

New Insights Into The Role of Kit Ligand During Mouse Germ Cell Development

by

Aparna Mahakali Zama

(Under the direction of Mary Bedell)

ABSTRACT

Kit ligand [KITL, also known as mast cell growth factor (MGF), stem cell factor (SCF), and Steel factor] and its receptor, KIT, are essential for embryonic and postnatal development of both male and female germ cells. While germ cells express KIT at multiple stages in their development, somatic cells that support the growth and differentiation of germ cells express KITL. Numerous studies have revealed that KITL promotes proliferation and suppresses apoptosis of germ cells. However, many details of the molecular mechanisms by which KITL signaling affects proliferation, apoptosis, migration and differentiation of germ cells are not known. Our laboratory recently identified hypomorphic mutations in the *Steel* locus (*Kitl^{Sl}*) that allowed fine dissection of the role of KITL during germ cell development. *Kitl^{Sl}* homozygous mutants that were previously analyzed exhibited perinatal lethality or had severe germ cell defects thus limiting the understanding of the role of KITL. Therefore, the newly identified hypomorphic mutations that exert graded mild effects on germ development were able to provide new insights into additional and novel roles of KITL at multiple stages of germ cell development.

Index Words: Kit ligand, *Steel* locus, primordial germ cells, apoptosis, proliferation, differentiation, migration, spermatogonia, spermatocytes, meiosis

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Aparna Mahakali Zama

B.Sc, Genetics, Chemistry, Zoology, Osmania University, India, 1994

M.Sc, Animal Sciences, Hyderabad Central University, India, 1997

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Aparna Mahakali Zama

Major Professor: Mary Bedell

Committee: Michael Bender
Nancy Manley
J. David Puett
Edward Kipreos

Electronic Version Approved:
Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2004

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Chapter 1
Introduction And Literature Review¹

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Germ cells are totipotent progenitor cells that are essential for the maintenance of the species by propagating genetic material from generation to generation. Germ cell development in mammals involves a complex process of lineage specification, survival, proliferation, migration, interaction with the somatic components of the gonad and further differentiation to give rise to the haploid gametes (Figure 1.1). Cell-cell signaling is critical for all of these events and many different cytokines have been identified that are required at one or more stages of germ cell development. Kit ligand (KITL) is one such cytokine that has been shown in numerous studies to be essential not only for the normal development of primordial germ cells (PGCs) but also for the normal progression of adult spermatogenesis and oogenesis. In this introductory Chapter, I provide an overview of the current understanding of germ cell development in mice and the prevalent questions in the field regarding the role of KITL during germ cell development. This Chapter is comprised of four sections:

Section I – an overview of the *Kitl* gene, gene products, mutations in the *Kitl* gene and KITL mediated signaling in germ cells.

Section II - an overview of the role of KITL during PGC development.

Section III - an overview of the recent advances in understanding the role of KITL during adult spermatogenesis.

Section IV - a review of the current understanding of the functions of KITL during oogenesis and folliculogenesis.

Section I: The *Kitl* gene and gene products

Kitl is located on chromosome 10 of mice and its coding region is comprised of 9 exons. KITL is expressed as either a membrane-bound protein or as a soluble protein, and these two forms arise from alternative RNA splicing and posttranslational processing (Flanagan et al., 1991; Huang et al., 1992) (see Figure 1.2). Two *Kitl* mRNAs that differ by the presence or absence of exon 6 [(+) E6 and (-) E6, also called KL-1 and KL-2, respectively (Huang et al., 1992)] are expressed in a developmentally regulated fashion in the testis (Manova et al., 1993; Mauduit et al., 1999; Rossi et al., 1993). The expression patterns of these alternatively spliced products and the potential significance of their differential expression are discussed in the following sections. Both *Kitl* mRNAs are translated into proteins that contain four functionally important regions (Figure 1.2 and reviews by Besmer et al., 1993 and Lev et al., 1994); a signal sequence, an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The N-terminal signal sequence of 25 amino acids (aa) is removed during intracellular trafficking and is similar to that of many other proteins targeted for the cell surface. The extracellular domain contains the major structural elements of KITL and contains sequences that bind to KIT with high specificity. A hydrophobic domain of 26 aa functions as the transmembrane domain of KITL and is typical of other single-pass integral membrane proteins. The cytoplasmic tail of MB-KITL is highly conserved and two studies revealed that intracellular trafficking and stability of KITL require sequences in its cytoplasmic domain (Tajima et al., 1998; Wehrle-Haller and Weston, 1999). While it was previously thought that the KITL cytoplasmic domain has no recognizable motifs shared with other transmembrane proteins, a recent study has revealed a new motif in this domain that targets KITL and other transmembrane growth factors to the baso-

lateral surface of polarized cells (Wehrle-Haller and Imhof, 2001). In the testes, such baso-lateral targeting may have important functional consequences for KITL (see Section III).

The (+) E6 *Kitl* mRNA encodes a 248 aa transmembrane precursor (Pre-KITL) that is processed at the primary cleavage site in exon 6 to produce a soluble isoform (S-KITL) of 165 aa (Figure 1.2). Unlike other transmembrane cytokines such as transforming growth factor- β , normal proteolysis of KITL does not require sequences in the cytoplasmic domain (Cheng and Flanagan, 1994). Despite the fact that Pre-KITL cleavage could be an important mechanism for regulating KITL function, there is little information about KITL processing enzymes. Currently, the only proteins known to cleave KITL are a chymase expressed on mast cells (Longley et al., 1997) and matrix metalloproteinase-9 found in bone marrow [MMP-9, (Heissig et al., 2002)]. The alternatively spliced *Kitl* mRNA, (-) E6 *Kitl* mRNA, lacks the primary cleavage site and encodes a predominantly membrane-bound KITL (MB-KITL) of 220 aa (Figure 1.2). It is clear from many *in vitro* studies that both S-KITL and MB-KITL are biologically active; these studies were conducted with PGCs (see reviews by De Felici, 2000; Donovan, 1994), and spermatogonia (Feng et al., 2002; Rossi et al., 1993; Yan et al., 2000b), as well as with other cells that require KITL signaling, such as oocytes, hematopoietic cells, and melanocytes. However, studies with cultured hematopoietic cells have shown that MB-KITL has greater bioactivity *in vitro* and promotes a more persistent activation of KIT than does S-KITL (Kapur et al., 1998; Miyazawa et al., 1995). Furthermore, genetic studies with mice (see below) suggest that MB-KITL plays the predominant role in many tissues *in vivo*, including the testis. While both S-KITL and MB-KITL form non-covalently linked dimers (Arakawa et al., 1991; Hsu et al., 1997; Tajima et al., 1998) and are heavily glycosylated at both N- and O-linked sites

(Huang et al., 1992; Lu et al., 1991; Lu et al., 1992), the roles of dimerization and glycosylation in KITL functions are currently not known.

Crystallographic studies revealed that KITL is a member of the short-chain subgroup of helical cytokines (Jiang et al., 2000; Zhang et al., 2000), a family that shares significant structural similarities despite minimal conservation in primary amino acid sequence (Hill et al., 2002). The major structural features of these helical cytokines are four α -helical regions in the extracellular domain (Figure 1.2) that are oriented in an up-up-down-down topology with long loops between helices A and B and between helices C and D (Hill et al., 2002). Structural studies have so far been conducted only with S-KITL, and it will be of interest to determine if the extracellular domains of S-KITL and MB-KITL have the same tertiary structure.

Alternatively, the presence of additional sequences and/or tethering via its transmembrane domain may affect the folding of the extracellular domain of KITL. If so, such structural differences in the extracellular domains of MB-KITL and S-KITL could contribute to their functional differences.

Mutations in the *Kitl* and *Kit* genes of mice

In mice, KITL is encoded by the *Steel* (*Sl*) locus, and KIT is encoded by the *Dominant White Spotting* (*W*) locus (Besmer et al., 1993; Lev et al., 1994). Over the last 50 years, classical genetic techniques have been used to identify spontaneous, radiation-induced and chemically induced mutations at both loci. More information on *Kitl^{Sl}*, *Kit^W*, and many other mouse mutants may be found online at <http://www.informatics.jax.org/> (Mouse Genome Database, The Jackson Laboratory) and at <http://bio.lsd.ornl.gov/mouse> (Mutant Mouse Stock Database, Oak Ridge

National Laboratory). Although these mutations were revealed on the basis of semi-dominant pigmentation phenotypes, *Kitl^{Sl}* and *Kit^W* mutations disturb normal development of several diverse cell types. The characteristic phenotype of a *Kitl^{Sl}* or *Kit^W* homozygous mutant is identical, with sterility in either or both sexes, macrocytic anemia, mast cell deficiency, and reduced coat pigmentation, as well as perinatal lethality in the case of homozygous null mutations. These phenotypic defects are due to deficiencies in development of germ cells, hematopoietic cells (particularly hematopoietic stem cells, erythroid cells, and mast cells), and melanocytes, and the extent of deficiencies in each cell type are allele-specific (see below). Once the products of the *Sl* and *W* loci were identified, expression studies in wildtype mice revealed that both genes are expressed in many more tissues than would be expected from the mutant phenotypes. Accordingly, it is now known that several additional cell types are deficient in *Kitl^{Sl}* and *Kit^W* mice, including enteric neurons (Huizinga et al., 1995; Torihashi et al., 1995) and T lymphocytes (Di Santo and Rodewald, 1998). In addition, these mutant mice have been reported to have learning and memory deficiencies (Motro et al., 1996).

The molecular and phenotypic diversity of *Kitl^{Sl}* mutants provide a rich genetic resource for dissecting KITL functions. There are more than 80 different *Kitl^{Sl}* mutant alleles, all of which exert semi-dominant phenotypes. This semi-dominance, where heterozygous mutants display a milder phenotype than homozygous mutants, reveals that gene dosage is critical to KITL function. Another important aspect of *Kitl^{Sl}* mutations that allows for their use in detailed functional studies is that different *Kitl^{Sl}* mutations produce phenotypes that are graded with respect to severity; i.e., some *Kitl^{Sl}* mutations produce very severe phenotypes while other *Kitl^{Sl}* mutations produce very mild phenotypes. These milder phenotypes are often useful for fine-

dissection of gene functions that may be quite subtle or may be required at later developmental stages.

DNA sequence alterations have been identified in many *Kitl^{Sl}* mutant alleles (see Rajaraman et al., 2002a for references), including alleles that have deletions of the entire *Kitl* coding region, intragenic mutations in the *Kitl* coding region, and intergenic mutations that occur outside of the *Kitl* coding region. *Kitl^{Sl}* alleles with coding mutations include intragenic deletions, splicing defects, or nonsense mutations that result in the absence of specific KITL domains while other alleles have point mutations that cause missense mutations. *Kitl^{Sl}* null mutations, such as the complete *Kitl* deletions, some of the nonsense and missense mutations, and an intragenic duplication, cause prenatal or perinatal lethality with severe anemia (Chandra et al., 2003; Rajaraman et al., 2002b; Rajaraman et al., 2003; Russell, 1979). In addition, embryos homozygous for *Kitl^{Sl}* null mutations have severe deficiencies in PGC development (see Chapter 2) but they are not useful for studies of postnatal germ cell development because they fail to live beyond the first few days after birth. On the other hand, homozygous hypomorphic *Kitl^{Sl}* mutations allow viability but have milder effects on red blood cells (RBCs) (Rajaraman et al., 2002b) and slightly milder effects on PGCs (Brannan et al., 1992; Chapter 2). Two *Kitl^{Sl}* mutations, *Kitl^{Sl-d}* and *Kitl^{Sl-17H}*, both of which contain coding mutations that affect whole domains of KITL and are hypomorphic, have been used in a number of studies to define the *in vivo* roles of KITL.

The *Kitl^{Sl-d}* allele contains an intragenic deletion of 4 kb from genomic DNA that removes the transmembrane and cytoplasmic domains and potentially encodes a normal S-KITL (Figure

1.2) but completely lacks MB-KITL (Brannan et al., 1991; Flanagan et al., 1991). Although *Kitl*^{Sl-d} homozygous mice are viable and severely anemic, their anemia is milder than that caused by null *Kitl*^{Sl} mutations (Rajaraman et al., 2002b). This indicates that the *Kitl*^{Sl-d} allele encodes a partially functional KITL protein. *In vitro* studies have revealed that the KITL^{Sl-d} protein(s) is biologically active (Brannan et al., 1991; Dolci et al., 1991). However, little is known about the *in vivo* expression levels or posttranslational modification of *Kitl*^{Sl-d}-encoded proteins. On the basis of nucleotide sequence, three different KITL^{Sl-d} proteins may be expressed, of which all three lack the transmembrane and cytoplasmic domains and two contain additional sequences at the truncated C-terminus (Figure 1.2). The 5' end of the intragenic deletion in *Kitl*^{Sl-d} gene is in exon 7, and so two mRNAs that differ by the presence or absence of exon 6, as in wildtype *Kitl*, could be produced that express two truncated proteins. It is likely that the absence of the transmembrane domain and/or the presence of the abnormal sequences would result in greatly reduced expression of both of these proteins at the cell surface and/or abnormal folding and glycosylation. Significantly, if the abnormal Pre-KITL^{Sl-d} protein does reach the cell surface, proteolysis could occur at the normal cleavage site to produce a S-KITL whose sequence is identical to wildtype. However, spacing between the primary cleavage site and the transmembrane domain of Pre-KITL is critical for efficient proteolysis (Cheng and Flanagan, 1994), and so it is not clear whether this normal S-KITL would even be produced by the *Kitl*^{Sl-d} allele *in vivo*.

The *Kitl*^{Sl-17H} allele contains a point mutation that affects splicing (Brannan et al., 1992) such that a MB-KITL and a Pre-KITL with abnormal cytoplasmic domains are encoded (Figure 1.2), but the mutation does not affect the proteolytic cleavage site for the release of S-KITL.

Although the normal cytoplasmic domain is not required for dimerization of KITL, the abnormal cytoplasmic domain in the KITL^{SI-17H} protein interferes with its dimerization (Tajima et al., 1998). In addition, there is evidence that the *Kitl*^{SI-17H} mutation is a gain-of-function mutation, because the KITL^{SI-17H} cytoplasmic domain contains sequences for abnormal routing of the mutant protein to lysosomes (Wehrle-Haller and Imhof, 2001). Interestingly, Wehrle-Haller and Weston (Wehrle-Haller and Weston, 1999) reported that of the KITL^{SI-17H} that reaches the cell surface, most of it localizes to the apical compartment, rather than to the baso-lateral compartment, of polarized epithelial cells. These authors suggested that such mislocalization could also occur in Sertoli cells, and because less KITL would be available at the baso-lateral surface for presentation to spermatogonia (see Chapter 3), such a model provides a plausible explanation for spermatogenesis defects in *Kitl*^{SI-17H} homozygotes. A possible, alternative explanation for reduced KITL function in these mutants is that the normal KITL cytoplasmic domain may elicit a signal(s) within Sertoli cells.

The KITL/KIT signaling pathway

KITL signaling is required for the normal development of germ cells, erythroid cells, mast cells and melanocytes. While KITL is a helical cytokine (see above), KIT is a type III receptor tyrosine kinase (RTK) that has sequence and structural similarities to the platelet-derived growth factor receptor, beta polypeptide (PDGFRB) family of RTKs (Besmer et al., 1993; Lev et al., 1994). Upon binding of KITL, KIT undergoes ligand-induced dimerization and activation of its intracellular kinase domain. Activated KIT is autophosphorylated on tyrosine residues and phosphorylates and/or binds to a number of cytoplasmic signaling molecules. A

wide variety of such downstream signaling molecules have been identified in KIT-dependent cell types (see the following papers and references cited therein: De Miguel et al., 2002; Dolci et al., 2001; Feng et al., 2000; Kissel et al., 2000), including phosphatidylinositol 3'-kinase (PI 3-K), the SRC non-receptor tyrosine kinase, the mitogen-activated protein kinase kinase (MEK, also known as MAPKK), the Janus-activated kinase 2 (JAK2), the SHP1 and SHP2 protein phosphatases, phospholipase C β , the GRB2 adaptor protein, the RAS protooncogene, SHC (an adaptor protein), and VAV (a GDP/GTP exchange factor). Based on the complexity of these downstream signaling pathways, it has been difficult to determine which pathway(s) is most critical to KITL/KIT functions *in vivo* and to determine whether events downstream of activated KIT are the same or different in different cell types. However, important information is emerging from recent *in vitro* and *in vivo* studies (see below) and many new resources have been developed that will be useful for understanding these complex processes.

An interesting aspect of KITL signaling in germ cells is that the pathway used to promote proliferation of PGCs may be slightly different from that used to promote proliferation of spermatogonia. Even though both PGCs and spermatogonia are dependent on AKT for their proliferative response to KITL, PI 3-K does not appear to play a role in activating AKT in PGCs (De Miguel et al., 2002) as it does in spermatogonia and in other cell types. De Miguel et al., (2002) showed that after stimulation of PGCs with S-KITL, AKT was activated by an SRC-dependent pathway and not by a PI 3-K-dependent pathway. Furthermore, proliferation of PGCs was inhibited with a specific chemical inhibitor of SRC, and not with an inhibitor of PI 3-K. Importantly, the *in vitro* studies of KITL-induced proliferation in PGCs and spermatogonia are consistent with *in vivo* studies described below, and it is now clear that signaling through PI 3-K

is important for this process in spermatogonia but not PGCs. The use of chemical inhibitors to dissect signaling pathways in cultured germ cells should continue to be a productive approach in future studies as in the studies described here. Important issues to be examined in future studies of KITL signaling are the pathway(s) used to suppress apoptosis of PGCs and spermatogonia, the stage in germ cell development in which the proliferative response to KITL switches from PI 3-K independence (in PGCs) to PI 3-K dependence (in spermatogonia), and the significance of that switch to the biological responses of PGCs and spermatogonia to KITL.

Genetic evidence for differences in KITL/KIT signaling between PGCs and spermatogonia has come from studies of mice carrying mutations in the *Kit* gene. The *Kit*^{W^f} mutation is one of only a few *Kit* or *Kitl* mutations that allow fertility in homozygous mutants of both sexes. Koshimizu et al., (1992) showed that normal spermatogenesis occurs in *Kit*^{W^f}/*Kit*^{W^f} mutants despite the fact that the mice are born with only about 50% the normal number of gonocytes. Because the reduced number of gonocytes is likely caused by deficient PGC development, the preferential effect of the *Kit*^{W^f} mutation on gonocytes may reflect differences in KITL/KIT signaling between PGCs and spermatogonia. The nucleotide alterations in the *Kit*^{W^f} have not been identified to our knowledge, but could provide important clues about KIT signaling in these cell types.

Two studies that used mice carrying targeted mutations in KIT provided conclusive evidence that PI 3-K is an integral aspect of the KITL/KIT signaling pathway in spermatogenesis (Blume-Jensen et al., 2000; Kissel et al., 2000). Both labs independently generated mice in which tyrosine 719 in the KIT cytoplasmic domain was mutated to phenylalanine (Y719F). This

mutation abolished PI 3-K binding to activated KIT but left intact binding sites for other signaling molecules. Unlike mice that carry kinase-defective KIT mutations and consequently have white coat color, reduced RBCs, reduced skin mast cells and reduced numbers of PGCs (Besmer et al., 1993; Lev et al., 1994), the *Kit*^{Y719F} homozygous mice have normal coat pigment, normal peripheral blood, normal skin mast cells and normal PGC numbers. While fertility and oogenesis of homozygous mutant females were relatively unaffected, the homozygous mutant males were sterile. Testicular development in the *Kit*^{Y719F} mutants was normal up until P8, after which decreased proliferation and increased apoptosis of spermatogonia were observed and no meiotic or post-meiotic cells were observed. Thus, these studies clearly demonstrate that signaling through PI 3-K is required for KIT function in proliferation, survival, and differentiation of spermatogonia *in vivo*. In addition, KITL signaling through PI3-K may affect the steroidogenic capacity of Leydig cells, but not the numbers or morphology of these cells, and this steroidogenic alteration may have a deleterious effect on spermatogenesis (Rothschild et al., 2003). Although it is tempting to speculate that PI 3-K signaling is not required for KIT functions in melanocytes, RBCs, mast cells or PGCs, the possibility of redundant or compensatory factors that take the place of PI 3-K in these cells cannot be excluded.

Section II - Embryonic development of germ cells in mice

Origin and migration of PGCs

In mice, precursors of primordial germ cells (PGCs) originate in the proximal epiblast at around embryonic day (E) 6.0 – 6.5 and then move into the extraembryonic mesoderm where they become lineage restricted and are identifiable at around E7.25 – 7.5 as a small cluster of alkaline phosphatase (AP) positive cells (McLaren, 2003). By E8.5, PGCs are found near the

base of the allantois, then become enveloped into the developing hindgut diverticulum and remain associated with the hindgut endoderm until around E9.5, while the embryo undergoes a series of morphogenetic processes (Anderson et al., 2000; Anderson et al., 1999; Eddy et al., 1981; Tam and Snow, 1981). PGCs populate the hindgut all along its vertical axis by E9.5 and migrate within the hindgut from the ventral axis to the dorsal axis (Anderson et al., 2000; Anderson et al., 1999; Molyneaux et al., 2001). By E10.5, the PGCs mobilize from the hindgut and migrate into the genital primordia via the dorsal mesentery (Donovan et al., 1986; Molyneaux et al., 2001) (Figure 1.3) and by E11.5 are completely resident in the genital ridges. Once the PGCs are in the gonad, they begin to coalesce with the somatic cells of the gonad. Around the same time, E11.5, the somatic cells of the gonad sexually differentiate, thus further dictating the differentiation program for the germ cells (Karl and Capel, 1998; McLaren, 1981).

Growth factor regulation of PGC development

The PGC lineage arises under the control of multiple members of the bone morphogenic protein family (BMPs). BMPs function as homodimers or heterodimers and the combination of BMP4 and BMP2 secreted from the extraembryonic mesoderm and BMP4 and BMP8b from the extraembryonic ectoderm have been shown to initiate the specification of the PGC lineage from the epiblast (Chang and Matzuk, 2001; Lawson et al., 1999; Tremblay et al., 2001; Ying et al., 2001; Ying and Zhao, 2001; Zhao et al., 1996). Subsequent to lineage specification, PGC survival and proliferation are under control of multiple signaling molecules such as leukemia inhibitory factor (LIF) (Cheng et al., 1994; Pesce et al., 1993; Resnick et al., 1992), fibroblast growth factor (FGF) (Kawase et al., 2004; Matsui et al., 1992; Resnick et al., 1998), oncostatin M (OSM) (Hara et al., 1998), tumor necrosis factor- α (TNF α) (Kawase et al., 1994) and

interleukin-4 (IL4) (Cooke et al., 1996). Many aspects of the *in vivo* mechanisms of migration of PGCs, have been described; including the types of cell adhesion molecules (e.g. cadherins, integrins) expressed by the PGCs (Anderson et al., 2000; Anderson et al., 1999; Bendel-Stenzel et al., 2000), the type of extracellular matrix proteins (e.g. laminin, fibronectin) that PGCs associate with either before, during or after migration (Garcia-Castro et al., 1997) and the types of cell associations that the PGCs make during migration (Gomperts et al., 1994). Studies with mouse mutants have revealed genes that are essential to specific aspects of germ cell development. For example, *germ cell deficient (gcd)* and *proliferation of germ cells (pog)* mutants have defects in the PGC proliferation (AgoulNIK et al., 2002), *peptidyl prolyl isomerase-1 (Pin1)* mutants have defects in the timing of PGC proliferation (Atchison et al., 2003) and *CXCR4* mutants have defects in PGC migration (Molyneaux et al., 2003). Taken together, these reports provide useful information on the nature of cytokines and cell interactions involved during PGC development. However, many details of the regulation of each of these processes need to be further investigated.

***Kit^{Sl}* mutants for the study of PGC development**

An important cell-cell signaling mechanism during early germ cell development is mediated by the KITL and KIT ligand- receptor pair. KITL is produced by somatic cells along the pathway of PGC migration, in a gradient pattern, with the highest levels of expression in the genital ridges, while KIT is expressed on the surface of the PGCs (Keshet et al., 1991; Matsui et al., 1991). Evidence from previous *in vivo* and *in vitro* studies have shown that KITL and KIT interaction is required for the suppression of apoptosis and proliferation of germ cells and that KITL acts in synergy with numerous other cytokines (see above). Recent studies have revealed

that KITL-KIT signaling activates distinct signaling cascades or pathways (see Section I), thus mediating the different effects on cell survival, cell proliferation and cell differentiation.

In previous studies with *Kitl^{Sl}* and *Kit^W* mutants, most of the defects described were in numbers of PGCs rather than with migration of PGCs. However two reports suggested a role for KITL/KIT in PGC migration: *Kitl^{We}/Kitl^{We}* mutants were shown to have ectopic PGCs (Buehr et al., 1993) and *Kitl^{Sl-d}/Kitl^{Sl-d}* mutants were shown to have abnormal retention of PGCs in the hindgut (McCoshen and McCallion, 1975). In Chapter 2, the role of KITL as a cytokine that is essential for migration of PGCs, in addition to survival and proliferation of PGCs, is further investigated.

Section III: Postnatal male germ cell development

After migration and proliferation is complete at E13.5, PGCs enter a quiescent state; in males, PGCs become gonocytes arrested in G₀/G₁ of mitosis while in females, PGCs become oogonia arrested in prophase I of meiosis (McLaren, 2000). Postnatally, spermatogenesis is initiated between birth and postnatal day 5 (P5) when gonocytes differentiate into spermatogonia. A small percentage of spermatogonia become self-renewing stem cells while others begin the developmental program leading to production of spermatozoa (de Rooij and Grootegoed, 1998; Russell et al., 1990). This program is temporally and spatially regulated, and can be divided into three phases that take about five weeks in mice: a proliferative phase, in which numbers of diploid spermatogonia increase through multiple mitotic divisions; a meiotic phase, in which spermatocytes undergo two meiotic divisions to produce haploid spermatids; and a spermiogenic phase, where round spermatids undergo morphological transformations to

become spermatozoa (Figure 1.4). During spermatogenesis in the seminiferous tubules of the testis, the most immature germ cells (spermatogonia) are situated at the periphery of the seminiferous epithelium and the successive stages of developing germ cells leading to the terminally differentiated germ cells (spermatozoa) are situated closer to the lumen of the tubule (Figure 1.4).

There are three types of spermatogonia; type A, intermediate (In), and type B (de Rooij and Grootegoed, 1998; Russell et al., 1990). Type A spermatogonia are either undifferentiated [A-single (A_s), A-paired (A_{pr}), and A-aligned (A_{al})] or differentiated (A_1 , A_2 , A_3 , and A_4). A_s spermatogonia are most widely thought to be spermatogonial stem cells (SSCs), which either renew themselves, or divide into two A_{pr} cells. Multiple divisions of A_{pr} cells produce chains of A_{al} cells, which differentiate into A_1 spermatogonia. Successive rounds of mitosis and differentiation produce A_2 , A_3 , A_4 , In spermatogonia, type B spermatogonia, and preleptotene (PL) spermatocytes, the latter being the last spermatogenic cells to undergo S phase. In mice, pachytene spermatocytes of prophase I appear at about P10 – P12. After a lengthy prophase, subsequent stages of meiosis I and II occur rapidly, and haploid round spermatids are produced by about P21. Spermiogenesis takes place in 16 steps over the next two weeks (Russell et al., 1990) giving rise to the mature spermatozoa (Figure 1.4). During the first wave (or cycle) of spermatogenesis, germ cell differentiation is relatively synchronous, with equivalent stages of differentiation present in different tubules. However the process is continuous, such that new cycles of differentiation are initiated before completion of preceding cycles and continue in normal mice throughout most of adulthood. Because of the cyclical nature of this process,

different tubules in adult testes can be staged based on morphological progressions of the germ cells (Russell et al., 1990).

Expression and function of KITL and KIT during spermatogenesis

In the testes, Sertoli cells, but not germ cells, were shown to express *Kitl* mRNA as well as biologically-active KITL (Motro et al., 1991; Rossi et al., 1991; Tajima et al., 1991).

Although *Kitl* expression in Sertoli cells is under developmental, tissue-specific, stage-specific and hormonal regulation (Hakovirta et al., 1999; Manova et al., 1990; Rossi et al., 1993; Tajima et al., 1993; Yan et al., 1999), little is known of its transcriptional and posttranscriptional regulation. In studies using cultured Sertoli cells from mouse, rat, and human, sequences in the adjacent 5'-flanking region of *Kitl* have been identified that mediate transcriptional activation in response to stimulation by FSH or cAMP analogs (Grimaldi et al., 2003; Jiang et al., 1997; Taylor et al., 1996). However, it is not clear if these cultured cells reflect the transcriptional state of the gene *in vivo*. For example, DNA sequences from a 10 kb region upstream of the *Kitl* transcriptional start site were not sufficient to drive *lacZ* reporter gene expression in the gonads of transgenic mice (Yoshida et al., 1996). This absence of expression in the gonads occurred despite the fact that this same fragment, or an even smaller fragment containing only 2 Kb of sequence upstream to the start site, was able to direct reporter gene expression to neural tissue and skin in a pattern identical to that of the endogenous gene. Furthermore, chromosomal rearrangements located up to 200 Kb away from the *Kitl* transcription start site exert tissue-specific effects on *Kitl* mRNA expression (Bedell et al., 1995), indicating that transcriptional regulation of this gene is very complex and may require sequences located a large distance from the gene.

A potentially important aspect of *Kitl* mRNA expression in the adult testis is that it is stage-specific and as such, may reflect requirements for KITL function during different aspects of spermatogenesis. The first indication of this came from a study that used *in situ* hybridization of testes sections from mice at different ages (Manova et al., 1993). In this study, all tubules from testes of mice younger than postnatal day 9 (P9) had high levels of *Kitl* mRNA, but by P13, *Kitl* mRNA levels were greatly reduced in about half of the tubules. Interestingly, *Kitl* mRNA expression in P13 testes was maximal in tubules that contained proliferating spermatogonia but lacked meiotic cells, and was greatly reduced in tubules that contained meiotic cells but no proliferating spermatogonia. Stage-specific expression of *Kitl* mRNA was confirmed by analysis of rat testis, where maximal expression was again observed in tubules (specifically stages II – VI) that have proliferating spermatogonia (Hakovirta et al., 1999; Yan et al., 1999). Addition of FSH to culture media of dissected tubules from rat testes resulted in a stage-specific increase in *Kitl* mRNA levels, with maximal induction occurring in stages II - VI (Yan et al., 1999). In this same study, the FSH-induced increase in *Kitl* mRNA was shown to result from both increased transcription initiation and increased mRNA stability. Such dual regulatory mechanisms may provide a way for *Kitl* mRNA levels to be tightly fine-tuned in response to developmental needs during spermatogenesis. In support of the idea that regulation of *Kitl* mRNA expression may relate to the proliferative capacity of the epithelium, addition of recombinant KITL to culture media of dissected stage XII tubules from rat testes resulted in a dramatic increase in spermatogonial DNA synthesis (Hakovirta et al., 1999). In the postnatal testes, KITL is expressed by Sertoli cells while KIT is expressed by germ cells and by Leydig cells (reviewed by Besmer et al., 1993). Examination of the expression patterns of *Kitl* mRNA and subcellular

localization of KITL in Sertoli cells has provided important clues to the functions of KITL during spermatogenesis. Using co-cultures of mast cells with Sertoli cells as an assay system for KITL bioactivity, Tajima et al., (1991) showed that mast cell proliferation occurred only if there was direct cell-cell contact between the two cell types. This requirement for cell-cell contact suggested that the biologically active form of KITL expressed from these Sertoli cells, which were from P16 – P20 mice, was predominantly membrane-bound. Additional studies revealed that the ratios of the two alternatively-spliced *Kitl* mRNAs, as well as total amount of *Kitl* mRNA, are altered at distinct developmental stages of the testis (Manova et al., 1993; Marziali et al., 1993; Mauduit et al., 1999). These findings suggest that there is a preferential requirement for either S-KITL or MB-KITL at different stages of spermatogenesis. Splicing of exon 6 is affected by pH, such that the (-) E6 *Kitl* mRNA, which expresses MB-KITL, is expressed in an acidic environment at a much higher level than (+) E6 *Kitl* mRNA (Mauduit et al., 1999). As discussed by Mauduit et al., it is conceivable that the pH of the Sertoli cell environment could influence KITL function by altering the relative amounts of alternatively-spliced *Kitl* mRNA in favor of the (-) E6 *Kitl* mRNA. Based on severe defects in spermatogenesis observed with a mouse mutant that lacks MB-KITL (*Kitl*^{Sl-d}, see Section I), it has often been suggested that MB-KITL is functionally more important than S-KITL in the postnatal testis. However, as discussed below, it seems unlikely that *Kitl*^{Sl-d} encodes a S-KITL that is normal in sequence and/or expression levels. Furthermore, the functions of S-KITL during later stages of spermatogenesis could be obscured by the clearly predominant, and earlier-acting, functions of MB-KITL. Indeed, S-KITL has been shown to promote both proliferation (Packer et al., 1995; Rossi et al., 1993) and meiotic progression (Feng et al., 2002; Vincent et al., 1998) of cultured primary spermatogonia. The localization of KITL in Sertoli cells also suggests that KITL

functions may vary at different stages of spermatogenesis. Prior to P5, KITL is concentrated in the apical cytoplasm of Sertoli cells, but is concentrated in the basal region of Sertoli cells in juvenile testes and in specific stages of tubules of mature testes (Manova et al., 1993).

Furthermore, a radial staining pattern for KITL in these cells was observed in stages VII and VIII of mature testes, with signal extending from the periphery to the adluminal compartment (Vincent et al., 1998). These studies suggest that the localization of KITL could be altered in response to a specific requirement during different stages of spermatogenesis; i.e. basally-concentrated KITL would be available for promoting spermatogonial proliferation while radially-concentrated KITL would be available for promoting the initiation of meiosis.

An important reagent for identifying KIT-expressing cells and for dissecting KITL and KIT function is the ACK2 monoclonal antibody (Nishikawa et al., 1991). This antibody is specific for an epitope in the extracellular domain of KIT and neutralizes signaling of KITL by blocking its binding to KIT. Nishikawa and co-workers demonstrated conclusively that injection of ACK2 into mice causes a depletion of differentiating type A spermatogonia but does not affect other types of spermatogonia (undifferentiated, intermediate and type B), or spermatocytes, spermatids, or spermatozoa (Yoshinaga et al., 1991). The specificity of ACK2-neutralizing activity was related directly to expression of KIT in these various cell types; i.e. in adults, all spermatogonia except undifferentiated spermatogonia are positive for ACK2-immunoreactivity while spermatocytes and spermatids are negative for ACK2-immunoreactivity.

Many studies have confirmed that KIT is expressed on differentiating but not undifferentiated spermatogonia and that S-KITL promotes the proliferation and suppresses

apoptosis of cultured spermatogonia (Packer et al., 1995; Schrans-Stassen et al., 1999; Yan et al., 2000b). The distinction between undifferentiated and differentiated spermatogonia is particularly important because the former are thought to be spermatogonial stem cells (SSCs). Brinster and colleagues developed a powerful functional test for SSC activity that involves transplantation of testes cells into recipient testes (for recent reviews see Brinster, 2002; Johnston et al., 2000). In one such study, flow cytometry was used to purify specific populations of spermatogonia from adult testes and the results demonstrated conclusively that only KIT (-) spermatogonia, and not KIT (+) spermatogonia, have stem cell activity (Shinohara et al., 2000). Interestingly, the age of either donor or recipient for transplantation has an effect on the repopulating ability of testes cells. The testes of mice at age P5 to P12 were found to provide a much better environment for repopulation than were testes from adult mice at 14 – 20 weeks of age (Shinohara et al., 2001). A possible explanation for this age-related difference is that about 80% of the total *Kitl* mRNA in the young testes is (-) E6 *Kitl* mRNA while the proportion of this mRNA relative to (+) E6 *Kitl* mRNA is greatly decreased in mature mice (Manova et al., 1993). Thus, more MB-KITL should be present in the young testes environment than in the older testes environment; however, that possibility remains to be tested directly. Recently, it was reported that unlike KIT (+) spermatogonia from adults, KIT (+) spermatogonia from neonatal mice have repopulating ability upon transplantation into adult recipients (Ohbo et al., 2003). Since KIT expression is considered a marker of differentiating spermatogonia, it will be of considerable interest to determine if the repopulating ability of neonatal KIT (+) spermatogonia reflects a reversion of at least some of these cells to an undifferentiated state. Alternatively, neonatal KIT (+) spermatogonia may be true stem cells whose properties differ from those of adult KIT (-)

SSCs or KITL and/or KIT may have significant functional differences in the neonatal versus adult testes.

In addition to the now well-established role in differentiating spermatogonia, evidence has been accumulating that KITL signaling is involved in initiating and/or maintaining meiosis. However, there have been conflicting reports on whether KIT is expressed on meiotic and post-meiotic cells. These differences may depend on the methods of detection (i.e., *in situ* hybridization versus immunostaining, and the type of antibody used for immunostaining) or on the preparation of germ cells (i.e., testes sections versus purified cell populations). For example, early studies used *in situ* hybridization (Manova et al., 1990) and immunostaining with ACK2 (Yoshinaga et al., 1991) to detect *Kit* mRNA and KIT protein, respectively, in mouse testes sections. These results are in basic agreement, with the highest levels found in differentiating spermatogonia and early primary spermatocytes. In both cases, KIT expression was not detected in late primary spermatocytes, secondary spermatocytes, spermatids, or spermatozoa. Despite this apparent lack of KIT expression in meiotic and post-meiotic cells, experiments with cultured spermatogonia have provided compelling evidence that KITL and KIT have roles in inducing meiosis. Through use of RT-PCR and flow cytometry of purified testes cell populations, the presence of both *Kit* mRNA and KIT protein in pachytene spermatocytes was demonstrated (Vincent et al., 1998). In addition, progression of germ cells through meiosis in culture could be achieved using an immortalized Sertoli cell line (15P-1), and this meiotic progression was shown to be dependent on KITL and KIT (Vincent et al., 1998). An important advance in understanding spermatogenesis has come from the generation of a cell line of type A spermatogonia that was immortalized by overexpression of the catalytic subunit of telomerase

(Feng et al., 2002). These authors showed that S-KITL induces the appearance of both meiotic and postmeiotic germ cells from the immortalized spermatogonia. Although both studies provide convincing evidence that KITL can initiate meiosis, there is a formal possibility that the purified cells or the culture conditions used in these *in vitro* experiments may not precisely mimic the *in vivo* situation.

If KITL does play a role in meiosis *in vivo*, then an important issue to be resolved is whether or not KIT is expressed in meiotic cells. Strong evidence that KIT is indeed expressed in meiotic and postmeiotic germ cells in testes sections has been provided by two reports that used methods of detection that were different from those used previously. Through use of a polyclonal antibody and electron microscopy, KIT expression was found on type A spermatogonia, round spermatids and spermatozoa (Sandlow et al., 1999). This demonstration of KIT-immunoreactivity in cells that were previously shown to lack ACK2- immunoreactivity suggests that the KIT epitope recognized by ACK2 may be masked or otherwise not expressed on meiotic and postmeiotic cells. The most definitive evidence that KIT is indeed expressed at high levels on these cells *in vivo* has been provided by genetic evidence. Guerif et al., (2002) used mice in which one allele of the *Kit* gene was disrupted by a targeted, in-frame insertion of a *lacZ* reporter gene. Thus expression of the reporter was under all of the cis-acting elements in their proper context for *Kit* transcriptional regulation and so should mimic precisely the pattern of endogenous *Kit* transcription. In the adult testis, intense reporter expression was observed in type A spermatogonia, as well as late primary spermatocytes, secondary spermatocytes and round spermatids. Furthermore, detailed histological analyses of the testes of these mice, which have reduced KIT function because they are heterozygous for the *Kit* null mutation caused by

insertion of the *lacZ* gene, revealed a significant delay in meiosis (Guerif et al., 2002). Whether this indicates a delay in initiation or a delay in progression of meiosis remains to be determined.

Collectively, all available data indicate that KITL and KIT are required for proliferation and survival of spermatogonia and for either initiation and/or progression of meiosis in the testis. The challenge now is to understand the molecular basis for these quite disparate processes. Do KITL and KIT have different functions in mitotic, meiotic, and post-meiotic cells? If so, are these functions mediated through differences in downstream KIT signaling pathways? Or, are there other factors expressed in these cells or in somatic cells of the testes that directly modulate the activities of KITL and KIT? Clearly, some of these questions could be addressed through a detailed understanding of the KITL/KIT signaling pathway and of the structure and function of these proteins.

Chemicals that specifically inhibit different signaling molecules have been valuable reagents to dissect complex signaling pathways in a variety of cultured cells. This approach was used in two recent investigations of KITL signaling in cultures of purified mouse spermatogonia (Dolci et al., 2001; Feng et al., 2000). In both studies, addition of S-KITL to spermatogonial cultures resulted in stimulation of DNA synthesis. Furthermore, both studies showed that the proliferative response of these cells to S-KITL was abolished by addition of chemicals that specifically inhibit signaling through PI 3-K and that AKT, a serine threonine kinase, is required downstream of PI 3-K activation. Retinoblastoma protein (RB), a well-documented regulator of the G1/S checkpoint, was shown in both studies to be phosphorylated in response to cyclin D3 activity in response to S-KITL. Thus, the importance of PI3-K and other major downstream

players in activating spermatogonial cell cycle regulators in response to KITL are indicated by both studies. Despite their general agreement, there are some differences in the two reports. In particular, Dolci et al., (2001) found that S-KITL induced a proliferative response in spermatogonia from P8 mice, but not spermatogonia from P5 mice. This observation is consistent with previous observations about the expression of KIT in germ cells at these ages (Manova et al., 1990; Yoshinaga et al., 1991); i.e., P5 spermatogonia are predominantly undifferentiated and so should be KIT (-) and not responsive to S-KITL, while P8 spermatogonia are predominantly differentiated and so should be KIT (+) and responsive to S-KITL. Feng et al., (2000) used spermatogonia from P5 animals for their study, and these cells would not be expected to be responsive to KITL if they really were KIT (-). Since all available evidence indicates that a cellular response to KITL requires its interaction with KIT, it is likely that the P5 spermatogonia used by Feng et al., (2000) were in fact KIT (+). In support of this supposition, the proliferation of spermatogonia from P5 mice (but not younger mice) was shown to be dependent on KIT (Tajima et al., 1994). Several factors, such as the strain of mice used, the method of purifying spermatogonia, and the culture conditions could affect the numbers of KIT (+) and KIT (-) cells in these testes cell preparations from young mice. In future studies of KITL signaling, it should be possible to avoid potentially contradictory results if spermatogonial preparations are enriched for KIT (+) cells or if the specific responses of KIT (-) and KIT (+) cells are examined.

The studies by Dolci et al., (2001) also provided evidence that KITL-mediated suppression of apoptosis in spermatogonia does not occur through pathways involving PI 3-K, MEK, or JAK2. Although the apoptotic pathway was not revealed in this study, the results

suggest that KITL signaling in spermatogonia may proceed through different pathways to suppress apoptosis than to promote cell proliferation. Apoptosis is known to be a critical aspect of normal spermatogenesis and its control is subject to a number of paracrine and endocrine signals (reviewed by Print and Loveland, 2000). Although a number of known regulators of apoptosis have been shown to function during spermatogenesis, little is known of the molecular interactions leading from KITL signaling to apoptosis in testes cells. Recently, addition of S-KITL to cultures of rat seminiferous tubules was shown to cause increased expression of BCL-xL (also known as Bcl2-like, BCL2L) and BCL-w (also known as Bcl2-like 2, BCL2L2), which have pro-survival functions, and to cause decreased expression of BAX, which has pro-apoptotic functions (Yan et al., 2000a). In addition, genetic evidence suggests that the increased apoptosis that occurs in the testes of mice with KIT mutations is dependent on p53 ((Jordan et al., 1999), a tumor suppressor protein (also known as transformation related protein 53, TRP53), and FAS (Sakata et al., 2003), a death receptor family member (also known as tumor necrosis factor receptor superfamily, member 6, TNFRSF6). However, using transplantation of p53-deficient spermatogonia, Ohta et al., provided evidence that loss of germ cells from *Kitl^{Sl-d}* testes is independent of p53 function (Ohta et al., 2003). Although these studies provide good starting points for more detailed studies, much more work will be required to gain a complete understanding of the mechanisms by which KITL affects the timing or frequency of apoptosis in the testes.

Hypomorphic *Kitl^{Sl}* mutations for the study of spermatogenesis

We recently described the nucleotide sequence alterations in ten *Kitl^{Sl}* mutant alleles, of which eight have little or no functional activity for mouse survival or development of peripheral

blood cells, while two of the alleles (*Kitl*^{SI-36R} and *Kitl*^{SI-39R}) are hypomorphic for these activities (Rajaraman et al., 2002a; Rajaraman et al., 2002b; Rajaraman et al., 2003). In the *Kitl*^{SI-36R} allele a point mutation in exon 5 was identified that creates a nonsense mutation in codon 147 (Rajaraman et al., 2002a). Thus, a mutant product of only 146 aa of S-KITL (Figure 1.2) would be produced by premature termination of both (+) E6 *Kitl* and (-) E6 *Kitl* mRNAs. In addition, abnormal splicing (skipping) of exon 5 was observed and is expected to produce a second KITL^{SI-36R} isoform of only the first 96 aa of S-KITL with 25 C-terminal aa out-of-frame (Figure 1.2). Evidence to date indicates that this second isoform is likely to be null functionally (Rajaraman et al., 2002b) and so it is likely that the 146 aa form is the only biologically active form expressed by the *Kitl*^{SI-36R} allele. The *Kitl*^{SI-39R} mutation results from a point mutation that causes a S122F missense mutation in the fourth α -helix and disrupts one of the four N-linked glycosylation sites (Asn120) identified in mouse KITL (Figure 1.2). While KITL expressed from tissue culture cells is known to be glycosylated at Asn120 (Huang et al., 1992; Lu et al., 1991; Lu et al., 1992), the effect (if any) that the absence of this glycan has on KITL function *in vivo* is not known.

Further characterization of KITL function using existing or newly-generated mouse strains with mutations in either KITL or KIT, combined with *in vitro* culture and testis cell transplantation, will ultimately allow dissection of questions such as: What are the molecular mechanisms that underlie the KITL functional differences between promoting proliferation and survival of differentiating spermatogonia? What role, if any, does KITL play in SSCs? Is KITL not required at all in these cells, or is it required only under certain circumstances, such as in neonatal mice or in germ cell-deficient adults? Does KITL directly regulate initiation and/or

progression of meiosis? If so, what are the molecular differences that underlie its functions in mitotic versus meiotic germ cells? The studies described in Chapter 3 are a starting point in evaluating whether new *Kitl^{Sl}* mutants could be useful to address these questions.

Section IV: Requirement of KITL during oogenesis and folliculogenesis

After PGC differentiation and colonization of the genital ridges, the oogonia undergo massive proliferation and by E13.5, enter meiosis and remain arrested in the prophase of the first meiotic division. At birth, there are a finite number of oocytes found in the mouse ovary (McLaren, 2000). Each oocyte is surrounded by two types of somatic cells: granulosa cells that are the supporting cells of the oocytes. At later stages thecal cells that produce the androgens form the outer layer of the follicles (Figure 1.5). During further development of the follicles i.e., folliculogenesis, cell-cell signaling takes place between the granulosa cells and oocyte and the granulosa cells and thecal cells (Cecconi et al., 1996; Joyce et al., 2000; Joyce et al., 1999; Matzuk, 2000; Matzuk et al., 2002). As a result, the oocyte grows to a final diameter of 80μ , finally achieving meiotic competence just before ovulation. During this process, several growth factors have been implicated to be acting locally within the ovary to regulate the orderly progression of folliculogenesis through the primordial, primary, secondary, pre-antral, antral and pre-ovulatory follicle stages (Figure 1.5). Upon maturing to the pre-antral stage, the follicles become responsive to gonadotropins and the subsequent development into antral and ovulatory follicles are dependent on an interplay of gonadotropins, steroid hormones and locally available cytokines (Richards et al., 2002).

***Kitl* and *Kit* expression during folliculogenesis in the mouse**

During embryogenesis *Kitl* and *Kit* expression is high while the oogonia proliferate from E11.5 to E13.5. However there is no detectable expression of *Kit* mRNA after the oogonia enter meiosis during the remainder of fetal development. In the newborn ovary *Kitl* is highly expressed in central cords whose cells contribute to the formation of central growing follicles. *Kitl* expression is low in follicle cells of small primordial follicles and increases to high levels in secondary to pre-antral follicles during late oocyte growth. Large amounts of the receptor *Kit* are found within growing oocytes (Manova et al., 1993). Studies of *Kitl* mRNA expression and protein localization (Manova et al., 1990; Motro and Bernstein, 1993; Packer et al., 1994) have shown that granulosa cells of primary follicles express *Kitl* while the receptor *Kit* is expressed on both the oocyte as well as the thecal cells. After oocyte growth ceases, at the transition of the follicle into antral follicle stages, expression of *Kitl* continues only in the outer layers of mature follicles i.e., in the mural granulosa cells next to the thecal cells and not in the cumulus granulosa cells which are the closest to the oocyte (Motro and Bernstein, 1993) (Figure 1.5). Both isoforms of KITL, membrane bound and soluble are found at all stages of the follicles with the variation being only in the ratios of each isoform (Packer et al., 1994).

Functions of KITL during ovarian folliculogenesis

As mentioned above, KITL expression is compartmentalized and modulated based on the size of the oocytes and follicles suggesting a role for the KITL signaling pathway in oocyte growth and in meiotic arrest. Observations with *Kitl*^{Sl-pan} (Bedell et al., 1995; Packer et al., 1994) and *Kit*^W mutants (Driancourt et al., 2000; Reynaud et al., 2001) have suggested that KITL and KIT are required for follicular initiation and the growth of oocytes. In *in vitro* follicle culture

experiments, addition of S-KITL resulted in a delay in the progression of meiotic maturation in fully grown follicles as measured by the indices of spontaneous germinal vesicle breakdown and polar body extrusions. Additionally, decreased expression of *Kit* is associated with an increased ability of these oocytes to resume meiosis (Ismail et al., 1997; Ismail et al., 1996; Joyce et al., 1999).

Regulation of *Kitl* expression by paracrine factors

Bidirectional signaling between the oocyte and GC is mediated by oocyte-specific factors such as TGF β family members, GDF-9 and BMP-15 and granulosa cell-specific factors such as KITL. It has been demonstrated in mouse knockouts of GDF-9 that the growth of primary follicles is inhibited. *Kit* expression is not detectable in these mutants while *Kitl* mRNA levels are elevated in the granulosa cells (Elvin et al., 1999; Joyce et al., 2000). In a study of partially and fully grown follicles in culture, it was shown that KITL negatively regulates secretion of BMP-15 from the oocyte while in turn BMP-15 upregulates *Kitl* in granulosa cells (Otsuka and Shimasaki, 2002). Taken together these data suggest that KITL is in a negative feed-back loop with the TGF β family members, GDF-9 and BMP-15.

Regulation of *Kitl* expression by gonadotropins and steroid hormones

It has been established by Motro et al., (1993) that the cyclic leutinizing hormone (LH) surge during the estrous cycle upregulates *Kitl* expression in the mural granulosa cells in the pre-antral to antral follicles while decreasing the levels of the *Kit* transcripts. These results were confirmed by other studies showing that LH induces the alternative splicing of the *Kitl* mRNA and that MB-KITL is localized more in the mural granulosa cells after the antral stages. Also, the

LH surge induces meiotic resumption that is consistent with the reduced levels of *Kit* in the oocyte. Similarly, hCG induces meiotic resumption in oocytes accompanied by a switch in expression from MB-KITL to S-KITL and a loss of expression of both isoforms in cumulus granulosa cells (Ismail et al., 1996).

KITL and its role in thecal cell differentiation

KITL can directly stimulate theca cell growth and steroid production during follicular development in bovine ovarian follicles (Parrott and Skinner, 2000). The observation that KITL stimulated androstenedione production, but not progesterone production, suggested that KITL promoted thecal cell differentiation. Therefore KITL could be one of the first granulosa cell-derived growth factor that can directly stimulate theca cell growth and differentiation in the absence of gonadotropins. Additional evidence comes from cultures of thecal cells from hypophysectomized rats wherein KITL was shown to act synergistically with insulin growth factor 1 (IGF-1) in stimulating hormone-independent androgen synthesis (Huang et al., 2001). This aspect of KITL requirement for normal thecal cell differentiation in mice will be further explored in Appendix I.

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Figure 1.1 Life cycle of germ cells. During the first week after fertilization, the PGC lineage emerges separate from the somatic cell lineage. After migration and proliferation between E9.5 to E11.5, the PGCs become completely resident in the gonads by E11.5. The somatic cells of the gonad sexually differentiate, thus further dictating the differentiation program for the germ cells between E11.5 - E12.5. The female germ cells complete all their mitotic cell divisions and enter meiotic arrest by E13.5 and remain in meiotic prophase until after birth. On the other hand, the male germ cells undergo cell divisions and enter mitotic arrest at E13.5 and continue in that state until after birth. Postnatally, the oocytes grow and achieve meiotic competence and mature with the first ovulation occurring around 4-5 weeks of age. The gonocytes in the testis resume mitosis and give rise to differentiated spermatogonia and are followed by meiosis and spermiogenesis with the first spermatozoa being produced by 6-7 weeks.

Figure 1.1

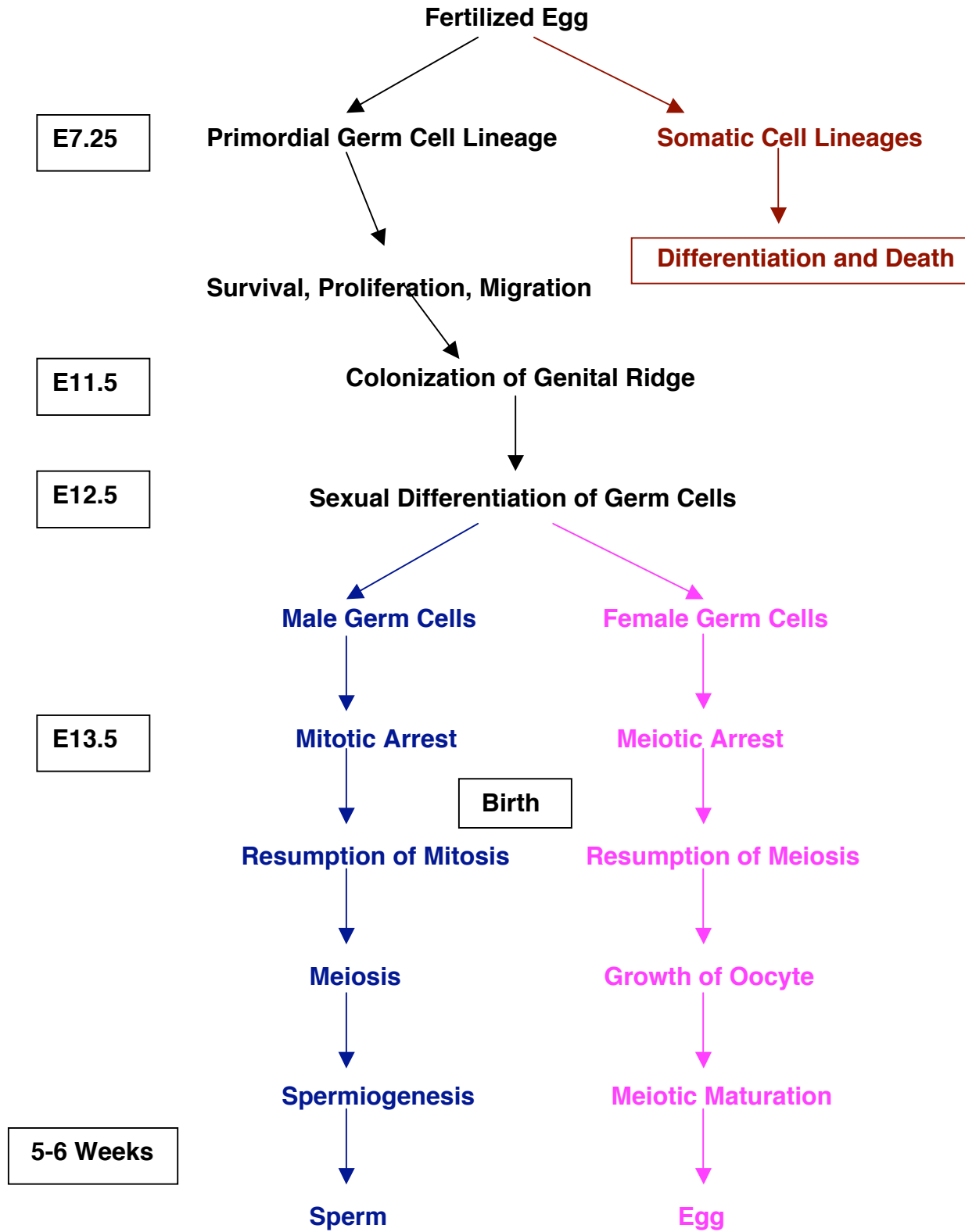


Figure 1.2. Wildtype and mutant KITL proteins [membrane-bound (MB), precursor (Pre), and soluble (S) forms]. The mRNAs encoding each form are described briefly in the text. The original reference describing each mutation is as follows: *Kitl*^{SI-39R} and *Kitl*^{SI-36R} (Rajaraman et al., 2002a), *Kitl*^{SI-17H} (Brannan et al., 1992), and *Kitl*^{SI-d} (Brannan et al., 1991; Flanagan et al., 1991). For each mutant, MB*, Pre* and S* refer to KITL proteins that have altered sequences. For wildtype and mutant proteins, the following symbols are use: solid black rectangles are the four α -helical domains; the light grey rectangle is the alternately-spliced exon 6 that contains the proteolytic cleavage site, shown as a jagged line; black rectangles with dots are transmembrane domains, the ovals and numbers to the right of each protein are the sites of N-linked glycosylation sites, and the amino acid number (with 1 at the N-terminus), respectively. For KITL^{SI-39R}, the white asterisk indicates the position of the S122F missense mutation. For KITL^{SI-17H}, KITL^{SI-d}, and KITL^{SI-136R}, the numbers corresponding to the C-terminal KITL sequence plus the numbers of out-of-frame residues are shown with out-of-frame sequences indicated as cross-hatched rectangles. (Figure reproduced with permission from Bedell MA, Mahakali Zama A. *J. Andrology*. 2004. Mar-Apr; 25(2): 188-99)

Figure 1. 2

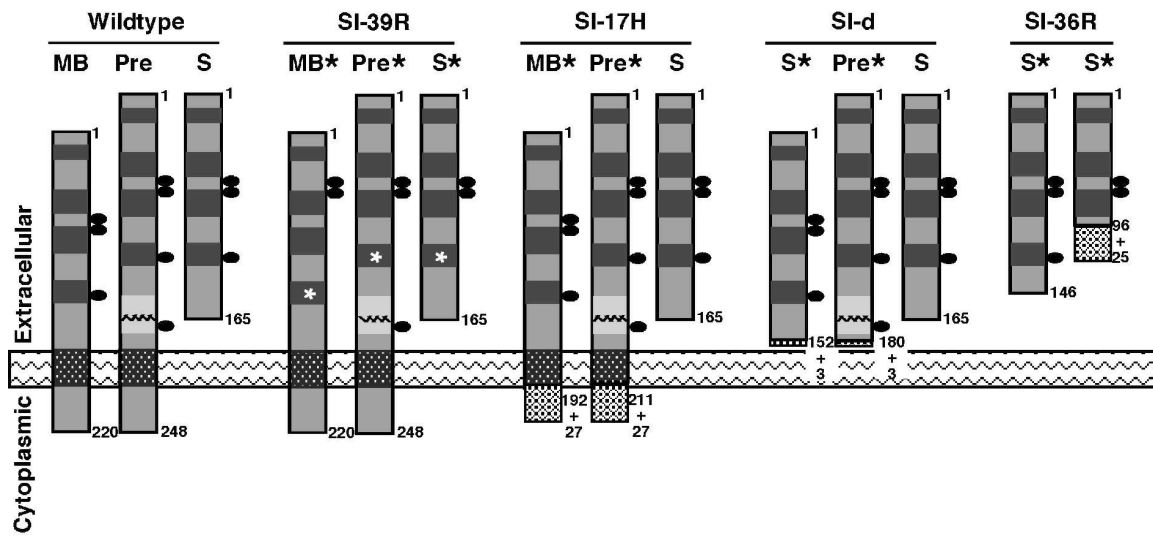


Figure 1. 3. Origin and migration of PGCs. PGCs arise as a small group of cells (red circles) at the base of the allantois (A) at E7.25 and become incorporated into the invaginating hindgut diverticulum (HGD) by E8.5. The PGCs spread along the vertical axis of the hindgut (HG) by E9.5 and begin to migrate away from the hindgut via a sheet of connective tissue, the dorsal mesentery (D) by E10.5. PGCs subsequently colonize the early primordia (G) which at this stage is associated with the mesonephros (M). Note: yellow shaded areas represent the embryo proper in the E7.5 and E8.5 embryo schematics. Only the caudal regions of the embryos are shown in the E9.5 and E10.5 schematics.

Figure 1.3

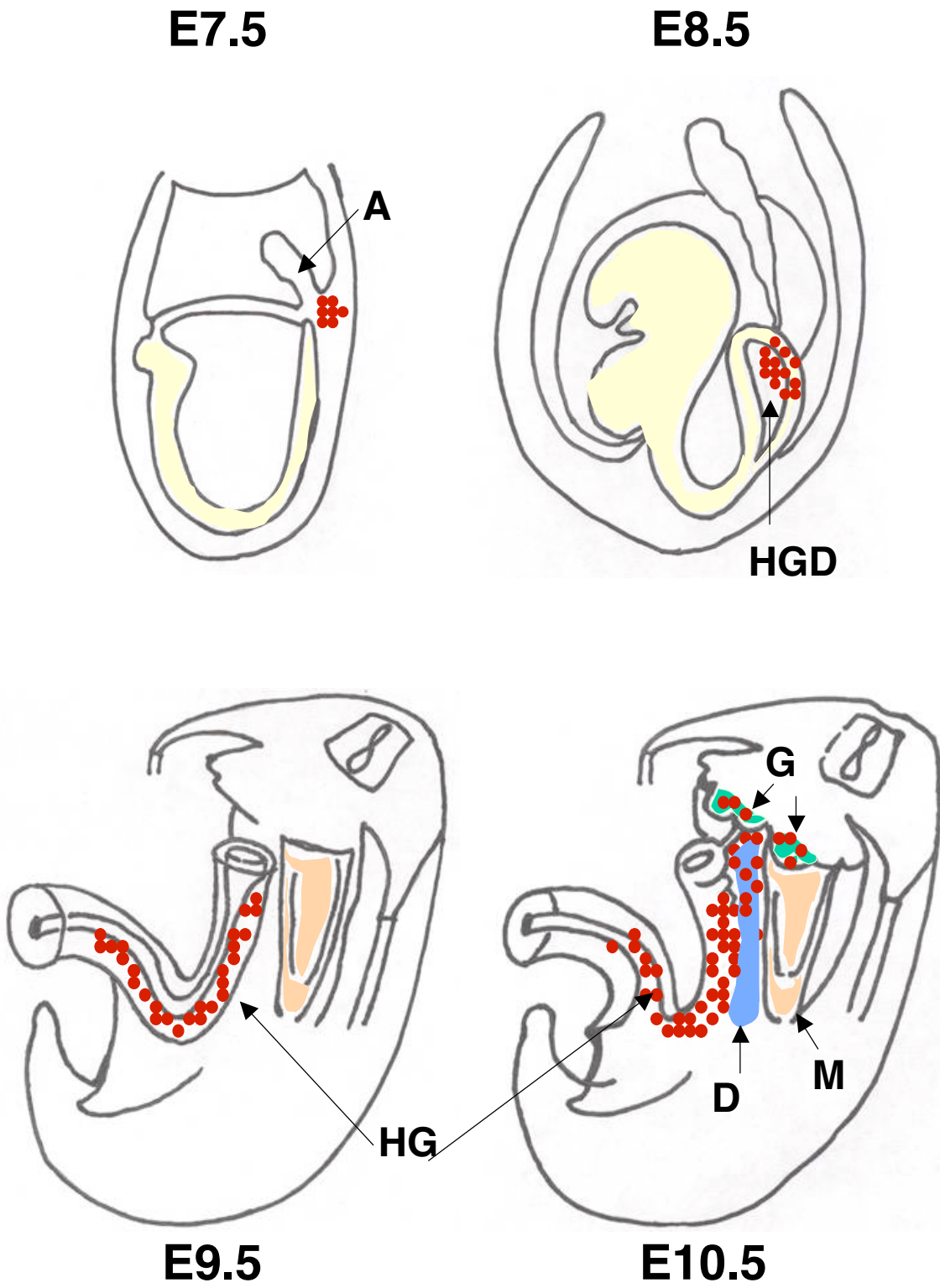


Figure 1.4. Sertoli cells with associated germ cells within a portion of a mature seminiferous epithelial tubule. Shown are two Sertoli cells with the most immature germ cells towards the basement membrane and the most advanced germ cells towards the lumen of the seminiferous epithelial tubule. The undifferentiated and differentiated type A spermatogonia and type B/Intermediate spermatogonia are situated in the basal compartment while meiotic germ cells are situated in the adluminal compartment and round and elongate spermatids in the luminal compartment. The Sertoli cells have nuclei with prominent nucleoli and the cytoplasm extending up to the luminal compartment. The Sertoli cells communicate with the germ cells via tight junctions (thatched lines) that also form a physical barrier between the basal and adluminal compartments.

Figure 1.4

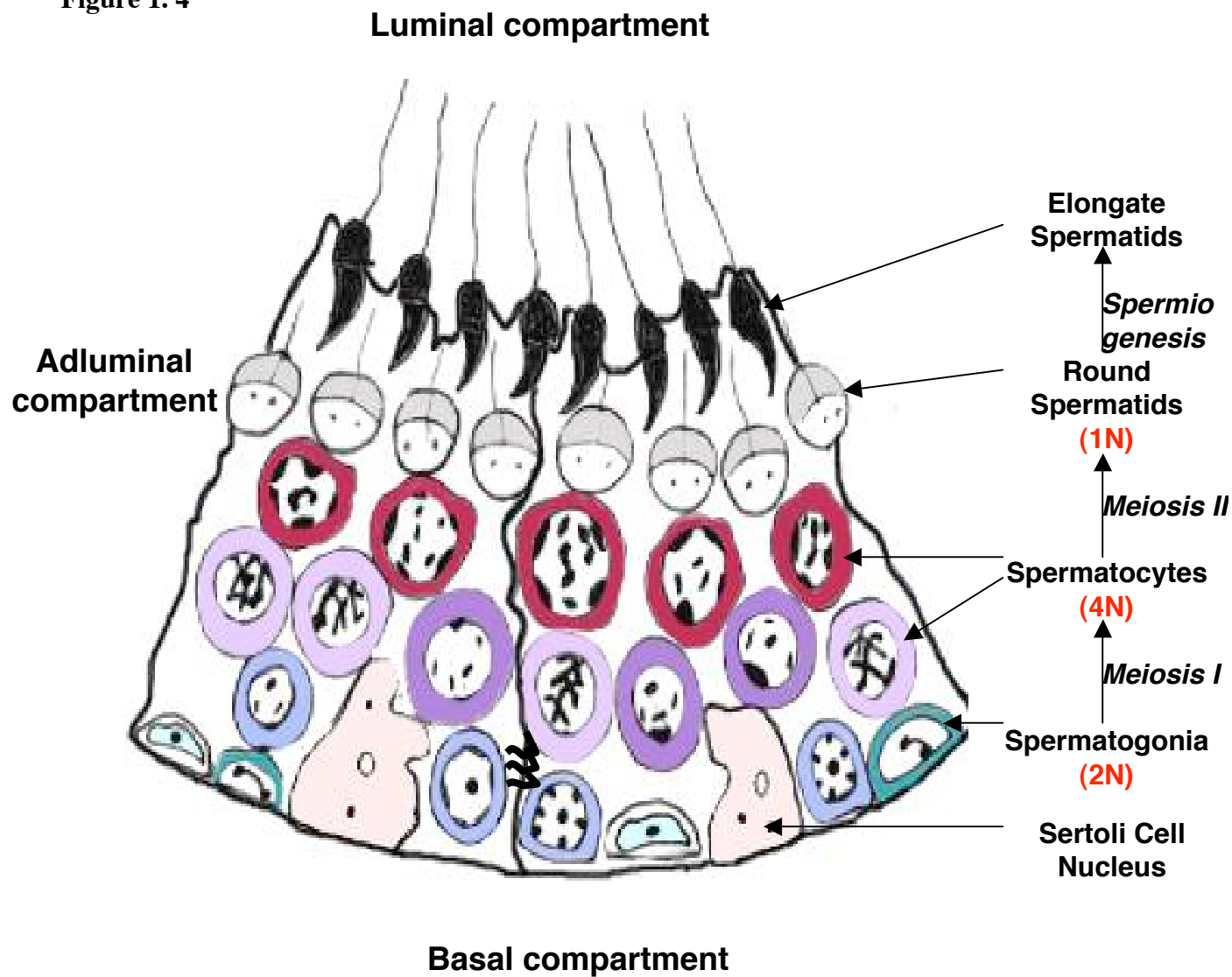
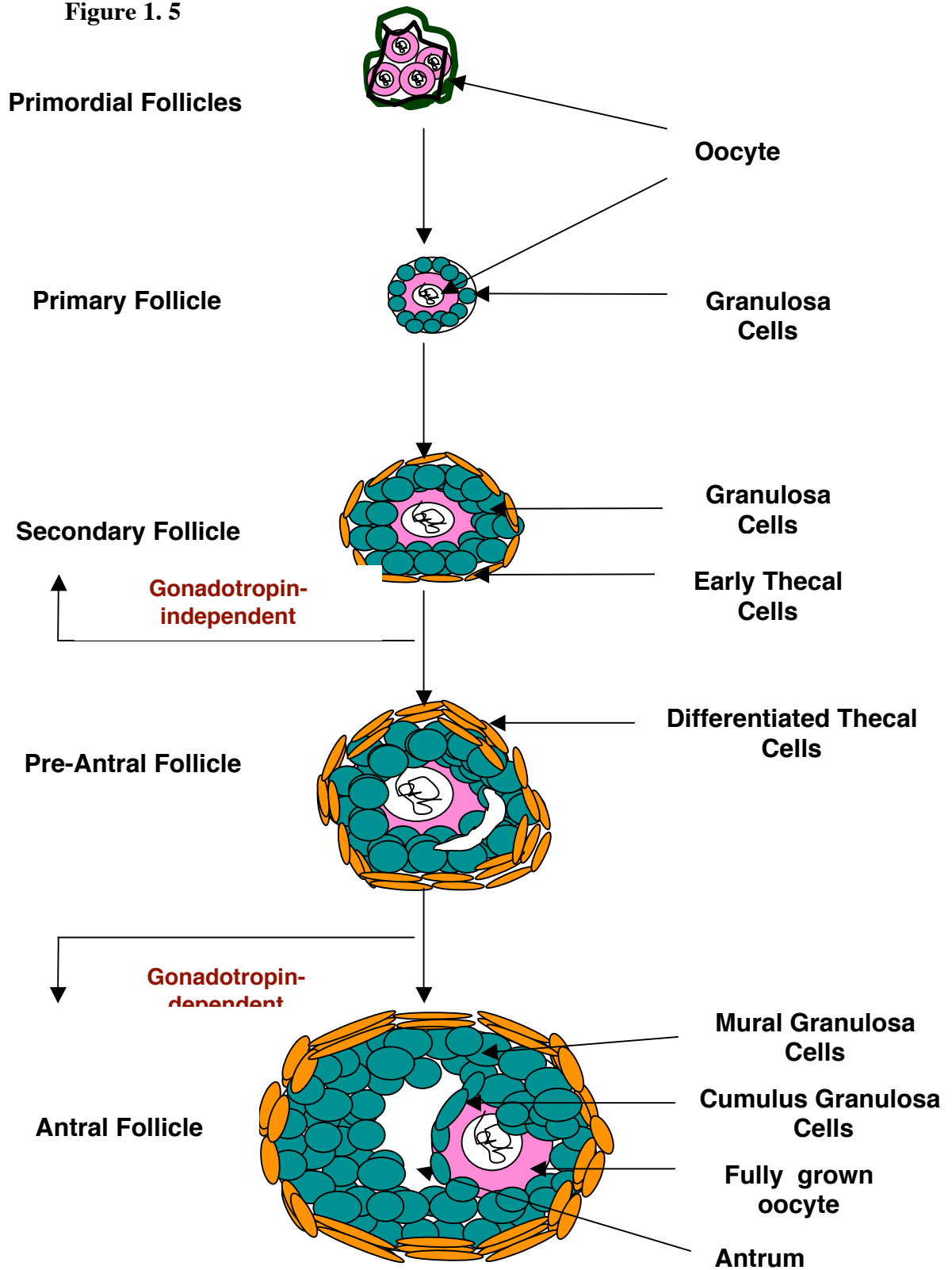


Figure 1.5. Ovarian follicle development in mice. At birth, oocytes, arrested in the dictyate stage, are enveloped by squamous epithelial cells to form primordial follicles. As the oocyte grows in the primary follicle, the granulosa cells (green) proliferate, differentiate and become multilayered. Thecal cells (orange) are recruited from the surrounding stroma at the secondary follicle stage and proliferate and differentiate by the pre-antral stage. The process of gonadotropin-independent thecal cell recruitment and differentiation occurs in the presence of locally available cytokines such as KITL. Further oogenesis and folliculogenesis are dependant on circulating gonadotropins, LH and FSH.

Figure 1.5



Chapter 2

Kit Ligand Is Required For Active Migration Of Primordial Germ Cells From The Embryonic Hindgut¹

¹Mahakali Zama. A, Hudson FP, Bedell MA, to be submitted to *Developmental Biology*

ABSTRACT

Germ cells give rise to gametes through a complex process involving lineage specification, survival, proliferation, migration, mitotic and meiotic cell cycle regulation, and differentiation. In mice, these processes are initiated when a small number of primordial germ cells (PGCs) are set aside from somatic cells during gastrulation. In the next 4 to 5 days, PGCs enter the hindgut, undergo a directed migration away from the hindgut into the developing gonads and proliferate. It is well established that *Kitl* ligand (KITL, also known as stem cell factor and mast cell growth factor) is required for the survival and proliferation of PGCs. However, there is little information on a role for KITL in PGC migration. In this report we provide genetic evidence that KITL is required for PGC migration and that KITL function(s) in migration may differ from those used in proliferation. By comparing the effects on PGC development of multiple *Kitl* mutations, including both null and hypomorphic mutations, we were able to distinguish stages that are preferentially affected by certain mutations. This study illustrates the usefulness of an allelic series of mutations to dissect developmental processes and suggests that these mutants may be useful for further studies of molecular mechanisms of KITL functions in PGC development.

INTRODUCTION

Germ cells are unique in that they are the only cells to undergo meiosis and form the gametes that are essential for transmission of genetic information from generation to generation. In mice and other organisms, primordial germ cells (PGCs) are set aside from somatic cell lineages early during embryogenesis and migrate from their site of origin to the site of the developing gonads (Starz-Gaiano and Lehmann, 2001; Wylie, 1999). Precursors of mouse primordial germ cells (PGCs) originate in the proximal epiblast at around embryonic day (E) 6.0 – 6.5 and then move into the extraembryonic mesoderm where they become lineage restricted at around E7.25 – 7.5 (McLaren, 2003). At this time PGCs are identifiable as a small cluster of alkaline phosphatase (AP) positive cells that subsequently move into the mesoderm of the posterior portion of the primitive streak, near the base of the allantoic bud (Ginsburg et al., 1990). Active migration of PGCs into the adjacent endoderm then occurs (Anderson et al., 2000), after which the cells remain associated with the endoderm during its folding ventrally to form the hindgut diverticulum. This association with the hindgut endoderm continues during further development of the hindgut such that PGCs are carried along passively into the embryo and become distributed along the anterior-posterior axis of the hindgut. Although PGCs in the hindgut were previously thought to be non-motile, an elegant study using time-lapse photography has shown these cells to be highly motile but their movements are restrained within the hindgut (Molyneaux et al., 2001). Beginning around E9.0 – E9.5, PGCs move to the dorsal axis of the hindgut and then leave the hindgut to undergo an active, directed migration towards the genital ridges through either the body wall or dorsal mesentery (Bendel-Stenzel et al., 2000; Molyneaux et al., 2001). Upon entry into the genital ridges, which occurs between E10.5 – E11.5, PGCs become non-motile and coalesce with somatic cells that have begun to sexually

differentiate. In male genital ridges, PGCs become arrested in G0/G1 of mitosis and in female genital ridges, PGCs enter meiotic prophase and arrest in diplotene (McLaren, 2003). Between the time when PGCs are lineage specified to their cell cycle arrest in the gonad, they proliferate from an initial population of about 45 cells to a final population of about 25,000 cells (McLaren, 1981). Thus, two crucial aspects of early PGC development are their movements in various parts of the extraembryonic and embryonic tissues and their proliferation.

Many aspects of PGC development between E6.0 and E13.5 are determined primarily by cell-cell signaling and cell-cell interactions. For the development of PGC precursors in the proximal epiblast, genetic studies of mice revealed that bone morphogenic protein 2 (BMP2), BMP4, and BMP8B (Lawson et al., 1999; Ying et al., 2001; Ying and Zhao, 2001) and their downstream signaling targets, SMAD1 and SMAD2 (Chang and Matzuk, 2001; Tremblay et al., 2001), are required. Specific adhesion molecules such as E-cadherin are required for formation of PGC precursors in the extraembryonic mesoderm (Okamura et al., 2003) and E-cadherin and α 1 integrin (Anderson et al., 2000; Bendel-Stenzel et al., 2000), are required during PGC migration. In addition, migrating PGCs exhibit extensive associations with each other (Gomperts et al., 1994) and with components of the extracellular matrix, such as laminin and fibronectin (Garcia-Castro et al., 1997). Migration of PGCs is affected in mice with mutations in the gene encoding CXCR4, which is the receptor for the chemokine SDF1 (Molyneaux et al., 2003) and the timing of PGC proliferation is affected in mice with mutations in the gene encoding peptidyl prolyl isomerase-1 (PIN1), which may respond to signaling molecules (Atchison et al., 2003). Last, many growth factors have been shown in both *in vitro* and *in vivo* studies to control the survival, proliferation, and migration of PGCs (Donovan, 1994; Wylie,

1999). Some of the first growth factors shown to promote these *in vitro* activities are Kit ligand (KITL, also known as stem cell factor, mast cell growth factor, and Steel factor) (Dolci et al., 1991; Pesce et al., 1993), leukemia inhibitory factor (LIF) (Cheng et al., 1994; Matsui et al., 1991; Pesce et al., 1993; Resnick et al., 1992), fibroblast growth factor (FGF) (Kawase et al., 2004; Matsui et al., 1992), and interleukin-6 (IL6) (Gearing et al., 1992).

The most widely known cell-cell signaling molecules required for PGC development in mice were first identified through classical genetic techniques. Several decades of research have shown that the *Steel* (*Sl*) locus, which encodes KITL, and the *Dominant white spotting* (*W*) locus, which encodes KIT, the only known receptor for KITL, are essential for proliferation of PGCs (Besmer et al., 1993). KITL is a member of the short-chain helical cytokine family (Jiang et al., 2000; Zhang et al., 2000) and KIT is a member of the PDGFRB superfamily of receptor tyrosine kinases (Besmer et al., 1993; Lev et al., 1994). In mouse embryos, the expression patterns of KITL and KIT are consistent with a role for this signaling pathway in migrating PGCs. *Kitl* is expressed in the somatic tissue all along the pathway of migration of PGCs in a gradient pattern with the highest levels in the genital ridges, while *Kit* is expressed on PGCs throughout this period (Keshet et al., 1991; Matsui et al., 1991). Although many studies have described cytoplasmic signaling pathways downstream of activated KIT in other cell types (for example, see (Blume-Jensen et al., 2000)), there is still much to be learned of KITL/KIT signaling in PGCs. Interestingly, recent studies with mouse mutants have shown that while PI3-K is clearly involved in KITL/KIT signaling in some cell types, it does not appear to be required for signaling in PGCs (Blume-Jensen et al., 2000; Kissel et al., 2000). Furthermore, PI3-K does not appear to be

involved in KITL-dependent proliferation and suppression of apoptosis in PGCs *in vitro* (De Miguel et al., 2002).

The large allelic series of mutants available at both the *Kitl^{Sl}* and *Kit^W* loci (ORNL and JAX websites) provide valuable genetic resources to gain more information about PGC development *in vivo*. Defects in PGC numbers in embryos carrying *Kitl^{Sl}* and *Kit^W* mutations have been previously described (Bedell et al., 1995; Brannan et al., 1992; Buehr et al., 1993; McCoshen and McCallion, 1975) however, those studies did not examine a possible effect of the mutations on PGC migration. To our knowledge, there are only two studies with mouse mutants that made observations suggesting a specific role for KITL and KIT in PGC migration. In *Kitl^{Sl-d}/Kitl^{Sl-d}* embryos, ectopic PGCs that had migrated off the normal route were reported (McCoshen and McCallion, 1975), and *Kit^{W-e}/Kit^{W-e}* embryos, abnormal retention of PGCs in the hindgut and delayed migration of PGCs were reported (Buehr et al., 1993).

Our laboratory recently identified eight intragenic *Kitl^{Sl}* mutations that exert graded effects on peripheral blood cell counts (Rajaraman et al., 2002a; Rajaraman et al., 2002b; Rajaraman et al., 2003). In the present study, we used these novel mutations, as well as several other *Kitl^{Sl}* mutations previously used in studies of PGC development, to provide insights into the roles of KITL during PGC migration. We describe evidence suggesting that KITL is required for active migration of PGCs as well as for their survival and proliferation. In addition, our data suggest that different levels of KITL function are required to support migration and proliferation of PGCs.

MATERIALS AND METHODS

Mice

The molecular defects in each *Kitl*^{Sl} mutant used in these experiments have been described previously (Bedell et al., 1996b; Brannan et al., 1992; Brannan et al., 1991; Rajaraman et al., 2002a; Rajaraman et al., 2002b; Rajaraman et al., 2003). For each allele, the nucleotide sequence alterations in the *Kitl* gene and associated homozygous phenotype for effects on viability and anemia are summarized in Table 2.1. Mice heterozygous for each of the *Kitl*^{Sl} mutations were maintained on a C3H/HeNCr background in a pathogen-free, AALAC-accredited facility at the University of Georgia. All procedures with mice were approved by the IACUC of the University of Georgia. Matings were set up between heterozygotes carrying each of the mutations and females checked daily for vaginal plugs. The morning that the plug was found was designated E0.5. Embryos were dissected at E9.5, E10.5, and E11.5 and the caudal portions processed for PGC analyses and the rostral portions used for genotyping. To reduce inter-embryo variation in development at E9.5 and E10.5, somites were counted and only embryos with 19-21 somites at E9.5 and 35-37 somites at E10.5 were used for experiments. At least three embryos from each genotype and age were analyzed for each experiment and *Kitl*^{+/+}/*Kitl*⁺ littermates were used as controls.

DNA genotyping

DNA extractions and PCR-based genotyping methods were done as previously described (Rajaraman et al., 2002a; Rajaraman et al., 2003). An allele-specific PCR method was performed for genotyping *Kitl*^{Sl7H} embryos (details are available upon request).

Whole mount histochemistry

Embryo dissection, fixation and staining were done essentially as previously described (Buehr and McLaren, 1993; Cooke et al., 1993). Briefly, the caudal portions of E11.5 embryos were fine dissected, the internal organs removed to expose the genital ridges. Embryos were fixed in an ethanol and acetic acid mixture (8:1) for 30 minutes at 4° C, then washed with cold 100% ethanol twice and stored in 100% ethanol at -20°C until further use. Before staining for AP activity, the tissues were rehydrated for 2 hours in 70% ethanol and washed in distilled water for 30 min. The embryos were stained for 15-20 minutes with freshly made AP staining solution (see below). Staining in the neural tube was used as an internal control.

Cryostat sectioning

Embryos were fixed in 4% paraformaldehyde (PFA) in PBS for one hour, then washed twice in PBS and stored overnight in 5% sucrose at 4°C. Next, the samples were transferred to 15% sucrose in PBS overnight at 4°C, then placed in OCT (Tissue Tek) and frozen at -80°C. Frozen sections of 14µ were prepared on slides and stained for AP activity or for BrdU immunodetection (see below).

Alkaline phosphatase (AP) staining

Whole mount embryos or frozen sections were stained with freshly made AP staining solution consisting of 0.5mg/ml Fast Red (Sigma) and 0.1mg/ml alpha-naphthyl phosphate (Sigma), 4 mM magnesium chloride and 10 mM borax (Donovan et al., 1986). The embryos were incubated in the staining solution for 15 to 30 minutes at room temperature (RT) and the reaction stopped by placing the slides in distilled water. Sections were cover-slipped, while

whole-mounts were stored in 50% glycerol with 0.01% sodium azide. Staining was monitored by inspection of samples with a stereomicroscope (Zeiss Stemi SV 11) and digital images captured with a Zeiss Axiocam or an Optronics MagnaFire camera and slight modifications for brightness and contrast made using Adobe Photoshop software.

BrdU labeling and immunodetection

Pregnant females were injected intra-peritoneally with BrdU at 10mg/kg body weight (Zymed). After 2 hours, the mice were euthanized by CO₂ asphyxiation and the embryos dissected and processed for frozen sections as described above, except that they were fixed in 4% PFA at 4°C overnight. The slides were dried thoroughly at room temperature and stained for AP for 15 minutes, then washed in PBS until the OCT was completely removed. Detection of incorporated BrdU was according to manufacturer's instructions. Briefly, the sections were treated sequentially with 0.25mg/ml trypsin solution and 4N HCl, then washed thoroughly in PBS and incubated with blocking solution (CAS block, Zymed) followed by an incubation with anti-BrdU monoclonal antibody (1:100 dilution) for 1 hour at RT. After washing in PBS, the sections were incubated in streptavidin -FITC (Vector Labs) at 20mg/ml for 30 minutes at RT, washed in PBS and mounted in Prolong antifade medium (Molecular Probes, OR). Analysis was done on a Leica TS2 Laser Scanning Confocal System. PGCs were visualized at 580-647nm for Fast Red (AP-positive) and 520nm for FITC (BrdU-positive).

Statistics

Unpaired, two-tailed t-tests were performed on all values obtained from 3-5 embryos of each age and genotype using Prism software (Graphpad). *P* values of < 0.05 were considered to be significant.

RESULTS

Screening of new *Kitl^{Sl}* mutants for defects in post-migratory PGCs

Our previous studies revealed the effects of eight newly characterized *Kitl^{Sl}* mutations on peripheral blood cells and mouse viability (Rajaraman et al., 2002b; Rajaraman et al., 2003). In homozygous mutant mice, six of these mutations cause severe anemia and perinatal lethality while two of the mutations allow viability with less severe anemia. As the first step in characterizing the effects of these mutations on PGC development, we screened all eight *Kitl^{Sl}* mutations for their effects on numbers of PGCs in E11.5 embryos. PGC migration in wildtype embryos is completed by E11.5, therefore any differences in PGC numbers between mutant and wildtype embryos at this age would reflect defects in survival, proliferation, or migration of PGCs, or a combination of all three factors. This screen was considered important for two reasons. First, it would reveal whether mutants that are hypomorphic with respect to anemia and viability are also hypomorphic for migrating PGCs. Second, it would identify mutants that could be used for more detailed analyses of PGC development.

The strategy for the screen was to use staining of whole-mount preparations of embryos for AP activity and examination of genital ridges as a way to rapidly assess the effects of the large number of mutations on PGCs. However, prior to screening all *Kitl^{Sl}* mutations, it was

necessary to first establish whether the whole-mount staining method was suitable for semi-quantitative assessment of PGC numbers. This was accomplished by examining embryos homozygous for the *Kitl*^{Sl-gb} deletion, which are null for KITL function because of removal of the entire *Kitl* coding region (Bedell et al., 1996a), and for *Kitl*^{Sl-17H}, which is a hypomorphic mutation in which E11.5 PGC numbers in homozygous mutants are only 22% that of *Kitl*⁺/*Kitl*⁺ littermates (Brannan et al., 1992). In *Kitl*⁺/*Kitl*⁺ embryos, numerous aggregates of AP-positive PGCs were found distributed along the length of the genital ridges (Figure 2.1A), while no AP-positive PGCs were observed in genital ridges of *Kitl*^{Sl-gb}/*Kitl*^{Sl-gb} embryos (Figure 2.1D). Due to the semi-dominant effects of *Kitl*^{Sl} mutations (for example, see Rajaraman et al., 2002b), we were also able to identify most, but not all, *Kitl*^{Sl-gb}/*Kitl*⁺ embryos because of their intermediate levels of AP-positive PGCs (not shown). In *Kitl*^{Sl-17H}/*Kitl*^{Sl-17H} embryos, the numbers of PGC aggregates observed were intermediate between those of *Kitl*^{Sl-gb}/*Kitl*^{Sl-gb} and *Kitl*^{Sl-gb}/*Kitl*⁺ embryos (not shown). However, it was not possible to reliably distinguish between *Kitl*^{Sl-17H}/*Kitl*⁺ embryos and *Kitl*⁺/*Kitl*⁺ mice. Nonetheless, we concluded that the whole-mount staining method is a reliable, semi-quantitative method for determining PGC defects in homozygous *Kitl*^{Sl} mutant embryos. In analyzing the remaining mutants, the effects on PGCs were considered to be “moderate” if genital ridges had about the same number of AP-positive PGCs as that found in *Kitl*^{Sl-17H}/*Kitl*^{Sl-17H} embryos or “severe” if genital ridges had only a few PGCs or were devoid of PGCs. Representative images of genital ridges with moderate and severe effects on PGCs are shown in Figure 2.1B, a *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} embryo, and Figure 2.1C, a *Kitl*^{Sl-30R}/*Kitl*^{Sl-30R} embryo.

The results of the screen for PGC defects at E11.5 in all *Kitl*^{Sl} mutants are summarized in Table 2.1. Embryos homozygous for the *Kitl*^{Sl-5R}, *Kitl*^{Sl-22R}, *Kitl*^{Sl-28R}, *Kitl*^{Sl-30R}, *Kitl*^{Sl-31R} and *Kitl*^{Sl-42R}

mutations were found to have severe effects on PGCs at this stage while embryos homozygous for the *Kitl*^{Sl-36R}, *Kitl*^{Sl-39R} and *Kitl*^{Sl-d} mutations, as well as the *Kitl*^{Sl-17H} mutation, were found to have moderate effects on PGCs at this stage. Thus, we concluded that the mutations that are null with respect to anemia and viability are also null for PGC development while the mutations that allow viability and cause less severe anemia are also hypomorphic for PGC development. Although the correlation between different aspects of the *Kitl*^{Sl} phenotype was not entirely surprising, the results provided us justification to focus on the hypomorphic mutations for more detailed analyses of PGC development.

Hypomorphic *Kitl*^{Sl} mutations have graded effects on PGC numbers in E11.5 embryos

We examined the effects of three hypomorphic mutations (*Kitl*^{Sl-39R}, *Kitl*^{Sl-36R} and *Kitl*^{Sl-d}) and the null mutation (*Kitl*^{Sl-gb}) on PGC development in more detail. The whole-mount staining method was useful to screen all *Kitl*^{Sl} mutants, however it was considered semi-quantitative and did not provide detailed comparisons of effects on PGC numbers caused by the different mutations. To quantify post-migratory PGCs, serial cryostat sections were prepared from the caudal regions of E11.5 embryos spanning the entire genital ridges, sections stained for AP activity, and AP-positive PGCs counted by light microscopy. Representative images of stained sections are shown in Figure 2.2A – 2.2E, and the total number of PGCs per embryo tabulated in Figure 2.2F. In *Kitl*^{+/+}/*Kitl*^{+/+} embryos at E11.5, virtually all PGCs had migrated to the genital ridge and there were only a few scattered PGCs in the mesentery (Figure 2.2A). An average of 1166 +/- 91 PGCs were found in *Kitl*^{+/+}/*Kitl*^{+/+} genital ridges (Figure 2.2A and 2.2F). No PGCs were found in genital ridges of embryos homozygous for the *Kitl*^{Sl-gb} mutation (Figure 2.2B and 2.2F), a finding that is consistent with the absence of PGCs in whole-mount preparations of these null

mutants (see above). In contrast, PGC numbers in genital ridges of embryos homozygous for the *Kitl*^{Sl-d} (Figure 2.2C), *Kitl*^{Sl-39R} (Figure 2.2D), and *Kitl*^{Sl-36R} (Figure 2.2E) were all greatly reduced from those of *Kitl*^{+/+} embryos but were more than those found in the *Kitl*^{Sl-gb/Kitl}^{Sl-gb} embryos. The total number of PGCs in genital ridges of *Kitl*^{Sl-36R/Kitl}^{Sl-36R}, *Kitl*^{Sl-39R/Kitl}^{Sl-39R} and *Kitl*^{Sl-d/Kitl}^{Sl-d} embryos averaged 302, 238, and 38, respectively. In *Kitl*^{Sl-36R/Kitl}^{Sl-36R} and *Kitl*^{Sl-39R/Kitl}^{Sl-39R} embryos, these average PGC counts were not significantly different from each other, but were only 20% and 26%, respectively, that of *Kitl*^{+/+} embryos. These values are similar to those reported previously for E11.5 *Kitl*^{Sl-17H/Kitl}^{Sl-17H} embryos (Brannan et al., 1992). Furthermore, the PGC counts in these embryos are significantly higher than those of *Kitl*^{Sl-d/Kitl}^{Sl-d} embryos, which had only about 3% of the *Kitl*^{+/+} PGC counts. Thus, the effects of homozygous *Kitl*^{Sl-39R}, *Kitl*^{Sl-36R} and *Kitl*^{Sl-17H} mutations on numbers of postmigratory PGCs are equivalent to each other, but are milder than those of *Kitl*^{Sl-d}. The reduced number of PGCs in the genital ridges of *Kitl*^{Sl} mutants at E11.5 could be a result of the loss of directionality of migrating PGCs or due to reduced survival and/or proliferation of PGCs.

Defects in total numbers of PGCs in *Kitl*^{Sl} mutants at E9.5

Previous studies showed that PGCs begin to leave the hindgut around E9.5 (Bendel-Stenzel et al., 2000; Molyneaux et al., 2001). Accordingly, to investigate whether early stages of PGC migration are affected in *Kitl*^{Sl} mutant embryos, the numbers and locations of PGCs in E9.5 (19 – 21 somite) embryos were determined. Representative images of E9.5 sections stained for AP are shown in Figure 2.3 and the total numbers of AP-positive PGCs per embryo tabulated in Figure 2.4A. PGCs in *Kitl*^{+/+} E9.5 embryos were found primarily within the hindgut, either associated closely with the hindgut epithelium (Figure 2.3A) or in the dorsal axis of the hindgut

(Figure 2.3B). The latter cells moved from the hindgut and were en route to the presumptive genital ridges. In *Kitl⁺/Kitl⁺* embryos, an average of 401 ± 73 PGCs per embryo were found (Figure 2.4A) and these cells were distributed along the anterior and posterior portions of the hindgut. In E9.5 embryos homozygous for *Kitl^{Sl-gb}* (Figure 2.3C) and *Kitl^{Sl-d}* (Figure 2.3D), PGCs were found predominantly in the ventral axis of the hindgut and many aggregated in the posterior portion of the hindgut, suggesting that these mutants have defects in migration early during PGC development. The total numbers of PGCs in E9.5 embryos homozygous or each of the *Kitl^{Sl}* mutations were significantly decreased compared to those of *Kitl⁺/Kitl⁺* embryos (Figure 2.4A). These values in *Kitl^{Sl-gb}/Kitl^{Sl-gb}* and *Kitl^{Sl-d}/Kitl^{Sl-d}* embryos are not significantly different from each other and average only 80 and 90 PGCs per embryo, respectively (Figure 2.4A), which is only 20% and 22% of the values in *Kitl⁺/Kitl⁺* embryos. Interestingly, PGCs were found in the proper locations in both *Kitl^{Sl-39R}/Kitl^{Sl-39R}* and *Kitl^{Sl-36R}/Kitl^{Sl-36R}* E9.5 embryos (Figure 2.3E and 2.3F). However, the average number of PGCs per embryos in these mutants was 126 and 177, respectively (Figure 2.4A), which is 31% and 44% of the *Kitl⁺/Kitl⁺*. As with the PGC numbers in the E11.5 genital ridge (see above), the PGC numbers in the E9.5 hindguts of the *Kitl^{Sl-39R}* and *Kitl^{Sl-36R}* mutants were both significantly ($P=0.002$) higher than those of the other two mutants, but were not significantly different from each other.

Defects in migration and total numbers of PGCs in *Kitl^{Sl}* mutants at E10.5

Between E9.5 and E11.5, PGCs actively migrate away from the hindgut by moving dorsally and laterally towards the genital ridges (Molyneaux et al., 2001) and expand their numbers by rapid proliferation. Numerous studies have shown that the survival and proliferation of these migrating PGCs require KITL (Donovan, 1994). To determine whether the numbers and

location of migrating PGCs were affected in *Kitl^{Sl}* mutants, we examined sections of 35-37 somite embryos at E10.5. Representative images of E10.5 sections stained for AP activity are shown in Figure 2.5, the total numbers of PGCs per embryo are summarized in Figure 2.4B, and the fold expansions in PGC numbers between E9.5 and E10.5 are shown in Figure 2.4C.

In *Kitl⁺/Kitl⁺* embryos, there was a total of 1237 ± 169 PGCs per E10.5 embryo (Figure 2.4B), which represents a significant 3-fold increase over the numbers seen in E9.5 embryos (Figure 2.4C). With all of the *Kitl^{Sl}* mutations, the total numbers of PGCs in E10.5 homozygous mutants were significantly reduced from those of *Kitl⁺/Kitl⁺* embryos. However, there are interesting differences between the effects of different mutations on the total numbers of PGCs. In *Kitl^{Sl-gb}/Kitl^{Sl-gb}* embryos, few PGCs were found outside of the hindgut and of those, most have an abnormal morphology suggesting that undergoing cell death (Figure 2.5C). Furthermore, the PGC numbers in these mutant embryos averaged only 78 PGCs per embryo (Figure 2.4B), which is not a significant change from that seen at E9.5 (Figure 2.4C). Surprisingly, the numbers of total PGCs in *Kitl^{Sl-d}/Kitl^{Sl-d}* embryos at E10.5 were only about 50% the numbers seen at E9.5 (Figure 2.4C). This value for *Kitl^{Sl-d}/Kitl^{Sl-d}* embryos at E10.5 is significantly less than that of *Kitl^{Sl-gb}/Kitl^{Sl-gb}* embryos at the same age and of *Kitl^{Sl-d}/Kitl^{Sl-d}* embryos at E9.5 ($P=0.012$ and $P=0.008$, respectively). In contrast, the PGC numbers in *Kitl^{Sl-36R}/Kitl^{Sl-36R}* embryos are significantly ($P = 0.034$) increased at E10.5 over those of E9.5. In E10.5 *Kitl^{Sl-36R}/Kitl^{Sl-36R}* embryos, there was an average of 291 PGCs per embryo, which is significantly greater than any of the other mutants at E10.5 (Figure 2.4B), and represents a 1.6 fold increase over the E9.5 value (Figure 2.4C). In *Kitl^{Sl-39R}/Kitl^{Sl-39R}* embryos, although the average number of PGCs per embryo at E10.5 is 156 (Figure 2.4B), the fold increase from E9.5 to E10.5 in these mutants is

only 1.2 (Figure 2.4C). As with the PGC numbers at E9.5, the total PGC numbers found in E10.5 *Kitl*^{SL-36R}/*Kitl*^{SL-36R} and *Kitl*^{SL-39R}/*Kitl*^{SL-39R} embryos are significantly greater than those of E10.5 *Kitl*^{SL-gb}/*Kitl*^{SL-gb} embryos.

To investigate the effects of the *Kitl*^{SL} mutations on migration of PGCs, we examined the localization of PGCs in E10.5 embryos. In *Kitl*⁺/*Kitl*⁺ embryos at E10.5, virtually all (93%) of the PGCs were found to have emigrated from the hindgut (Figure 2.4D), with many found in the dorsal portions of the mesentery (Figure 2.5A) and within/or near the genital ridges (Figure 2.5B). A similar percent of migrating cells was observed in *Kitl*^{SL-36R}/*Kitl*^{SL-36R} embryos, where 83% of the PGCs had migrated out of the hindgut (Figure 2.5F). Thus, in the *Kitl*^{SL-36R}/*Kitl*^{SL-36R} embryos, even though the numbers of PGCs were reduced, they were able to migrate properly. In contrast, the numbers of migrating PGCs observed in embryos homozygous for *Kitl*^{SL-gb}, *Kitl*^{SL-d}, and *Kitl*^{SL-39R} mutations (Figures 2.5C-2.5E) were significantly reduced compared to *Kitl*⁺/*Kitl*⁺, with only 50%, 45%, and 31% of PGCs, respectively, found in locations outside of the hindgut at E10.5 (Figure 2.4D).

In summary, all four *Kitl*^{SL} mutants had significantly reduced numbers of PGCs at both E9.5 and E10.5 compared to wildtype, *Kitl*^{SL-36R}/*Kitl*^{SL-36R} and *Kitl*^{SL-39R}/*Kitl*^{SL-39R} embryos had significantly more PGCs at both ages than *Kitl*^{SL-gb}/*Kitl*^{SL-gb} embryos, only *Kitl*^{SL-d}/*Kitl*^{SL-d} embryos had a significant decrease in PGC numbers between E9.5 and E10.5, and only *Kitl*^{SL-36R}/*Kitl*^{SL-36R} embryos had a significant increase in PGC numbers between E9.5 and E10.5. Furthermore, movement of PGCs in *Kitl*^{SL-36R}/*Kitl*^{SL-36R} embryos proceeds relatively normally but is impaired equally in *Kitl*^{SL-39R}/*Kitl*^{SL-39R}, *Kitl*^{SL-d}/*Kitl*^{SL-d}, and *Kitl*^{SL-gb}/*Kitl*^{SL-gb} embryos. Together, these studies of

PGC numbers and localization in E10.5 embryos suggest that KITL is required for emigration and/or mobilization of PGCs from the hindgut, as well as for proliferation and/or survival of migrating PGCs.

Rates of proliferation of PGCs in different regions of the migratory pathway

The reduced number of PGCs and the increased number of dying cells in the *Kitl^{Sl}* mutants suggested that the survival of the PGCs was affected by all the mutations, particularly at E10.5 and E11.5. However, these studies did not reveal if the reduced numbers of PGCs was due to a higher level of apoptosis or to a lower level of proliferation. Indeed, large numbers of PGCs that appear to be undergoing apoptosis were observed in the hindguts of the *Kitl^{Sl-gb}/Kitl^{Sl-gb}*, *Kitl^{Sl-d}/Kitl^{Sl-d}* and *Kitl^{Sl-39R}/Kitl^{Sl-39R}* embryos (Figures 2.5C-2.5E) but not in *Kitl⁺/Kitl⁺* embryos (Figure 2.5A, 2.5B) or in *Kitl^{Sl-36R}/Kitl^{Sl-36R}* embryos (Figure 2.5F and not shown). Earlier reports suggested that non-motile PGCs are rounded and intensely stained for alkaline phosphatase and that apoptotic PGCs disintegrate and disappear (De Felici, 2000; Donovan et al., 1986; Gomperts et al., 1994; Molyneaux et al., 2001; Molyneaux et al., 2003; Stallock et al., 2003).

To determine if PGC proliferation was affected in the *Kitl^{Sl}* mutant embryos, we assessed BrdU incorporation as a measure of DNA synthesis. Sections of *Kitl⁺/Kitl⁺*, *Kitl^{Sl-gb}/Kitl^{Sl-gb}*, *Kitl^{Sl-d}/Kitl^{Sl-d}*, *Kitl^{Sl-39R}/Kitl^{Sl-39R}* and *Kitl^{Sl-36R}/Kitl^{Sl-36R}* embryos at E10.5 and E11.5 (Figures 2.5C-2.5E) were double-stained to reveal AP-positive PGCs and anti-BrdU-positive cells. Double-stained sections were examined by confocal microscopy and representative images of these sections are shown in Figure 2.6. The proliferative index, i.e. the number of BrdU-positive

PGCs versus the total number of PGCs counted, was calculated for PGCs in the hindgut and in the dorsal mesentery plus genital ridges, and the results summarized in Figure 2.7.

In E10.5 *Kitl*⁺/*Kitl*⁺ embryos, the majority of PGCs that were mobilized from the hindgut and reached the genital ridges and/or dorsal mesentery were BrdU-positive (Figure 2.6A, 2.6a and 2.6a') while few of the PGCs remaining in the hindgut were BrdU-positive (Figure 2.6B and Figure 2.6b). At E11.5, numerous PGCs that were resident in the genital ridges were BrdU-positive in both *Kitl*⁺/*Kitl*⁺ and *Kitl*^{SL-39R}/*Kitl*^{SL-39R} embryos (Figure 2.6C and 2.6E). However, in E10.5 *Kitl*^{SL-39R}/*Kitl*^{SL-39R} embryos, a large number of the PGCs in the dorsal mesentery as well as in the hindgut were BrdU-negative (Figures 2.6D, d, F and f). From our confocal analyses, we also confirmed the presence of a large number of disintegrating and apoptotic PGCs in the hindgut of the *Kitl*^{SL-39R}/*Kitl*^{SL-39R} embryos (see arrows in Figure 2.5E and barbed arrows in Figure 2.6 f').

In the hindguts of E10.5 embryos, 39% of PGCs in *Kitl*⁺/*Kitl*⁺ embryos were BrdU-positive, while only 13-19% of PGCs in embryos homozygous for each of the *Kitl*^{SL} mutations were BrdU-positive (Figure 2.7A). While none of the values in homozygous mutants were significantly different from those of *Kitl*⁺/*Kitl*⁺ embryos, there was a trend for PGCs that remain in the hindguts of the mutants to have a lower proliferative index than those remaining in hindguts of the *Kitl*⁺/*Kitl*⁺ embryos. In contrast, embryos homozygous for each of the *Kitl*^{SL} mutations had significantly reduced proliferative indices of migratory and postmigratory PGCs, which were found either within the mesentery or within the genital ridge (Figure 2.7B). Compared to PGCs remaining in the hindgut at E10.5, PGCs in the mesentery and genital ridge

had proliferative indices that were increased 2 to 3 fold in *Kitl*⁺/*Kitl*⁺, *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R}, *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R}, and *Kitl*^{Sl-d}/*Kitl*^{Sl-d} embryos, but not in *Kitl*^{Sl-gb}/*Kitl*^{Sl-gb} embryos (Figure 2.7B). However, these percentages in all mutants were significantly reduced from that of *Kitl*⁺/*Kitl*⁺ embryos. While there were no BrdU-positive PGCs observed in *Kitl*^{Sl-gb}/*Kitl*^{Sl-gb} embryos, there were 40% to 46% BrdU-positive PGCs in embryos homozygous for each of the other three *Kitl*^{Sl} mutations. However, pairwise comparisons between the latter mutants did not reveal any significant differences between them.

At E11.5, post-migratory PGCs in the genital ridges from *Kitl*⁺/*Kitl*⁺, *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R}, and *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} embryos had slightly reduced proliferative indices (Figure 2.7C) compared to the corresponding values at E10.5. These values in *Kitl*⁺/*Kitl*⁺ embryos averaged 59%, but were only 32% and 33%, respectively, in *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} and *Kitl*^{Sl-d}/*Kitl*^{Sl-d} embryos (Figure 2.7C). No PGCs were identified in genital ridges of *Kitl*^{Sl-gb}/*Kitl*^{Sl-gb} embryos. Interestingly, the proliferative index of PGCs in *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} embryos was 46%, which was significantly greater than the values seen in *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} and *Kitl*^{Sl-d}/*Kitl*^{Sl-d} embryos.

DISCUSSION

We screened a large allelic series of *Kitl*^{Sl} mutations (Table 2.1) for PGC deficiencies in genital ridges at E11.5, by which time PGC migration is nearly completed in normal embryos. Because all of these mutations are congenic on a common strain background, comparisons of mutants phenotypes are not confounded by possible inter-strain differences. This allelic series includes six *Kitl*^{Sl} mutations that were shown previously to be null, or nearly null, for peripheral RBCs and mouse viability, and four that are hypomorphic for these phenotypes (Rajaraman et

al., 2002b; Rajaraman et al., 2003). The present study shows that all *Kitl^{Sl}* mutations null for RBCs are also null for postmigratory PGCs (Table 2.1). Although it is possible that some of these *Kitl^{Sl}* mutations could have interesting effects on early stages of PGCs, we focused the remainder of our studies on three hypomorphic and an unambiguous null *Kitl^{Sl}* mutation.

One of the most useful aspects of *Kitl^{Sl}* mutations is that different hypomorphic alleles exhibit graded effects. The hypomorphic *Kitl^{Sl}* mutations used in this study all allow viability to homozygotes and have different molecular defects (see Table 2.1). In addition, graded effects on RBCs in newborn and juvenile mice were observed, with effects being mild with *Kitl^{Sl-36R}*, intermediate with *Kitl^{Sl-39R}* and most severe with *Kitl^{Sl-d}* (Rajaraman et al., 2002b). In the present studies of PGC development, graded effects were also observed in embryos homozygous for *Kitl^{Sl}* mutations, with either or both of the *Kitl^{Sl-36R}* and *Kitl^{Sl-39R}* mutations consistently having milder effects than *Kitl^{Sl-d}*. In some cases, such as for movement of PGCs from the hindgut (Figure 2.4D), total numbers of PGCs at E10.5 (Figure 2.4B), and fold-increase at E10.5 versus E9.5 (Figure 2.4C), the *Kitl^{Sl-36R}* mutation has significantly milder effects than the *Kitl^{Sl-39R}* mutation. Interestingly, the relative effects of these mutations on BrdU-positive indices in E11.5 PGCs are reversed, with *Kitl^{Sl-39R}* having significantly milder effects than *Kitl^{Sl-36R}* (Figure 2.7C). This variation in allele strength at different stages of germ cell development could reflect alterations in expression or function of mutant KITL proteins, altered interactions of other proteins with KITL mutants, or altered responses of germ cells at different developmental stages to the mutant KITL proteins. Detailed studies of KITL mutant proteins will be needed to resolve these issues. Nonetheless, even in the absence of such information, the *Kitl^{Sl}* mutations provide valuable genetic resources for understanding KITL functions during germ cell development.

Early studies demonstrated that PGC numbers in *Kitl^{Sl}* (McCoshen and McCallion, 1975) and *Kit^W* (Mintz and Russell, 1957) mutants are similar to those of wildtype in E8 and E9 embryos; therefore it is generally thought that allocation and early migration of PGCs are independent of KITL and KIT (Donovan, 1994; Wylie, 1999). However, PGCs express KIT at E7.5 (Manova and Bachvarova, 1991), raising the possibility that KITL-KIT signaling could be involved at this stage of development. In the present studies, we found that embryos homozygous for null or hypomorphic *Kitl^{Sl}* mutations had significantly reduced numbers of PGCs in the E9.5 hindgut (Figure 2.4A). If early PGCs are KITL-independent, this observation suggests that PGC survival and/or proliferation become at least partially KITL-dependent shortly after their arrival in the hindgut. However, *Kitl^{Sl-gb}/Kitl^{Sl-gb}* embryos have some PGCs at E9.5, albeit with only about 20% of wildtype embryos, indicating that PGCs at this stage are not completely dependent on KITL. Many PGCs with abnormal morphology were observed in E10.5 *Kitl^{Sl-gb}/Kitl^{Sl-gb}* and *Kitl^{Sl-d}/Kitl^{Sl-d}* embryos (Figure 2.5C and 2.5D). Since KITL is known to suppress apoptosis in PGCs and because previous studies have shown that PGCs with similar abnormal morphology are apoptotic, it is likely that the abnormal PGCs in the *Kitl^{Sl}* mutants are apoptotic. If so, the main function of KITL in the E9.5 hindgut may be to suppress apoptosis as well. Between E9.5 and E10.5, the PGC numbers are constant in *Kitl^{Sl-gb}/Kitl^{Sl-gb}* embryos, in contrast to the 3-fold expansion seen in *Kitl⁺/Kitl⁺* embryos (Figure 2.4C). Although the trend for the proliferative-indices of PGCs in the E10.5 hindgut is to be reduced in all *Kitl^{Sl}* mutants, these values are not significantly different from those of *Kitl⁺/Kitl⁺* embryos (Figure 2.7A). This situation is strikingly different in PGCs that have migrated away from the hindgut in the E10.5 embryos, where proliferative indices of PGCs in the mesentery and genital ridges of all *Kitl^{Sl}*

homozygotes are significantly less than in *Kitl*⁺/*Kitl*⁺ embryos (Figure 2.7B). More importantly, in *Kitl*^{Sl-gb}/*Kitl*^{Sl-gb} embryos we detected no BrdU-positive PGCs once they left the hindgut (Figure 2.7B). Although the numbers of migrating PGCs are greatly reduced in these null mutants they are about the same as seen in *Kitl*^{Sl-d}/*Kitl*^{Sl-d} embryos and migrating PGCs in the latter have detectable numbers of BrdU-positive PGCs (Figure 2.7B). Therefore, the lack of BrdU-positive migrating PGCs in E10.5 *Kitl*^{Sl-gb}/*Kitl*^{Sl-gb} mutants is probably not due to an indirect effect of reduced PGC numbers.

Three main conclusions about KITL functions for affecting PGC numbers in E9.5 – E10.5 embryos can be made from the above observations. First, KITL is required to maintain normal numbers of PGCs in the hindgut at E9.5. Second, KITL has minimal effect on proliferation of PGCs in the hindgut at E9.5 but may act on these cells primarily through suppression of apoptosis. Third, there is an absolute requirement of KITL for PGC proliferation once the cells start migrating away from the hindgut. It has been demonstrated that synergy between KITL and other cytokines such as LIF, FGF, IL-4 is essential for abundant growth of PGCs in vitro (Dolci et al., 1991; Matsui et al., 1991; Resnick et al., 1998). Therefore we hypothesize that in the hindgut of *Kitl*^{Sl}/*Kitl*^{Sl} embryos, other cytokines are able to partially compensate for the absent or reduced KITL functions. However, once PGCs begin migrating away from the hindgut, the cells become critically dependent on KITL and it does not appear that there are any compensatory mechanisms that occur *in vivo* in the absence of KITL.

It is often reported that MB-KITL is more important for supporting PGC growth than S-KITL. *In vivo* evidence has come predominantly from analysis of *Kitl*^{Sl-d} mice, which lack MB-KITL because of an intragenic deletion. However, the *Kitl*^{Sl-36R} mutation is also predicted to

lack MB-KITL because of a nonsense mutation (Rajaraman et al., 2002a) located just a few codons upstream to the *Kitl*^{Sl-d} deletion breakpoint. Despite the similarities in their predicted encoded proteins, the *Kitl*^{Sl-36R} mutation has milder effects than the *Kitl*^{Sl-d} mutation on peripheral blood cells (Rajaraman et al., 2002b) and PGCs (present study). A possible explanation for this apparent discrepancy may be found once more information about proteins (e.g. localization, stability, processing, etc) encoded by both mutant alleles is known. The present studies also reveal that E10.5 PGC numbers are significantly lower in *Kitl*^{Sl-d}/*Kitl*^{Sl-d} embryos than in *Kitl*^{Sl-gb}/*Kitl*^{Sl-gb} embryos (Figure 2.4A and 2.4B). These observations suggest that KITL^{Sl-d} protein may inhibit other factors required for PGC numbers. If so, the inhibitory effect is likely to be on survival, and not proliferation, as the percentages of BrdU-positive PGCs in the hindgut of E10.5 *Kitl*^{Sl-d}/*Kitl*^{Sl-d} and *Kitl*^{Sl-gb}/*Kitl*^{Sl-gb} embryos are equivalent (Figure 2.7A). Notwithstanding the possible inhibitory effect of KITL^{Sl-d} protein, recent evidence suggests that responses of cultured PGCs to S-KITL and MB-KITL requires a delicate balance of the two isoforms. When added to cultures of PGCs, S-KITL was found to synergize with FGF to promote their proliferation but MB-KITL actually inhibited their proliferation (Kawase et al., 2004). Although these studies may not directly explain the differences noted between the *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} and *Kitl*^{Sl-d}/*Kitl*^{Sl-d} phenotypes, they do illustrate the need for more structure-function analyses of wildtype and mutant isoforms of KITL.

With the exception of BrdU indices in the E11.5 genital ridge, all aspects of PGC development in *Kitl*^{Sl-36R} and *Kitl*^{Sl-39R} mutants were either equally affected, or more severe in the latter. These observations are consistent with the previously described effects of these mutations on peripheral RBCs (Rajaraman et al., 2002b). Interestingly, the percentage of BrdU-positive

PGCs in the E10.5 genital ridges of *Kitl*^{SI-39R}/*Kitl*^{SI-39R} embryos is significantly higher than in *Kitl*^{SI-36R}/*Kitl*^{SI-36R} embryos (Figure 2.7C). While this observation will need to be confirmed and extended, it is significant that the effects of the *Kitl*^{SI-39R} mutation on postnatal spermatogenesis (A.M.Z and M.A.B., Chapter 3) and postnatal oogenesis (unpublished observations) are milder than those of the *Kitl*^{SI-36R} mutation. Thus KITL^{SI-39R} function appears to improve relative to that of KITL^{SI-36R}, in sexually differentiated gonads, but the reason for this is not currently known.

Some of the most significant observations in this study are regarding the role of KITL in PGC migration. Defects in PGC migration in *Kitl*^{SI} embryos are apparent in the E9.5 hindgut, where PGCs in *Kitl*^{SI-gb}/*Kitl*^{SI-gb} and *Kitl*^{SI-d}/*Kitl*^{SI-d} embryos do not undergo ventral to dorsal migration (Figure 2.3C and 2.3D). At E10.5, defects in PGC migration in these mutants, as well as in *Kitl*^{SI-39R}/*Kitl*^{SI-39R} mutants, were even more pronounced (Figure 2.4D). Interestingly, the percentage of PGCs that migrated from the hindgut was equivalent between *Kitl*^{SI-36R}/*Kitl*^{SI-36R} and *Kitl*⁺/*Kitl*⁺ embryos. This significant difference in movement of PGCs from the hindgut of *Kitl*^{SI-36R}/*Kitl*^{SI-36R} embryos and lack of this movement in *Kitl*^{SI-39R}/*Kitl*^{SI-39R} and *Kitl*^{SI-d}/*Kitl*^{SI-d} embryos occurs at a time when the percentage of BrdU-positive PGCs in the three mutants are virtually identical (Figure 2.7A and 2.7B). Thus, migration and proliferation of PGCs may require different levels of KITL function. Together, these observations on localization of PGCs in the E9.5 and E10.5 embryos provide compelling evidence that KITL plays an active role in migration of these cells. While previous *in vitro* studies indicate that KITL does not act as a chemotropic factor for PGCs (Godin et al., 1991), it has been shown to be a chemokinetic factor for melanocyte migration (Jordan and Jackson, 2000). Thus, KITL may act to mobilize PGCs

for their migration out of the hindgut, and other factors may be directly involved in guiding PGCs along the correct path.

What other factors could either directly or indirectly affect KITL-mediated migration of PGCs? These cells express various cell adhesion molecules, including E- and P-cadherins (Bendel-Stenzel et al., 2000; De Felici, 2000; Di Carlo and De Felici, 2000; Okamura et al., 2003) that are required for the proper migration of the PGCs from the base of the allantois into the genital ridges. Use of neutralizing antibodies to E-cadherin in cultured PGCs provided evidence that E-cadherin interactions are linked to motility as well as survival of these cells (Di Carlo and De Felici, 2000). Studies with transgenic mice have shown that KITL-dependent movement of melanoblasts may be mediated via modified expression of E- and P-cadherins (Nishimura et al., 1999). Considering all these observations, it is tempting to speculate that KITL is directly mediating the expression patterns of cadherins and thereby migration of PGCs. Another player in KITL signaling during migration could be another ligand/receptor pair, SDF1/CXCR4 (Molyneaux et al., 2003). SDF-1 is the ligand for CXCR4 and is expressed in the mesenchyme of the body wall while CXCR4 is expressed on PGCs. *CXCR4* mutants have normal early PGC development, but reduced numbers of PGCs that are probably due to reduced proliferation and not to increased apoptosis. Furthermore, CXCR4 function is required for PGCs to colonize the gonad. There are many reports that KITL and SDF1 act synergistically in mediating migration of other cells, including hematopoietic progenitors (Drayer et al., 2000; Dutt et al., 1998; Kijima et al., 2002; Lapidot and Petit, 2002; Minamiguchi et al., 2001). In addition, in the bone marrow stroma, SDF-1 activity is required for activity of a matrix metalloproteinase (MMP-9) (Heissig et al., 2002). MMP9 has been shown to cleave the KITL precursor for release

of S-KITL, and these processes are required for mobilization of hematopoietic stem cells during regenerative hematopoiesis (Heissig et al., 2002). These few examples illustrate how complex the complete range of biological and molecular interactions that may take place to mediate KITL function. Additional studies that combine genetic resources in the mouse with elegant tissue (e.g. Molyneaux et al., 2001) and cell culture (e.g. De Miguel et al., 2002) experiments should prove to reveal even more aspects of KITL function during PGC development.

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Table 2.1. Summary of *Kitl*^{SI} mutations used in the present study and results of screening all mutations for their effects on E11.5 PGCs.

<i>Kitl</i> allele ^a	Effects on KITL sequences ^b	Viability of homozygous mice ^c	Screen for defects in PGCs at E11.5 ^d	
			No. homozygous mutants examined	Effects on PGCs ^e
<i>Sl-gb</i>	Deletion of all coding sequences	Perinatally lethal	5	Severe
<i>Sl-5R</i>	Deletion of 2 amino acids (delN31,Y32)	Perinatally lethal	6	Severe
<i>Sl-22R</i>	Missense mutation (L54P)	Perinatally lethal	6	Severe
<i>Sl-28R</i>	Missense mutation (I118N)	Perinatally lethal	5	Severe
<i>Sl-30R</i>	Missense mutation (L18R)	Perinatally lethal	11	Severe
<i>Sl-31R</i>	Missense mutation (P23L)	Perinatally lethal	4	Severe
<i>Sl-42R</i>	Truncated S-Kitl (96 + 2 aa)	Perinatally lethal	7	Severe
<i>Sl-36R</i>	2 truncated S-Kitl (147aa; 96 + 25 aa)	Viable	8	Moderate
<i>Sl-39R</i>	Missense mutant (S122F)	Viable	9	Moderate
<i>Sl-d</i>	Truncated S-Kitl (180 + 3 aa)	Viable	3	Moderate
<i>Sl-17H</i>	Truncated Pre-KITL (211 + 27 aa) Truncated MB-KITL (192 + 27 aa)	Viable	2	Moderate

^aDescriptions of the *Kitl* sequence alterations for the mutant alleles are in the following papers:

Kitl^{Sl-gb} (Bedell et al., 1996a); *Kitl*^{Sl-5R} (Rajaraman et al., 2003); *Kitl*^{Sl-22R}, *Kitl*^{Sl-28R}, *Kitl*^{Sl-30R},

Kitl^{Sl-31R}, *Kitl*^{Sl-42R}, *Kitl*^{Sl-36R}, and *Kitl*^{Sl-39R} (Rajaraman et al., 2002a); *Kitl*^{Sl-d} (Brannan et al., 1991;

Flanagan et al., 1991), and *Kitl*^{Sl-17H} (Brannan et al., 1992). The *Kitl*^{Sl-gb} and *Kitl*^{Sl-d} alleles arose

spontaneously, and all other alleles were induced by ENU mutagenesis. For the present studies, all of the mutations are congenic on a C3H background.

^bEach of the missense mutations and the deletion in *Kitl*^{Sl-5R} affect sequences in both S-KITL and MB-KITL isoforms (see text). However, the KITL^{Sl-36R} and KITL^{Sl-d} mutants have truncated C-termini such that no MB-isoform would be expected. In KITL^{Sl-17H}, the cytoplasmic domains of Pre-KITL, which is a precursor to S-KITL, and MB-KITL are out-of-frame.

^cInformation on the viability of mice homozygous for each mutation is from previous reports (Brannan et al., 1992; Rajaraman et al., 2002b; Rajaraman et al., 2003).

^dEffects of *Kitl*^{Sl} mutations on PGCs in genital ridges of E11.5 embryos are summarized from the present study. Severe effects on PGC numbers were defined as similar effects to those of *Kitl*^{Sl-gb} mutation and moderate effects similar to those in *Kitl*^{Sl-17H} mutation. For each mutation, at least two litters of E11.5 embryos were dissected, whole mount preparations stained for AP activity, and phenotypes with respect to PGC numbers in genital ridges recorded prior to genotyping. For embryos homozygous for each *Kitl*^{Sl} mutation, the phenotypes and genotypes were in agreement.

Table 2.2. Relative activities of *Kitl*^{Sl} mutations for different stages of PGC development in homozygous embryos^a.

Stage	Total numbers of PGCs ^b	BrdU-positive PGCs in hindgut ^c	Migration of PGCs from hindgut ^d	BrdU-positive PGCs in mesentery ^e	BrdU-positive PGCs in genital ridge ^f
E9.5	<i>Sl-36R</i> = <i>Sl-39R</i> <i>Sl-d</i> = <i>Sl-gb</i>	ND	NA	NA	NA
E10.5	<i>Sl-36R</i> <i>Sl-39R</i> <i>Sl-gb</i> <i>Sl-d</i>	<i>Sl-36R</i> = <i>Sl-39R</i> = <i>Sl-d</i> = <i>Sl-gb</i>	<i>Sl-36R</i> <i>Sl-39R</i> = <i>Sl-d</i> = <i>Sl-gb</i>	<i>Sl-36R</i> = <i>Sl-39R</i> = <i>Sl-d</i> <i>Sl-gb</i> (none)	<i>Sl-36R</i> = <i>Sl-39R</i> <i>Sl-d</i> (none) <i>Sl-gb</i> (no PGCs)
E11.5	<i>Sl-36R</i> = <i>Sl-39R</i> <i>Sl-d</i> (<i>Sl-gb</i> , no PGCs)	NA	NA	NA	<i>Sl-39R</i> <i>Sl-36R</i> = <i>Sl-d</i> <i>Sl-gb</i> (no PGCs)

^a*Kitl* mutant alleles are shown in order of activity in homozygotes from highest to lowest. Equal signs indicate equivalent activities and mutations with different activities are on different lines. All mutations have effects that are significantly different from those seen in *Kitl*^{+/+}/*Kitl*^{+/+} embryos, except for those that are shown in boxes. Note that no PGCs were observed in genital ridges of *Kitl*^{Sl-gb}/*Kitl*^{Sl-gb} embryos. NA, not applicable; ND, not determined.

^bSummarized from data in Figure 4A (E9.5), Figure 4B (E10.5), and Figure 2F (E11.5).

^cSummarized from data in Figure 7A.

^dSummarized from data in Figure 4D.

^eSummarized from data in Figure 7B.

^fSummarized from data in Figure 7C.

FIGURE LEGENDS

Figure 2.1. Whole-mount embryos at E11.5 stained for AP activity. E11.5 embryos were exposed at the genital ridges and AP histochemistry performed. Arrow point to clumps of PGCs and the asterisk indicates a tear in the tissue. GR, genital ridge; NT, neural tube. Numerous clumps of PGCs are found in the genital ridges of *Kitl*⁺/*Kitl*⁺ embryos (A) while *Kitl*^{SL-39R}/*Kitl*^{SL-39R} embryos (B) have reduced number of PGCs similar to *Kitl*^{SL-36R}/*Kitl*^{SL-36R} and *Kitl*^{SL-d}/*Kitl*^{SL-d} embryos (not shown). Either none or only an occasional PGC was seen in *Kitl*^{SL-30R}/*Kitl*^{SL-30R} embryos (C) and in *Kitl*^{SL-gb}/*Kitl*^{SL-gb} (null) embryos (D). Embryos homozygous for each of the other five *Kitl*^{SL} mutations also had virtually no PGCs at this age (images not shown, see Table 1 for summary).

Figure 2.1

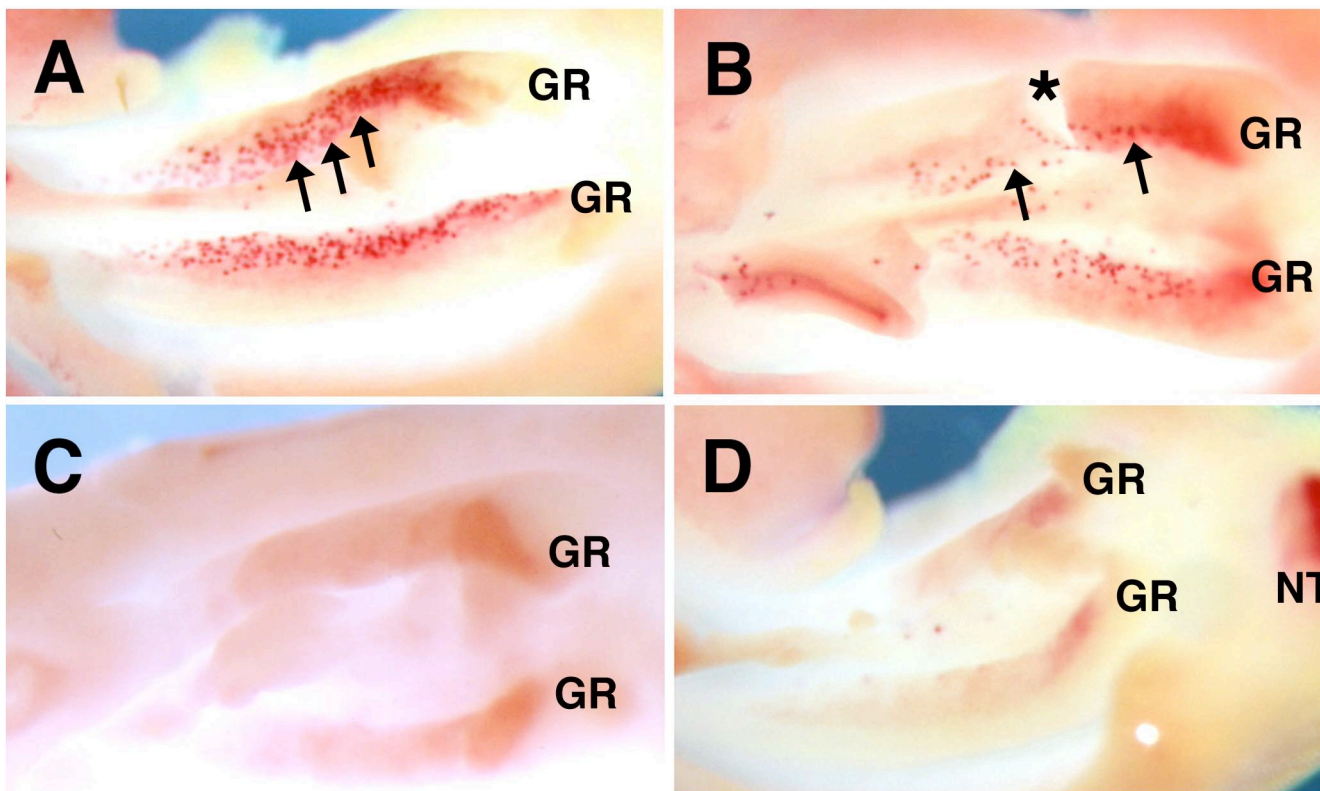


Figure 2.2. Quantitation of PGCs in genital ridges of E11.5 embryos. Serial transverse sections were prepared from the region of embryos containing the genital ridges and stained for AP activity. Numerous AP-positive PGCs were found in *Kitl*⁺/*Kitl*⁺ embryos (A) while *Kitl*^{Sl-gb}/*Kitl*^{Sl-gb} embryos (B) had no AP-positive PGCs. Embryos homozygous for each of the hypomorphic mutations [*Kitl*^{Sl-d}/*Kitl*^{Sl-d} (panel C), *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} (panel D), and *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} (panel E)] have PGC numbers that are substantially reduced compared to *Kitl*⁺/*Kitl*⁺ embryos. The total number of genital ridge PGCs per embryo was determined for at least three embryos of each genotype and the mean and SEM are shown in panel F. Compared to *Kitl*⁺/*Kitl*⁺ embryos, embryos homozygous for each *Kitl*^{Sl} mutation had significantly reduced numbers of PGCs in the genital ridge, and were 0% in *Kitl*^{Sl-gb}/*Kitl*^{Sl-gb}, 3% in *Kitl*^{Sl-d}/*Kitl*^{Sl-d}, and 20-25% in *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} and *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R}.

Figure 2.2

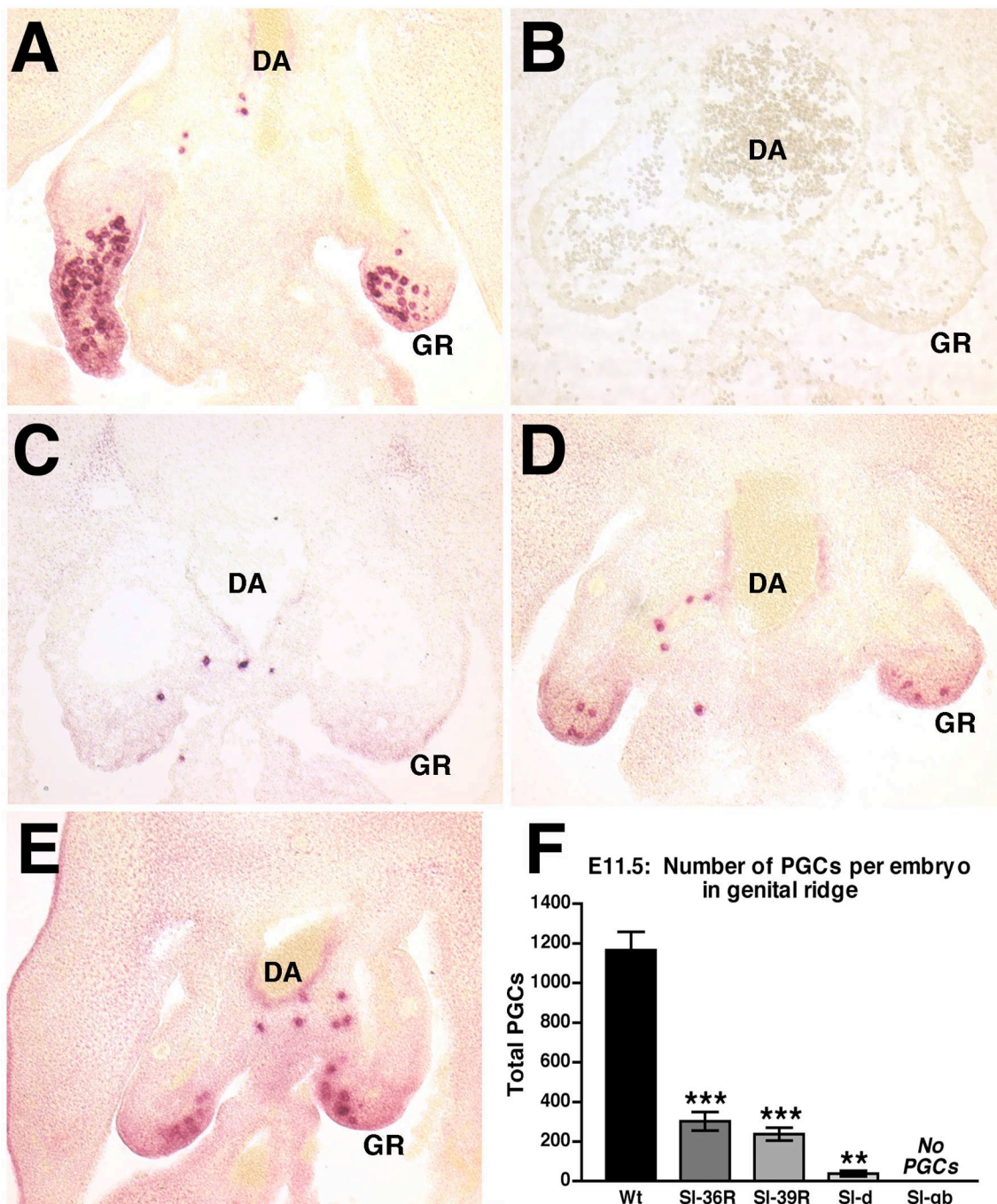


Figure 2.3. Localization of PGCs in E9.5 in *Kitl*⁺/*Kitl*⁺ (A, B) and *Kitl*^{Sl}/*Kitl*^{Sl} (C – D)

embryos. PGCs in normal location are indicated with arrowheads and PGCs in ectopic locations are indicated with arrows. In panel A, the direction of the dorsal (D) and ventral (V) axis is shown as a double headed arrow. HG, hindgut. In *Kitl*⁺/*Kitl*⁺ embryos, most PGCs were found associated with the hindgut epithelium (A) or located in the dorsal axis of the hindgut with only a few in ectopic sites (B). In *Kitl*^{Sl-gb}/*Kitl*^{Sl-gb} (C) and *Kitl*^{Sl-d}/*Kitl*^{Sl-d} (D) embryos, PGCs were in the ventral axis of the hindgut or in ectopic sites, but were not found in the dorsal axis of the hindgut. In *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} (E) and *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} (F) embryos, PGCs are distributed in a pattern similar to that of *Kitl*⁺/*Kitl*⁺ embryos, either associated with the hindgut epithelium or in the dorsal axis.

Figure 2.3

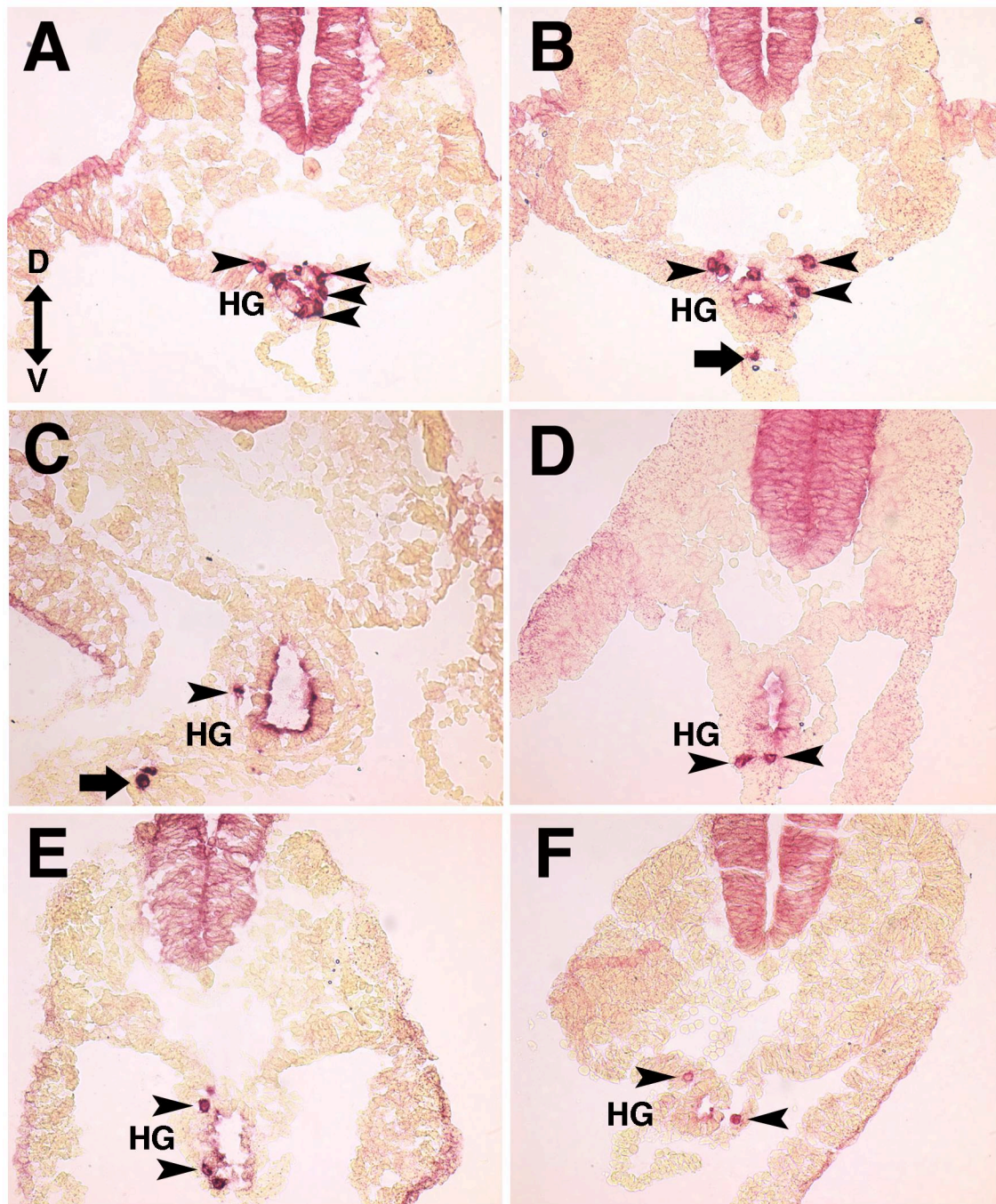


Figure 2.4. Numbers and locations of PGCs in E9.5 and E10.5 embryos. In panels A and B, PGCs the total number of PGCs per embryo (mean and SEM) are shown for E9.5 and E10.5, respectively. In panel C, the fold increase between PGC numbers at E9.5 and E10.5 are shown for each genotype. In panel D, the percentage of PGCs (mean and SEM) mobilized from the hindgut were calculated by counting the number of PGCs in locations other than hindgut versus the total number of PGCs. In all panels, asterisks indicated significant differences from unpaired t-tests (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$). In all panels except panel C, pair-wise comparisons were made between wildtype and each mutant, while in panel C, pair-wise comparisons between PGC numbers at E10.5 versus E9.5 for each genotype were made-. Note that all mutants at both ages had PGC numbers that are significantly reduced from those of wild-type embryos (panels A and B). Pairwise comparisons between each hypomorphic mutant and $Kitl^{Sl-gb}/Kitl^{Sl-gb}$ were also conducted (not shown). At E9.5, none of the values in mutants were significantly different from each other while at E10.5, values for $Kitl^{Sl-36R}/Kitl^{Sl-36R}$ and $Kitl^{Sl-39R}/Kitl^{Sl-39R}$ mutants, but not $Kitl^{Sl-d}/Kitl^{Sl-d}$, are significantly different from those of $Kitl^{Sl-gb}/Kitl^{Sl-gb}$. Significant differences in PGC numbers at E10.5 versus E9.5 (panel C) were observed in $Kitl^+/Kitl^+$, $Kitl^{Sl-36R}/Kitl^{Sl-36R}$ and $Kitl^{Sl-d}/Kitl^{Sl-d}$ mutants, but not in $Kitl^{Sl-39R}/Kitl^{Sl-39R}$ and $Kitl^{Sl-gb}/Kitl^{Sl-gb}$. In addition, the percentage of PGCs at E10.5 that had migrated from the hindgut (panel D) were equivalent between $Kitl^+/Kitl^+$ and $Kitl^{Sl-36R}/Kitl^{Sl-36R}$ embryos, but were significantly reduced in the other three mutants.

Figure 2. 4

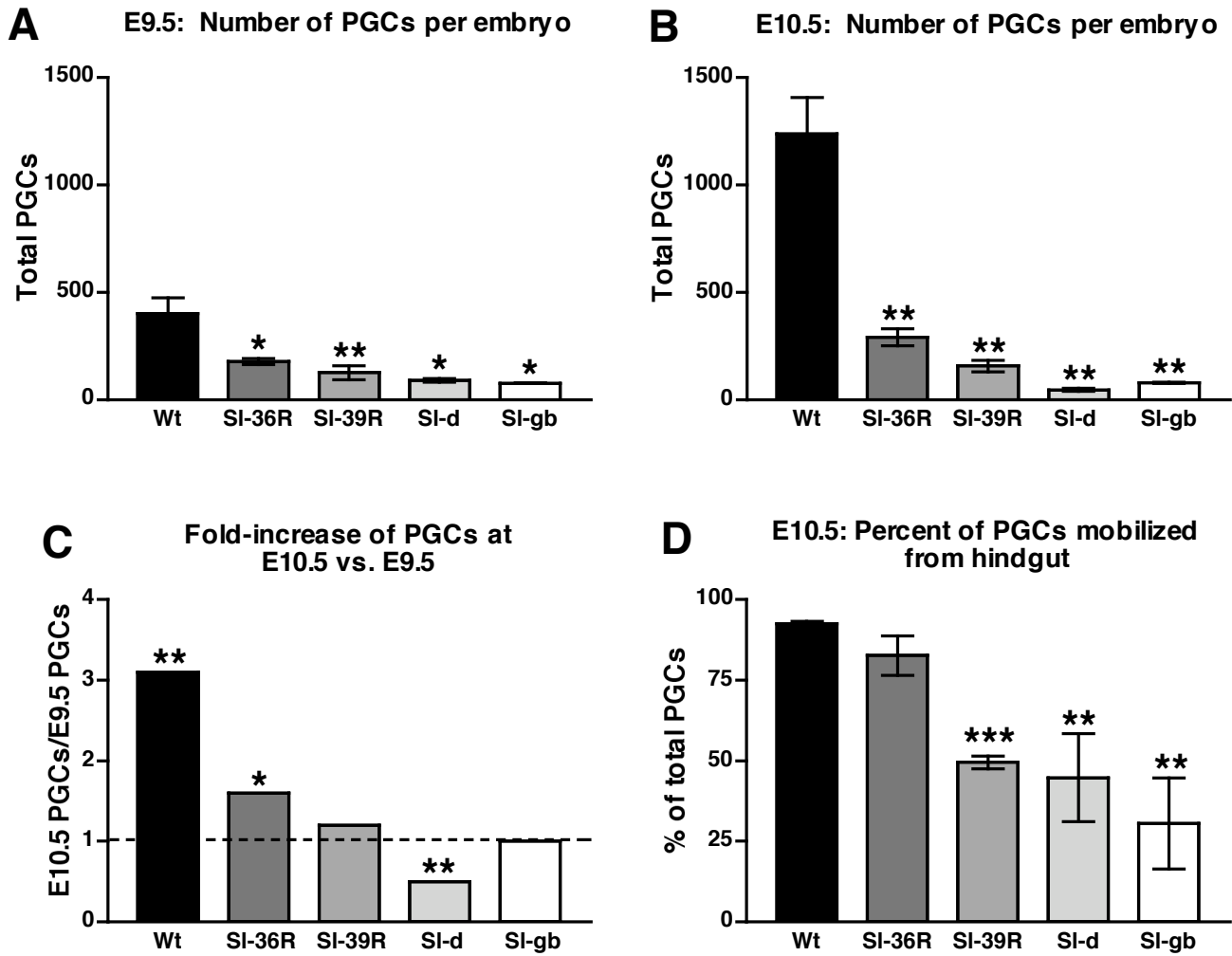


Figure 2.5. Localization of PGCs in E10.5 in $Kit^+/Kitl^+$ (A, B) and $Kit^{Sl}/Kitl^{Sl}$ (C – D)

embryos. PGCs with normal morphology are indicated with arrowheads and PGCs that are disintegrating and appear apoptotic are shown with arrows. HG, hindgut; DM, dorsal mesentery; GR, genital ridge. In $Kit^+/Kitl^+$ embryos at E10.5 (A, B), all PGCs have left the hindgut and are located either within the dorsal mesentery or are about to enter the genital ridge (A); or have arrived at the genital ridge and have begun to coalesce with the somatic cells of the presumptive gonad (B). In $Kit^{Sl-gb}/Kitl^{Sl-gb}$ (C) and $Kit^{Sl-d}/Kitl^{Sl-d}$ (D) embryos, most PGCs are found in the hindgut and only a few are on the normal migratory route. In $Kit^{Sl-39R}/Kitl^{Sl-39R}$ embryos (E), a large number of PGCs are retained in the HG but a few migrate through the DM. In $Kit^{Sl-36R}/Kitl^{Sl-36R}$ embryos (F), the pattern of PGC migration is similar to that of $Kit^+/Kitl^+$ embryos. Note the large number of apoptotic and disintegrating cells (arrows) in $Kit^{Sl-gb}/Kitl^{Sl-gb}$ (C), $Kit^{Sl-d}/Kitl^{Sl-d}$ (D) and $Kit^{Sl-39R}/Kitl^{Sl-39R}$ (E) embryos.

Figure 2.5

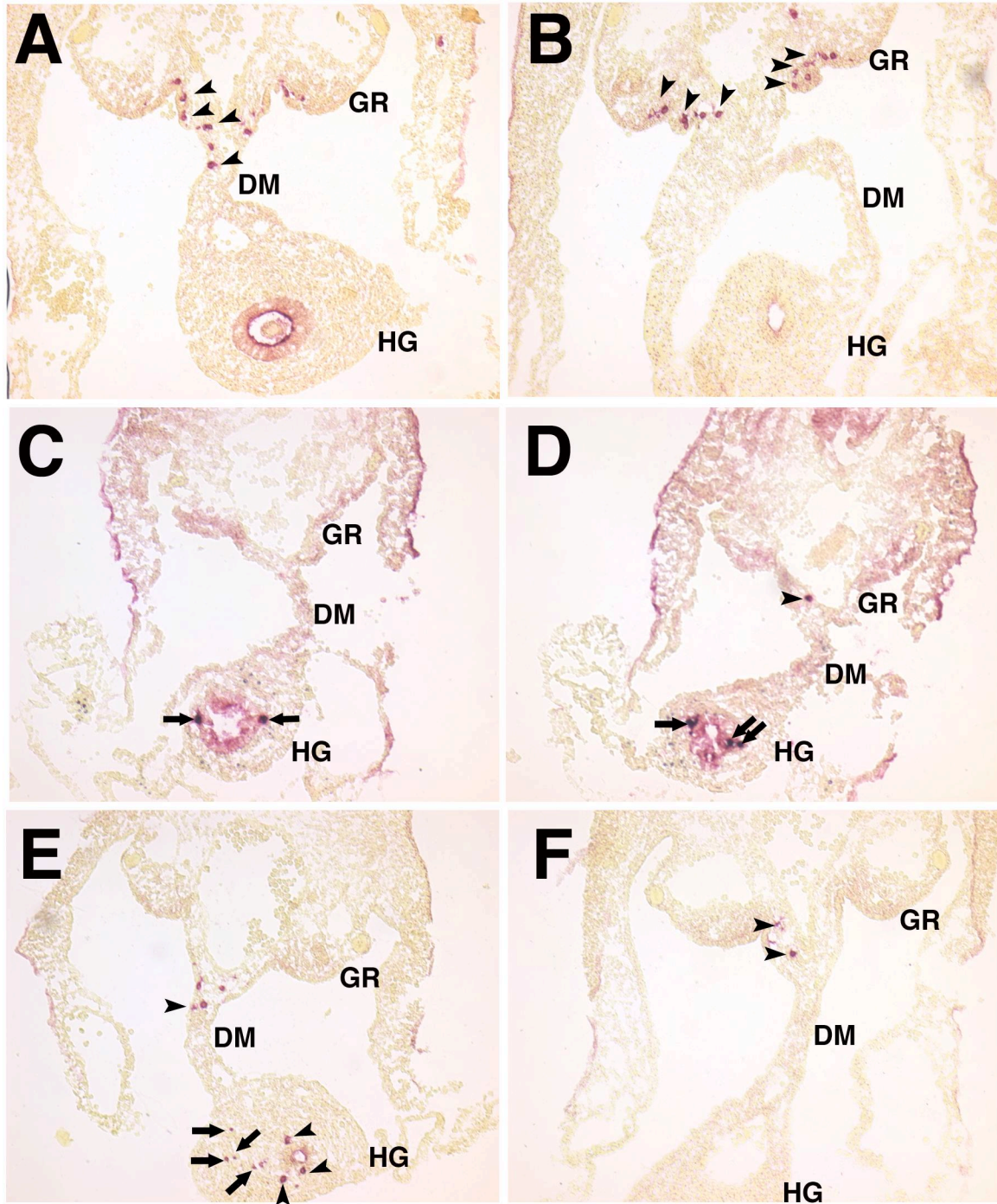


Figure 2.6. BrdU incorporation in PGCs in different locations of embryos at E10.5 and E11.5. Pregnant females were injected with BrdU and sections of their embryos stained with Fast Red for AP activity (red) and with a FITC-labeled antibody to BrdU (green). Confocal images are shown with both channels merged. Images of *Kitl*⁺/*Kitl*⁺ embryos are shown in the top two rows and images of *Kitl*^{SL-39R}/*Kitl*^{SL-39R} embryos are shown in the bottom two rows. Arrowheads point to BrdU-positive PGCs and arrows point to BrdU-negative PGCs. Barbed arrows point to disintegrating, BrdU-negative PGCs that are probably apoptotic (panel f' only). GR, genital ridges; DM, dorsal mesentery; HG, hindgut; HGL, hindgut lumen. In panels A and B, low power images of an E10.5 *Kitl*⁺/*Kitl*⁺ embryo are shown. In panels D and F, low power images of an E10.5 *Kitl*^{SL-39R}/*Kitl*^{SL-39R} embryo are shown. High power images of the boxed regions in panels A, B, D, and F are shown in panels a, a', b, d, f, and f'. In panels C and E, high power images of genital ridges from *Kitl*⁺/*Kitl*⁺ embryos and *Kitl*^{SL-39R}/*Kitl*^{SL-39R} embryos, respectively, at E11.5 are shown. At all locations and ages, numerous BrdU-positive PGCs were observed but their frequency is generally higher in *Kitl*⁺/*Kitl*⁺ embryos than in *Kitl*^{SL-39R}/*Kitl*^{SL-39R}. Note the many disintegrating, BrdU-negative PGCs (barbed arrows) in the *Kitl*^{SL-39R}/*Kitl*^{SL-39R} hindgut at E10.5 (f').

Figure 2.6

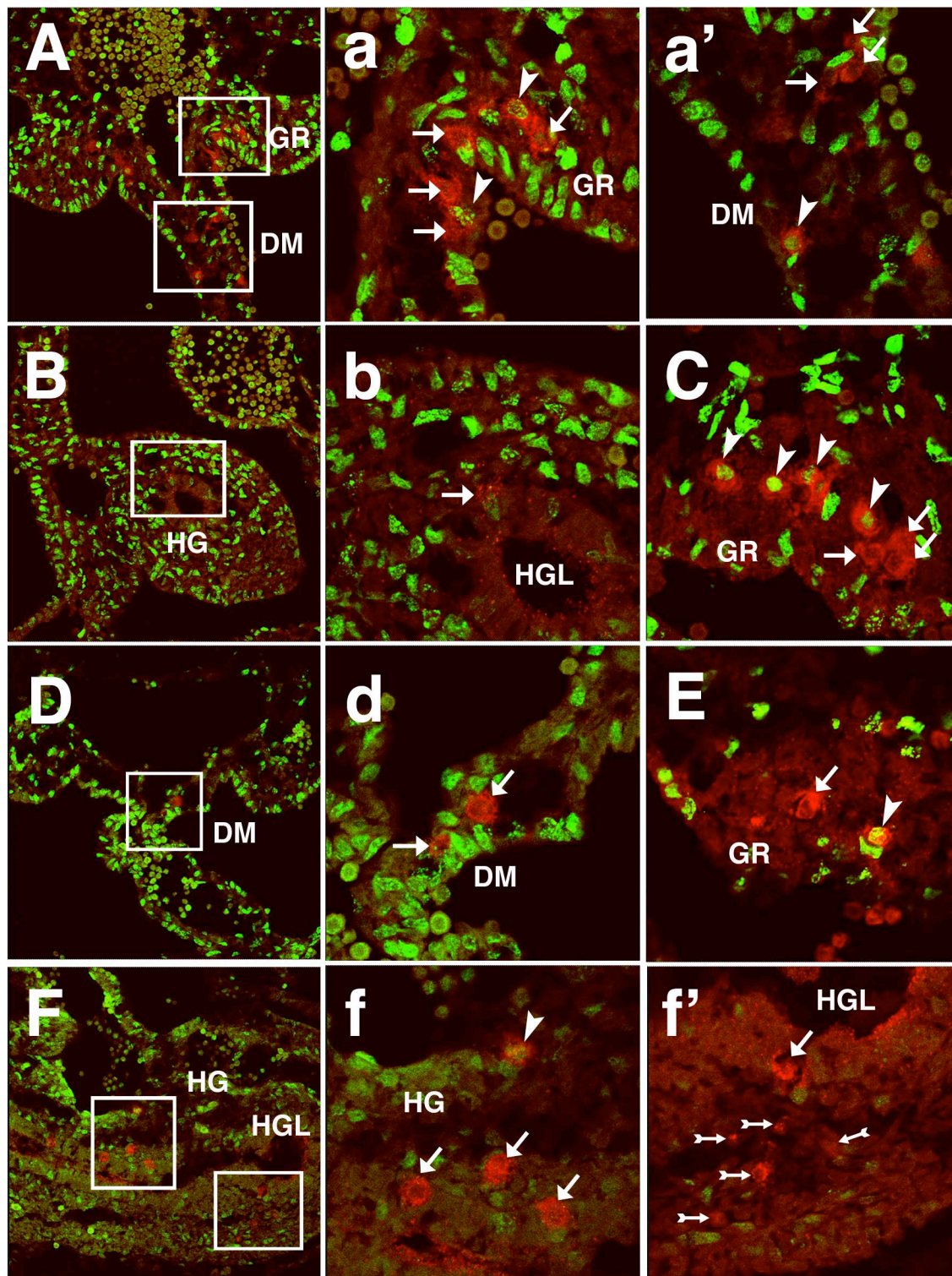
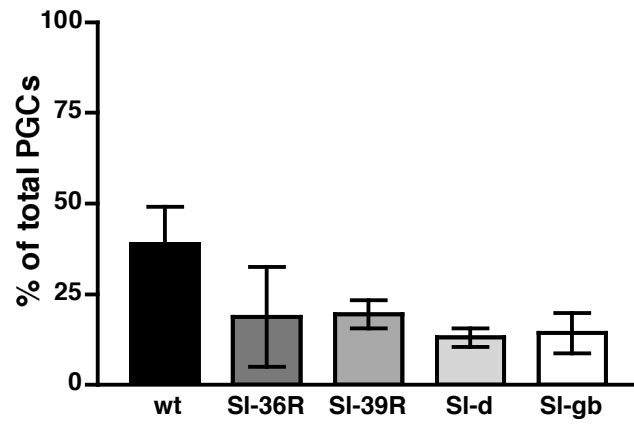


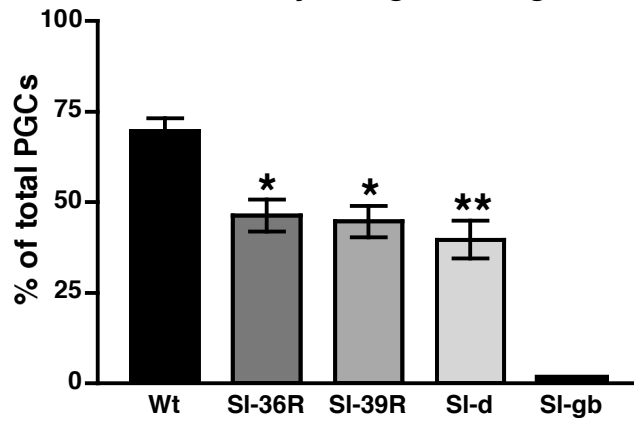
Figure 2.7. Proliferation indices of PGCs along their migratory pathway in E10.5 and E11.5 embryos. Serial sections of $Kitl^+/Kitl^+$ and $Kitl^{Sl}/Kitl^{Sl}$ embryos at each age were processed for AP activity and BrdU immunodetection (see Figure 2.6 for examples) and the percentage of BrdU-positive PGCs versus the total number of PGCs calculated for each embryo. A range of 35-95 total PGCs in mutant embryos and 100-200 total PGCs were scored in this way and the means and SEM for at least 3 embryos of each genotype and age are shown. For data in each panel, asterisks indicate significant differences between $Kitl^+/Kitl^+$ embryos and embryos homozygous for each of the $Kitl^{Sl}$ mutations in unpaired t-tests (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$). Note that in the E10.5 hindgut (panel A), none of the mutants have BrdU-positive indices that are significantly different from those of $Kitl^+/Kitl^+$ while in PGCs that have left the hindgut (panel B), all of the mutants have BrdU-positive indices that are significantly lower than in $Kitl^+/Kitl^+$. Interestingly, these values at E10.5 in the three hypomorphic mutations are all substantially higher than in the null mutant ($Kitl^{Sl-gb}/Kitl^{Sl-gb}$) but none of these values are significantly different from each other (not shown). Similarly to E10.5, the BrdU-positive indices of PGCs in the E11.5 genital ridges (panel C) of all mutants are significantly lower than in $Kitl^+/Kitl^+$ embryos (note that no PGCs were found in genital ridges of $Kitl^{Sl-gb}/Kitl^{Sl-gb}$ embryos at this age). Interestingly, the BrdU-positive indices of PGCs in the $Kitl^{Sl-39R}/Kitl^{Sl-39R}$ genital ridges at E11.5 are significantly higher (not shown) than the corresponding values in the other two hypomorphic mutants.

Figure 2. 7

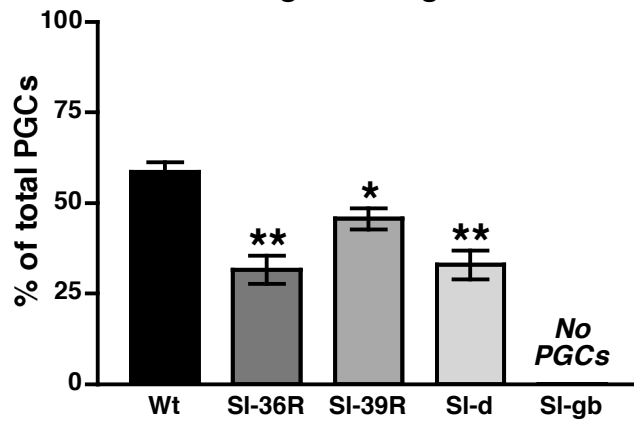
A E10.5: BrdU-positive PGCs in hindgut



B E10.5: BrdU-positive PGCs in mesentery and genital ridge



C E11.5: BrdU-positive PGCs in genital ridge



Chapter 3

Effects Of A Missense Mutation In The *Kit Ligand* Gene On Proliferation, Apoptosis And Meiosis During Spermatogenesis In Mice¹

¹Mahakali Zama A, Bedell MA. To be submitted to *Development*

ABSTRACT

Mammalian spermatogenesis is a tightly regulated process that involves successive waves of proliferation, meiosis and spermiogenesis. Many studies in mice have shown that spermatogenesis is dependent on Kit ligand (KITL), which is a member of the short chain helical cytokine family, and its receptor KIT, which is a member of the PDGFR α superfamily of receptor tyrosine kinases. While the major requirement for KITL and KIT during spermatogenesis is for the survival and proliferation of type A spermatogonia, it is unclear what role this signaling pathway has in post-mitotic male germ cells. We recently identified an ENU-induced hypomorphic mutant, *Kitl*^{SL-39R}, which encodes a missense mutation in KITL. In this report, we describe the effects of the *Kitl*^{SL-39R} on spermatogenesis and show that they are milder than those previously reported in *Kitl* mutants. Our studies of *Kitl*^{SL-39R}/*Kitl*^{SL-39R} mice provide evidence that KITL function is also required for the survival of Type In/B spermatogonia and the normal progression of meiosis in spermatocytes.

INTRODUCTION

Development of the male germ line in mammals is a complex process that initiates in the embryo and continues through postnatal life. In mice, proliferating primordial germ cells (PGCs) begin migrating from the hindgut into the presumptive genital ridge around embryonic day 9 (E9) (Donovan, 1994; Wylie, 1999). After migration is complete at E13.5, PGCs enter a quiescent state; in males, PGCs arrest in G_0/G_1 of mitosis and become gonocytes, while in females, PGCs arrest in prophase I of meiosis and become oogonia (McLaren, 2000). Spermatogenesis initiates between birth and postnatal day 5 (P5) when gonocytes differentiate into spermatogonia. Some spermatogonia become self-renewing stem cells while others begin the developmental program leading to production of spermatozoa (de Rooij and Grootegoed, 1998; Russell et al., 1990). This program requires about five weeks in mice and consists of three phases: a proliferative phase, in which diploid spermatogonia increase their number through multiple mitotic divisions; a meiotic phase, in which spermatocytes undergo two meiotic divisions to produce haploid spermatids; and a spermiogenic phase, where round spermatids are transformed into spermatozoa.

The spatial organization of germ cells within the seminiferous epithelium of adults reflects their differentiation state. Spermatogonia are situated near the basement membrane at the periphery of the seminiferous tubules. There are three types of spermatogonia; type A, intermediate (In), and type B (de Rooij and Grootegoed, 1998; Russell et al., 1990). Type A spermatogonia are undifferentiated [A-single (A_s), A-paired (A_{pr}), and A-aligned (A_{al})] or differentiated (A_1 , A_2 , A_3 , and A_4). A_s spermatogonia are most widely thought to be spermatogonial stem cells (SSCs), which either renew themselves, or divide into two A_{pr} cells.

Multiple divisions of A_{pr} cells produce A_{al} cells, which differentiate into A_1 spermatogonia. Successive rounds of mitosis and differentiation produce A_2 , A_3 , A_4 . In spermatogonia, type B spermatogonia, and preleptotene (PL) spermatocytes, the latter being the last spermatogenic cells to undergo S phase. PL and leptotene spermatocytes move through the blood testis barrier (BTB), and subsequent stages of meiosis takes place in the adluminal compartment. After a lengthy prophase, subsequent stages of meiosis occur rapidly, which produce haploid round spermatids. Spermiogenesis takes place in 16 steps (Russell et al., 1990) resulting in mature spermatozoa in the luminal compartment of tubules. During the first wave of spermatogenesis, germ cell differentiation is relatively synchronous, with equivalent stages of differentiation present in different tubules. Thus pachytene spermatocytes of prophase I appear at about P12, round spermatids appear at about P21, and the first wave of spermatogenesis is completed at about P35. However the process is continuous, such that new cycles of differentiation are initiated before completion of preceding cycles. Because of the cyclical nature of this process, different tubules in adult testes can be staged based on morphological progressions of the germ cells (Russell et al., 1990).

Sertoli cells are essential to spermatogenesis by providing structural support, nutrients, and cytokines to the germ cells (Griswold, 1998; Russell et al., 1990). One of the cytokines expressed by Sertoli cells is Kit ligand [KITL; also known as mast cell growth factor (MGF) and stem cell factor (SCF)]. KITL and its receptor KIT, which in mice are encoded by the *Steel* (*Sl*) and *Dominant spotting* (*W*) locus, respectively, are required for spermatogenesis as well as for PGC development and oogenesis (Besmer et al., 1993). In the postnatal testis, full-length KIT is expressed on all differentiated spermatogonia (Dym et al., 1995; Schrans-Stassen et al., 1999;

Yoshinaga et al., 1991), in certain meiotic cells (Vincent et al., 1998), and in Leydig cells (Rothschild et al., 2003; Yoshinaga et al., 1991). KITL is expressed as a soluble isoform (S-KITL), which is produced from a membrane-bound precursor by proteolytic cleavage, or as a membrane-bound isoform (MB-KITL), which lacks the primary cleavage site (Majumdar et al., 1994). Many *in vivo* and *in vitro* studies have demonstrated that KITL is required to promote proliferation and suppress apoptosis in differentiating type A spermatogonia, KITL (reviewed by Bedell and Mahakali Zama, 2004; de Rooij and de Boer, 2003; Rossi et al., 2000). In addition, there is accumulating evidence that KITL may affect the survival and/or differentiation of meiotic cells in the testis (see also Bedell and Mahakali Zama, 2004). Despite intense study, the molecular and biological responses elicited by KITL in spermatogenic cells are poorly understood.

The analysis of postnatal spermatogenesis in homozygous *Kitl^{Sl}* mutant mice is limited to only a few alleles that allow viability. Mice homozygous for null *Kitl* mutations die around the time of birth with severe hypoplastic anemia (Rajaraman et al., 2002b; Russell, 1979). With regard to spermatogenesis, the mildest reported *Kitl^{Sl}* allele is *Kitl^{Sl-17H}*, which has an abnormal cytoplasmic domain of KITL due to a splicing defect (Brannan et al., 1992). Although a normal S-KITL^{Sl-17H} isoform could potentially be expressed, both the precursor of S-KITL^{Sl-17H} and the MB-KITL^{Sl-17H} would contain an out-of-frame cytoplasmic domain. *Kitl^{Sl-17H}* homozygotes are viable but were reported to undergo one wave of spermatogenesis with cessation of spermatogenesis by eight weeks of age (Brannan et al., 1992). Examination of older *Kitl^{Sl-17H}/Kitl^{Sl-17H}* mutants revealed the complete absence of spermatogenic cells past the A_{al} stage (de Rooij et al., 1999).

We recently described a novel *Kitl* mutation, *Kitl*^{Sl-39R}, which contains a missense mutation (S122F) in the fourth α -helical domain that disrupts an N-linked glycosylation site in KITL (Rajaraman et al., 2002a). *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} mice are viable and anemic (Rajaraman et al., 2002b), and this anemia is considerably more severe than that of *Kitl*^{Sl-17H}/*Kitl*^{Sl-17H} mice (unpublished observations). In mice homozygous for either mutation, embryos have only about 20% of the wildtype numbers of PGCs, adult females are fertile, albeit with reduced fecundity, and males are sterile (Brannan et al., 1992 and unpublished observations).

In the present study, we report a detailed comparative analysis of spermatogenesis defects in *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} males and *Kitl*^{Sl-17H}/*Kitl*^{Sl-17H} males. Our studies revealed that the numbers, proliferation, and apoptosis of type A spermatogonia are relatively unaffected in both mutants. However, in In and B spermatogonia of both mutants, apoptosis was greatly increased while proliferation was mildly decreased; thus both mutants have severe depletion of these differentiating spermatogonia and subsequent stages of spermatogenic cells. Interestingly, defects in meiotic cells were milder in *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} mice than in *Kitl*^{Sl-17H}/*Kitl*^{Sl-17H} mice, and in the former mice, a defective progression through meiosis was observed.

MATERIALS AND METHODS

Mice

The *Kitl*^{Sl-17H} and *Kitl*^{Sl-39R} alleles both contain ENU-induced point mutations (Brannan et al., 1992; Rajaraman et al., 2002a) and are congenic on a C3H/HeNCr background. The mice are maintained in a pathogen-free, AALAC-accredited facility at the University of Georgia by

backcrossing to inbred C3H/HeNCr mice maintained in the same facility. Mice homozygous for each mutant allele were generated by intercrossing heterozygous mice and identified by their white coats. *Kitl⁺/Kitl⁺* siblings from these same litters were used as controls. At least three mice of each age and genotype were analyzed. All procedures with mice were approved by the IACUC of the University of Georgia.

Histological analysis

Mice were euthanized by CO₂ asphyxiation, then testes excised, fixed in Bouin's fixative or in 10% neutral buffered formalin (NBF), embedded in paraffin, and sectioned at 5 microns. Germ cell types were identified after staining of Bouin's-fixed sections with hematoxylin and eosin (H&E) using conventional methods and of NBF-fixed sections with Hoechst 33342. For Hoechst staining, sections were deparaffinized, dried briefly at 55°C, rehydrated in PBS, and stained with an aqueous solution of Hoechst (1.6 µg/ml) for 10 minutes. The stained sections were washed in PBS, mounted in Prolong Anti-fade Mounting Media (Molecular Probes, OR), and then cross-sections of seminiferous tubules examined using a Zeiss Axiophot microscope. Digital images were captured with an Optronics MagnaFire camera or a Zeiss AxioCam, and slight modifications for brightness and contrast made using Adobe Photoshop software.

For H&E sections, cross-sectioned tubules were scored based on the following parameters: the presence of at least one germ cell at any stage, the most advanced stage of germ cell (i.e. spermatogonia, primary spermatocyte, round spermatid, or elongate spermatid); and germ cell associations and/or stages. On average, 160 tubules from each *Kitl⁺/Kitl⁺* and mutant mouse were scored this way.

Hoechst-stained sections were used for quantitative analyses of germ cell types. Using an optical micrometer, sections were divided into grids and tubules examined in a consistent pattern and orientation in order to avoid re-counting the same tubule. Previous descriptions of germ cell nuclear morphologies (Chiarini-Garcia and Russell, 2001; Russell et al., 1990) were used to identify different types of spermatogonia and spermatocytes (see Results). Germ cells from at least 30 tubules from each of three animals of each genotype and age were counted and expressed as numbers of each cell type per germ cell-containing tubule.

Analysis of proliferation and apoptosis

Incorporation of BrdU and TUNEL staining were used to detect cells in S-phase and undergoing apoptosis, respectively. Mice were euthanized two hours after intraperitoneal injection with BrdU (10 mg/kg of body weight) and the testes dissected, fixed in NBF, embedded in paraffin, sectioned, deparaffinized, and rehydrated as described above. To control for consistent BrdU injections, intestines from the same animals were prepared and stained in the same manner as for testes. BrdU detection was accomplished after antigen retrieval, where slides were microwaved in citrate buffer, followed by washing at room temperature in PBS. The sections were treated with trypsin, washed, and denatured for 30 min at room temperature. After blocking, sections were incubated with biotinylated anti-BrdU antibody (Zymed) for 90 min and antibody visualized using Streptavidin-FITC (Vector Labs). For TUNEL detection, deparaffinized and rehydrated sections were treated with Proteinase K, washed with PBS, and equilibrated in reaction buffer. The sections were incubated with terminal deoxynucleotidyl transferase (TdT) and FITC-labeled dUTP (Promega) for 60 minutes. After multiple washes in

PBS, sections were counterstained with Hoechst and mounted in Prolong Anti-fade mounting solution as described above. As positive controls for TUNEL detection, additional sections were treated with DNase I and stained the same way.

BrdU-positive cells and TUNEL-positive cells were counted in 30-35 tubules per animal from three animals at each age and genotype. Two groups of germ cells were identified by morphology, size, and shape of the nuclei and location relative to the basement membrane: all type A spermatogonia were combined into one group and In spermatogonia, type B spermatogonia, and PL spermatocytes were combined into the second group. For each group of germ cells, the numbers of BrdU-positive and TUNEL-positive cells per tubule in mutants were normalized to *Kitl⁺/Kitl⁺* numbers in the same age to account for the relative differences in total cell numbers in mutant and wildtype testes (see Figure 3.4). Therefore, the data in Figure 3.7 represent the numbers of BrdU-positive cells per tubule (Figure 3.7A and 3.7B) and TUNEL-positive cells per tubule (Figure 3.7C and 3.7D) as if the numbers of each cell type were the same in mutant and wildtype testes.

Immunodetection of CDC25C

Bouin's fixed sections were processed and antigen-retrieved as described above. Sections were blocked with 10% goat serum, incubated with DNaseI for 20 min, then washed, and incubated overnight at 4°C with a 1:50 dilution of rabbit polyclonal anti-CDC25C antibody (Santa-Cruz). After washing, the sections were incubated with biotinylated goat anti-rabbit IgG (Zymed), followed by Streptavidin-FITC and counterstaining with ethidium homodimer -2

(Molecular Probes). The stained sections were analyzed and images captured on a Leica confocal microscope.

Statistics

All statistical analyses were conducted using Prism software (GraphPad, CA). In each group, pair-wise comparisons between mutant and wildtype values were made using an unpaired, two-tailed t-test. *P* values < 0.05 were considered to be statistically significant.

RESULTS

Spermatogenesis defects are milder in *Kitl*^{SL-39R}/*Kitl*^{SL-39R} mice than in *Kitl*^{SL-17H}/*Kitl*^{SL-17H} mice

Representative micrographs of H&E-stained testes sections from *Kitl*^{+/+}/*Kitl*⁺, *Kitl*^{SL-39R}/*Kitl*^{SL-39R}, and *Kitl*^{SL-17H}/*Kitl*^{SL-17H} mice at 3, 5, and 8 weeks of age are shown in Figure 3.1. In *Kitl*^{+/+}/*Kitl*⁺ testes, pachytene spermatocytes or round spermatids are the most advanced germ cells at 3 weeks (Figures 3.1A), and round spermatids and elongate spermatids are the most advanced stages at 5 weeks (Figures 3.1B). Of note, all tubules in 5-week *Kitl*^{+/+}/*Kitl*⁺ testes have progressed past the spermatocyte stage, and contain post-meiotic cells. In *Kitl*^{SL-39R}/*Kitl*^{SL-39R} testes at 3 and 5 weeks, many tubules are devoid of meiotic and post-meiotic cells, but some tubules have these cells at less advanced stages than in *Kitl*^{+/+}/*Kitl*⁺ testes (Figures 3.1D and 3.1E, respectively). Elongate spermatids appear in some tubules of the *Kitl*^{SL-39R}/*Kitl*^{SL-39R} testes by 5 weeks (Figure 3.1E) indicating that the first wave of spermatogenesis can be completed. However, at 8 weeks (Figure 3.1F), most tubules of the *Kitl*^{SL-39R}/*Kitl*^{SL-39R} lack meiotic and post-meiotic cells, and subsequent waves of spermatogenesis are absent from the few tubules that

contain elongate spermatids. In *Kitl^{Sl-17H}/Kitl^{Sl-17H}* testes, no meiotic and post-meiotic germ cells were observed at any of the three ages (Figure 3.1G – 3.1I).

These data reveal that in both mutants, spermatogenesis ceases by 8 weeks after birth. However, at least one wave of spermatogenesis occurs in juvenile *Kitl^{Sl-39R}/Kitl^{Sl-39R}* mutants while we observed no spermatogenesis in juvenile *Kitl^{Sl-17H}/Kitl^{Sl-17H}* mice. These effects of the *Kitl^{Sl-17H}* mutation are slightly more severe than those reported previously (Brannan et al., 1992). Nonetheless, the direct comparison of the two mutants maintained on identical strain backgrounds reveal that the effects of the *Kitl^{Sl-39R}* mutation on spermatogenesis are milder than those of the *Kitl^{Sl-17H}* mutation.

Analysis of germ cell types in *Kitl^{Sl-39R}* and *Kitl^{Sl-17H}* mutants

Hoechst dye, which binds to DNA with high affinity and reveals pronounced differences in chromatin configurations of the germ cells, was used to identify germ cell types. In Hoechst-stained sections, germ cells were classified according to their positions relative to the basement membrane, nuclear size, nuclear morphology, and cell associations using previous descriptions that used other staining methods (Chiarini-Garcia and Russell, 2001; Russell et al., 1990). We found that compared to H&E staining, Hoechst staining revealed more details of the nuclear morphology, and allowed easier discrimination of nuclei from Sertoli cells and germ cells, and easier identification of germ cell types.

Representative images of Hoechst-stained testis sections from *Kitl⁺/Kitl⁺* mice at 3, 5, and 8 weeks of age are shown in Figure 3.2. We identified two classes of type A spermatogonia.

Small type A spermatogonia (SA) are located on the basement membrane and have small oblong nuclei with a pale or dark nucleoplasm and none to very little heterochromatin on the rim of the nucleus (Figures 3.2B, 3.2E, and 3.2G, thin arrows). Large type A spermatogonia (LA) have a large oblong or round nucleus with varying amounts of heterochromatin rimming the nucleus (Figures 3.2C, 3.2E – 3.2I, barbed arrows). SA spermatogonia may be undifferentiated Type A spermatogonia (i.e. A_s , A_{pr} , and A_{al}), while LA spermatogonia may be differentiating type A spermatogonia (i.e. A_1 , A_2 , A_3 , and A_4). However, further analysis using more detailed morphological criteria [such as staining of whole mount preparations (de Rooij et al., 1999) or with more refined histological methods (Chiarini-Garcia et al., 2001; Chiarini-Garcia and Russell, 2001; Dettin et al., 2003)] and molecular markers [such as RET, GFR α , and KIT (Schrans-Stassen et al., 1999; Tadokoro et al., 2002)] will be needed to establish the identities of SA and LA spermatogonia. With Hoechst staining, In spermatogonia, type B spermatogonia, and PL spermatocytes all have small, round to oval nuclei with a much brighter fluorescence signal than that of type A spermatogonia (Figure 3.2A, 3.2C, and 3.2D). While both In and type B spermatogonia have prominent heterochromatin clumps in the nucleoplasm as well as heterochromatin rimming the nucleus (Figure 3.2C, thick arrows), the two cell types could not be reliably distinguished and so were counted together as In/B spermatogonia. With Hoechst staining, In/B spermatogonia are distinguishable from PL spermatocytes (Figure 3.2D, arrowheads) on the basis of the greater extent of chromosome condensation in the latter.

We identified pre-meiotic, meiotic, and post-meiotic germ cells in Hoechst-stained sections from $Kitl^+/Kitl^+$, $Kitl^{Sl-39R}/Kitl^{Sl-39R}$, and $Kitl^{Sl-17H}/Kitl^{Sl-17H}$ mice at 3, 5, and 8 weeks of age and determined the fraction of tubules that contained at least one cell of each cell type (Figure

3.3). Pre-meiotic germ cells include all Type A and In/B spermatogonia and PL spermatocytes (Figure 3.3A). Tubules with meiotic and post-meiotic germ cells were combined together (Figure 3.3B); meiotic cells include all spermatocytes except PL spermatocytes and post-meiotic germ cells include round spermatids and elongate spermatids. At all three ages, all tubules in *Kitl*⁺/*Kitl*⁺ contained pre-meiotic (Figure 3.3A), and meiotic and post-meiotic (Figure 3.3B) germ cells. In both mutants, the numbers of tubules with pre-meiotic germ cells at each age were only marginally reduced (Figure 3.3A). However, the two mutants differed considerably in the fraction of tubules containing meiotic and post-meiotic germ cells (Figure 3.3B). In the *Kitl*^{SL-17H}/*Kitl*^{SL-17H} testis, only 5% tubules at 3 weeks contained meiotic cells and no tubules with these cells were found at 5 and 8 weeks of age. In the *Kitl*^{SL-39R}/*Kitl*^{SL-39R} mutants at 3 and 5 weeks, 60% of tubules contained meiotic and post-meiotic germ cells. Significantly, this fraction was greatly reduced in the *Kitl*^{SL-39R}/*Kitl*^{SL-39R} mutants at 8 weeks, where only 9% of the tubules contained meiotic or post-meiotic cells. Thus, these quantitative analyses reveal that the *Kitl*^{SL-39R} and *Kitl*^{SL-17H} mutations preferentially affect meiotic and post-meiotic germ cells. However, in immature mice, these defects are milder in *Kitl*^{SL-39R}/*Kitl*^{SL-39R} mice than in *Kitl*^{SL-17H}/*Kitl*^{SL-17H} mice while in mature mice, these defects are equivalent in the two mutants.

In *Kitl*^{SL-39R} and *Kitl*^{SL-17H} mutants, numbers of type A spermatogonia are relatively unaffected but numbers of other pre-meiotic germ cells are greatly reduced

To determine if the *Kitl*^{SL} mutations had preferential effects on particular pre-meiotic cell types, we counted the numbers of each type of pre-meiotic cell per tubule from at least 30 tubules at each age and genotype (Figure 3.4). While the numbers of pre-meiotic germ cells in *Kitl*⁺/*Kitl*⁺ mice ranged from 16 to 29 per tubule, these values in both mutants ranged from 7 to

12 per tubule (Figure 3.4A). Thus, the numbers of pre-meiotic cells per tubule in both mutants are only about 30% to 40% that of *Kitl⁺/Kitl⁺* mice. Interestingly, the average numbers of type A spermatogonia per tubule in mutant mice are only marginally altered from those observed in *Kitl⁺/Kitl⁺* mice (Figures 3.4B – 3.4D). Although the values for total type A spermatogonia per tubule in both mutants are not different statistically from those of *Kitl⁺/Kitl⁺* mice (Figure 3.4D), there is a trend for SA spermatogonia per tubule to be slightly reduced (Figure 3.4B) and LA spermatogonia numbers per tubule to be slightly elevated (Figure 3.4C) in the mutants compared to *Kitl⁺/Kitl⁺* at all ages. In contrast to the mild defects in type A spermatogonia, both mutants displayed dramatically reduced numbers of In/B spermatogonia (Figure 3.4E) and PL spermatocytes (Figure 3.4F) compared to *Kitl⁺/Kitl⁺*. At all ages, the numbers of In/B spermatogonia per tubule in *Kitl⁺/Kitl⁺* mice ranged from 11 to 12 per tubule while these numbers in both mutants were less than 2 per tubule (Figure 3.4E). Thus the mutants have only about 2% to 14% the number of In/B spermatogonia per tubule in *Kitl⁺/Kitl⁺* mice. A similar effect was seen with PL spermatocytes (Figure 3.4F) as *Kitl^{SI-39R}/Kitl^{SI-39R}* mutants at 3 weeks had only 40% the numbers seen in *Kitl⁺/Kitl⁺*, which decreased to 8% and 0% of *Kitl⁺/Kitl⁺* by 5 and 8 weeks, respectively. In *Kitl^{SI-17H}/Kitl^{SI-17H}* mutants there was a nearly complete absence of PL spermatocytes with less than 0.5% of *Kitl⁺/Kitl⁺* at 3 weeks and none observed at 5 and 8 weeks. Thus, the major deficiencies in numbers of pre-meiotic cells in the *Kitl^{SI-17H}/Kitl^{SI-17H}* and *Kitl^{SI-39R}/Kitl^{SI-39R}* mutants are in In/B spermatogonia and PL spermatocytes.

In order to assess the progressive differentiation of pre-meiotic germ cells, we used the data shown in Figure 3.4 to calculate the proportions of each type of pre-meiotic cell versus the total number of pre-meiotic cells in *Kitl⁺/Kitl⁺* and both mutants at 3, 5, and 8 weeks (Figure

3.5). As expected, In/B spermatogonia make up the predominant pre-meiotic cell type in *Kitl⁺/Kitl⁺* testes at all ages (columns 1, 4, and 7 of Figure 3.5), ranging from 44% to 70% of the total number of pre-meiotic cells per tubule. In contrast, type A spermatogonia, particularly LA spermatogonia, make up the predominant pre-meiotic cell type in both mutants, ranging from 50% to 68% of the total number of pre-meiotic cells per tubule (*Kitl^{SL-39R}*, columns 2, 5, and 8 of Figure 3.5; *Kitl^{SL-17H}*, columns 3, 6, and 9 of Figure 3.5). Notably, In/B spermatogonia make up only a small fraction of the total number of pre-meiotic cells in both mutants. Although we cannot distinguish between In and B spermatogonia, the latter are derived from the former, and so the effect on the latter is probably secondary to an effect on the former. Not surprisingly, the fraction of PL spermatocytes, which are derived from B spermatogonia, is greatly reduced in both mutants at 5 and 8 weeks of age. However, at 3 weeks of age, the fraction of PL spermatocytes in the *Kitl^{SL-39R}/Kitl^{SL-39R}* testes is nearly identical to that of *Kitl⁺/Kitl⁺* testes (compare columns 1 and 2 of Figure 3.5), despite the fact that proportion of B spermatogonia is greatly reduced in the former.

In *Kitl^{SL-39R}* and *Kitl^{SL-17H}* mutants, proliferation of pre-meiotic germ cells and apoptosis of type A spermatogonia are relatively unaffected, but apoptosis of In/B spermatogonia and PL spermatocytes is greatly increased

The reduced numbers of In/B spermatogonia and PL spermatocytes observed in *Kitl* mutant mice could occur if type A spermatogonia did not proliferate and differentiate into In spermatogonia, if In/B spermatogonia underwent apoptosis, or if a combination of decreased proliferation and increased apoptosis occurred. To test these possibilities, we estimated the

extent of proliferation by BrdU incorporation and the extent of apoptosis by TUNEL assay for each pre-meiotic cell type.

Representative images of testes sections stained with antibody to BrdU are shown in Figure 3.6 and the normalized numbers of BrdU-positive cells per tubule are summarized in Figure 3.7. At all ages, the normalized numbers of BrdU-positive type A spermatogonia, In/B spermatogonia, and PL spermatocytes in both mutants are either equivalent to, or are reduced by about half, compared to those of *Kitl⁺/Kitl⁺* mice (Figures 3.7A and 3.7B). Thus, the frequency of germ cells in S-phase in the testes of both mutants was either similar to that of *Kitl⁺/Kitl⁺* mice, or was moderately reduced.

Representative images of testes sections stained for TUNEL-positive cells are shown in Figure 3.8. Consistent with previous reports (Rodriguez et al., 1997; Wang et al., 1998), we found that the majority of TUNEL-positive germ cells are spermatocytes and round spermatids and occurred mainly in tubules wherein spermatocytes are undergoing meiosis I and II (Figure 3.8B). In *Kitl⁺/Kitl⁺* testes at 3 weeks of age (Figure 3.8A and 3.8B) and other ages (not shown), many TUNEL-positive mid-to-late pachytene spermatocytes were observed. However only a very small number of TUNEL-positive type A spermatogonia (Figure 3.7C) and TUNEL-positive In/B spermatogonia and PL spermatocytes (Figure 3.7D) were found in *Kitl⁺/Kitl⁺* mice at all ages. As in *Kitl⁺/Kitl⁺* mice, there were very few TUNEL-positive type A spermatogonium in the testes of both mutants (Figure 3.7C). In striking contrast, the normalized numbers of TUNEL-positive In/B spermatogonia and PL spermatocytes per tubule in both mutants were increased from 6 to 14 times that of *Kitl⁺/Kitl⁺* mice (Figure 3.7D).

In summary, the *Kitl*^{SL-17H}/*Kitl*^{SL-17H} and *Kitl*^{SL-39R}/*Kitl*^{SL-39R} mutants exhibit moderately decreased numbers of BrdU-positive cells, but greatly increased numbers of TUNEL-positive cells. These results suggest that KITL functions for suppressing apoptosis are severely compromised but that sufficient function is retained for promoting proliferation. The virtual absence of apoptotic type A spermatogonia, and the greatly increased numbers of apoptotic In/ B spermatogonia and PL spermatocytes are consistent with the cell numbers (see Figure 3.4) and suggest that the numbers of the latter cells are reduced because of increased apoptosis. Of interest, the effects on total numbers, apoptosis, and proliferation of pre-meiotic cells are nearly identical between *Kitl*^{SL-17H}/*Kitl*^{SL-17H} and *Kitl*^{SL-39R}/*Kitl*^{SL-39R} mice. Thus, the major differences observed between the two mutants are on the numbers of meiotic and post-meiotic germ cells, where the testes of *Kitl*^{SL-17H}/*Kitl*^{SL-17H} mutants are devoid of these cells and the testes of juvenile *Kitl*^{SL-39R}/*Kitl*^{SL-39R} mice contain significant numbers of these cells (Figure 3.3B).

Completion of the first wave of spermatogenesis is delayed in *Kitl*^{SL-39R} mutants

To examine the temporal appearance of post-meiotic spermatids, H&E-stained sections from all ages were examined and germ cell-containing tubules scored for the most advanced stage of germ cells present irrespective of their numbers. On average, 160 tubules from each *Kitl*⁺/*Kitl*⁺ and *Kitl*^{SL-39R}/*Kitl*^{SL-39R} mutant animal were scored in this way, and the data are represented as the percentage of germ cell-containing tubules with spermatocytes, round spermatids, or elongate spermatids as the most advanced stage (Figure 3.9). In the *Kitl*⁺/*Kitl*⁺ testis at 3 weeks, about half of the tubules had advanced to the spermatocyte stage while the other half had advanced to the round spermatid stage (Figure 3.9A). In the *Kitl*^{SL-39R}/*Kitl*^{SL-39R} testis

at 3 weeks, nearly all (93%) of the tubules had spermatocytes as the most advanced stage and only a small fraction (7%) of the germ cell-containing tubules had advanced to the round spermatid stage (Figure 3.9A). In the *Kitl*⁺/*Kitl*⁺ testis at 5 and 8 weeks (Figure 3.9B and 3.9C, respectively), all of the tubules had advanced past the spermatocyte stage, a small percentage of tubules had round spermatids as the most advanced stage, and the majority of tubules had elongate spermatids or mature spermatids as the most advanced stage. In contrast, in the *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} testis at 5 and 8 weeks (Figure 3.9B and 3.9C, respectively), 15% to 16% of the tubules contain spermatocytes as the most advanced stage, while ~38% and ~52% of tubules have round spermatids and elongate spermatids, respectively, as the most advanced stage. Thus, these analyses reveal that the appearance of haploid cells is delayed in the *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} mutant testis. Furthermore, in tubules at 5 and 8 weeks of age that have spermatocytes as the most advanced stage, the spermatocytes are likely to be from the second wave of spermatogenesis, and may in fact be blocked from further stages of meiosis.

Evidence for impaired meiotic competence of primary spermatocytes in *Kitl*^{Sl-39R} testes

To investigate further the possibility that meiotic progression could be delayed or impaired in the *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} testis, we used CDC25C as a marker to identify pachytene spermatocytes that are competent to continue through meiosis. CDC25C activates the cyclin B/p34^{Cdc2} (MPF) complex during cell cycle progression from G2-M in mitotic cells. In the testis, CDC25C is expressed at high levels in late pachytene to diplotene spermatocytes and in round spermatids (Wu and Wolgemuth, 1995) and its appearance in mid-pachytene spermatocytes coincides with their acquiring competence to enter metaphase (Cobb et al., 1999).

In the *Kitl*^{+/+}/*Kitl*^{+/+} testis at 3 weeks, tubules with early-mid pachytene spermatocytes as the most advanced stage displayed punctate cytoplasmic staining of anti-CDC25C with no detectable signal in the nucleus (Figure 3.10A-3.10C). However, intense nuclear staining was observed in tubules with mid-late pachytene spermatocytes ((Figure 10D-10F) and in round spermatids (not shown), suggesting that CDC25C is translocated from the cytoplasm to the nucleus of the spermatocytes as they are achieving meiotic competence. In the *Kitl*^{+/+}/*Kitl*^{+/+} testis at 5 weeks (Figure 3.10J – 3.10L) and 8 weeks (not shown), primary spermatocytes in stage VII-VIII tubules (which have PL spermatocytes and mid-to-late pachytene spermatocytes), have intense, nuclear staining (Figure 3.10L) while spermatocytes in stages I – VI (where there are no PL spermatocytes but there are early pachytene spermatocytes) have punctate cytoplasmic staining (not shown). In the *Kitl*^{SL-39R}/*Kitl*^{SL-39R} testes at both 3 weeks and 5 weeks, tubules that have round spermatids as the most advanced stage have intense, nuclear staining in the round spermatids (Figure 3.10G-3.10I and 3.10M-3.10O). However, nuclear staining was not observed in the tubules of mutants that contain spermatocytes as the most advanced stage (Figure 3.10G-3.10I and 3.10M-3.10O). In particular, the *Kitl*^{SL-39R}/*Kitl*^{SL-39R} tubule shown in Figure 3.10M-3.10O contains leptotene spermatocytes, which indicates that the tubule is comparable to stage IX-X and should therefore have late pachytene spermatocytes. Thus, in the *Kitl*^{SL-39R} testis, the aberrant or substantially delayed translocation of CDC25C into the nucleus of some spermatocytes is consistent with there being impaired progression of meiosis in pachytene spermatocytes. This defect in the *Kitl*^{SL-39R}/*Kitl*^{SL-39R} mutant is however leaky, at least during the first wave of spermatogenesis, because some tubules are able to progress to the haploid stage and the round spermatids display nuclear localization of CDC25C (see Figure 3.10N and 3.10O).

DISCUSSION

The present study is the first report of spermatogenesis defects in mice homozygous for a missense mutation in KITL. In *Kitl^{SI-39R}/Kitl^{SI-39R}* mutants, which carry a S122F mutation (Rajaraman et al., 2002a), the defects in spermatogenesis are milder than those observed in mice homozygous for other coding *Kitl^{SI}* mutations, thus allowing analysis of later stages of spermatogenesis. The main conclusions from this study are: 1) KITL function is required for survival of In/B spermatogonia and perhaps PL spermatocytes; 2) the effects of KITL on proliferation and survival of spermatogonia are separable; and 3) KITL is required during prophase I of male germ cells. The present observations on the *Kitl^{SI-39R}* phenotype contribute to understanding the complexities of KITL-dependent aspects of spermatogenesis and suggest that *Kitl^{SI-39R}* mutant mice may provide a valuable genetic model for more mechanistic studies of KITL functions.

Previous studies provided strong evidence that reduced KITL or KIT function results in a block to the transition of undifferentiated to differentiated type A spermatogonia and to increased apoptosis and decreased proliferation in the latter cells (reviewed by Bedell and Mahakali Zama, 2004). Some of this evidence came from analyses of mice injected with ACK2 (Packer et al., 1995; Yoshinaga et al., 1991), an antibody that neutralizes KITL signaling, and of mice carrying mutations in *Kit* (Blume-Jensen et al., 2000; Kissel et al., 2000; Ohta et al., 2003) or in *Kitl* (de Rooij et al., 1999; Ohta et al., 2000). At first glance, our observations that *Kitl^{SI-39R}/Kitl^{SI-39R}* mutants have relatively normal numbers of type A spermatogonia, but are deficient in In/B spermatogonia appears to contradict the earlier studies. However, effects of reduced KITL-KIT function on In/B spermatogonia but not on type A spermatogonia have also been observed in

Kitl^{Sl-d/+} mice during regenerative spermatogenesis after reversal of cryptorchidism (Tajima et al., 1991) and in *Kit*-haploinsufficient mice (Guerif et al., 2002). These observations can be reconciled if the different strategies for affecting KITL-KIT function have different quantitative or qualitative effects on the signaling pathway, i.e. mutants that affect early steps in spermatogenesis result from a greater reduction in KITL function than mutants that affect later steps in the pathway (see Figure 3.11). In support of this, *Kitl*^{Sl} mutations exert gene dosage effects and graded phenotypes with respect to peripheral red blood cells and pigmentation (Bedell et al., 1996; Rajaraman et al., 2002b).

The spermatogenesis defects we observed in *Kitl*^{Sl-17H}/*Kitl*^{Sl-17H} mutants are slightly different than those reported previously, with our results either more severe or milder than in previous studies (Brannan et al., 1992; de Rooij et al., 1999). However, the three studies differ with respect to the substrain and strain backgrounds. In the first study (Brannan et al., 1992), the *Kitl*^{Sl-17H} mutants had been on a C3H/HeJ background for only a few generations after rederivation from a C3H/HeH background. In the present study, the *Kitl*^{Sl-17H} mutants had been on a C3H/HeNCr background for >15 generations. Regardless of possible C3H substrain differences, the spermatogenesis defects caused by *Kitl*^{Sl-17H} on a C3H background in both studies are milder than those reported on a C57Bl/6 background, where *Kitl*^{Sl-17H}/*Kitl*^{Sl-17H} mutants were shown to be arrested at the A_{al} to A₁ transition (de Rooij et al., 1999). Confirmation of the possible strain differences is important because it would suggest the action of genetic modifiers of the *Kitl*^{Sl} phenotype in the testis. However, in our study we did not make a distinction between different types of A spermatogonia. Using Hoechst dye staining we were able to distinguish two classes of type A spermatogonia. Although it will be important to determine the

true identity of these cells, they clearly are type A spermatogonia. It is possible that a subset of differentiating type A spermatogonia, particularly A3 and A4, could be affected in the *Kitl^{Sl}* mutants but the presence of even the few In/B spermatogonia and PL spermatocytes reveal a more advanced stage of differentiation than in previous studies.

KITL regulates both proliferation and apoptosis of spermatogonia, but the mechanisms by which the same signaling pathway regulates these opposing biological responses are not known. We observed that in comparison to wildtype mice, both *Kitl^{Sl-39R}* and *Kitl^{Sl-17H}* mutants had marginally affected BrdU incorporation in all types of spermatogonia (Figure 3.7A and 3.7B), no change in apoptosis of type A spermatogonia (Figure 3.7C), but greatly increased apoptosis of other pre-meiotic cells (Figure 3.7D). Therefore, the levels of functional KITL required to suppress apoptosis may be lower in type A spermatogonia than in other types of pre-meiotic cells, and the levels of functional KITL required to promote proliferation may be lower than levels required to suppress apoptosis. While further studies will be required to examine these possibilities, there is evidence that enhanced proliferation and suppression of apoptosis in cultured spermatogonia in response to KITL are mediated through different downstream signaling pathways (Dolci et al., 2001). While this observation provides an explanation for the differential effects of KITL, it will be essential to understand the molecular basis by which these differences are initiated.

There is also some evidence that the KITL-KIT pathway is also required in spermatocytes during meiosis in vivo (Guerif et al., 2002; Tajima et al., 1991; Vincent et al., 1998) and in vitro (Feng et al., 2002). In the *Kitl^{Sl-39R}* testis, our observations that the appearance of meiotic and

post-meiotic germ cells during the first wave of spermatogenesis is delayed (Figure 3.9), and that late-pachytene spermatocytes do not have the normal localization pattern of CDC25C (Figure 3.10), are consistent with a role for KITL during prophase I of male meiosis. If KITL does have a role in meiosis, it will be important to determine the stage at which it is required; i.e. for entry into meiosis, during progression through the long meiotic prophase, or for the transition out of prophase. Because there are cells in the juvenile *Kitl^{Sl-39R}* testis that have a nuclear morphology like that of primary spermatocytes, we think there is sufficient KITL function for the cells in the first wave to enter into meiosis. However, massive apoptosis causes reduced numbers of In/B spermatogonia and PL spermatocytes and it is not known if every PL spermatocyte in the *Kitl^{Sl-39R}* mutants becomes a pachytene spermatocyte. Therefore we cannot exclude the possibility that entry into meiosis is adversely affected in the mutants. Once the cells enter meiosis, it is very likely that their progress through prophase and/or the transition out of prophase is affected by KITL function. In the future, these issues of progression through prophase and into the division phases in spermatogenic cells of the *Kitl^{Sl}* mutants will be addressed using more molecular markers and chromosome preparations (Inselman et al., 2003).

A concern in interpreting the results of the present study, and all studies involving germ cell-deficient mice, is that some of the effects we observed could result from disrupted germ cell-Sertoli cell interactions. In the case of the existing *Kitl^{Sl}* mutants, it is difficult to separate direct effects on postnatal spermatogenesis with indirect effects due to PGC deficiency. Clearly, reduced numbers of germ cells cannot explain all defects in *Kitl^{Sl}* mutants because transplantation of wildtype testicular germ cells into *Kitl^{Sl-17H}* and *Kitl^{Sl-d}* testes results in colonies of undifferentiated type A spermatogonia that do not progress further (Ohta et al., 2000).

Furthermore, reduced KIT function causes arrested spermatogenesis even when there are no PGC deficiencies (Blume-Jensen et al., 2000; Kissel et al., 2000). Nonetheless, there are a number of factors that could be affected by severely reduced numbers of germ cells and exert multiple indirect effects on spermatogenesis. For example, tight junctions between adjacent Sertoli cells make up the blood testis barrier (BTB), which physically separates the seminiferous epithelium into basal and adluminal compartments and is essential to spermatogenesis (reviewed by Lui et al., 2003). These tight junctions are loosened transiently to allow passage of PL and leptotene spermatocytes at specific stages of spermatogenesis and there is speculation that germ cells may produce cytokines that regulate tight junction dynamics (Lui et al., 2003). Intriguingly, KITL shifts from the basal compartment of Sertoli cells to radial aspects of the adluminal compartment (Vincent et al., 1998) during the same stages which germ cells traverse the BTB. Thus it will be of interest to determine if there is a direct or indirect connection between KITL function and/or localization to the BTB.

In addition to Sertoli cell architecture, the intricate balance of hormone production and responsiveness that takes place in the testis could result from germ cell deficiencies and adversely affect spermatogenesis. High levels of intratesticular testosterone (ITT) occur in some mouse mutants with spermatogenesis defects but differentiation was restored when the high ITT levels were suppressed by gonadotropin-releasing hormone antagonists (Matsumiya et al., 1999; Meistrich and Shetty, 2003; Shetty et al., 2001). However we observed defects in spermatogenesis in *Kitt^{Sl}* mutants at 3 weeks of age, which is before the surge of testosterone that occurs at puberty. Thus, it seems that elevated ITT may not contribute to spermatogenesis

defects in juvenile mice. However, it could contribute to defective spermatogenesis in adult mice, and could explain the lack of meiotic cells in the 8 week old *Kitl*^{SI-39R} mutants.

A large number of *Kitl*^{SI} mutations, including other missense mutations (Rajaraman et al., 2002a) have been identified (Jackson Laboratory and Oakridge National Laboratory) and provide an extremely valuable genetic resource for dissecting KITL structure and function. However, most of these mutations cause perinatal lethality in the homozygous condition and so are of limited use for studying postnatal spermatogenesis. Indeed, the true null phenotype for either KITL or KIT function in spermatogenesis is not known because of the perinatal lethality. In order to establish the null phenotype in the postnatal, it will be necessary to engineer conditional mutations in the *Kitl* or *Kit* genes that would leave KITL or KIT functions intact in the embryo, but would affect only postnatal spermatogenesis. To our knowledge, the only *Kitl*^{SI} coding mutations that allow viability to homozygous mice are *Kitl*^{SI-d}, *Kitl*^{SI-17H}, and *Kitl*^{SI-39R}. The *Kitl*^{SI-d}, *Kitl*^{SI-17H}, and *Kitl*^{SI-39R} mutations are predicted to have distinctly different effects on KITL structure but little is known of the molecular mechanisms by which they affect KITL function (Bedell and Mahakali Zama, 2004). Possible reasons for different functional activities of protein include different expression levels, subcellular localization, or signaling properties. While nothing is known of subcellular localization of KITL^{SI-39R}, there is evidence that KITL^{SI-17H} is mislocalized in polarized epithelial cells and it was suggested that the inappropriate amounts of this KITL mutant protein in the basal aspect of Sertoli cells could contribute to its reduced function (Wehrle-Haller and Weston, 1999). Interestingly, the relative effects of the two mutations on spermatogenesis and on peripheral blood cells are reversed; i.e. the anemia of *Kitl*^{SI-17H} mutants is milder than that of *Kitl*^{SI-39R} (unpublished observations). Whether these

phenotypic differences are due to different responses of erythroid progenitors and spermatogenic cells to the mutant proteins, or to differences in expression or localization of mutant proteins in hematopoietic tissues versus Sertoli cells remains to be determined.

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FIGURE LEGENDS

Figure 3.1. Representative H&E-stained testes sections from *Kitl*⁺/*Kitl*⁺ (A – C), *Kitl*^{SI-39R}/*Kitl*^{SI-39R} (D – F), and *Kitl*^{SI-17H}/*Kitl*^{SI-17H} (G – I) mice. Sections are from mice at 3 weeks (A, D, and G), 5 weeks (B, E, and H), and 8 weeks (D, F, and I) after birth. At 3 weeks, the most advanced cells in *Kitl*⁺/*Kitl*⁺ tubules (A) are either pachytene spermatocytes or round spermatids while in *Kitl*^{SI-39R}/*Kitl*^{SI-39R} mice (D), some tubules are devoid of differentiating germ cells (asterisk) while others have a nearly normal complement of meiotic germ cells. At 5 weeks, there are numerous tubules with elongate spermatids (arrowheads) in the *Kitl*^{SI-39R}/*Kitl*^{SI-39R} testes (E) indicative of progression of spermatogenesis. However, spermatogenesis ceases by 8 weeks of age in both *Kitl*^{SI-39R}/*Kitl*^{SI-39R} testes (F) similar to the *Kitl*^{SI-17H}/*Kitl*^{SI-17H} testes (I). A few elongate spermatids remain from the first wave of spermatogenesis (arrowheads). In contrast there are no differentiating germ cells in the *Kitl*^{SI-17H}/*Kitl*^{SI-17H} testes at 3 weeks (G), 5 weeks (H), or 8 weeks (I). Pachytene spermatocytes (P), round spermatids (R), elongate spermatids (arrowheads), tubules with no meiotic or post-meiotic cells (asterisk). In B and C, roman numerals indicate stages of spermatogenesis (Russell et al., 1990).

Figure 3.1

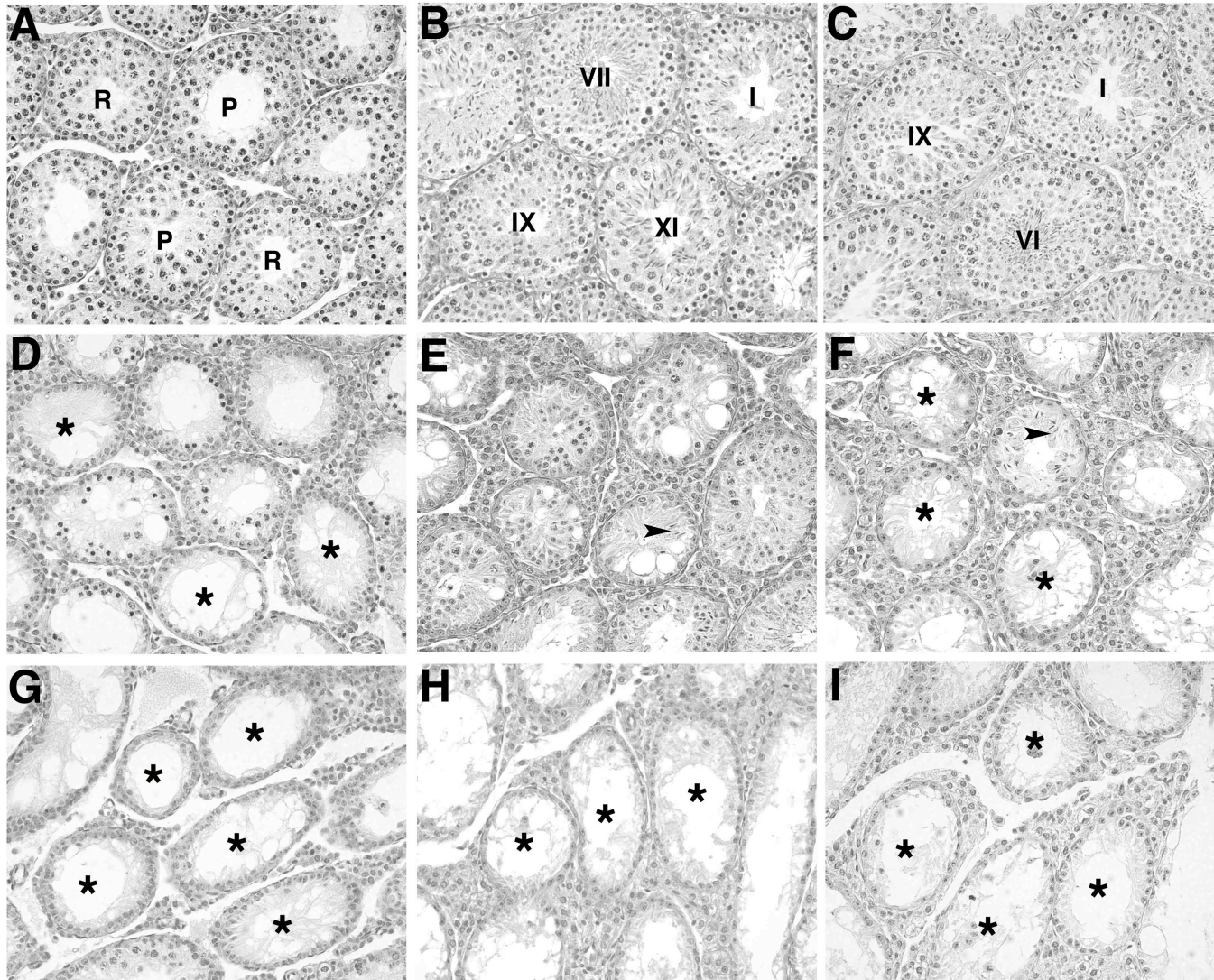


Figure 3.2. Representative Hoeschst-stained testes sections from *Kitl*^{+/+}/*Kitl*⁺ (A – C), *Kitl*^{SL-39R}/*Kitl*^{SL-39R} (D – F), and *Kitl*^{SL-17H}/*Kitl*^{SL-17H} (G – I) mice. The cell types are (see complete description in Results): SA spermatogonia, thin arrows; LA spermatogonia, barbed arrows; In/B spermatogonia, thick arrows; PL spermatocytes, arrowheads; pachytene spermatocytes, P; diplotene spermatocytes, D; round spermatids, R; elongate spermatids, E; and Sertoli cells, SC. (See text for further details)

Figure 3.2

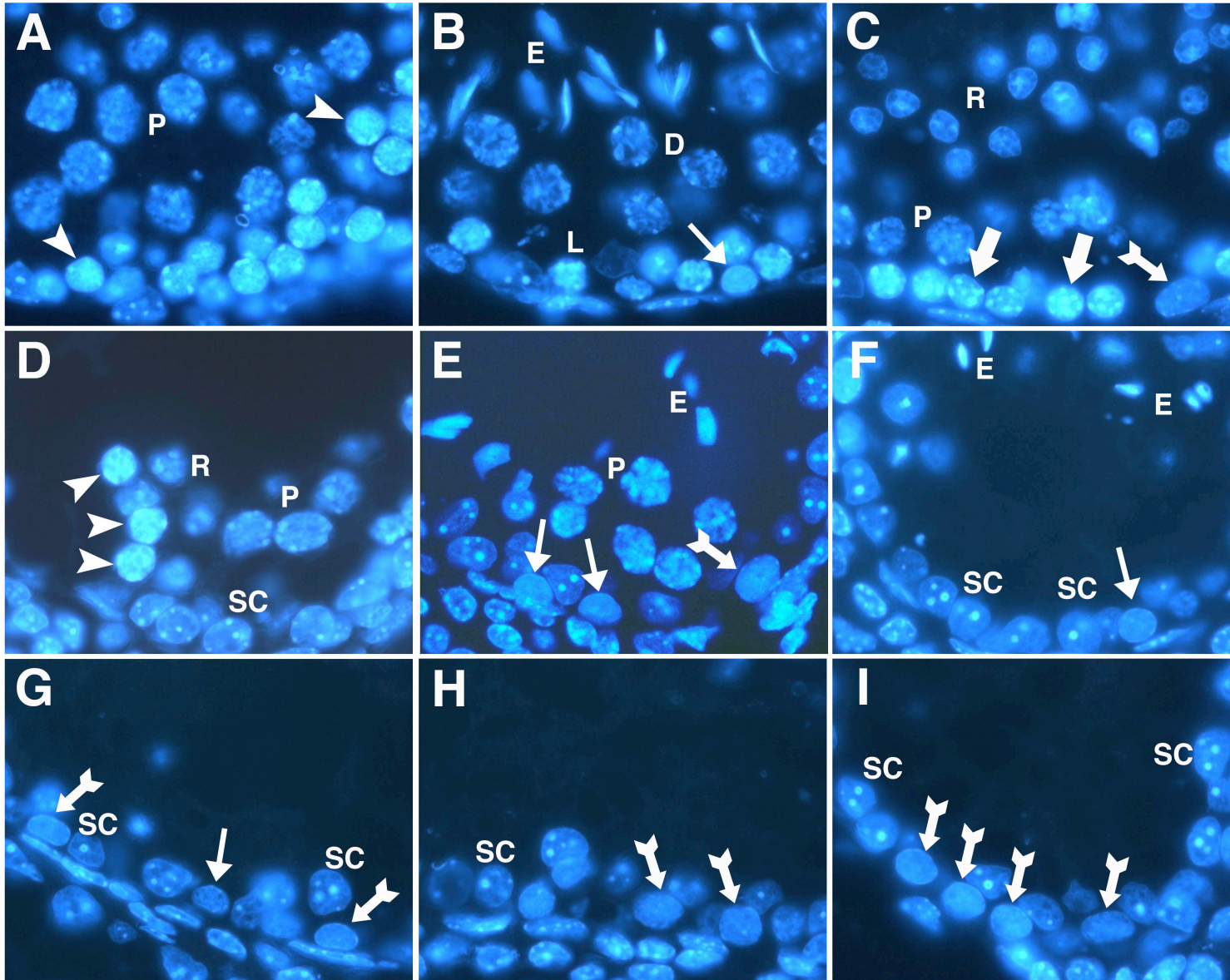
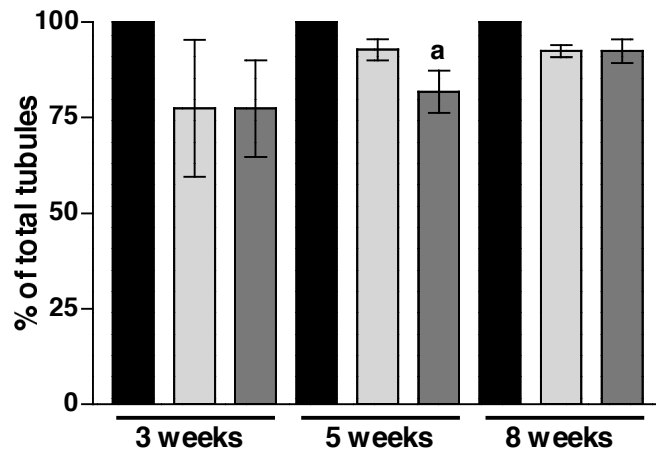


Figure 3.3. The percentage of tubules containing pre-meiotic (A) and meiotic and post-meiotic (B) germ cells in *Kitl*⁺/*Kitl*⁺ (black bars), *Kitl*^{SI-39R}/*Kitl*^{SI-39R} (light grey bars), and *Kitl*^{SI-17H}/*Kitl*^{SI-17H} (dark grey bars) mice. 30 – 35 tubules from Hoechst-stained testes sections of mice were scored and the numbers of tubules containing each type of cell expressed as a percentage of the total tubules scored. Pre-meiotic cells include all spermatogonia and PL spermatocytes. Meiotic and post-meiotic cells include all spermatocytes except PL, round spermatids, and elongate spermatids. The means and standard errors are shown for values from three animals at each age and genotype. Values from mutants were compared to *Kitl*⁺/*Kitl*⁺ values at the same age and lower case letters above the bars indicate significant differences (a, P<0.05; c, P<0.001).

Figure 3. 3

A. Pre-meiotic cells



B. Meiotic and post-meiotic cells

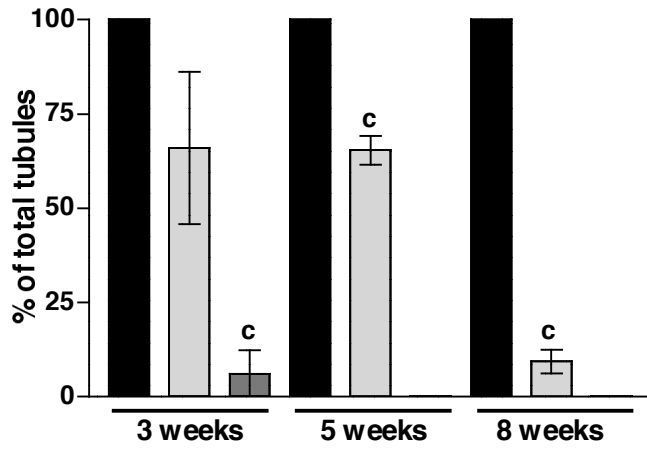
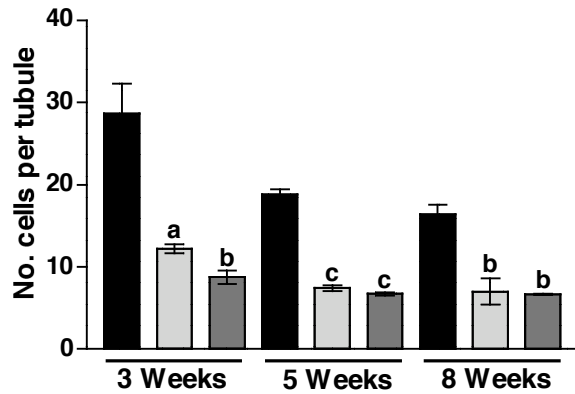


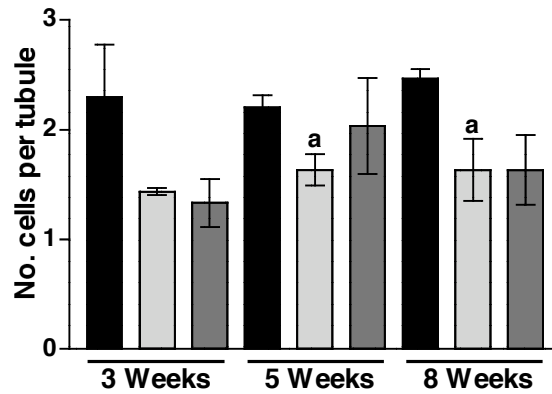
Figure 3.4. Quantification of pre-meiotic germ cell types per tubule in testes of *Kitl*⁺/*Kitl*⁺ (black bars), *Kitl*^{SL-39R}/*Kitl*^{SL-39R} (light grey bars), and *Kitl*^{SL-17H}/*Kitl*^{SL-17H} (dark grey bars) mice. Different types of pre-meiotic germ cells were identified by Hoechst staining and scored from 30-35 cross-sectioned tubules per section. The numbers of each cell type per tubule were calculated, and the means and standard errors are shown for values from three animals at each age and genotype. The cell types shown in each panel are (A) total pre-meiotic cells, including all types of spermatogonia and PL spermatocytes; (B) SA spermatogonia; (C) LA spermatogonia; (D) all type A spermatogonia, (i.e. SA plus LA spermatogonia; see Results for a description of SA and LA spermatogonia); (E) In/type B spermatogonia; and (F) PL spermatocytes. At each age, pair-wise comparisons were made between values for *Kitl*⁺/*Kitl*⁺ and each mutant. Lower case letters above the bars indicate significant differences between mutant and wildtype at each age (a, P<0.05; b, P<0.01; c, P<0.001).

Figure 3. 4

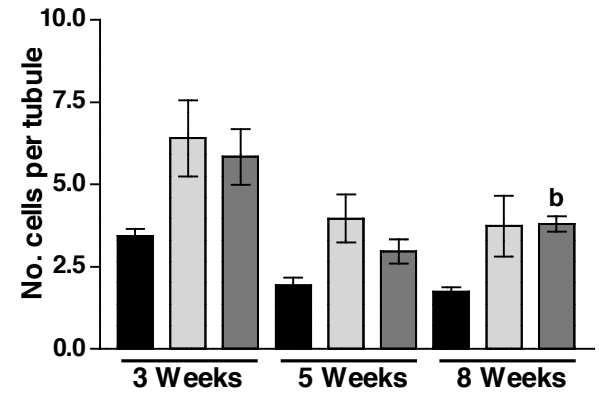
A. All pre-meiotic germ cells



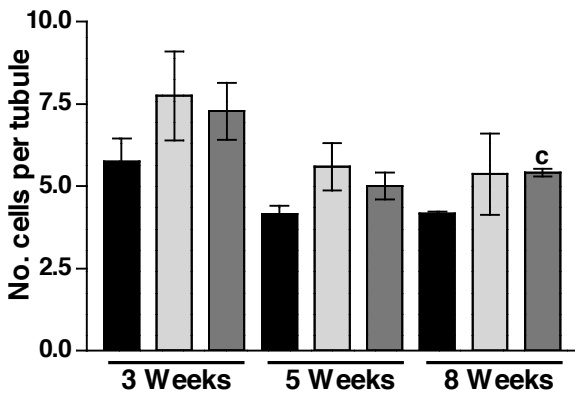
B. Small type A spermatogonia



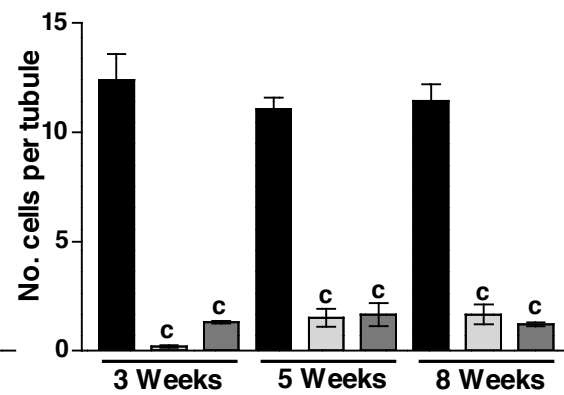
C. Large type A spermatogonia



D. All type A spermatogonia



E. Type B/Intermediate spermatogonia



F. Preleptotene spermatocytes

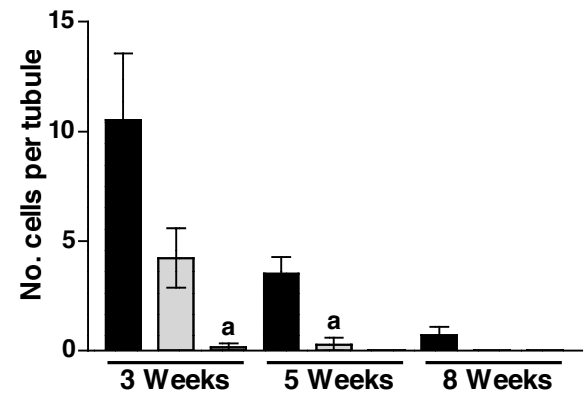


Figure 3.5. The distributions of the different types of pre-meiotic cells within the total number of pre-meiotic cells. In testes of *Kitl*⁺/*Kitl*⁺ (columns 1, 4, and 7), *Kitl*^{SL-39R}/*Kitl*^{SL-39R} (columns 2, 5, and 8), and *Kitl*^{SL-17H}/*Kitl*^{SL-17H} (columns 3, 6, and 9) mice at 3 (columns 1 – 3), 5 (columns 4 – 7), and 8 (columns 7 – 9) weeks of age. The cell types represented by each column are SA spermatogonia (black), LA spermatogonia (dark grey), In/B spermatogonia (light grey), and PL spermatocytes (diagonal lines).

Figure 3.5

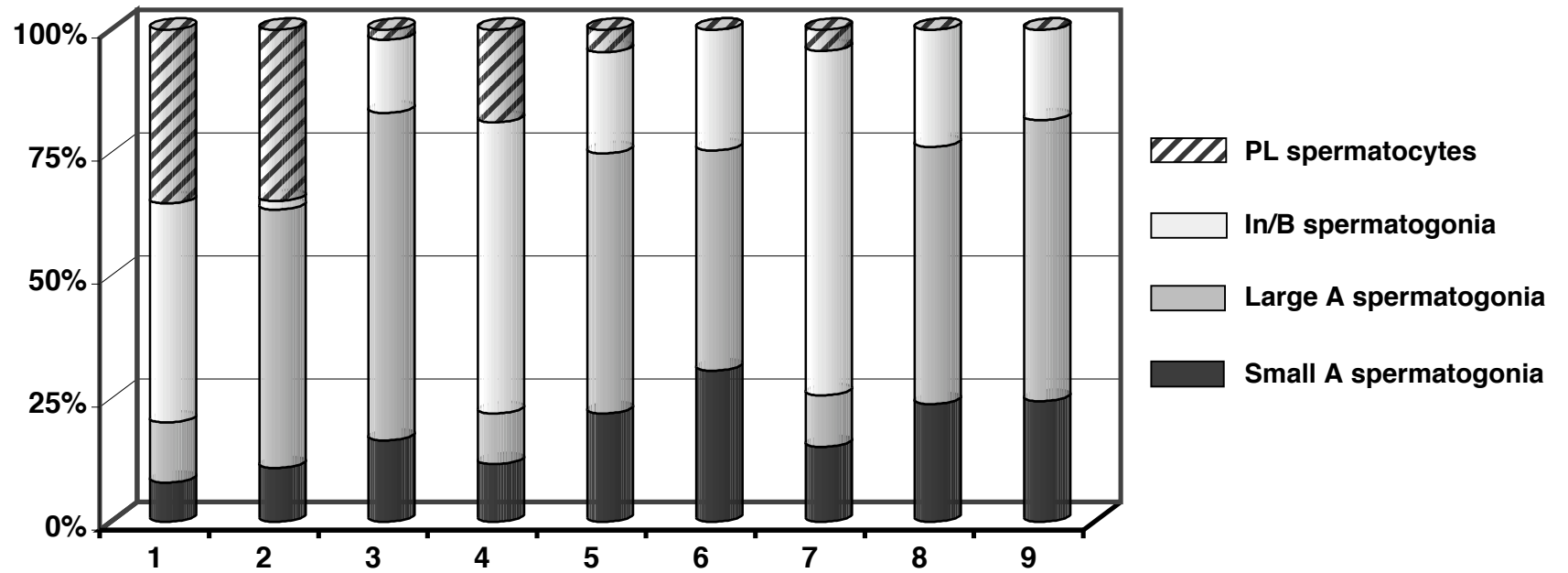


Figure 3.6. Incorporation of BrdU into testes from 3 week old *Kitl⁺/Kitl⁺* (A, B), *Kitl^{SL-39R}/Kitl^{SL-39R}* (C), and *Kitl^{SL-17H}/Kitl^{SL-17H}* testes (D). Testes were collected from mice 2 hours after intra-peritoneal injection of BrdU. BrdU-positive cells (green) were revealed by immunostaining and cell types were identified based on nuclear morphology and size. The cell types are indicated as follows: type A spermatogonia, thin arrows (A, C and D); In/B spermatogonia and PL spermatocytes, closed arrows (B). Note that in this analysis, a distinction was not made between the latter cell types.

Figure 3.6

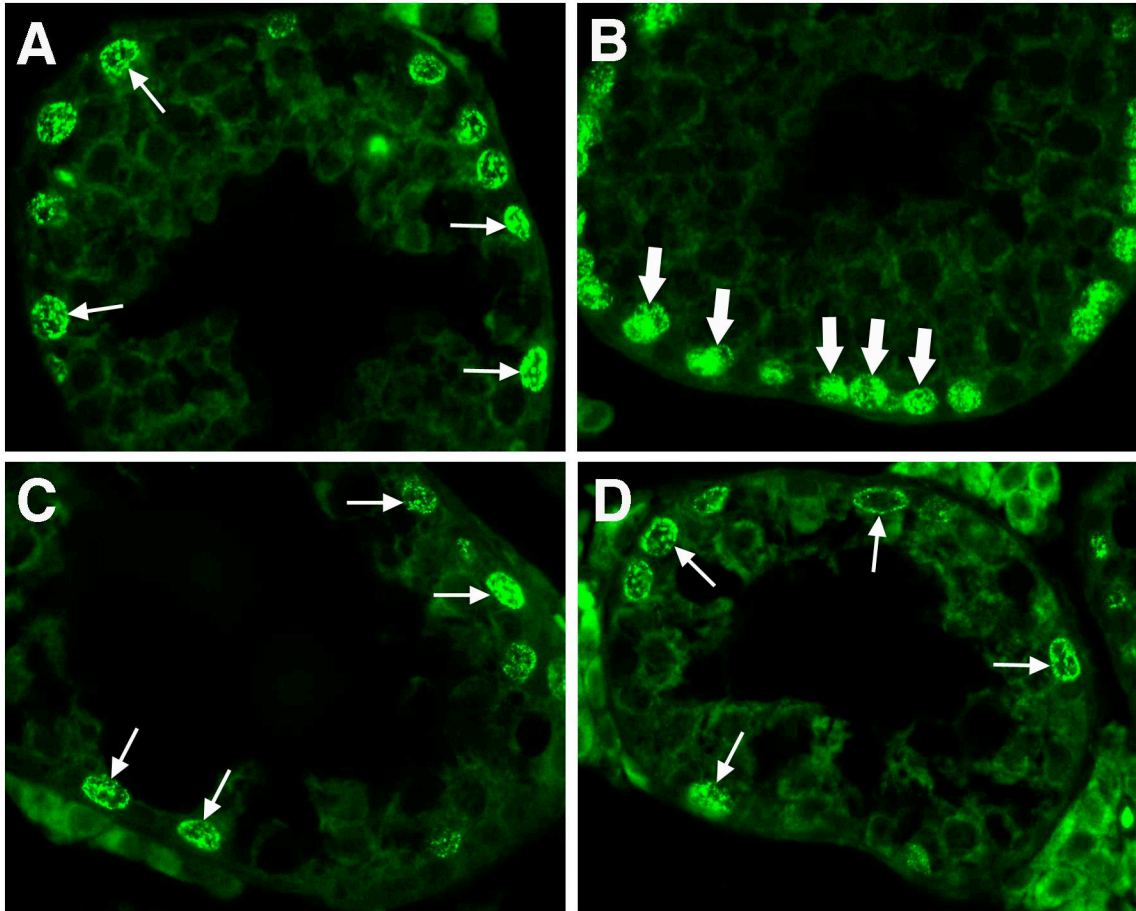


Figure 3.7. Normalized values for BrdU-positive (A, B) and TUNEL-positive (C, D) germ cells in testes of *Kitl*⁺/*Kitl*⁺ (black bars), *Kitl*^{SL-39R}/*Kitl*^{SL-39R} (light grey bars), and *Kitl*^{SL-17H}/*Kitl*^{SL-17H} (dark grey bars) mice at 3, 5, and 8 weeks of age. For BrdU- and TUNEL-stained sections, the numbers of each type of pre-meiotic germ cells per tubule were determined. Because there are considerable differences in total numbers of these cells (Figure 3.4), the BrdU-positive and TUNEL-positive values were normalized to account for the relative differences in total numbers of these cells found in mutant and wildtype testes (see Figure 3.4). These data therefore represent the numbers of BrdU-positive and TUNEL-positive cells per tubule if equal numbers of each cell type were present in mutant and wildtype testes. Values for all type A spermatogonia are in panels A and B, while values for all other pre-meiotic cells (In/B spermatogonia and PL spermatocytes) are in panels C and D. At each age, pair-wise comparisons were made between values for wildtype and each mutant. Lower case letters above the bars indicate significant differences between mutant and wildtype at each age (a, P<0.05; b, P<0.01; c, P<0.001).

Figure 3. 7

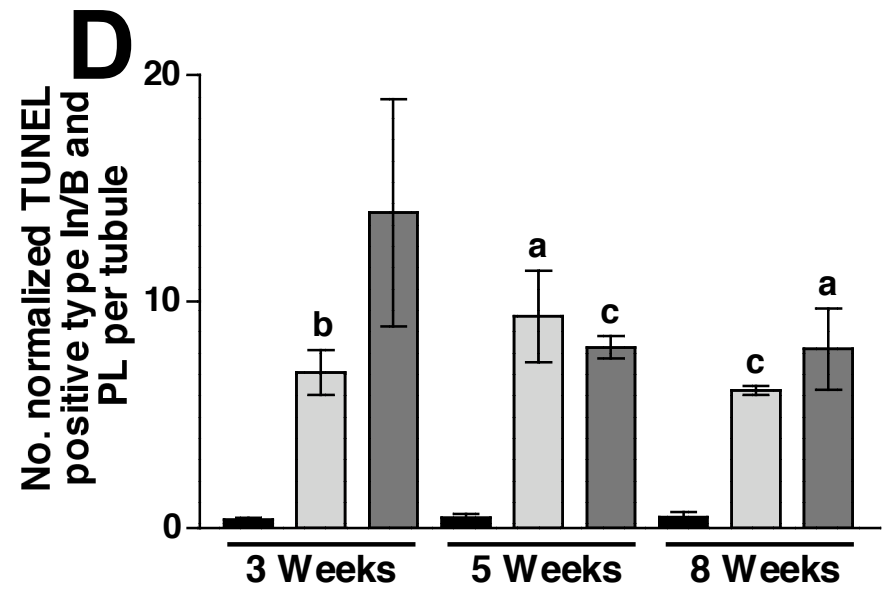
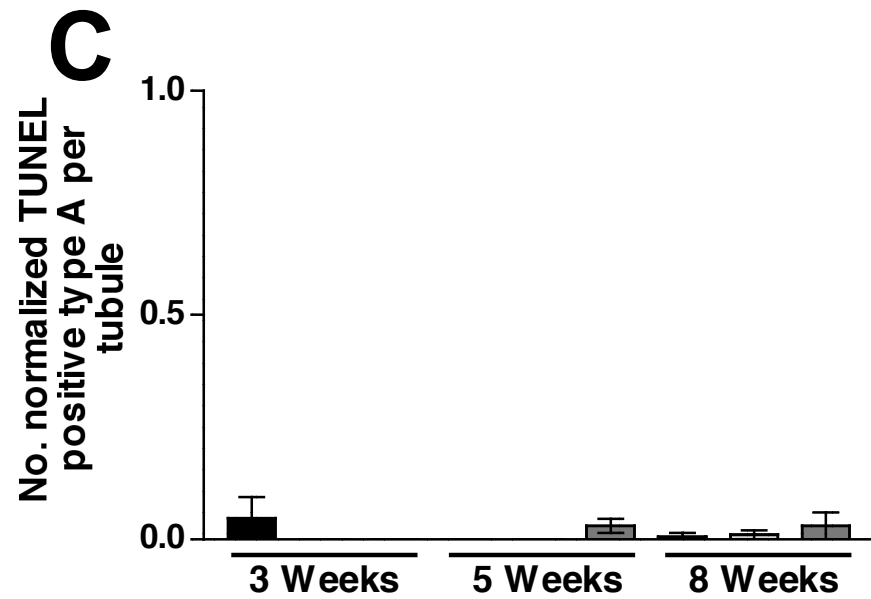
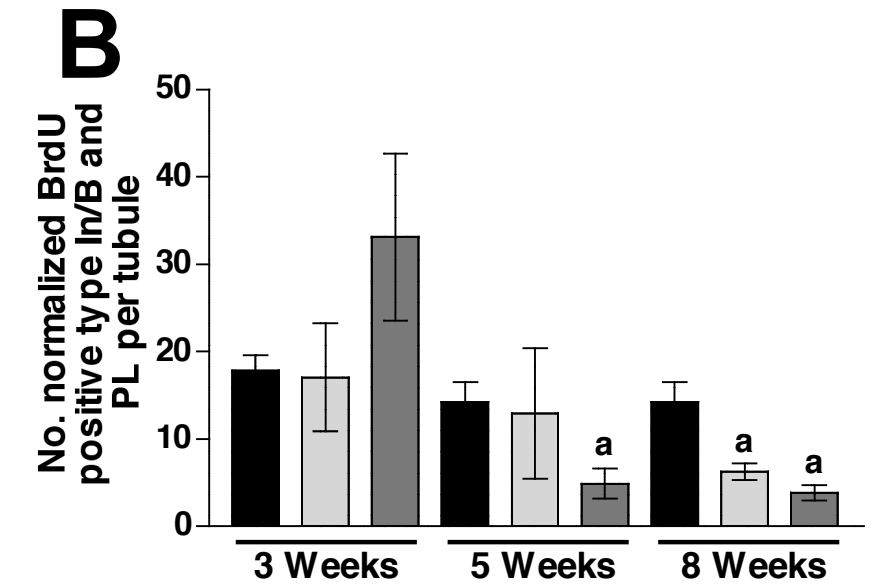
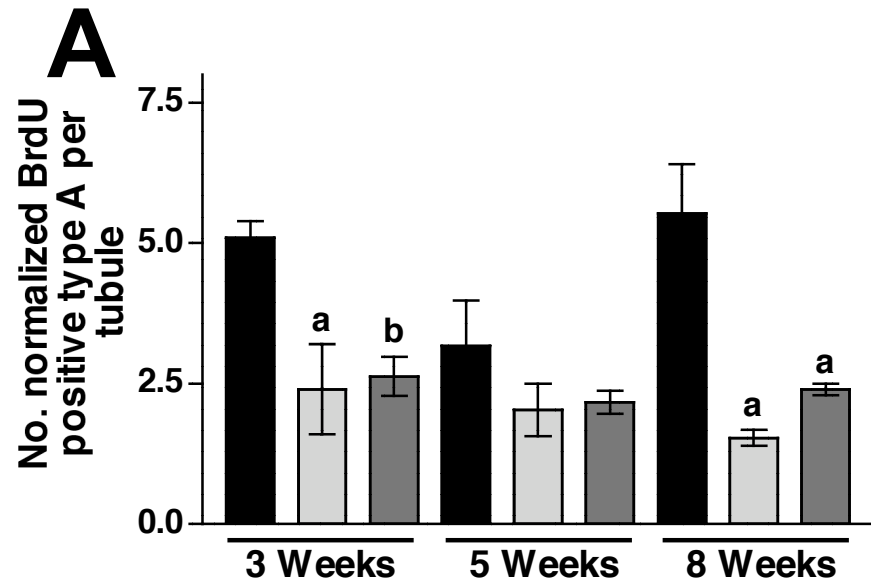


Figure 3.8. Sections of testes from 3 week old *Kitl⁺/Kitl⁺* (A, B), *Kitl^{SL-39R}/Kitl^{SL-39R}* (C), and *Kitl^{SL-17H}/Kitl^{SL-17H}* testes (D) mice stained for TUNEL activity (green) and Hoechst (blue).

The cell types are indicated as follows: all spermatocytes other than PL spermatocytes, arrowheads; In/B spermatogonia and PL spermatocytes, thick arrows. In *Kitl⁺/Kitl⁺* tubules, large number of spermatocytes in meiosis I and II, undergo apoptosis (B) while a few type In/B spermatogonia and pachytene spermatocytes undergo apoptosis in other tubules (A). Conversely, the predominant number of apoptotic cells are In/B spermatogonia and PL spermatocytes in *Kitl^{SL-39R}/Kitl^{SL-39R}* (C), and *Kitl^{SL-17H}/Kitl^{SL-17H}* (D) tubules.

Figure 3.8

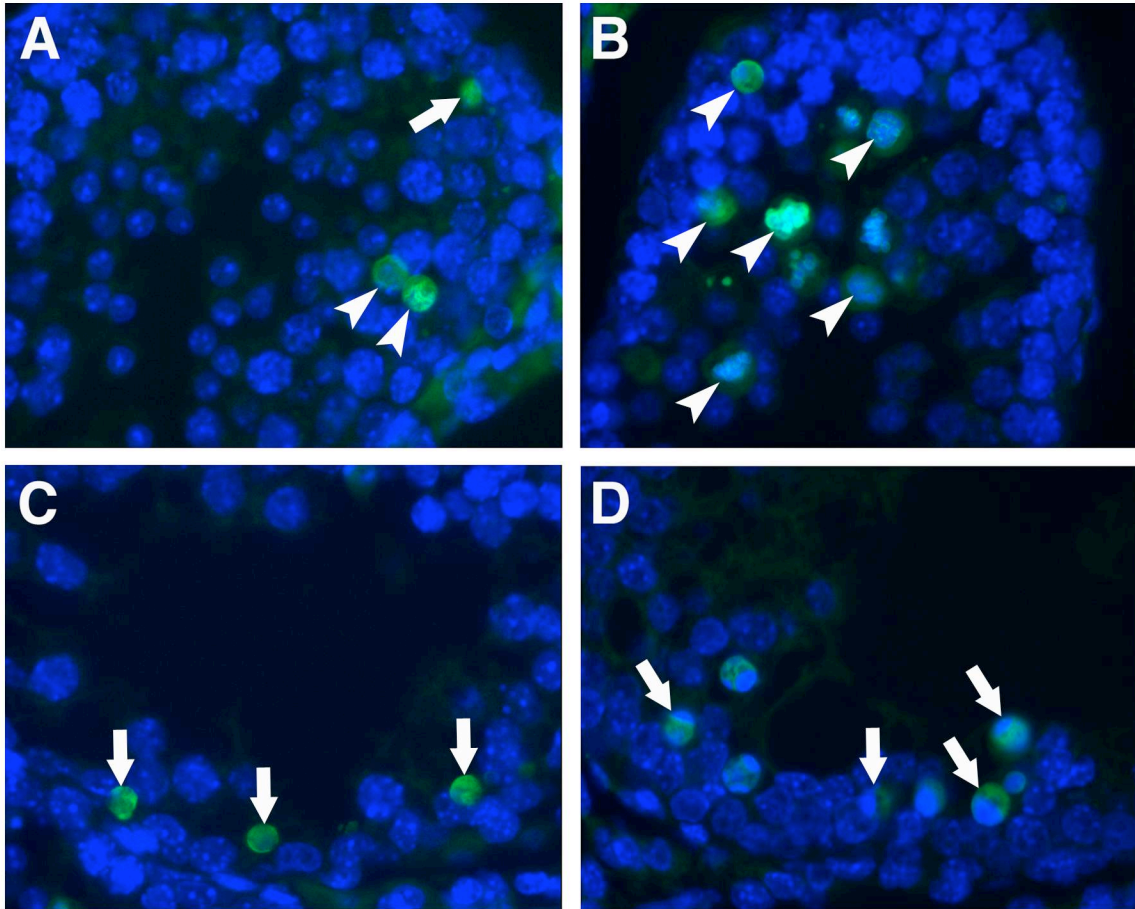


Figure 3.9. Progression of meiotic and post-meiotic cells in *Kitl*⁺/*Kitl*⁺ (black) and *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} (light grey) testes at 3 weeks (panel A), 5 weeks (panel B), and 8 weeks (panel C). Germ cell-containing tubules were scored for the most advanced stage of germ cell present and values expressed as a percentage of the tubules with a given germ cell type versus the total tubules scored. At 3 weeks (panel A), the most advanced stages in the *Kitl*⁺/*Kitl*⁺ tubules are spermatocytes and round spermatids while there are predominantly spermatocytes in *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} tubules. In *Kitl*⁺/*Kitl*⁺ testes at 5 weeks (panel B) and 8 weeks (panel C), the majority of tubules contain elongate spermatids as the most advanced stage and there are no tubules with spermatocytes as the most advanced stage. In contrast, in the *Kitl*^{Sl-39R}/*Kitl*^{Sl-39R} testes at 5 weeks (panel B) and 8 weeks (panel C), many tubules have not advanced as far in *Kitl*⁺/*Kitl*⁺, with a smaller percentage of tubules containing elongate spermatids but a higher percentage containing round spermatids and spermatocytes.

Figure 3.9

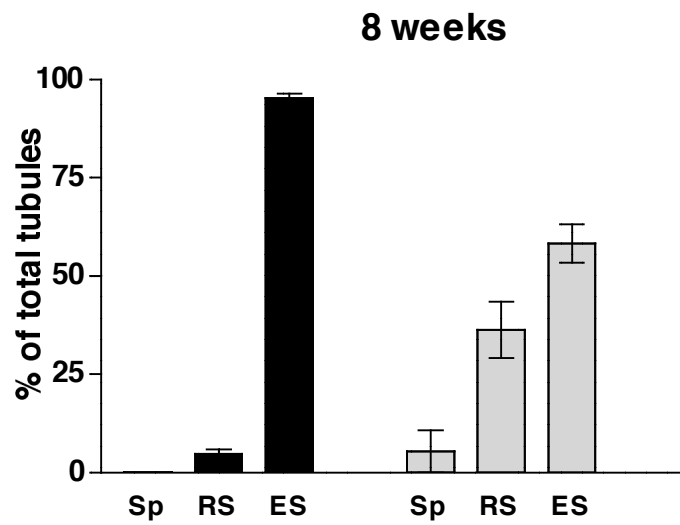
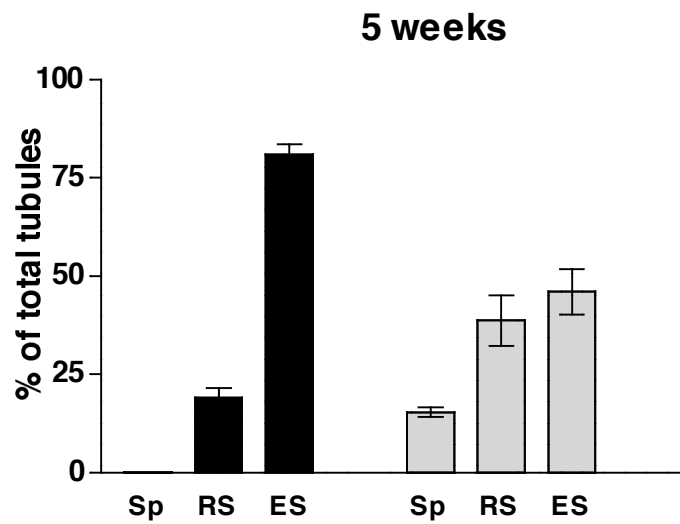
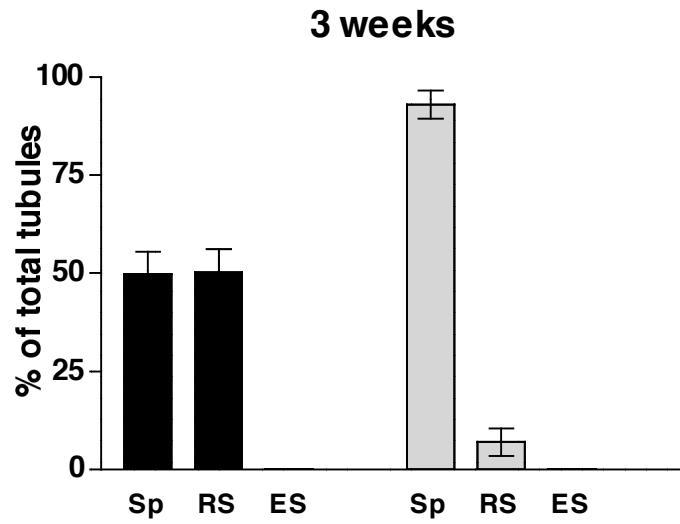


Figure 3.10. Confocal images of CDC25C localization in *Kitl*^{+/+}/*Kitl*^{+/+} (A – F, J – L) and *Kitl*^{SL-39R}/*Kitl*^{SL-39R} (G – I, M – O) testes from mice at 3 weeks (A – I) and 5 weeks (J – O) of age. Left column, ethidium homodimer-2 stain, red; middle column, anti-CDC25C, green; right column, merged images of red and green channels. Enlarged images of 1 or 2 cells from each merged panel are shown as insets in the right column. Early-mid pachytene spermatocytes, arrows; mid-late pachytene spermatocytes, arrowheads; leptotene spermatocytes, thin arrow; round spermatids, barbed arrows. In *Kitl*^{+/+}/*Kitl*^{+/+} testes at 3 weeks, anti-CDC25C staining appears in a punctate pattern in the cytoplasm of early-mid pachytene spermatocytes (A – C) but in an intense nuclear pattern in more advanced tubules with mid-late pachytene spermatocytes (D – F). Anti-CDC25c staining is also seen in the nucleus of late pachytene spermatocytes in stage IX-X tubules of *Kitl*^{+/+}/*Kitl*^{+/+} testes at 5 weeks (J – L) and 8 weeks (not shown). In the *Kitl*^{SL-39R}/*Kitl*^{SL-39R} testis at 3 weeks (G – I) and at 5 weeks (M – O), anti-CDC25C staining is punctate and in the cytoplasm in mid-late pachytene spermatocytes, but round spermatids in the most advanced tubules have nuclear anti-CDC25c staining.

Figure 3.10

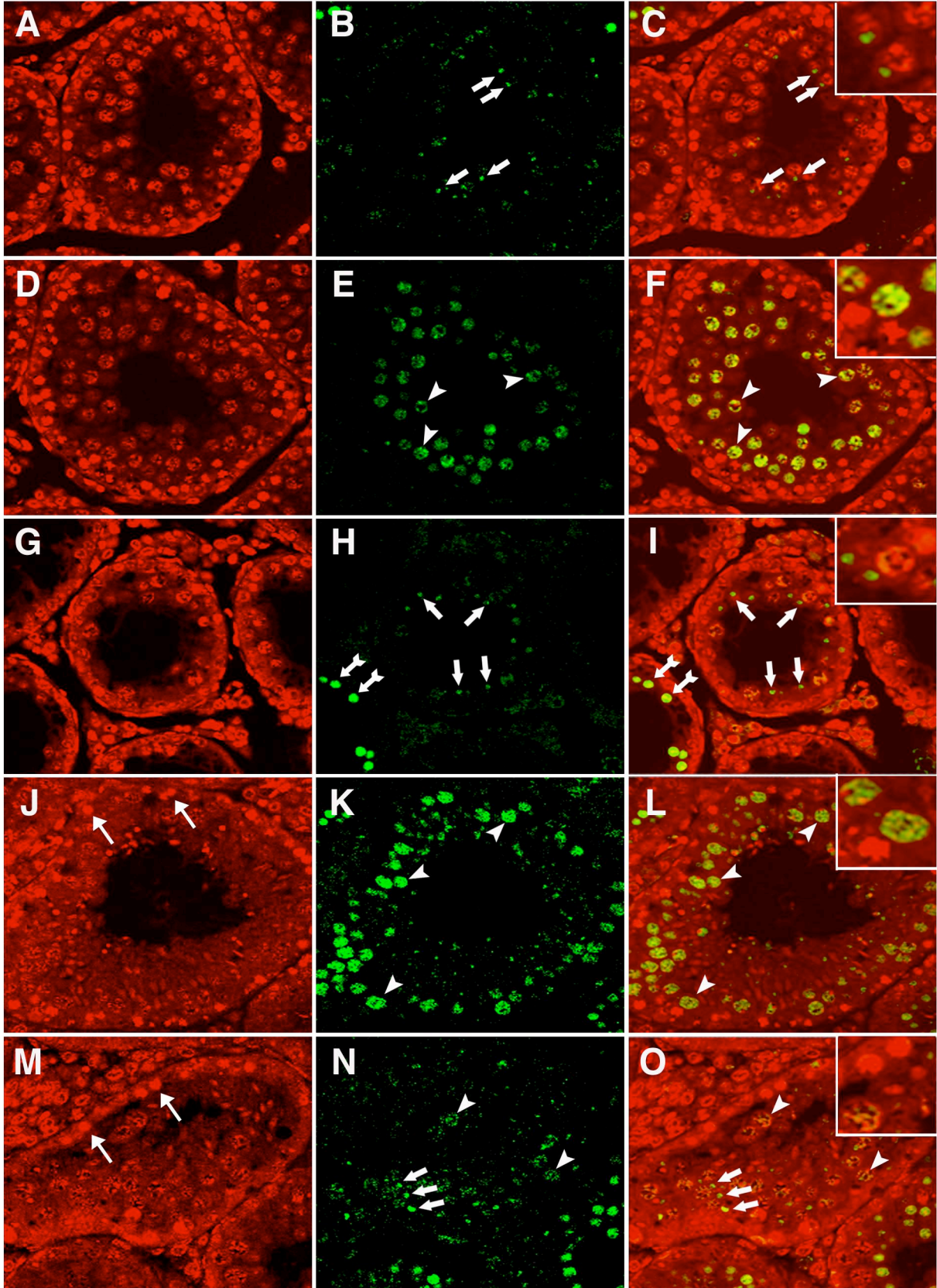
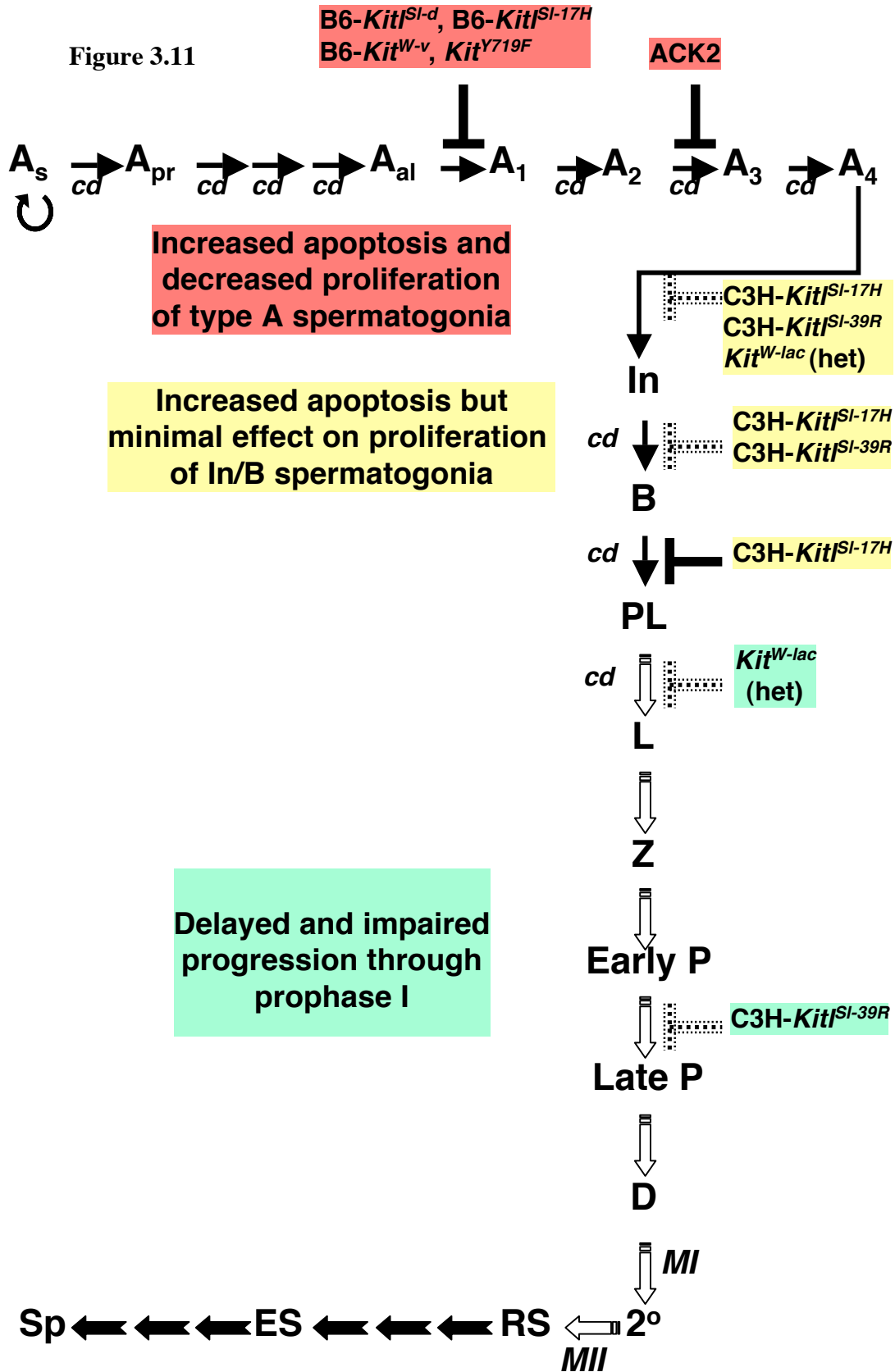


Figure 3.11. Schematic of spermatogenesis and processes affected by reduced KITL-KIT function. Differentiation steps between cell types are shown as arrows: solid arrows are for mitotic cells, solid arrows; striped open arrows are for meiotic cells; and notched solid arrows are for spermiogenic cells. Complete and partial inhibition by different conditions are shown as solid T-bars and stippled T-bars, respectively. Abbreviations for spermatogonial types are type A (A), intermediate (In) and type B (B); for spermatocytes are preleptotene (PL), leptotene (L), zygotene (Z), pachytene (P), diplotene (D), secondary (2°); and for spermiogenic cells are round spermatids (RS), elongating spermatids, and spermatozoa (Sp). Other abbreviations are cell division (cd), meiosis I (MI), and meiosis II (MII).

Figure 3.11



Chapter 4
Conclusions and Future Directions

CONCLUSIONS AND FUTURE DIRECTIONS

The study of the molecular mechanisms involved in germ cell development is essential for understanding the basis of infertility and for the development of methods for contraception in humans. In addition, more information about normal germ cell development will contribute to understanding how germ-cell tumors arise and progress. Through use of a mouse model system, my work will be a stepping-stone towards accomplishing these goals.

Germ cell development in mice is very similar to that of humans, and in both species involves a complex process of lineage specification, survival, proliferation, migration, interaction with the somatic components of the gonad and further differentiation to give rise to the haploid gametes (McLaren, 1999; McLaren, 2003; Wylie, 1999). Although much information about these processes has been obtained in the last several decades, there are numerous questions that are yet to be answered. In particular, many aspects of germ cell development are guided by signaling pathways that exist between these cells and their surrounding somatic cells. Previous studies have shown that many different cytokines are required at specific stages of germ cell development (Donovan, 1994; Kierszenbaum and Tres, 2001). In this thesis, I describe my work with one such cytokine known as Kit ligand (KITL). Taking a molecular genetic approach using multiple mouse strains with different mutations in the *Kitl* gene (an allelic series of *Kitl*^{Sl} mutations), I have finely dissected the role of KITL during germ cell development. The results of my study are good examples of the power of a genetic approach involving an allelic series of mutations in furthering the understanding of molecular mechanisms involved in complex developmental processes.

In Chapter 2, the prevalent questions in the field about the role/s of KITL during PGC development are addressed. We describe evidence that KITL is required for directed migration of

PGCs in addition to its well-known role in supporting the survival and proliferation of these cells. In our studies, *Kitl*^{Sl-gb}/*Kitl*^{Sl-gb}, the null mutant embryos, had PGCs at early stages of PGC development suggesting that KITL is required at early stages but not sufficient by itself in supporting the normal development of PGCs. Additionally, *Kitl*^{Sl-d}/*Kitl*^{Sl-d} embryos, which produce only S-KITL, were unable to support PGC migration and proliferation confirming that MB-KITL is essential for PGC migration and proliferation.

Interesting observations were also made regarding the relative strengths of each of the *Kitl*^{Sl} mutant alleles for their effects on PGC proliferation versus PGC migration. The *Kitl*^{Sl-36R} mutation had only minor effects on migration of PGCs but had significant effects on proliferation. On the other hand, the *Kitl*^{Sl-39R} mutation had mild effects on proliferation but had severe effects on PGC migration. These data suggest that there are different signaling pathways that are being activated by KITL to mediate its different functions. Whether these pathways are being co-activated is an immediate question that needs to be addressed. Further investigations also need to be conducted on the localization and stability of the mutant allele encoded proteins within the somatic cells that they are being expressed in and their ability to activate the receptor, KIT. The migration of PGCs is via different solid tissue environments and is very similar to that of metastasizing cancer cells (Stott and Wylie, 1986). There have been recent reports that numerous extra-cellular matrix proteins and cell adhesion molecules are differentially expressed during PGC migration (Anderson et al., 1999; Bendel-Stenzel et al., 2000; Garcia-Castro et al., 1997; Gomperts et al., 1994). For example, laminin is expressed at high levels in the genital ridges at E11.5 and at lower levels along the migratory pathway. Interestingly the expression of *Kitl* is in a similar pattern. After the PGCs arrive in the genital ridges, they associate with a strip of laminin in the developing genital ridges that aids in the coalescence of the PGCs with the

somatic portion of the gonad. It will be interesting to know if the expression of laminin is affected in *Kitl*^{Sl} mutant embryos. If so, it would suggest that KITL has a role in the colonization of PGCs in the early gonads. Another important question to be addressed is whether the actions of KITL are mediated via specific cell adhesion molecules such as E-cadherin. Cadherin mediated homotypic interactions are required for motility as well as survival of PGCs similar to melanocyte migration and survival, the same functions that are regulated by KITL (Bendel-Stenzel et al., 2000; Di Carlo and De Felici, 2000; Nishimura et al., 1999).

In Chapter 3, the functions of KITL are finely dissected by comparative analyses in mice carrying two hypomorphic mutants to reveal that KITL is essential for the suppression of apoptosis of In/B spermatogonia and pre-leptotene spermatocytes and also for the normal progression of meiosis of spermatocytes. While these studies highlight the role of KITL at more advanced stages of male germ cell development, they do not address the question of the involvement of hormones in these processes. Since the *Kitl*^{Sl-39R} mutants are sterile in spite of exhibiting less severe defects than *Kitl*^{Sl-17H} mutants, it will be informative to study the effects of high levels of testosterone on germ cells in juvenile animals (Meistrich and Shetty, 2003). It will be exciting to know whether the suppression of testosterone at early ages would allow long-term spermatogenesis in the *Kitl*^{Sl-39R} mutant males. Such work could have very important implications for treatment of infertility in men.

In appendix I, I describe preliminary evidence that supports a role for KITL during thecal cell differentiation. Numerous reproductive diseases in women are associated with defects in androgen synthesis. Therefore understanding the nature of defects in the *Kitl*^{Sl-36R} mutant females

will shed light on the involvement of KITL functions in the differentiation of androgen synthesizing thecal cells.

This is an exciting time for studying genetic, molecular, and developmental aspects of reproductive biology in general, and germ cell biology in particular due to the relationship between embryonic stem cells and germ cells. This thesis lays the foundation for many future studies on the role of KITL and other cytokines in embryonic and postnatal germ cell development.

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Appendix I

Effects Of A Nonsense Mutation In The *Kit Ligand* Gene On Thecal Cell Differentiation In Mice

INTRODUCTION

During ovarian follicle development after birth, the growth of the oocyte is one of the first events to occur shortly after which the granulosa cells begin to proliferate and differentiate in the primary follicles (Erickson and Danforth, 1995; Richards et al., 2002). In primordial and primary follicles there are no thecal cells associated with the follicles. Adjacent to the basal lamina, fibroblastic stromal cells are present which differentiate into thecal cells once the granulosa cells achieve two to three layers of cell growth (see Figure 1.5). As the follicles grow, there is active proliferation of both thecal cells and granulosa cells (Gougeon, 1996). Granulosa cells of pre-antral follicles secrete proteins that diffuse into the stromal compartment adjacent to growing follicles that can stimulate the differentiation of pre-thecal cells into androgen producing thecal cells (Magoffin, 2002). Throughout this process, the thecal cells do not express luteinizing hormone (LH) receptors, therefore the process of thecal cell recruitment and differentiation is presumably independent of gonadotropin stimulation.

When follicle stimulating hormone (FSH) enters the follicle, the steroidogenic enzyme, aromatase, is expressed by the granulosa cells and estrogen begins to be produced (Hillier et al., 1995; Smyth et al., 1993). This causes a negative feedback loop on the circulating FSH. A variation of the FSH concentration causes a dominant follicle to be selected, to grow and become an ovulatory follicle. In a similar timeline the thecal cells become LH responsive and produce androgen precursors that diffuse into the granulosa cells, wherein they are converted into estrogen derivatives (Hillier et al., 1994) (see Figure 4.1). The estrogens further allow follicle selection to occur and eventually

trigger mid cycle LH surge and ovulation. Therefore the regulation of thecal cell androgen production is not only an important component of the selection of the dominant follicle for normal ovulation but is also essential for the negative feedback loops of the hypothalamic-pituitary-gonadal axis to be activated.

Kit ligand (KITL) and its receptor, Kit, are required for multiple stages of embryonic and postnatal germ cell development (Besmer et al., 1993). During embryogenesis, from E11.5 to E13.5, high levels of *Kitl* and *Kit* expression are found in the gonads. However, there is no detectable expression of *Kit* mRNA after the oogonia complete proliferation and enter meiotic arrest in the later stages of fetal development. After birth, *Kitl* is highly expressed in central cords whose cells contribute to the formation of central growing follicles. Expression of *Kitl* is low in granulosa cells of small primordial follicles and increases to high levels in secondary to pre-antral follicles during late oocyte growth. While granulosa cells express *Kitl*, the receptor *Kit* is expressed on both the oocyte as well as the thecal cells (Manova et al., 1990; Motro and Bernstein, 1993; Packer et al., 1994). After oocyte growth ceases, at the transition of the follicle into antral follicle stages, *Kitl* expression continues only in the outer layers of mature follicles i.e., in the mural granulosa cells next to the thecal cells and not in the cumulus granulosa cells which are the closest to the oocyte (Motro and Bernstein, 1993) (Figure 1.5).

As mentioned above, KITL expression is compartmentalized and modulated based on the size of the oocytes and follicles suggesting a role for the KITL signaling

pathway in oocyte growth and in meiotic arrest. Studies with *Kitl*^{Sl-pan} (Bedell et al., 1995; Packer et al., 1994) and *Kitl*^W mutants (Driancourt et al., 2000; Reynaud et al., 2001) have suggested that there is reduced follicular initiation and very little growth of the oocytes in the absence or reduced function of KITL. In *in vitro* follicle culture experiments, addition of KITL resulted in a delay in the progression of meiosis/meiotic maturation in fully grown follicles as measured by the indices of spontaneous germinal vesicle breakdown and polar body extrusions (Ismail et al., 1996). These data were further validated with observations that decreased expression of *Kit* is associated with an increased ability of these oocytes to resume meiosis (Ismail et al., 1997; Ismail et al., 1996). Administration of the neutralizing antibody ACK2 to different ages of mice disrupted follicular development at specific stages suggestive of the requirement of KITL-KIT signaling during transitions of different stages of follicles (Yoshida et al., 1997). Since all the above mentioned studies were conducted under conditions of complete abrogation of KITL/KIT function or in cultures with disrupted follicles or in mutants with severe effects on *Kitl* expression, investigation of follicular development in with mutant mice carrying hypomorphic *Kitl*^{Sl} mutations would be more informative.

Among the recently identified hypomorphic mutations in the *Kitl* gene (Rajaraman, 2002b), *Kitl*^{Sl-39R} and *Kitl*^{Sl-36R} allowed viability of the mice although with severe effects on PGC development and spermatogenesis (see Chapters 2 and 3). In preliminary fertility tests (M.A.Bedell, unpublished), the females homozygous for each mutation produced less than 2 litters with only 2-3 pups per litter. Preliminary histological analysis (M.A.Bedell, unpublished), revealed a reduced number of follicles

in both *Kitl*^{SI-39R}/*Kitl*^{SI-39R} and *Kitl*^{SI-36R}/*Kitl*^{SI-36R} ovaries that was consistent with the reduced number of PGCs during embryogenesis (see Chapter 2). However, *Kitl*^{SI-36R}/*Kitl*^{SI-36R} ovaries had reduced number of growing and maturing follicles and therefore were analyzed further for defects in follicular development.

In the *Kitl*^{SI-36R} allele a point mutation in exon 5 was identified that creates a nonsense mutation in codon 147 (Rajaraman et al., 2002a). Thus, a mutant product of only 146 aa of S-KITL (Figure 1.2) would be produced by premature termination of two alternatively spliced *Kitl* mRNAs. In addition, abnormal splicing (skipping) of exon 5 was observed and is expected to produce a second KITL^{SI-36R} isoform of only the first 96 aa of S-KITL with 25 C-terminal aa out-of-frame (Figure 1.2). Evidence to date indicates that this second isoform is likely to be null functionally (Rajaraman et al., 2002b) and so it is likely that the 146 aa form is the only biologically active form expressed by the *Kitl*^{SI-36R} allele.

The requirements for factors that are able to initiate differentiation of thecal cells are that they must be able to induce the expression of LH receptors and steroidogenic enzymes in the thecal cells (Magoffin, 2002) (see Figure 4.1). Recent work from the Magoffin lab has identified several candidate proteins required for thecal cell differentiation. From *in vitro* studies with cultures of ovarian thecal cells from hypophysectomized rats, it was shown that the combination of insulin growth factor 1 (IGF1) and KITL was sufficient for androgen production, independent of LH stimulation (Huang et al., 2001). Addition of neutralizing antibodies to KITL and IGF1 in the

cultures abrogated androgen synthesis, which further supported the notion that KITL and IGF1 are involved in thecal cell differentiation. Furthermore, preliminary data from *in vitro* culture studies with thecal cells isolated from *Kitl*^{SL-36R}/*Kitl*^{SL-36R} ovaries that suggested that the *Kitl*^{SL-36R}/*Kitl*^{SL-36R} thecal cells were hyperandrogenic upon LH stimulation (D. Magoffin, personal communication).

The most common endocrine disorder in women, polycystic ovarian syndrome (PCOS), is characterized by excessive androgen production by thecal cells but the mechanism stimulating this production is not clearly understood (Erickson et al., 1992). Therefore to define the role of KITL in thecal differentiation would further the understanding on the pathogenesis of PCOS and other androgen hyperproduction syndromes.

MATERIALS AND METHODS

Mice

Mice heterozygous for the *Kitl*^{SL-36R} mutation were maintained on a C3H/HeNCR background in a pathogen-free, AALAC-accredited facility at the University of Georgia. All procedures with mice were approved by the IACUC of the University of Georgia. Homozygotes were obtained by intercrossing heterozygotes and identified by the white coat color. Mice were euthanized by CO₂ asphyxiation.

Histology

At least five pairs of ovaries each from 3, 6 and 12 week, *Kitl*^{+/+}/*Kitl*⁺ and *Kitl*^{SL-36R}/*Kitl*^{SL-36R} female mice were collected and fixed in Bouin's fixative, dehydrated, processed and embedded in paraffin. Five micron sections were cut from the paraffin blocks and stained with hematoxylin and eosin (H&E) by standard protocols.

Immunohistochemistry

Ovaries were collected from at least two, 3 week and 6 week old females, snap frozen in OCT (Tissue Tek) and stored at -80°C. Five micron thick frozen sections were cut and thoroughly air dried before storage at -20°C. Slides were transferred out of the -20°C freezer just before immunostaining and dried at 37°C for 20 min to avoid condensation on the sections. For detection of cytochrome P450 cholesterol side chain cleavage enzyme (SCC), rabbit anti-rat SCC polyclonal antibody (Chemicon #AB1244) was used. For detection of P450 17 α hydroxylase (17-alpha), rabbit anti-porcine 17-alpha polyclonal antibody (provided by Dr. D. Magoffin) was used. For detection of P450 aromatase (arom), rabbit anti-human arom polyclonal antibody (Hauptman-Woodward # R-10-2) was used. After blocking the sections for non-specific binding, incubations with primary antibodies were carried out as follows: anti-SCC at a dilution of 1:200 for 40 min at room temperature, anti-17-alpha and anti-arom at dilutions of 1:100 overnight at 4°C. For detection of primary antibody binding, biotinylated secondary antibody and streptavidin conjugated horseradish peroxidase was used as per manufacturer's instructions (Zymed Histostain Plus Kit (85-9943)).

RESULTS

Disruption of early folliculogenesis in *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} ovaries

To investigate further the defects caused by *Kitl*^{Sl-36R} mutation on ovarian follicle development, histology of *Kitl*⁺/*Kitl*⁺ and *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} ovaries from 3 and 6 week old females was examined. In *Kitl*⁺/*Kitl*⁺ ovaries, at 3 weeks of age, there were a large number of growing oocytes within primary and secondary follicles that were well organized (Figure 4.2A). In the preantral and antral follicles, multiple layers of thecal cells were observed (Figure 4.2A). At 6 weeks of age, there are numerous antral follicles and a few corpora lutea signifying previous ovulations (Figure 4.2B). In *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} ovaries, at 3 weeks, there were only a few oocytes that had grown to a similar size as in the primary and secondary follicles of the wildtype ovaries. Numerous apoptotic oocytes were found (arrows in Figure 4.2C) and the follicles were disorganized with no clear definition of thecal cells (Figure 4.2C). By 6 weeks of age, a few follicles were able to reach the ovulatory stage in the *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} ovaries that presumably resulted in mature ova being released. However the follicles from the next cohort were in early primary and secondary follicle stages (Figure 4.2D). Interestingly, a large number of corpora lutea-like structures were found in the *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} ovaries. Further analyses need to be carried out to investigate whether these lutea like structures are true corpora lutea since very few antral follicles were observed. Also at this age, numerous fluid filled, non-hemorrhagic cysts were found in the *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} ovaries that were not observed in the *Kitl*⁺/*Kitl*⁺ ovaries.

Disruption of steroidogenic enzyme expression in *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} ovaries

There is a temporal and spatial compartmentalization of the expression of the enzymes involved in steroidogenesis (Figure 4.1): the androgen precursors to the estrogens are synthesized in the thecal cells and diffused into the granulosa cells wherein they are converted into estrogens. The enzymes involved in steroidogenesis are therefore compartmentalized: SCC, 17-alpha and BHSD are expressed in the thecal cells while Arom is expressed in the granulosa cells (Figure 4.1). However after ovulation, both granulosa cell-specific and thecal cell-specific enzymes are expressed in the corpora lutea aiding in the production of progesterone (Gougeon, 1996). To investigate the patterns of steroidogenic enzyme expression that are required to initiate differentiation in granulosa cells and thecal cells, immunohistochemistry was performed to detect SCC, 17-alpha and aromatase. In wildtype follicles, the SCC expression was highest in the thecal cells of secondary, preantral and antral follicles as well as corpora lutea (Figure 4.3A). In the *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} ovaries, there is very little expression of SCC in the thecal cells of the follicles or the lutea like structures (Figure 4.3C) suggesting that the reduced function of *Kitl*^{Sl-36R}- encoded KITL is not sufficient to initiate steroidogenesis in the *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} follicles. 17-alpha expression was similar to the expression pattern of SCC in the wildtype (Figure 4.3E). However, the expression was limited to only a thin layer of thecal cells in the mutants (Figure 4.3G). The aromatase enzyme expression was consistently in the granulosa cells of pre-antral and antral follicles and corpora lutea. However we found some expression in the thecal cells was observed. The significance of such an expression pattern is not yet known.

DISCUSSION

Histological examination of juvenile *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} ovaries revealed that there is an early defect in follicular organization and that the growth of the oocytes was impaired. However by 6 weeks of age we observed a few antral follicles that presumably lead to ovulations, explaining the single litters from the fertility tests. Our data suggests that the reduced function of *Kitl*^{Sl-36R}- encoded KITL is not sufficient for the normal growth of the oocytes. We hypothesize that in turn, the oocytes are unable to generate the appropriate signal for granulosa cell proliferation. Therefore, there is less growth/recruitment of thecal cell layers resulting in a delay in follicular development.

Our investigation of the expression of various steroidogenic enzymes in the *Kitl*^{Sl-36R}/*Kitl*^{Sl-36R} ovaries suggested that the initiation of steroidogenesis is affected in the mutants. However further studies using a fluorescence-based detection method might yield better sensitivity and it may be possible to better discern the compartmentalization of the steroidogenic enzyme expression patterns. Additionally, the circulating levels of androgens, estrogens and gonadotropins in these mutants must be assayed to confirm that the expression of the steroidogenic enzymes are consistent with the output of androgen products. Furthermore, investigation of the expression patterns of these enzymes at earlier ages (3 weeks) is essential to gaining insights into the timeline of events.

The preliminary data from the thecal cell cultures from the Magoffin lab (personal communication) suggested that *Kitl*^{Sl-36R} thecal cells are hyperandrogenic upon LH stimulation. However, our data suggests that there are reduced levels of steroidogenesis

in the *Kitl*^{SI-36R}/*Kitl*^{SI-36R} ovaries. In addition, even though these ovaries developed multiple cysts they were non-hemorrhagic unlike in the polycystic ovaries. While our evidence indicates that KITL may be involved in thecal cell differentiation, it does not suggest that the *Kitl*^{SI-36R}/*Kitl*^{SI-36R} ovaries are an appropriate model to study PCOS.

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Figure 4.1. Steroidogenic enzymes required for the synthesis of androgen precursors in ovarian follicles. Upon differentiation, thecal cells begin to express SCC and 17alpha.

The import and cleavage of cholesterol is the rate-limiting step in steroid biosynthesis that is mediated by SCC while 17alpha is required to convert pregnenolone to hydroxy pregnenolone. BHSD isomerizes the hydroxy precursors to give rise to the androgens.

Arom is expressed in the granulosa cells of antral and ovulatory follicles and corpora lutea. It converts androgen precursors that are shuttled into the granulosa cells to estradiol and other estrogen derivatives.

Figure 4.1

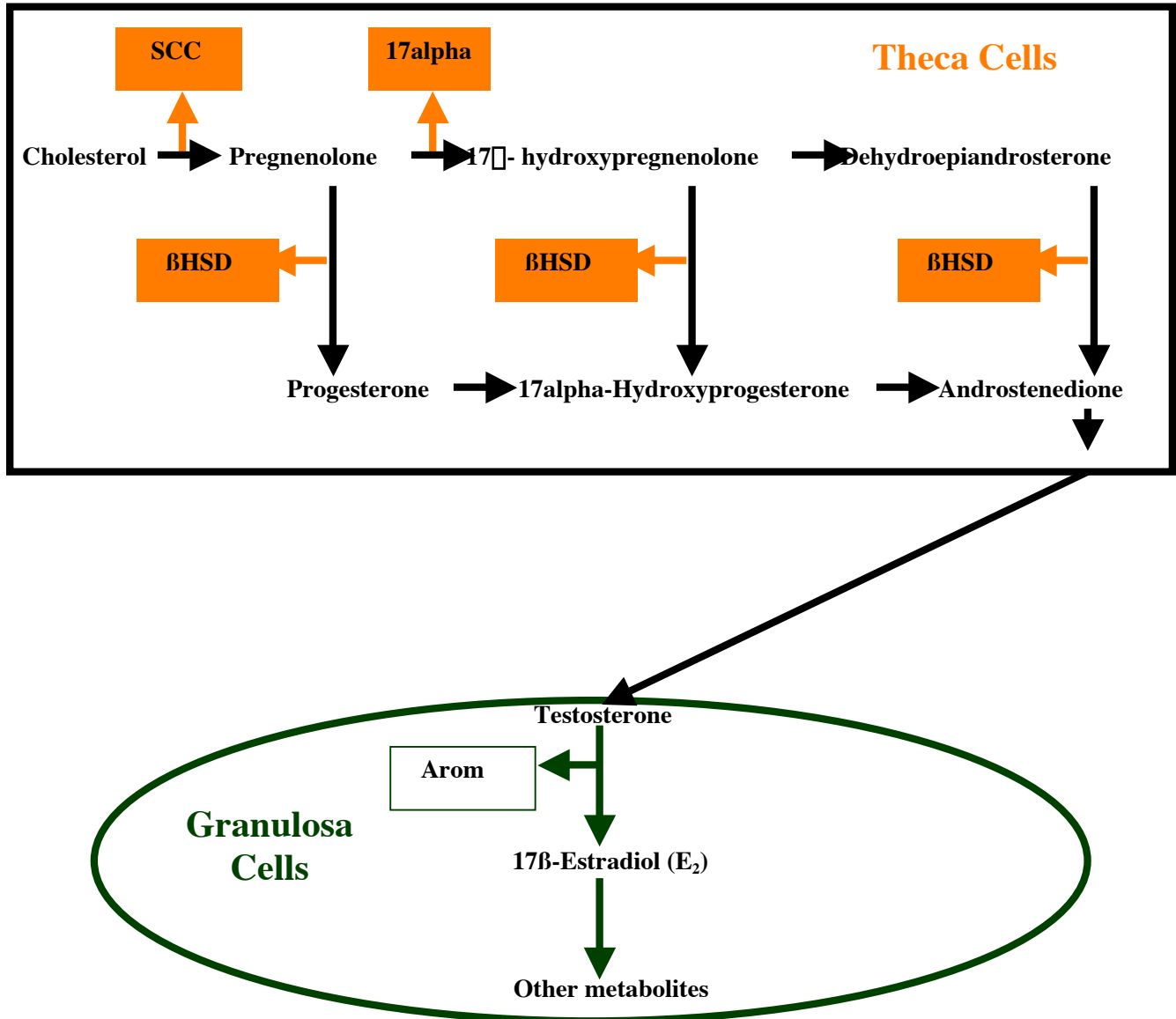


Figure 4.2. Disorganization and disruption of ovarian follicle development in *Kitl^{SL-36R}/Kitl^{SL-36R}* ovaries. At 3 and 6 weeks in *Kitl⁺/Kitl⁺* (A) and *Kitl^{SL-36R}/Kitl⁺* ovaries (B) there are numerous follicles at different stages of development. Shown are antral follicles (An) that are well organized with many layers of thecal cells. Corpora lutea (CL) are present suggestive of previous ovulations. In *Kitl^{SL-36R}/Kitl^{SL-36R}* ovaries, at 3 weeks (C) follicles are disorganized and many oocytes undergo apoptosis (arrows, densely stained nuclei). Also, follicles do not grow to the same size as in *Kitl⁺/Kitl⁺* (A). By 6 weeks, a few antral follicles are observed in the *Kitl^{SL-36R}/Kitl^{SL-36R}* ovaries (D). However no growing follicles from the next cohort were observed (arrows).

Figure 4.2

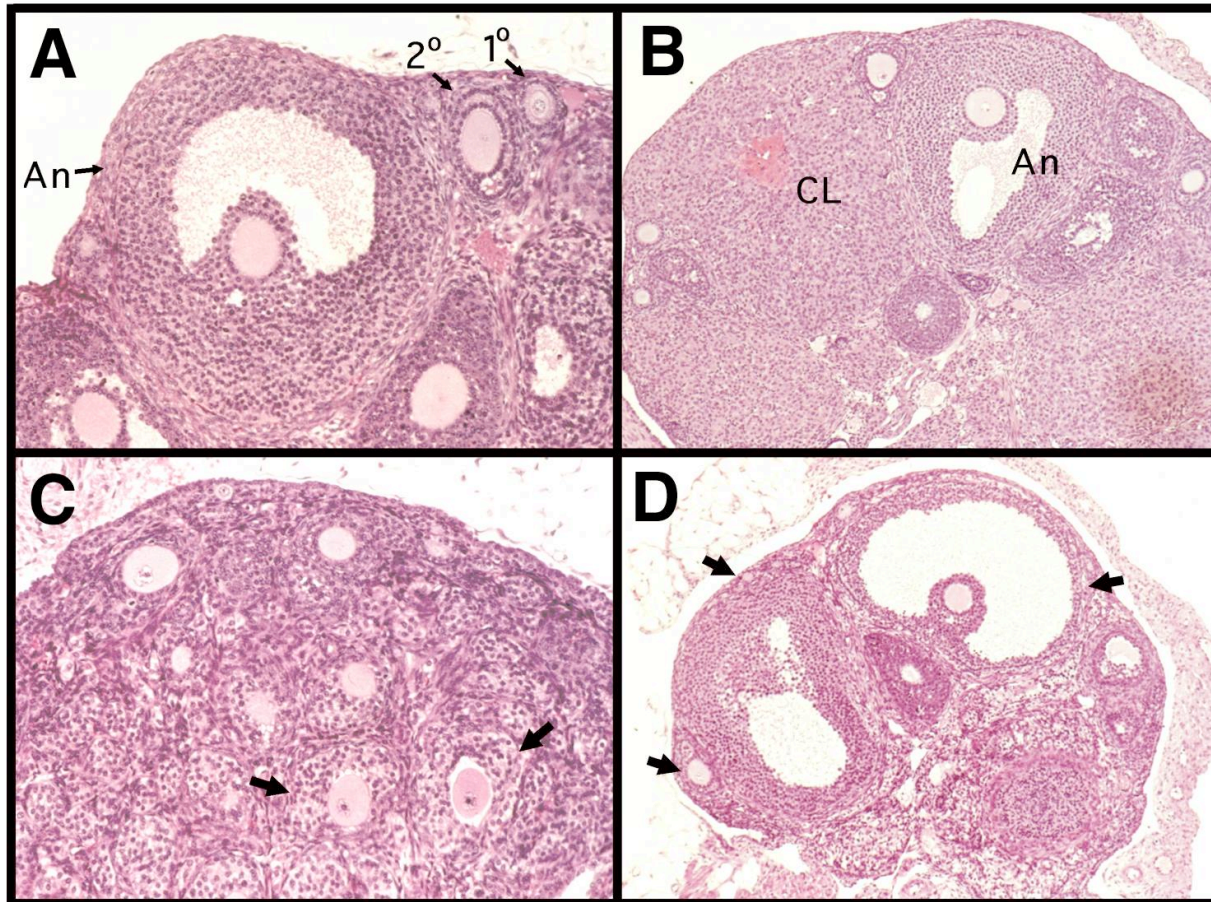


Figure 4.3. Steroidogenic enzyme expression patterns in *Kitl*⁺/*Kitl*⁺ and

***Kitl*^{SL-36R}/*Kitl*^{SL-36R} at 6 weeks of age.** In *Kitl*⁺/*Kitl*⁺ follicles (A, B, E, F, I and J) SCC expression is high in preantral and antral follicles, predominantly in the thecal cells (A). Corpora lutea express SCC as well (A). In *Kitl*^{SL-36R}/*Kitl*^{SL-36R} ovaries (C, D, G, H, K and L), there is reduced expression of SCC in the thecal cells and very faint expression in corpora lutea like structures. 17alpha expression pattern is similar to SCC in *Kitl*⁺/*Kitl*⁺ (E) and *Kitl*^{SL-36R}/*Kitl*^{SL-36R} follicles (G). Aromatase expression is predominantly in the corpora lutea (I). Expression is seen in granulosa cells as well as thecal cells suggestive of cross-reactivity (I and K). Negative controls are sections with only secondary antibody staining: SCC (B and D), 17alpha (F and H) and Arom (J and L).

Figure 4.3

