogenesis. While Foxo3 was found to be necessary for maintenance of the primordial follicles, the precise mechanisms initiating PFA remain to be elucidated. Given the high number of ovarian genes discovered, it was likely that many of these would be involved in oogenesis, through Foxo3 or otherwise.

Many oocyte-specific genes were attractive candidates to pursue further analysis, especially given what was known of their functions in other contexts. IGF1R and IR are both known to act through the PI3K pathway and Foxo proteins in cell growth, and so their requirement for PFA and oogenesis was entirely plausible. Single ablation of either *IR* or *IGF1R* had no discernible effect on PFA. However, given the probable redundancy and overlapping function between the two receptors, it remains possible that insulin signaling is in fact required for oocyte growth. The extensive involvement of insulin signaling through PI3K/Akt in reproductive function throughout multiple species, as outlined in Chapter II, no doubt reinforces this possibility. Whether acting through the Foxos in the context of mammalian oogenesis or not, a double oocyte-specific knockout of both *IR* and *IGF1R* merits investigation. Additionally, given the role of Foxo1 in spermatogenesis, germ-cell specific ablation of both *IR* and *IGF1R* may also provide further insight into the upstream mechanisms responsible for Foxo shuttling in the testis. The slight hypotrophy in the *IGF1R* testis, though free of obvious spermatogenesis defects, hints of the importance of insulin signaling in testis development.

The functional characterization of Disp1 has uncovered its essential role in mediating secretion and signaling of sonic hedgehog (Shh) (Burke, Nellen et al. 1999; Kawakami, Kawcak et al. 2002; Ma, Erkner et al. 2002; Etheridge, Crawford et al. 2010), though extensive work on this protein is somewhat lacking. The expression analysis revealed its relatively high expression in the juvenile ovary, as well as laser-capture microdissected oocytes, prompting hypotheses of an oocyte-specific role in PFA. The lack of any reproductive defects in *Disp1* females was therefore somewhat surprising; ovaries developed normally and adults were fertile. However, Shh signaling is known to

affect the growth and development of follicles, granulosa, and theca cells in mammalian ovaries (Russell, Cowan et al. 2007; Nguyen, Lin et al. 2009; Spicer, Sudo et al. 2009). Disp1, along with canonical effectors of Shh signaling, such as patched homologs (Ptch) and smoothened (Smo), may act in enhancing paracrine signaling between ovarian cell types. However, given the hypomorphic allele used in this study, there is either functional redundancy within this signaling pathway, or the low levels of Disp1 are still sufficient to promote oogenesis. Additional studies utilizing a true null *Disp1* allele will definitively confirm a necessary role in oogenesis.

While the genes analyzed in this study did not prove to be requisite factors for oogenesis and fertility, other ovarian genes uncovered through the screen have since been found to play important roles in follicle development. For example, fibroblast growth factor 8 (Fgf8) is a secreted mitogenic signaling protein that was highly expressed in oocytes, specifically within the maturing oocytes (Partanen, Vainikka et al. 1992; Mason 1994; MacArthur, Shankar et al. 1995; Valve, Penttila et al. 1997; Gallardo, John et al. 2007). Conditional germline knockout of Fgf8 was undertaken along with the studies described above, but phenotypic analysis was precluded by recent studies confirming the requirement of Fgf8 signaling between oocytes and granulosa cells in the development of follicles, and a role in ovarian tumorigenesis (Valve, Penttila et al. 1997; Valve, Martikainen et al. 2000; Buratini, Teixeira et al. 2005; Sugiura, Su et al. 2007). These results have further validated the microarray expression analysis and the potential importance of the numerous ovarian factors discovered through these efforts.

The genetic basis of female infertility and premature menopause underscores the importance of these gene discovery efforts, as ovarian and especially oocyte-specific

genes are obvious possibly causal factors (Matzuk and Lamb 2002; Kumar 2007). The large number of ovarian factors uncovered that remain to be further evaluated is a starting point for additional elucidation of the precise mechanisms of PFA and oogenesis. These initial experiments in oogenesis comprised the beginning of my graduate studies, and as the specific genes analyzed during this time did not prove essential to PFA, further investigations into oocyte-specific factors were postponed and my primary focus was shifted to the studies of the Foxos in spermatogenesis.

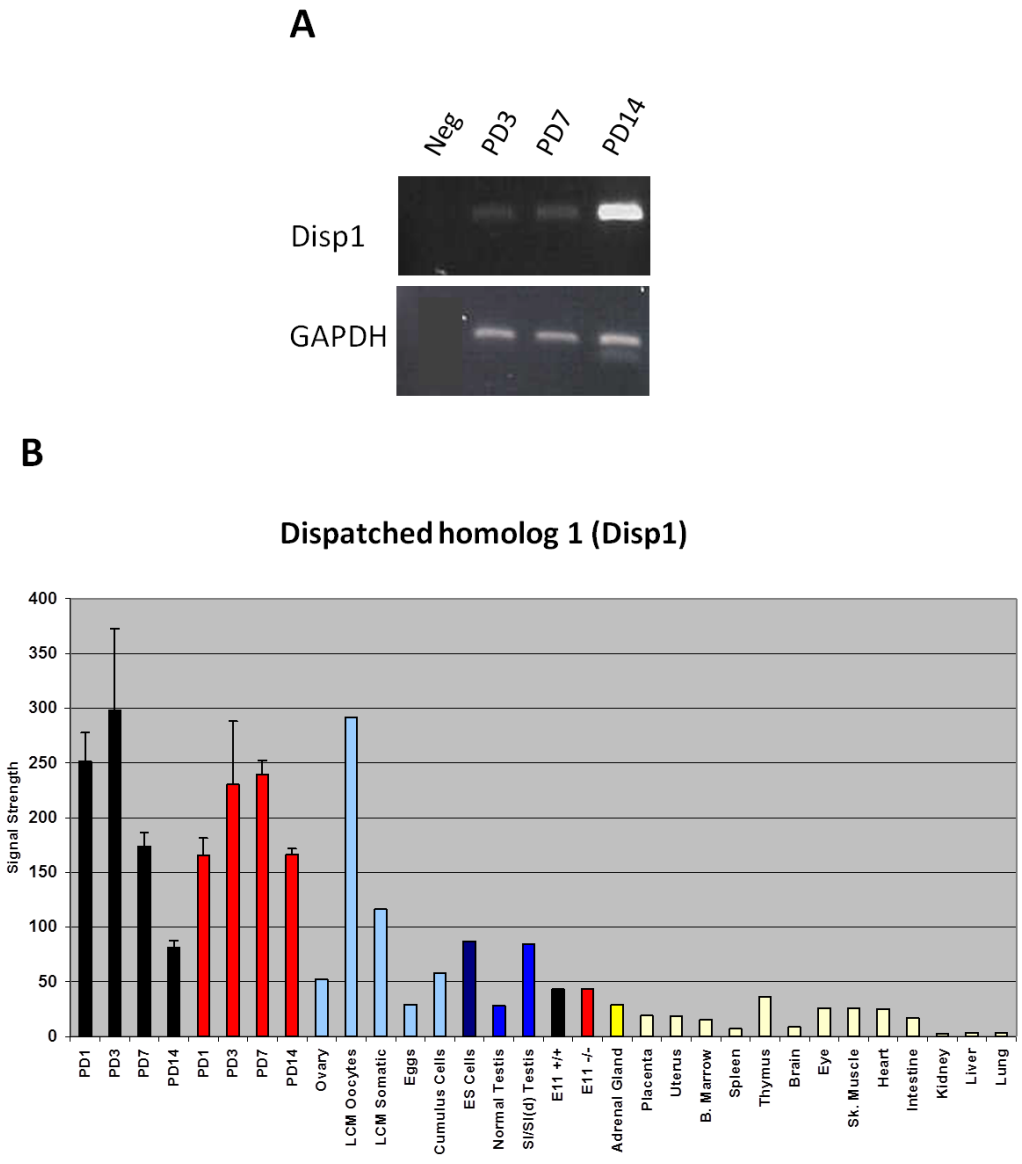

Figure 6.1: Disp1 expression profile
(A) Semi-quantitative RT-PCR from neonatal ovaries. Neg = negative control with no input RNA. (B) Digital graph shows relative expression levels of single probe set for Disp1 across multiple samples; error bars represent SEM. Samples are (left to right) *Foxo3* +/+ PND1, -3, -7, -14 (black), *Foxo3* −/− PND1, -3, -7, -14 (red), adult ovary, LCM primary and secondary oocytes, LCM somatic cells (primary plus secondary granulosa cells and surrounding stroma), eggs, cumulus cells, ES cells, normal adult testis, Sl/Sl^{d} (germ cell-depleted) adult testis, *Foxo3* +/+ E11 embryos, *Foxo3* −/− E11

embryos, adrenal gland, placenta, uterus, bone marrow, spleen, thymus, brain, eye, skeletal muscle, heart, intestine, kidney, liver, and lung.

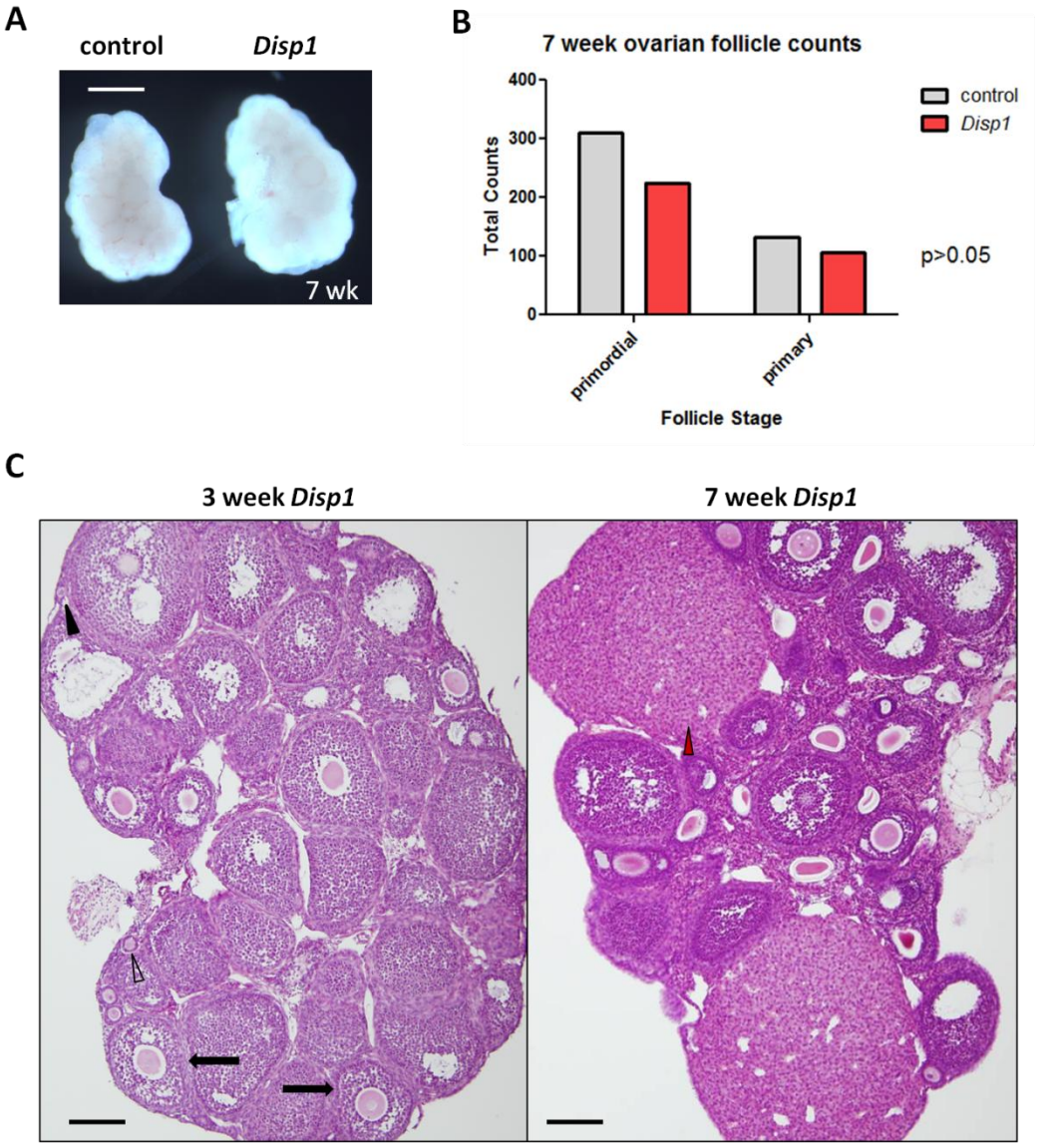

Figure 6.2: Disp1 is not required for normal oogenesis
(A) Ovaries from 7 week old females. Bar=1 mm. (B) Quantification of primordial and primary follicles from 7 week ovaries. (C) H&E stained-sections from 3 week old (left panel) and 7 week old (right panel) *Disp1* ovaries. 3 week ovary shows presence of primordial follicles (closed triangle), primary follicles (open triangle), as well as more mature oocytes (arrow). 7 week ovary has corpora lutea (red triangle). Bar=100 µm.

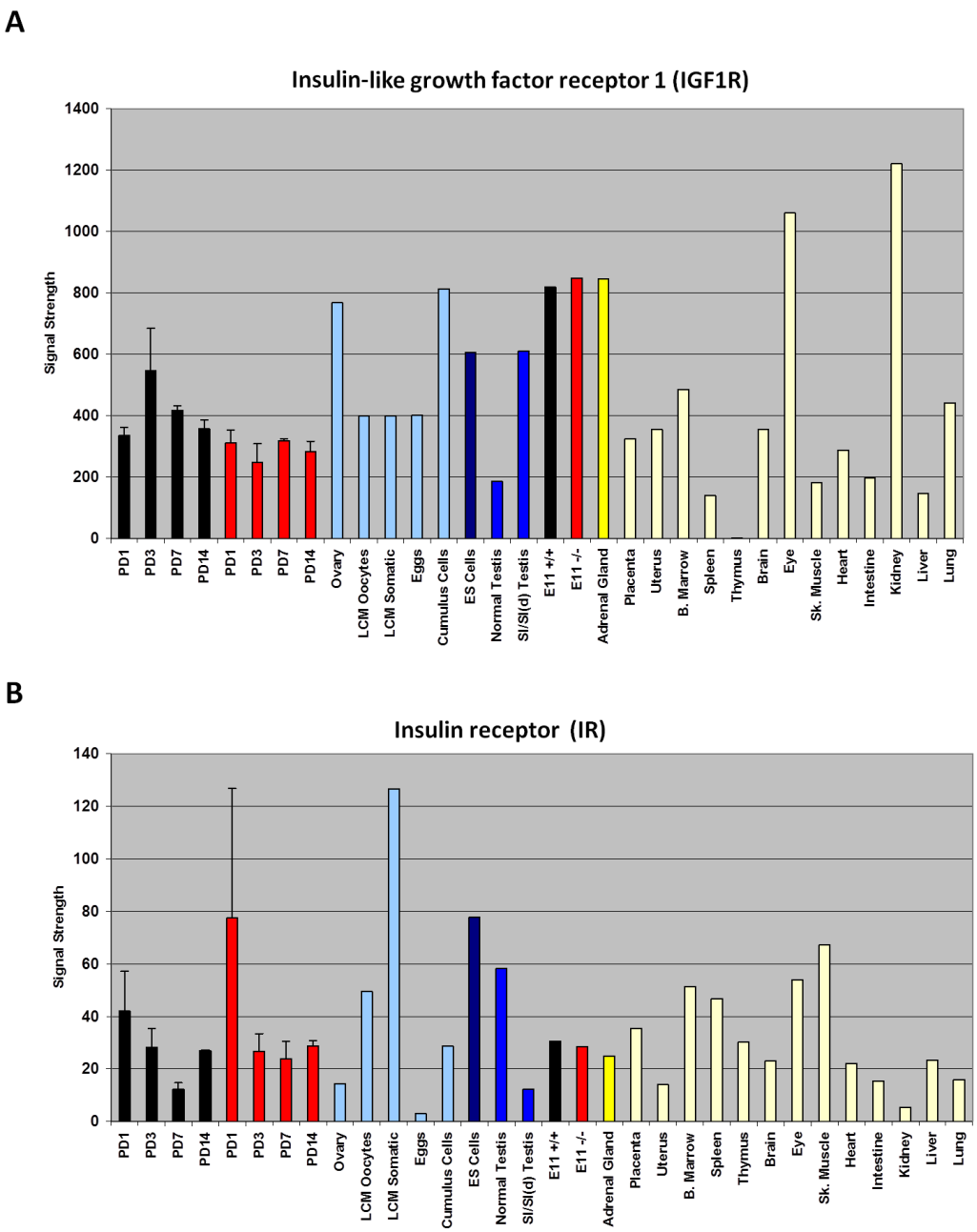

Figure 6.3: IGF1R and IR expression profiles
(A) IGF1R expression. (B) IR expression. Digital graph shows relative expression levels of single probe set for IGF1R and IR across multiple samples; error bars represent SEM. Samples are (left to right) *Foxo3* +/+ PND1, -3, -7, -14 (black), *Foxo3* −/− PND1, -3, -7, -14 (red), adult ovary, LCM primary and secondary oocytes, LCM somatic cells (primary

plus secondary granulosa cells and surrounding stroma), eggs, cumulus cells, ES cells, normal adult testis, *Sl/Sl*^{d} (germ cell-depleted) adult testis, *Foxo3* +/+ E11 embryos, *Foxo3* −/− E11 embryos, adrenal gland, placenta, uterus, bone marrow, spleen, thymus, brain, eye, skeletal muscle, heart, intestine, kidney, liver, and lung.

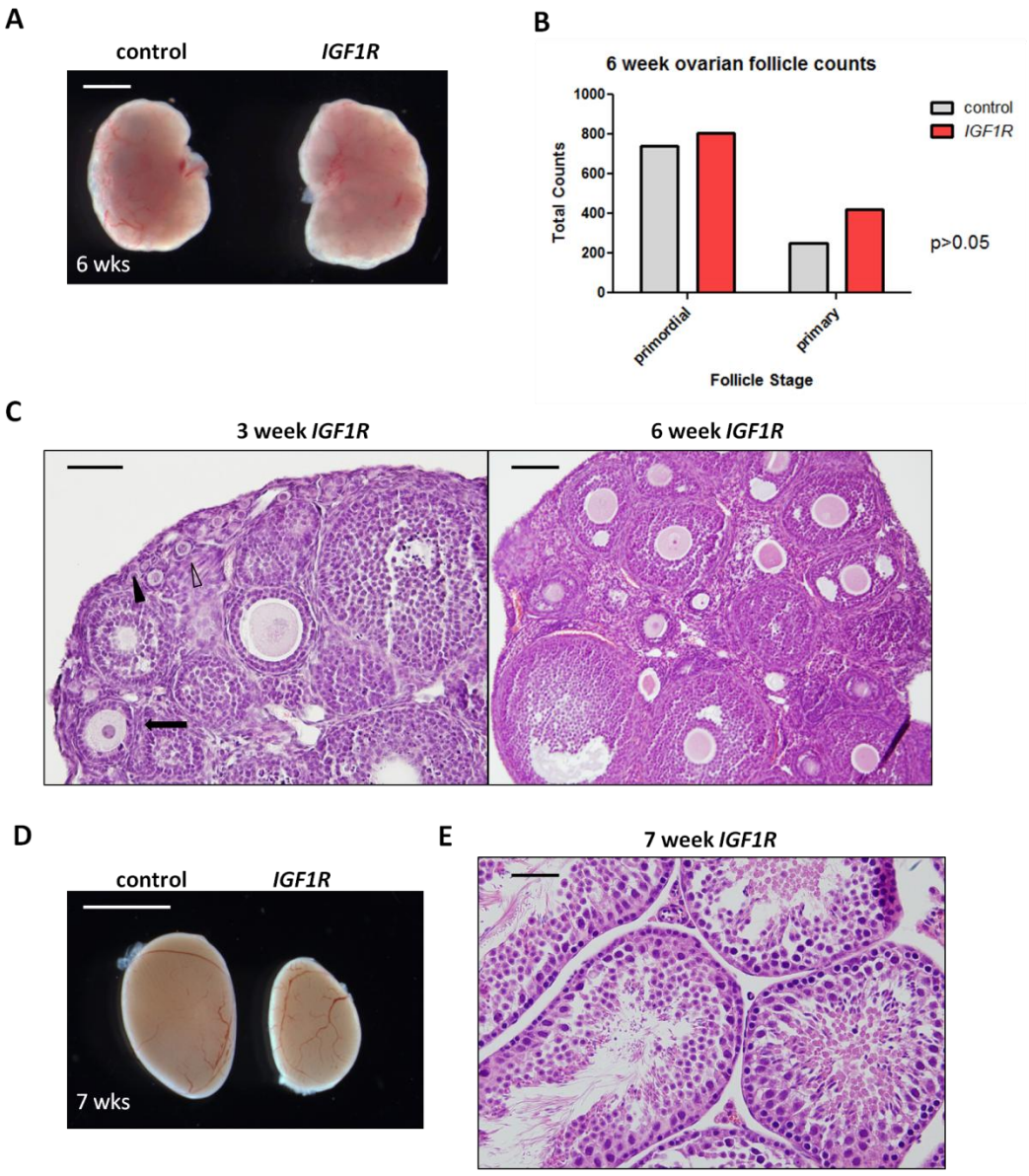

Figure 6.4: IGF1R is dispensable for both oogenesis and spermatogenesis
(A) Ovaries from 6 week old females. Bar=1 mm. (B) Quantification of primordial and primary follicles from 6 week ovaries. (C) H&E-stained sections from 3 week old (left panel) and 7 week old (right panel) *IGF1R* ovaries. 3 week ovary shows presence of primordial follicles (closed triangle), primary follicles (open triangle), as well as more mature oocytes (arrow). Bar=100 µm. (D) Intact testes from 7 week males. Bar=3mm. (E) H&E-stained section from 7 week *IGF1R* testis shows multilayer spermatogenesis. Bar=20 µm.

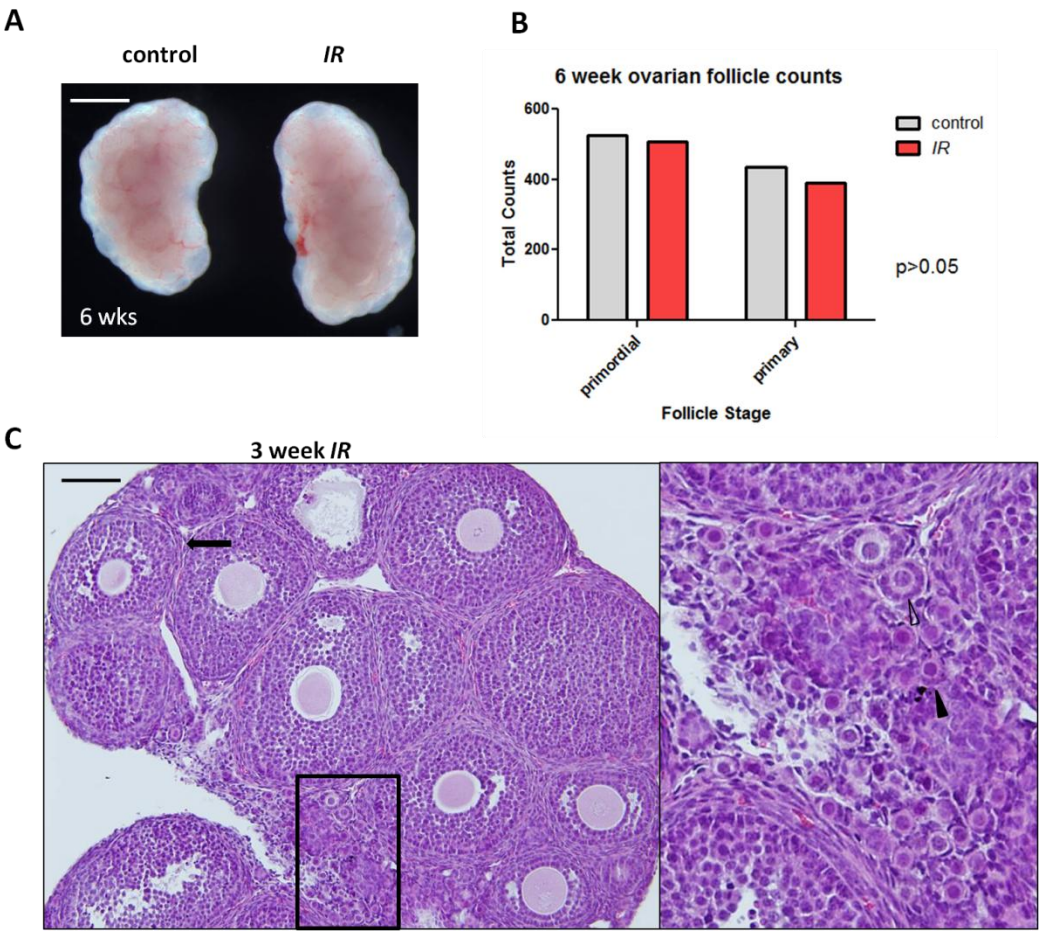

Figure 6.5: IR is not required for PFA and oogenesis
(A) Ovaries from 6 week old females. Bar=1 mm. (B) Quantification of primordial and primary follicles from 6 week ovaries. (C) H&E stained-sections from 3 week old *IR* ovaries. Right panel represents inset from left panel. 3 week ovary shows presence of primordial follicles (closed triangle), primary follicles (open triangle), as well as more mature oocytes (arrow). Bar=100 µm.

CHAPTER SEVEN
Conclusions and Recommendations

The studies outlined in this dissertation demonstrate that the Foxo forkhead transcription factors, particularly Foxo1, serve critical roles in maintaining mammalian spermatogonial stem cells. Prior to the studies outlined in this thesis, the function and pattern of expression of the Foxos within the mammalian testis was unknown. Foxo1 germ cell-specific expression paralleled Foxo3 expression within oocytes, as did the cytoplasmic-to-nuclear translocation in neonatal germ cells. The primary focus of this dissertation outlines the discovery of Foxo1 as an essential factor in both spermatogonial stem cell self-renewal and differentiation. Through the use of multiple germ cell-specific knockouts, Foxo1 was found to act downstream of PI3K/Akt signaling in spermatogonia, confirming the mechanistic conservation of germ line maintenance between species.

Additionally, experiments elucidating the downstream targets of Foxo1 in the context of spermatogenesis, and the mechanism responsible for Foxo1 nuclear to cytoplasmic shuttling were performed to further understand Foxo function. Gene expression analysis of *Foxo1* testes revealed down-regulation of both pluripotency genes, most notably Ret, as well as genes necessary for differentiation. Described in Chapter II, insulin signaling is responsible for reproductive maintenance through the Foxos in multiple species, but the upstream signaling factors in the mouse testis remain to be uncovered.

Finally, Foxo3 is required for maintaining primordial follicles, but the process of PFA is still relatively poorly understood. Here, conditional ablation in oocytes of various ovarian genes was undertaken in order to identify additional PFA and oogenesis factors. These experiments found that Disp1, an essential mediator of hedgehog secretion and signaling, may not be required for oogenesis, and ruled out insulin and insulin-like growth factor signaling as upstream of PI3K/Akt and Foxo3 in the context of PFA. Together, the work presented in this dissertation provides the first evidence of the requirement of the Foxo forkhead transcription factors in spermatogenesis, and further elucidation of the role of PI3K signaling in the maintenance of the germ line.

Foxo1in spermatogonial stem cells

In the absence of the Foxos, particularly Foxo1, spermatogonial stem cells exhibit defects in both self-renewal and differentiation, leading to infertility. This work underscores the similar functions of the Foxos within the male and female germline, as well as Foxo functional conservation in multiple stem cell populations (see Chapter II). While ablation of either Foxo3 or Foxo4 causes no fertility phenotype in the male, *Foxo1* males are infertile, demonstrating that Foxo1 is the most functionally important of the Foxos in the testis. Conversely, Foxo3 is highly expressed in primordial oocytes within the ovary (John, Gallardo et al. 2008), where Foxo1 and Foxo4 are less abundant and functionally relevant. Foxo1 acts to maintain the SSC pool in males, while Foxo3 acts to maintain quiescence of the primordial follicles in females. Primordial follicles are not a pluripotent cell population, but similarly to SSCs, are the foundation for a lifetime of

reproduction, and so the onset of differentiation must be tightly regulated. The unique genetic requirement for *Foxo1* in males and *Foxo3* in females mirrors their high expression at discrete cellular stages in spermatogenesis or oogenesis. Moreover, both Foxo1 and Foxo3 undergo relocalization from the cytoplasm to the nucleus postnatally, suggesting that the timing of their activation is critical for normal germline development and function in juveniles. These studies suggest that the Foxos have evolved to adopt distinct but controlling roles in oogenesis and spermatogenesis, two processes with many similarities.

Loss of Foxo1 in the male germline led to obvious defects in spermatogenesis, with overall decreased germ cells per tubule and complete absence of elongating spermatids and spermatozoa. The pleiotropic *Foxo1* phenotype showed varying degrees of spermatogenic defects between seminiferous tubules, but knockout of all three Foxos increased the severity of defects, and tubules were depleted of differentiating germ cells. The difference in spermatogenic defects between these models indicates that there is some functional redundancy between the three Foxos in spermatogonia. Expression analysis of *Foxo1* testes provided potential transcription targets of Foxo1, but additional expression analyses and ChIP-Seq using *Foxo1/3/4* testes or SSCs will reveal both overlapping targets and targets specific to each of the Foxos.

Given the well-described function of both Pten and the Foxos as tumor suppressors, the phenotypes seen in these conditional knockouts are somewhat surprising, as no animals developed testicular germ cell tumors. The Foxos were initially discovered as chromosal translocations with Pax proteins in rhabdomyosarcomas (Mercado and Barr 2007), and more recently, have been implicated in additional cancer models, as

widespread deletion of *Foxo1/3/4* in adult somatic tissues leads to the formation of aggressive lymphomas and hemangiomas (Paik, Kollipara et al. 2007). The action of the Foxos as tumor suppressors in these systems likely reflects their role in the DNA-damage response and mitigating oxidative stress (for review see (Dansen and Burgering 2008)). The lack of tumor formation in the *Foxo1* testes may be due to the functional redundancy between the Foxos, as knockout of all three Foxos is required to observe, tumors in somatic tissues (Paik, Kollipara et al. 2007). However, even *Foxo1/3/4* testes failed to form tumors, underscoring the spermatogenesis-specific function of the Foxos within the testes. Lack of tumorigenesis in this model can also be explained by the rapid depletion of Foxo-deficient germ cells, and the additional mechanisms in place within the stem cell population to protect against DNA damage and oxidative stress. Instead, Foxo function in SSCs more closely resembles its role in other stem cell populations. The Foxos, particularly Foxo3, coordinately regulate neural stem cell homeostasis and hematopoietic stem cell differentiation (Paik, Ding et al. 2009; Renault, Rafalski et al. 2009). Moreover, Foxo1 is required to maintain pluripotency of both human and mouse embryonic stem cells, presumably through transcription of Oct4, though was not found to be acting downstream of the PI3K/Akt pathway (Zhang, Yalcin et al. 2011).

Moreover, germline ablation of *Pten*, also originally discovered due to its tumor suppressive role (Li, Yen et al. 1997; Steck, Pershouse et al. 1997), did not lead to testicular tumor formation in this study. However, Kimura *et al*. reported that germ cell-specific knockout of Pten leads to bilateral testicular teratomas in all mice, due to increased germ cell proliferation in PGCs (Kimura, Suzuki et al. 2003). The difference of this study as compared to what is reported here may be due to the timing of gene

deletion, with their *TNAP-cre* turned on in PGCs by E13.5, days earlier than *Vasa-cre*. All *Pten* animals generated with *Vasa-cre* exhibited normal numbers of germ cells at birth, with depletion evident by PD21, and no teratomas or other testicular germ cell tumors were ever observed. The lack of tumorigenesis in *Pten*-null SSCs seems to reflect its stem cell-specific role; *Pten* inactivation within hematopoietic stem cells reveals its function in maintaining self-renewal, paralleling its function in SSCs (Yilmaz, Valdez et al. 2006; Zhang, Grindley et al. 2006). Use of an earlier acting *Cre* line to delete the *Foxos* in the germline may similarly result in testicular germ cell tumors, reflecting separate developmental functions of this pathway, and merits further investigation. The work presented in this dissertation underscores the potentially conserved mechanisms of PI3K/Akt signaling and the Foxos in maintaining multiple stem cell populations.

Subcellular localization of Foxo1 in SSCs

In adult steady-state spermatogenesis, Foxo1 is exclusively in the cytoplasm in approximately 20% of Foxo1^{+} undifferentiated spermatogonia, begging the question of the molecular distinction between nuclear and cytoplasmic Foxo1 in SSCs. Canonically, nuclear Foxo1 is associated with its transcriptional activation, while cytoplasmic Foxo1 is associated with its inactivation and degradation. Retinoic acid signaling was deemed unrelated to Foxo1 subcellular localization (in contrast to *in vitro* data), nor was cell cycle found to reflect nuclear versus cytoplasmic Foxo1. However, all Gfrα1^{+} spermatogonia, purportedly marking the SSC population, had nuclear Foxo1. First, this co-expression suggests that GDNF signaling through Gfrα1/Ret receptor tyrosine kinases

does not control Foxo1 localization through the PI3K/Akt pathway (i.e. if Gfrα1/Ret were active, Foxo1 would be cytoplasmic). Also, Foxo1 is nuclear and active within the stem cell population, validating the importance of its transcriptional targets, such as *Ret*, in SSCs. However, the contrasting functions of the potential Foxo targets (described in Chapter IV) in both stem cell self-renewal and differentiation implies that nuclear Foxo1 itself does not demarcate the stem cell population. The nuclear status of Foxo1 alone is not necessarily indicative of pluripotency, since the majority of $Foxo1^{+}$ nuclear spermatogonia did not express Gfrα1. Additional studies differentiating these two populations of $Foxo1^{+}$ nuclear spermatogonia are needed to validate any differences in transcriptional targets. Taken together, there are three distinct populations of $Foxo1^{+}$ spermatogonia (nuclear Foxo1 co-expressed with Gfrα1; nuclear Foxo1 without Gfrα1; cytoplasmic Foxo1), which may represent the transition from stem cell to differentiating spermatogonia.

PI3K pathway activity in gametogenesis

An interesting and yet unknown aspect of the Foxos' role in reproduction, both Foxo1 in spermatogenesis and Foxo3 in oogenesis, is the upstream signaling ligands and activating receptor that trigger the pathway. Kit was ruled out as a possibility for multiple reasons. Foxo1 localization was unchanged in *KitY719F* testes, which abrogates Kit signaling through PI3K, indicating that SF and Kit are not acting upstream in this pathway to trigger Foxo1 shuttling. Additionally, Kit is expressed on differentiating spermatogonia and is essential for later stages of spermatogenesis, whereas Foxo1 is

expressed on undifferentiated spermatogonia. In ovaries, disruption of Kit signaling through PI3K/Akt had no effect on PFA, but seemed to be required for later stages of oocyte maturation. Another interesting possibility explored in these studies was GDNF signaling through Ret and Gfrα1 as acting upstream of Foxo1 in the testes. However, Foxo1 is exclusively nuclear in Gfrα1^{+} spermatogonia, whereas if signaling through Gfrα1 was in fact acting upstream of Foxo1, one would expect to find at least a subset of Gfrα1^{+} spermatogonia with cytoplasmic Foxo1.

Since IGF1R and IR are both known to act through the PI3K pathway and Foxo proteins in cell growth, their requirement for gametogenesis in either males or females is entirely plausible. Described in Chapter II, insulin signaling through PI3K and Foxo factors is an essential process in germ cell maintenance in both *Drosophila* and *C. elegans*. The identification of Foxo3 and the PI3K pathway in mammalian PFA (Castrillon, Miao et al. 2003), along with Foxo1 in spermatogenesis as presented here, certainly mirrors Foxo functions in other species, so insulin signaling is a likely upstream activator in mammalian gametogenesis as well. Surprisingly, single ablation of either *IR* or *IGF1R* had no discernible effect on PFA, and *IGF1R* males were fertile with no spermatogenic defects. However, given the probable redundancy and overlapping function between the two receptors, these results do not entirely exclude the possibility that insulin signaling is in fact required for oocyte growth and spermatogenesis. Studies from Nef *et al*. found that mutation of all three insulin signaling receptors *IR*, *IGFR*, and *IRR* (insulin receptor-related receptor) disrupted normal embryonic testis development and sex determination (Nef, Verma-Kurvari et al. 2003). These triple-knockout mice are not viable, precluding further analyses of gametogenesis and fertility, but clearly

emphasize the importance of insulin signaling in reproductive development. Whether acting through the Foxos in the context of mammalian gametogenesis or not, a double germline-specific knockout of both *IR* and *IGF1R* merits investigation. Additionally, given the role of Foxo1 in spermatogenesis, germ-cell specific ablation of both *IR* and *IGF1R* may also provide further insight into the upstream mechanisms responsible for Foxo shuttling in the testis.

Conclusion

The studies presented within this thesis provide critical new insights into the mechanisms of both spermatogonial stem cell maintenance and spermatogenic differentiation. The Foxos, acting through the PI3K signaling pathway, are intimately linked with reproductive function in both males and females, a process that seems to be highly conserved between species. My findings have demonstrated that Foxo1 expression in spermatogonia is indicative of a stem cell state, and Foxo1 has SSC-specific targets required for both maintenance and differentiation. Before the work presented here, the function and expression pattern of the Foxos within the mammalian testis was entirely unknown, and the molecular mechanisms responsible for SSC maintenance were poorly understood. These data serve to further clarify the SSC population and the processes regulating self-renewal versus differentiation, with the Foxos acting upon a complex hierarchy of transcriptional targets regulating SSC function. This novel discovery of the Foxo-dependent mechanism underlying SSC function and spermatogenesis has multiple implications for male infertility and testicular cancers due

to abnormalities in SSC function, and provides valuable insights into stem cell homeostasis.

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